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SK&F S-106203 inhibits leukotriene C_4, leukotriene D_4 and leukotriene E_4 vasopressor responses in the conscious rat

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1 The purpose of these experiments was to investigate the effects of the selective peptidoleukotriene receptor antagonist, SK&F S-106203, on leukotriene C_4 (LTC_4), LTD_4 and LTE_4 vasopressor responses in the conscious, normotensive rat. SK&F S-106203 was administered as a bolus followed by a continuous infusion in order to provide information on the relationship between antagonism of leukotriene responses and steady-state plasma concentrations.

2 Infusion of SK&F S-106203 at doses of $0.2\,mg\,kg^{-1} + 1\,mg\,kg^{-1}\,h^{-1}$, $1\,mg\,kg^{-1} + 3\,mg\,kg^{-1}\,h^{-1}$ or $2\,mg\,kg^{-1} + 10\,mg\,kg^{-1}\,h^{-1}$ produced dose-dependent steady-state plasma drug concentrations of 1.0, 3.2 and 23.8 $\mu g\,ml^{-1}$, respectively. Plasma SK&F S-106203 concentrations appeared to increase in a linear fashion at doses of 1 and $3\,mg\,kg^{-1}\,h^{-1}$; at the highest dose the increment in plasma drug concentrations (i.e., 7–8 fold) was greater than the increment in dose (i.e., 3 fold), suggesting saturation of the primary clearance mechanism(s) at this dose.

3 SK&F S-106203 ($2\,mg\,kg^{-1} + 10\,mg\,kg^{-1}\,h^{-1}$) had no effect on noradrenaline-, vasopressin-, isoprenaline-, or U 46619-induced responses.

4 SK&F S-106203 produced dose-dependent rightward shifts in the LTC_4 and LTE_4 dose-response curves. Administration of SK&F S-106203 at doses of $0.2\,mg\,kg^{-1} + 1\,mg\,kg^{-1}\,h^{-1}$, $1\,mg\,kg^{-1} + 3\,mg\,kg^{-1}\,h^{-1}$, or $2\,mg\,kg^{-1} + 10\,mg\,kg^{-1}\,h^{-1}$ produced dose-ratios of 1.0, 3.1 and 19.9, respectively, against LTC_4 responses, and dose-ratios of 1.6, 3.8 and 9.1, respectively, against LTE_4 responses.

5 Against LTD_4 responses, SK&F S-106203 at doses of $0.2\,mg\,kg^{-1} + 1\,mg\,kg^{-1}\,h^{-1}$, $1\,mg\,kg^{-1} + 3\,mg\,kg^{-1}\,h^{-1}$, or $2\,mg\,kg^{-1} + 10\,mg\,kg^{-1}\,h^{-1}$ produced dose-ratios of 2.5, 2.8, and 11.4, respectively. Administration of D-penicillamine, a non-competitive LTD_4 dipeptidase inhibitor, had no effect on LTD_4 responses.

6 The similarity in the LTD_4 dose-ratios at the two lower infusion rates, despite increases in the plasma drug concentrations, suggests the existence of pharmacologically heterogeneous LTD_4 receptors. These results indicate that SK&F S-106203 is a potent, selective and apparently competitive antagonist of LTC_4, LTD_4 and LTE_4 vascular responses in the intact rat.

Introduction

The investigation of the role of the peptidoleukotrienes leukotriene C_4 (LTC_4), LTD_4 and LTE_4 in asthma, allergic reactions and inflammatory cell responses is an area of intensive activity (Weiss *et al.*, 1982; Samuelsson, 1983). More recently, attention has focused on the role these putative mediators may have in cardiovascular disorders, such as myocardial reperfusion injury, renal failure and circulatory shock. LTC_4, LTD_4, and LTE_4 have been shown to increase vascular permeability (Dahlen *et al.*, 1981), and have potent effects on various smooth muscle preparations, including trachea (Fleisch *et al.*, 1982; Hay *et al.*, 1987), the small intestine (Fleisch *et al.*, 1982), and coronary and mesenteric vessels (Michelassi *et al.*, 1982; Woodman & Dusting, 1983; Roth & Lefer, 1983; Eimerl *et al.*, 1986). The pathophysiological responses to peptidoleukotrienes, such as contraction of smooth muscle and increased vascular permeability, would exacerbate conditions such as myocardial ischaemia, circulatory shock and inflammatory disorders.

In order to clarify the precise role of peptidoleukotrienes in cardiovascular or other disorders, highly potent and selective peptidoleukotriene receptor antagonists are required. A number of peptidoleukotriene receptor antagonists are available, including FPL 55712, LY 171883 and ONO 1078. However, some leukotriene receptor antagonists with relatively low potency have been shown to inhibit the contractile effects of other pressor agents, such as vasopressin and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Boyd *et al.*, 1983), and to inhibit cyclic nucleotide phosphodiesterase (Hay *et al.*, 1987). SK&F S-106203 is representative of a class of peptidoleukotriene receptor antagonists that are structurally similar to the natural agonist. Like similar molecules such as SK&F 102922 and SK&F 104353, SK&F S-106203 is a potent and selective receptor antagonist (Hay *et al.*, 1988). However, SK&F S-106203, unlike many of its structural analogues, appears to have high oral bioavailability (Newton *et al.*, 1988). A peptidoleukotriene receptor antagonist with high oral bioavailability, such as SK&F S-106203, could be useful for probing the role of leukotrienes in respiratory diseases, as well as other diseases where peptidoleukotrienes have been implicated as important mediators, such as endotoxic and septic shock, and myocardial reperfusion injury, where high systemic concentrations of antagonist may be required to antagonize the effect of leukotrienes. As a prelude to such studies, it is imperative to document the plasma concentrations of SK&F S-106203 required to antagonize a peptidoleukotriene response. Therefore, the purpose of this study was to characterize the effects of SK&F S-106203 on the vascular responses to LTC_4, LTD_4 and LTE_4. These studies were conducted in conscious, chronically instrumented rats in order to avoid the confounding influences of acute surgical preparation and anaesthetic agents.

Methods

Animal preparation

Male Sprague-Dawley rats, weighing 320–395 g, were anaesthetized with pentobarbitone sodium ($30\,mg\,kg^{-1}$, i.p.) and prepared, with modifications, as described previously (Smith *et al.*, 1988). Briefly, catheters were placed into the vena cava

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and abdominal aorta through the left and right femoral veins and left femoral artery, respectively. The catheters were led subcutaneously along the tail and exteriorized through an incision 5 cm from the base of the tail. A 10 ml syringe barrel, with the closed end removed, was placed over the tail and secured with tape. The syringe barrel served to protect the incision site and catheters. The animals were housed in individual cages and the syringe barrel used to tether the animals. Catheter patency was maintained with an infusion of 0.9% NaCl containing 2.4 u ml^{-1} heparin at a rate of 0.6 ml h^{-1}. Blood pressure was continuously monitored (Statham P23 DC pressure transducer) on a Grass Model 7D polygraph recorder. The animals were allowed to stabilize for 1–2 days following the surgical procedures. The animals were permitted access to food and water *ad libitum*.

Quantitation of SK&F S-106203 in plasma

SK&F S-106203 was administered as an initial bolus followed by a continuous infusion for 6 h at the following doses: 0.2 mg kg^{-1} + 1.0 mg kg^{-1} h^{-1}, 1 mg kg^{-1} + 3 mg kg^{-1} h^{-1}, or 2 mg kg^{-1} + 10 mg kg^{-1} h^{-1}. After initiation of the drug infusion, blood samples (approximately 0.5 ml) were collected into heparinized tubes and transferred to Eppendorf microtubes. The blood samples were placed immediately on ice.

Plasma concentrations of SK&F S-106203 were determined in a similar manner to methods previously described (Smith *et al.*, 1988). Briefly, the internal standard SK&F 104353 (2(S)-hydroxy-3(**R**)-(2-carboxyethylthio)-3-[2-(8-phenyloctyl) phenyl]propanoic acid) was added to the plasma sample (100 μl), and extracted twice (750 μl) with methyl formate containing 0.5% trifluoroacetic acid. The samples were vortexed, centrifuged at 1000 *g* and the supernatants combined. The combined supernatants were evaporated to dryness under nitrogen, and resuspended in h.p.l.c. mobile phase. The samples were quantitated on an h.p.l.c. system by a 5 μm NOVAPAK C_{18} cartridge (Waters Assoc., Inc., Milford, MA). H.p.l.c. effluent was monitored at 215 nm with a Kratos Model 783 u.v. detector. SK&F S-106203 and the internal standard were separated isocratically by a solvent system consisting of 59% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 2 ml min^{-1}. Plasma concentrations of SK&F S-106203 were determined from peak area ratios (compared to internal standard) that were compared to extracted authentic standard curves. The detection limit for quantitation of SK&F S-106203 in plasma was 0.25 μg ml^{-1}, and the standard curve of the assay was linear to 100 μg ml^{-1}.

Antagonism of leukotriene responses

A separate group of animals, prepared as described above, were used to determine antagonism of LTC_4, LTD_4 or LTE_4 vasopressor responses with SK&F S-106203. On the day of study, animals received LTC_4, LTD_4 or LTE_4 intravenously, at 60 min intervals, in one-half log increment doses ranging from 0.17 to 510 nmol kg^{-1} (i.e., approximately 0.1–300 μg kg^{-1}). In this manner, complete dose-response curves were obtained. The acute increase in mean arterial blood pressure following each injection was monitored. LTC_4, LTD_4 or LTE_4 were administered in a volume of 0.1 ml 100 g^{-1} body weight and followed with a 0.5 ml saline flush.

On the following day, animals were administered a bolus followed by an infusion of SK&F S-106203 at the same doses indicated above. At 1 h after the start of the infusion, the dose-response curve to LTC_4, LTD_4 or LTE_4 was repeated according to the procedures described above. The infusion of SK&F S-106203 was continued until the entire dose-response curve was completed. The highest dose of a peptidoleukotriene studied in the presence of an antagonist was 510 nmol kg^{-1}.

To investigate the specificity of SK&F S-106203 to antagonize vascular peptidoleukotriene responses, animals were injected with arginine vasopressin (30 and 100 ng kg^{-1}, i.v.), noradrenaline (100 and 300 ng kg^{-1}, i.v.) or isoprenaline (100 and 300 μg kg^{-1}, i.v.). Each animal was injected with only one agonist, and the two doses of the agonist were given at 1 h intervals. Following the administration of the agonist, SK&F S-106203 was administered as a dose of 2 mg kg^{-1} + 10 mg kg^{-1} h^{-1}. After 1 h, the two doses of the agonist were re-administered during continuous infusion of SK&F S-106203 and the change in blood pressure monitored.

To determine the effects of SK&F S-106203 on thromboxane receptor-mediated responses, animals were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.p.) and prepared with catheters as described above. After the animals were allowed to stabilize for 15 min, SK&F S-106203 was administered at a dose of 2 mg kg^{-1} + 10 mg kg^{-1} h^{-1}. One h after initiation of the infusion, the animals were injected intravenously with the thromboxane mimetic U 46619 (100 μg kg^{-1}). Another group of animals was injected with the thromboxane receptor antagonist, daltroban (10 mg kg^{-1}, i.v.), and served as a control group (Smith & McDonald, 1988).

Materials

The disodium salt of SK&F S-106203 (3(S)-(2-carboxyethylthio)-3-[2-(8-phenyloctyl)phenyl] propanoic acid) was dissolved in 0.9% NaCl. The dose of SK&F S-106203 refers to the free acid. LTC_4, LTD_4 and LTE_4 (synthesized at SmithKline Beecham Pharmaceuticals plc, King of Prussia, PA) were dissolved in distilled water and the stock stored at 4°C. On the day of use, a sample was removed, diluted in distilled water and scanned in a spectrophotometer (Beckman DU-50, Somerset, N.J.) from 200–350 nm. The absorbance at 282 nm was then measured, and the concentration of the sample determined with an extinction coefficient of 40,000 M^{-1} cm^{-1}. An aliquot of the stock was then diluted in 0.9% NaCl and stored on ice until used. D-Penicillamine and isoprenaline (Sigma Chemical Co., St. Louis, MO) were dissolved in 0.9% NaCl. Noradrenaline (Sigma Chemical Co., St. Louis, MO) was dissolved in 0.9% NaCl containing 100 μg ml^{-1} ascorbic acid. Arginine vasopressin (Sigma Chemical Co., St. Louis, MO) was dissolved in acetic acid at a concentration of 10 mg ml^{-1}, and further diluted in 0.9% NaCl. U 46619 (9,11-dideoxy-11α9α-epoxymethano-prostaglandin $F_{2\alpha}$, Upjohn Diagnostics, Kalamazoo, MI) was initially dissolved in 50% ethanol containing 1.5 mg ml^{-1} $NaHCO_3$, and further diluted in 0.9% NaCl. Daltroban (SmithKline Beecham Pharmaceuticals plc, King of Prussia, PA) was dissolved in 50 nM Tris buffer, pH 9.4. All solutions were made fresh on the day of use.

Statistics

All data in the text, figures and table are expressed as the mean ± s.e.mean of *n* observations. Since multiple samples were taken from individual animals, the average plasma concentrations over the course of an infusion were first calculated for individual animals, and then averaged to obtain mean group estimates. Steady-state plasma concentrations of SK&F S-106203 are expressed as the mean of the plasma concentrations of drug determined at 1, 2, 4 and 6 h of infusion. For LTC_4 and LTD_4 responses, the ED_{20} was calculated for individual dose-response curves, with the ED_{20} being the dose of peptidoleukotriene which produced an increase in blood pressure of 20 mmHg. Since LTE_4 appeared to be a partial agonist in these preparations, and produced a comparatively small response, the corresponding ED_{10} dose was calculated. Dose-ratios were calculated as the quotient of the ED_{20} (or ED_{10} for LTE_4) dose of an individual experiment in the presence of SK&F S-106203 to the mean ED_{20} determined from the peptidoleukotriene dose-response curves in the absence of SK&F S-106203.

Results

Plasma drug concentrations following an infusion of SK&F S-106203 are illustrated in Figure 1. Administration of doses

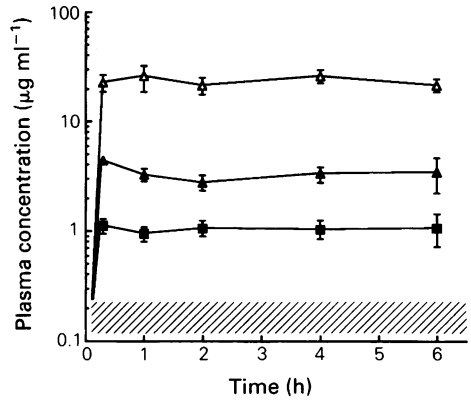

Figure 1 Steady-state plasma concentrations of SK&F S-106203. The doses of SK&F S-106203 were $0.2\,mg\,kg^{-1} + 1\,mg\,kg^{-1}\,h^{-1}$ (■), $1\,mg\,kg^{-1} + 3\,mg\,kg^{-1}\,h^{-1}$ (▲), or $2\,mg\,kg^{-1} + 10\,mg\,kg^{-1}\,h^{-1}$ (△). The detection limit of $0.25\,\mu g\,ml^{-1}$ is represented by the horizontal hatched bar. Each group is the result of 7 animals. Vertical lines show s.e.mean.

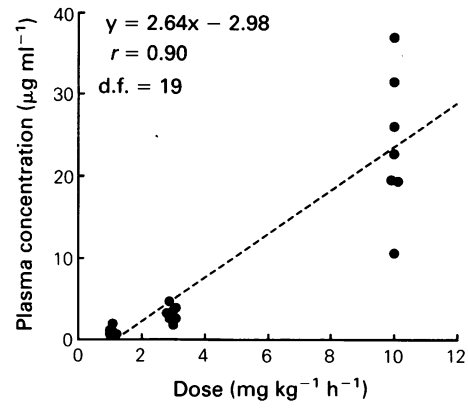

Figure 2 Relationship between the dose of SK&F S-106203 and plasma concentrations. Linear regression analysis performed on individual steady-state plasma concentration values, (i.e., the average value of plasma concentrations between 1 and 6 h).

ranging from $0.2\,mg\,kg^{-1} + 1\,mg\,kg^{-1}\,h^{-1}$ to $2\,mg\,kg^{-1} + 10\,mg\,kg^{-1}\,h^{-1}$ produced steady-state plasma concentrations within 30 min. Steady-state plasma concentrations of SK&F S-106203 were approximately $1\,\mu g\,ml^{-1}$ at the lowest dose, and at a dose of $10\,mg\,kg^{-1}\,h^{-1}$ steady-state plasma concentrations were approximately $24\,\mu g\,ml^{-1}$ (Table 1). At doses of $0.2\,mg\,kg^{-1} + 1\,mg\,kg^{-1}\,h^{-1}$ or $1\,mg\,kg^{-1} + 3\,mg\,kg^{-1}\,h^{-1}$, steady-state plasma concentrations of SK&F S-106203 appeared to increase linearly as a function of dose (Figure 2), although it appeared that at the highest dose of SK&F S-106203 the increment in plasma concentration was approximately 2–3 fold greater than the increment in dose.

The dose-response relationship for LTC_4 and the effect of SK&F S-106203 are illustrated in Figure 3 and summarized in Table 1. LTC_4 at a dose of $0.17\,nmol\,kg^{-1}$, i.v. ($0.3\,\mu g\,kg^{-1}$), produced a threshold vasopressor response and the ED_{20} was $2.3 \pm 0.5\,nmol\,kg^{-1}$ ($n = 10$). SK&F S-106203 inhibited the responses to LTC_4 in a manner which suggested competitive interaction at the peptidoleukotriene receptor site. Administration of SK&F S-106203 at doses ranging from 1.0–$10\,mg\,kg^{-1}\,h^{-1}$ produced dose-dependent, parallel and rightward shifts in the dose-response curve.

Table 1 Effect of SK&F S-106203 on steady-state drug plasma concentrations, and on the leukotriene C_4 (LTC_4), LTD_4 and LTE_4 dose-response relationship

	SK&F S-106203 ($mg\,kg^{-1} + mg\,kg^{-1}\,h^{-1}$)		
	0.2 + 1	*1 + 3*	*2 + 10*
	Steady-state plasma concentrations ($\mu g\,ml^{-1}$)		
	1.0 ± 0.2	3.2 ± 0.4	23.8 ± 3.3
(*n*)	(7)	(7)	(7)
	Dose ratios		
LTC_4	1.0 ± 0.3	3.1 ± 0.7	19.9 ± 3.2
(*n*)	(8)	(8)	(6)
LTD_4	2.5 ± 1.0	2.8 ± 0.9	11.4 ± 4.2
(*n*)	(6)	(7)	(8)
LTE_4	1.6 ± 0.7	3.8 ± 1.3	9.1 ± 2.1
(*n*)	(3)	(6)	(7)

SK&F S-106203 was administered as a bolus followed by an infusion at doses of: $0.2\,mg\,kg^{-1} + 1\,mg\,kg^{-1}\,h^{-1}$, $1\,mg\,kg^{-1} + 3\,mg\,kg^{-1}\,h^{-1}$, or $2\,mg\,kg^{-1} + 10\,mg\,kg^{-1}\,h^{-1}$. Steady-state plasma drug concentrations were determined from the mean of the plasma concentrations at 1, 2, 4 and 6 h. Steady-state plasma concentrations of SK&F S-106203 and dose-ratios to peptidoleukotriene vasopressor responses were determined in separate groups of animals.

The effect of SK&F S-106203 on the dose-response relationship to LTD_4 is illustrated in Figure 3 and summarized in Table 1. The ED_{20} for LTD_4 was $2.3 \pm 0.5\,nmol\,kg^{-1}$ ($n = 13$). Administration of increasing doses of SK&F S-106203 produced parallel and rightward shifts in the LTD_4 dose-response curve. At a dose of $0.2\,mg\,kg^{-1} + 1\,mg\,kg^{-1}\,h^{-1}$, SK&F S-106203 antagonized the LTD_4 vasopressor response, with a resultant dose-ratio of 2.5. A dose of $1\,mg\,kg^{-1} + 3\,mg\,kg^{-1}\,h^{-1}$, which produced a steady-state plasma concentration of $3.2\,\mu g\,ml^{-1}$, did not result in any further shift in the dose-response curve than that produced by a dose of $0.2\,mg\,kg^{-1} + 1\,mg\,kg^{-1}\,h^{-1}$. However, at a dose of $2\,mg\,kg^{-1} + 10\,mg\,kg^{-1}\,h^{-1}$, SK&F S-106203 produced a further shift in the dose-response curve to LTD_4, with a dose ratio of 11.4.

The dose-response relationship for LTE_4, and the effect of SK&F S-106203, is illustrated in Figure 3 and summarized in Table 1. LTE_4 at a dose of $5.1\,nmol\,kg^{-1}$, i.v., produced a threshold vasopressor response. The ED_{20} for LTE_4 was $30 \pm 5\,nmol\,kg^{-1}$ ($n = 13$). Although the dose-response curves for LTC_4, LTD_4 and LTE_4 were not parallel, the data indicate that LTE_4 was 10 to 30 fold less potent than LTC_4 or LTD_4 at increasing blood pressure in conscious, normotensive rats. Furthermore, within the range of doses used the maximal vasopressor response to LTE_4 appeared to be less than that achieved with LTC_4 or LTD_4. Administration of SK&F S-106203 at doses of $0.2\,mg\,kg^{-1} + 1\,mg\,kg^{-1}\,h^{-1}$, $1\,mg\,kg^{-1} + 3\,mg\,kg^{-1}\,h^{-1}$ or $2\,mg\,kg^{-1} + 10\,mg\,kg^{-1}\,h^{-1}$ produced dose-dependent rightward shifts in the LTE_4 dose-response curve. SK&F S-106203 at a dose of $1\,mg\,kg^{-1} + 3\,mg\,kg^{-1}\,h^{-1}$ did not appear to affect the maximal vasopressor response to LTE_4, whereas the maximal LTE_4 response following SK&F S-106203 at a dose of $2\,mg\,kg^{-1} + 10\,mg\,kg^{-1}\,h^{-1}$ did appear to be depressed. Unfortunately, higher doses of LTE_4 could not be administered due to the limited availability of this compound.

Table 2 and Figure 4 summarize the experiments to evaluate the specificity of SK&F S-106203 in antagonizing peptidoleukotriene vasopressor responses. Injection of arginine vasopressin or noradrenaline produced the expected increases in arterial blood pressure, and isoprenaline the expected decrease in arterial blood pressure. Administration of SK&F S-106203 ($2\,mg\,kg^{-1} + 10\,mg\,kg^{-1}\,h^{-1}$) had no significant effects on the cardiovascular responses to these agonists (Table 2), although these responses were blocked by specific receptor antagonists (i.e., $d(CH_2)_5Tyr(Me)AVP$, prazosin, and propranolol). Similarly, administration of SK&F S-106203 did not prevent U 46619-induced sudden death in rats ($n = 4$). Pretreatment with the thromboxane receptor antagonist, daltroban, at a dose of $10\,mg\,kg^{-1}$, i.v., prevented completely the U 46619-induced sudden death ($n = 4$).

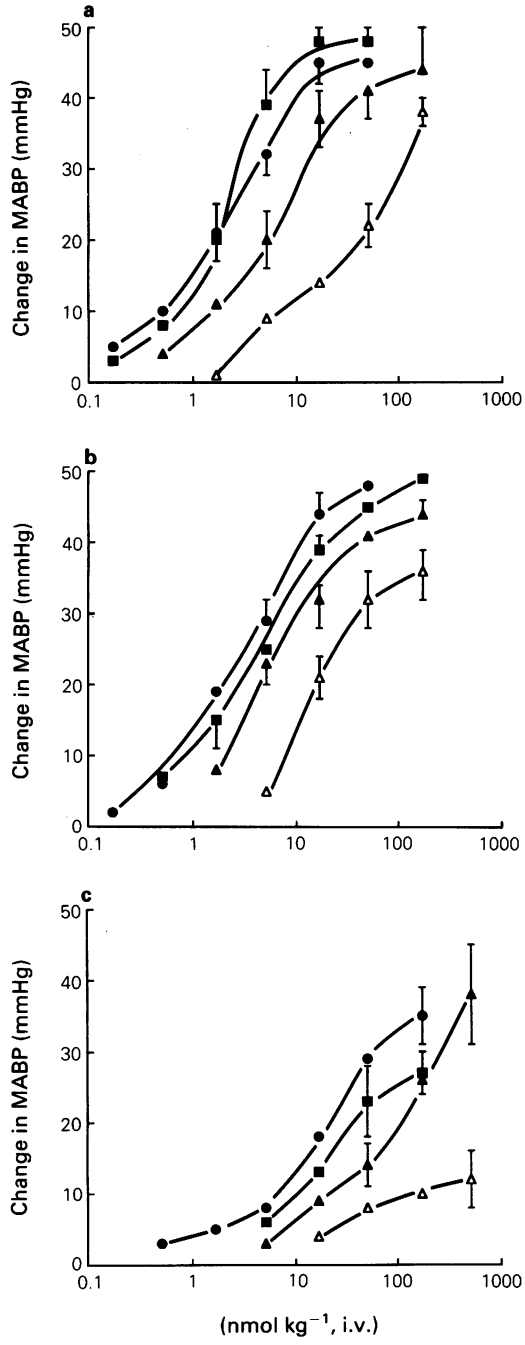

Figure 3 Effect of SK&F S-106203 on the leukotriene C_4 (LTC_4) (a), LTD_4 (b), LTE_4 (c) dose-response curves in conscious normotensive rats. MABP, mean arterial blood pressure. For the number of animals at each dose, see Table 1. (●) Peptidoleukotriene dose-response curves in the presence of a vehicle infusion: the doses of SK&F S-106203 were $0.2\,mg\,kg^{-1} + 1\,mg\,kg^{-1}\,h^{-1}$ (■), $1\,mg\,kg^{-1} + 3\,mg\,kg^{-1}\,h^{-1}$ (▲), or $2\,mg\,kg^{-1} + 10\,mg\,kg^{-1}\,h^{-1}$ (△). Vertical lines show s.e.mean.

The effects of an LTD_4 dipeptidase inhibitor, D-penicillamine (Huber & Keppler, 1987), on the LTD_4 vasoconstrictor response was investigated. Before the administration of D-penicillamine, an injection of $5.1\,nmol\,kg^{-1}$ LTD_4 produced an increase in blood pressure of 32 ± 3 mmHg ($n = 9$). At 5 and 50 min following the administration of $2\,nmol\,kg^{-1}$ D-penicillamine, LTD_4 increased blood pressure by 31 ± 3 ($n = 9$) and 32 ± 2 mmHg ($n = 9$), respectively. There was no statistically significant difference between the initial LTD_4 pressor response and those pressor responses elicited following the injection of D-penicillamine.

Table 2 Effect of SK&F S-106203 on the vasopressin-, noradrenaline- and isoprenaline-induced changes in blood pressure

Agonist	n	*Change in blood pressure* (mmHg) *Initial*	*Final*	*Initial*	*Final*
		$30\,ng\,kg^{-1}$		$100\,ng\,kg^{-1}$	
Vasopressin	4	46 ± 7	38 ± 5	60 ± 5	53 ± 6
		NS		NS	
		$100\,ng\,kg^{-1}$		$300\,ng\,kg^{-1}$	
Noradrenaline	4	12 ± 3	19 ± 4	31 ± 5	32 ± 4
		NS		NS	
		$100\,ng\,kg^{-1}$		$300\,ng\,kg^{-1}$	
Isoprenaline	5	-20 ± 4	-18 ± 6	-21 ± 2	-21 ± 1
		NS		NS	

SK&F S-106203 was administered as a bolus followed by an infusion, at a dose of $2\,mg\,kg^{-1} + 10\,mg\,kg^{-1}\,h^{-1}$, beginning 1 h before the final challenge. Vasopressin, noradrenaline and isoprenaline were administered intravenously at the indicated doses.
NS: not statistically different.

Discussion

The purpose of this study was to characterize the effects of the selective peptidoleukotriene receptor antagonist, SK&F S-106203, on the vasopressor responses to exogenously administered peptidoleukotrienes in the rat. SK&F S-106203 has been shown to be a potent and highly selective antagonist of peptidoleukotriene responses in guinea-pig tracheal preparations, with a pA_2 of 7.9 against LTD_4 responses, and a pK_B of 7.3 on LTE_4 contractions (Hay *et al.*, 1988). SK&F S-106203 does not inhibit guinea-pig tracheal contractions produced by carbachol, histamine, U 44069, potassium chloride, PGD_2 or $PGF_{2\alpha}$, and does not inhibit cyclic nucleotide phosphodiesterases (Hay *et al.*, 1988). Furthermore, SK&F S-106203 does not exhibit intrinsic agonist activity at leukotriene receptors. As described in the present study, SK&F S-106203 did not block arginine vasopressin, α- or β-adrenoceptor, or thromboxane receptor-mediated responses. Therefore, SK&F S-106203 is a potent and selective peptidoleukotriene receptor antagonist on pulmonary tissue and would appear to be an appropriate agent with which to characterize vascular peptidoleukotriene responses.

In comparison to numerous other investigations which have employed a bolus administration of a receptor blocker to demonstrate antagonism of peptidoleukotriene responses (Michelassi *et al.*, 1982; Ahmed *et al.*, 1986; Eimerl *et al.*, 1986; Filep *et al.*, 1987), this study investigated the effects of a continuous infusion of SK&F S-106203 on the vasopressor responses to LTC_4, LTD_4 and LTE_4. The inability to achieve steady-state pharmacokinetics following a bolus administration of drug would preclude *a priori* any conclusions on the relationship between plasma concentrations and drug effect.

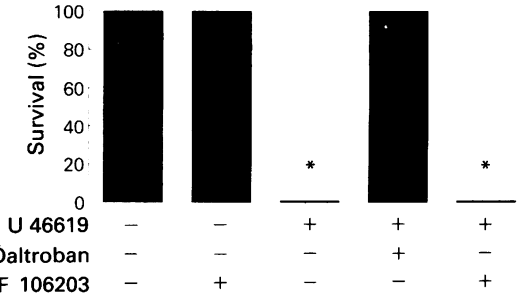

Figure 4 Effect of SK&F S-106203 ($2\,mg\,kg^{-1} + 10\,mg\,kg^{-1}\,h^{-1}$) or daltroban ($10\,mg\,kg^{-1}$) on U 46619-induced sudden death in anaesthetized rats. Pretreatment with the thromboxane receptor antagonist daltroban prevented sudden death, whereas SK&F S-106203 did not prevent sudden death. Each group is the result of 4 animals. * $P < 0.01$.

As depicted in Figure 1, the intent of achieving dose-related increases in steady-state plasma concentrations of SK&F S-106203 was in fact accomplished in these studies. At the lower doses of SK&F S-106203, a linear relationship appeared to exist between the dose and plasma concentrations. Thus, a 3 fold increase in dose from $1\,mg\,kg^{-1}\,h^{-1}$ to $3\,mg\,kg^{-1}\,h^{-1}$ was associated with a corresponding increase in steady-state plasma concentrations (i.e., 1 and $3.2\,\mu g\,ml^{-1}$, respectively). However, at a dose of $10\,mg\,kg^{-1}\,h^{-1}$ the corresponding plasma concentration was $23.8\,\mu g\,ml^{-1}$. The explanation for the deviation from linearity at the highest dose of SK&F S-106203 is not precisely known, but may possibly reflect saturation of the primary clearance mechanism(s) for the drug.

SK&F S-106203 inhibited the responses to LTD_4 in a manner which suggests competitive interaction at the peptidoleukotriene receptor site. Administration of SK&F S-106203 at doses of $1–10\,mg\,kg^{-1}\,h^{-1}$ produced parallel shifts to the right in the LTD_4 dose-response curve. Interestingly, increasing the dose of SK&F S-106203 from $0.2\,mg\,kg^{-1} + 1\,mg\,kg^{-1}\,h^{-1}$ to $1\,mg\,kg^{-1} + 3\,mg\,kg^{-1}\,h^{-1}$ did not result in a further shift in the LTD_4 dose-response curve. However, an increase in the SK&F S-106203 dose to $10\,mg\,kg^{-1}\,h^{-1}$ produced a further increase in the dose-ratio. The resultant dose-response curves, which resemble the theoretical curves obtained for a non-selective agonist acting in a two-receptor system (Lemoine & Kaumann, 1983; Kenakin, 1987), suggest the existence of two peptidoleukotriene receptor sites mediating the vasopressor effects of LTD_4. Similarities in the dose-ratios to the vasopressor responses of LTD_4 despite incremental increases in the dose (*vis a vis* plasma concentrations) of a peptidoleukotriene receptor antagonist has been previously obtained for SK&F S-104353 (Smith *et al.*, 1989). Additional support for the hypothesis of two peptidoleukotriene receptor sites mediating LTD_4 vasopressor responses is provided by the observation that ICI 198615 produced dose-ratios of approximately 20 against LTD_4 responses at doses of 1, 3 or $10\,mg\,kg^{-1}\,h^{-1}$ (Smith & Slivjak, 1989). These data with ICI 198615 corroborate the observations of Hand *et al.* (1989) in guinea-pig isolated trachea, and further support their suggestion of two LTD_4 receptor subtypes. Therefore, the results with SK&F S-106203 provide additional support for the proposal that the vasopressor responses to LTD_4 are mediated by activation of a specific, but possibly heterogeneous high and low affinity LTD_4 receptor population.

The possibility that LTD_4 receptors may not be comprised of a homogeneous population has been suggested previously (Fleisch *et al.*, 1984). Fleisch *et al.* (1982) showed that the pA_2 values for FPL 55712 against LTD_4 responses were similar in guinea-pig trachea and parenchyma, but different from those of the ileum. Krell *et al.* (1983) suggested the existence of two LTD_4 receptors in guinea-pig trachea based upon different affinities for FPL 55712. Aharony *et al.* (1989) suggested subtypes of LTD_4 receptors on guinea-pig lung membranes based on results from receptor binding studies. In conjunction, the results in pulmonary tissue provide considerable support for the proposal of heterogeneity within the LTD_4 receptor. Although thromboxane appears to be the final mediator of peptidoleukotriene responses in the lung (Folco *et al.*, 1981; Piper & Samhoun, 1981; Ueno *et al.*, 1982), the systemic effects of the peptidoleukotrienes appear to be independent of the effects of thromboxane (Ahmed *et al.*, 1986; Smith *et al.*, 1989). Furthermore, the vasopressor responses to LTD_4 in the rat do not appear to be explained by the contribution of prostaglandins, thromboxane, catecholamines, angiotensin or vasopressin since indomethacin, prazosin, phentolamine, saralasin, the thromboxane receptor antagonist daltroban, or the V_1-selective arginine vasopressin receptor antagonist $d(CH_2)_5Tyr(Me)AVP$ have no effect on the systemic responses to either LTC_4 or LTD_4 (Badr *et al.*, 1984; Filep *et al.*, 1987; Smith *et al.*, 1989). The present study provides further support for the supposition of LTD_4 receptor heterogeneity by demonstration of a similarity in LTD_4 dose-ratios despite increasing plasma concentrations of SK&F S-106203. The observations with SK&F 104353 (Smith *et al.*, 1989) and ICI 198615 (Hand *et al.*, 1989; Smith & Slivjak, 1989) are entirely consistent with this hypothesis. Therefore, the proposal for the existence of a heterogeneity of LTD_4 receptors (i.e., LTD_4 receptor subtypes) is supported by accumulating evidence in both pulmonary and vascular preparations.

SK&F S-106203 was a potent and apparently competitive antagonist of LTC_4-mediated pressor responses. SK&F S-106203 was also an effective antagonist of LTE_4 vasopressor responses, although within the limitations of this study (e.g., the maximal dose and amounts of LTE_4 available) it is not possible to conclude unequivocally that SK&F S-106203 competitively antagonized LTE_4-mediated responses. The antagonist shifted the dose-responses curves of LTC_4 and LTE_4 to the right in a dose-dependent fashion. At the highest dose of SK&F S-106203 studied (i.e., $2\,mg\,kg^{-1} + 10\,mg\,kg^{-1}\,h^{-1}$), the LTC_4 and LTE_4 dose-response curves were shifted rightward 20 and 9 fold, respectively, indicating effective blockade of both LTC_4 and LTE_4 responses in this preparation. Interestingly, Eimerl *et al.* (1986) showed that the bolus administration of the leukotriene receptor antagonist LY 171883 blocked LTD_4 vasopressor responses, but not those of LTE_4. These authors suggested that the differential effects of LY 171883 could be explained by assuming that the vascular LTE_4 receptor was pharmacologically distinct from the vascular LTD_4 receptor. Nonetheless, in comparison to the effects of LY 171883, SK&F S-106203 effectively antagonized both LTC_4 and LTE_4 responses.

The ED_{20} values for LTC_4 and LTD_4 were 2.3 ± 0.5 and $2.3 \pm 0.5\,nmol\,kg^{-1}$, respectively, whereas the ED_{20} of LTE_4 was $30 \pm 5\,nmol\,kg^{-1}$. Thus, relative to the LTC_4 or LTD_4 vasopressor responses, LTE_4 was less potent and produced smaller maximal responses. These observations agree with those of Roth & Lefer (1983), Chapnick (1984) and Ahmed *et al.* (1986). Moreover, the greater potency of LTC_4 and LTD_4, relative to LTE_4, suggests that quantitative metabolism of LTC_4 and LTD_4 to LTE_4 does not occur. If substantial bioconversion were to occur, the potency of the vasopressor responses to LTC_4 and LTD_4 would be expected to be more similar to that of LTE_4. Similarly, if LTD_4 to LTE_4 catabolism were relevant, prevention of LTD_4 to LTE_4 bioconversion would be expected to potentiate the LTD_4 vasopressor response. In the present study, administration of the non-competitive LTD_4 dipeptidase inhibitor, D-penicillamine (Huber & Keppler, 1987), had no effect on the vasopressor response to LTD_4. Similarly, in a previous study it was demonstrated that D-penicillamine had no effect on the LTD_4 vasopressor response produced in the presence of SK&F 104353 (Smith *et al.*, 1989). Therefore, at least for LTD_4 vasopressor responses, these appear to be relatively unaffected by the LTD_4 dipeptidase inhibitor D-penicillamine either in the absence or presence of a peptidoleukotriene receptor antagonist.

Ahmed *et al.* (1986) showed that LTC_4, LTD_4 and LTE_4 increased systemic vascular resistance in the conscious sheep, but only LTD_4 increased pulmonary vascular resistance. Neither LTC_4 nor LTE_4 was effective in increasing pulmonary vascular resistance. Thus, LTC_4, LTD_4 and LTE_4 increased systemic vascular resistance, but only LTD_4 was effective in the pulmonary circulation. In the present study, SK&F S-106203 at a dose of $0.2\,mg\,kg^{-1} + 1\,mg\,kg^{-1}\,h^{-1}$ had no effect on LTC_4 vasopressor responses (i.e., dose-ratio of 1), but this same dose of SK&F S-106203 produced a greater than 2 fold rightward shift in the LTD_4 dose-response curve. It is tempting to speculate that these data provide pharmacological evidence for distinct LTC_4 vs LTD_4 receptor sites, although it would be prudent to consider other interpretations. Nonetheless, these data not only support the evolving hypothesis of distinct peptidoleukotriene receptors in the sheep pulmonary and rat systemic circulation, but additionally suggest that pharmacologically relevant metabolism of peptidoleukotrienes *in vivo* does not occur. Therefore,

although catabolism of peptidoleukotrienes does occur *in vivo*, there is no definitive evidence that peptidoleukotriene metabolism *in vivo* is relevant to the pharmacological effect of peptidoleukotriene cardiovascular responses.

In summary, the results of this study indicate that SK&F S-106203 is an effective antagonist of LTC_4, LTD_4 and LTE_4 vascular responses in the conscious rat. A peptidoleukotriene receptor antagonist with high oral bioavailability, such as SK&F S-106203, could be useful for probing the role of peptidoleukotrienes in respiratory and cardiovascular disorders. Moreover, the results of the present study suggest that SK&F S-106203 pharmacologically differentiates two LTD_4 receptor subtypes on vascular smooth muscle: one characterized as a high affinity receptor site for LTD_4, and a second receptor subtype with somewhat lower affinity. This observation is consistent with previous studies with SK&F 104353 and ICI 198,615 (Hand *et al.*, 1989; Smith & Slivjak, 1989; Smith *et al.*, 1989). Although LTD_4 can activate both receptor subtypes, further studies will be required in order to determine the importance and significance of these receptor subtypes.

All animals were housed in accordance with the 'Guide for the care and use of laboratory animals', NIH publication No. 85–23. Procedures involving the use of laboratory animals were approved by the Institutional Animal Care and Use Committee of SmithKline Beecham Pharmaceuticals plc, and are in accordance with NIH guidelines for the use of experimental animals.

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Characterization of responses to cromakalim and pinacidil in smooth and cardiac muscle by use of selective antagonists

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1 In dog isolated coronary artery (precontracted with endothelin, 10 nM) cromakalim (0.1–30 μM) and pinacidil (1–30 μM) produced concentration-dependent vasorelaxant responses. The effects of these compounds could be blocked by glibenclamide (3 μM), phentolamine (30 μM) or alinidine (30 μM) to a similar extent, indicating that both agents alter vascular tone through the same mechanism in this preparation.

2 The ability of the antagonists glibenclamide, phentolamine and alinidine to block the response to cromakalim in a number of smooth muscle types from the guinea-pig was determined. Cromakalim (0.1–30 μM) produced concentration-dependent relaxant responses in thoracic aorta (precontracted with endothelin, 30 nM), ileum (precontracted with K^+, 25 mM) and trachea (spontaneously contracted). Responses to cromakalim in all tissues could be blocked by the three antagonists. However, significantly higher concentrations of the antagonists were required to block responses in the thoracic aorta than in the ileum or trachea. Given that the rank order of potency of the antagonists was similar in all tissues (i.e. glibenclamide > phentolamine = alinidine), this result may suggest vascular K^+ channels opened by cromakalim are quantitatively but not qualitatively different in vascular compared with non-vascular smooth muscle. Glibenclamide was approxmately 10 times more potent than phentolamine or alinidine.

3 Cromakalim had minimal functional effects on the rat spontaneously beating right atrial (rate) or electrically driven left ventricular strip (force) preparations. Similarly the three antagonists studied failed to alter force generation in the right ventricular strip. However alinidine and phentolamine did produce a dose-related bradycardia in the spontaneously beating right atria. This effect appears to be unrelated to blockade of the K^+ channel opened by cromakalim since glibenclamide, the most potent K^+ channel antagonist studied, failed to produce the same response.

4 It would appear that the K^+ channel opened by cromakalim is present in a number of vascular and non-vascular smooth muscle. Based on the potency of the three antagonists studied, there appears to be little heterogeneity in the process activated by cromakalim in vascular and non-vascular smooth muscle.

Introduction

Cromakalim (Hamilton *et al.*, 1986; Quast, 1987; Wilson *et al.*, 1988) and pinacidil (see Videbaek *et al.*, 1988) are two new antihypertensives which are thought to produce their vasodilator actions by opening K^+ channels in the plasma membrane of smooth muscle cells. However, the exact nature of the interaction between these compounds and the cell, the events leading to the observed efflux of K^+ and the mechanism resulting in relaxation are unclear. For example, detailed radioligand binding studies have failed to identify receptors for cromakalim in tissues where it causes relaxation (Coldwell & Howlett, 1987). In addition, some recent studies have suggested that while the hyperpolarization observed with these compounds is important in their mechanism of action, in some tissues at least it is not the subsequent closing of voltage-sensitive Ca^{2+} channels that produces the observed relaxation (Gillespie & Sheng, 1988).

Previous studies using vascular and non-vascular preparations have shown that phentolamine, alinidine (McPherson & Angus, 1989) and glibenclamide (Quast, 1988; Eltze, 1989; Buckingham *et al.*, 1989) can antagonize the response to cromakalim and pinacidil. The purpose of the present work was to determine whether the K^+ channels activated by cromakalim and pinacidil were functionally similar in terms of their sensitivity to the antagonists phentolamine, alinidine and glibenclamide. In addition we asked whether there were fundamental tissue differences in the K^+ channel activated by cromakalim in a number of vascular and non-vascular smooth muscle preparations, judged by their sensitivity to the three antagonists.

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Methods

General methods

A number of vascular, non-vascular and cardiac preparations were studied *in vitro*. All experiments were carried out in 25 ml organ baths containing a modified Krebs buffer (composition in mM: NaCl 119, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 1.17, $NaHCO_3$ 25, KH_2PO_4 1.18, $CaCl_2$ 2.5 and glucose 11) maintained at 37°C and gassed with 5% CO_2 in O_2. Changes in isometric force development of the preparations were monitored with Grass (FT03) force displacement transducers coupled to a Grass polygraph (model 7). Each preparation was placed under an initial passive force and allowed to equilibrate for 30 min before the addition of any drugs.

Vascular and non-vascular smooth muscle

Dog isolated coronary artery Ring segments (4 mm long) of circumflex coronary arteries were obtained from greyhound dogs (20–30 kg) killed by injection of pentobarbitone (60 mg kg^{-1}, i.v.). Each tissue segment was mounted on wire hooks and placed under an initial passive force of 4 g. Active tone was induced by the addition of endothelin (ET-1) at a concentration (3–10 nM) that caused approximately 50–80% of the maximum response to this agent. Vasorelaxant responses to increasing concentrations of cromakalim (0.3–30 μM), pinacidil (1–30 μM) or isoprenaline (1–300 nM) were assessed in the absence and in the presence of the antagonists glibenclamide (3 μM), phentolamine (30 μM) or alinidine (30 μM). The antagonists were added to the bath once the response to endothelin had reached a plateau. Ten minutes later one of the vasorelaxant drugs were tested. In this and other studies this period of exposure was found to cause maximal effects on the cromakalim response. The relaxant response to each agonist was

expressed as a percentage of the maximum response obtained to isoprenaline (10 μM) which was added at the end of each experiment.

Smooth muscle preparations from the guinea-pig

Guinea-pigs were killed by a blow to the back of the head and the thoracic aorta, ileum or trachea removed. In each case the experimental protocol was the same. Relaxant responses to cromakalim were assessed in the absence and in the presence of increasing concentrations of glibenclamide (0.1–10 μM), phentolamine (1–100 μM) or alinidine (1–100 μM). Each preparation acted either as a control or was treated with one concentration of an antagonist. In this latter case the antagonist was added to the bath once the contractile response had reached a plateau. The preparation was allowed to equilibrate for 10 min before the concentration-effect curve to cromakalim was obtained. The relaxant responses to cromakalim were expressed as a percentage of the maximum relaxant response to papaverine (0.15 mM) which was added at the end of each experiment.

In the case of thoracic aorta, tone was induced in each vascular segment (see dog coronary artery) by the addition of endothelin (3–10 nM). Segments (2 cm long) of ileum were mounted longitudinally under 1 g force and precontracted with 25 mM KCl. The trachea single ring segments were suspended under 0.5 g force. Under these conditions the trachea exhibited spontaneous tone and thus the relaxant effect of cromakalim could be assessed directly.

Cardiac muscle

Rat spontaneously beating right atrium Rats (250–300 g) were killed by a blow to the head and their hearts removed. The right atria was dissected free and suspended under 0.5 g force. Isometric force (FT03 transducer) recorded from the spontaneously beating atrium was used to trigger a Grass (model 7P4) tachograph. Chronotropic responses to cromakalim, glibenclamide, phentolamine and alinidine were then assessed directly.

Rat electrically driven right ventricle strip Segments (1 cm long) of rat right ventricle were obtained and mounted under 1 g force. The strips were stimulated with a Grass (SD9) stimulator at 0.5 Hz, 5 ms duration and at a voltage sufficient to produce maximal contractile responses. Responses to increasing concentrations of cromakalim (10–100 μM) were assessed. Responses to glibenclamide (30 μM), phentolamine (100 μM) and alinidine (100 μM) were also assessed.

Drugs

The following drugs were used: cromakalim (Beecham); pinacidil monohydrate (Leo Pharmaceuticals); endothelin (ET-1; porcine, human) (Auspep, Australia); (−)-isoprenaline bitartrate (Sigma); phentolamine mesylate (Ciba-Geigy); alinidine bromide (Boehringer-Ingelheim); glibenclamide (Hoechst).

Stock solutions (10 mM) of phentolamine and alinidine were made in distilled water and diluted each day in the same vehicle. The poor solubility of cromakalim and pinacidil limited, in some tissues, the construction of full concentration-effect curves due to the adverse effects the organic solvents used. Glibenclamide (10 mM) was prepared in 100% methanol. Cromakalim (10 mM) and pinacidil (10 mM) stock was made in 50% (v/v) methanol; any precipitation could be reversed by gently warming the mixture. Isoprenaline (10 mM) stock was made in 0.01 M HCl. Dilutions of all stock solutions were made with distilled water.

Data analysis and statistics

pD_2 ($-\log EC_{50}$ value) and concentration-ratios were generally (see below) estimated by standard graphical procedures. Statistical comparisons between two groups were made by Student's t test. Multiple comparisons of dependent samples were made by Bonferroni's t test. Multiple comparisons between independent samples were made by use of Scheffe's t test (see Wallenstein *et al.*, 1980). Results in the text are the mean ± s.e.mean for the specified number of experiments.

In some cases, concentration-effect curves were fitted by a non-linear curve fitting technique (McPherson *et al.*, 1983). The three parameter function gave estimates of the EC_{50}, maximum response and the slope factor (pseudo-Hill coefficient).

Results

Similarities in the actions of cromakalim and pinacidil on the dog coronary artery

Cromakalim (0.3–30 μM), pinacidil (1–30 μM) and isoprenaline (1–300 nM) caused concentration-dependent relaxation of the dog isolated coronary artery (Figure 1). In terms of the maximal relaxant response to isoprenaline (= 100%) cromakalim (30 μM) and pinacidil (30 μM) caused a relaxation of

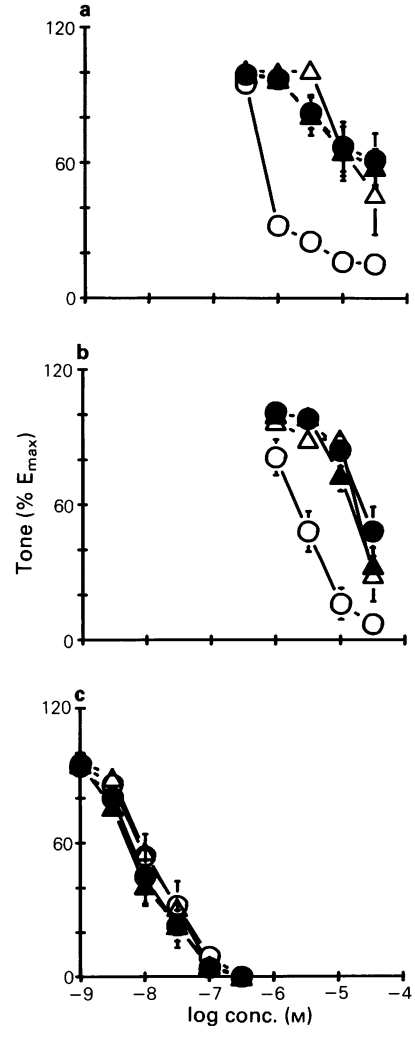

Figure 1 Mean concentration-effect curves for cromakalim (a), pinacidil (b) and isoprenaline (c) in dog coronary artery. Results are expressed as percentage of the contraction induced by endothelin (3 nM, 100%). The maximal possible tissue relaxant response was defined using isoprenaline (10 μM). Curves for each agonist were obtained in the absence (○) and in the presence of glibenclamide (△, 3 μM), phentolamine (●, 30 μM) and alinidine (▲, 30 μM). Results are the mean from 3–4 separate experiments; s.e.mean shown by vertical bars.

85 ± 4% ($n = 3$) and 93 ± 5% ($n = 3$) respectively. Cromakalim ($pD_2 \approx 6.3$) was approximately 50 and pinacidil ($pD_2 \approx 5.6$) 250 times less potent than isoprenaline ($pD_2 \approx 8$) (Figure 1.) The ability of glibenclamide (3 μM), phentolamine (30 μM) and alinidine (30 μM) to modify responses to the three agonists was tested. Isoprenaline vasorelaxant responses were unaffected by these compounds; however, those to cromakalim and pinacidil were markedly attenuated. Based on the concentration of agonist required to produce 50% relaxation (assuming that relaxation at 30 μM of each agonist = 100%) then the concentration-effect curves to cromakalim and pinacidil were both shifted 5–10 fold for each antagonist. The inability to construct full concentration-effect curves to the agonists (cromakalim and pinacidil) in the presence of higher concentrations of the antagonists precluded full quantitative analysis of the antagonism. However, the shift of the cromakalim concentration-effect curve by phentolamine and alinidine was accompanied by a significant reduction in the maximum response ($P < 0.05$, Scheffe's t test). In the case of pinacidil the shift produced by the three antagonists appeared more parallel (Figure 1) with little change in the maximal relaxation.

The effect of cromakalim on vascular and non-vascular smooth muscle from the guinea-pig

The effect of cromakalim and its interaction with glibenclamide, phentolamine and alinidine was assessed in a number of preparations from the guinea-pig. Cromakalim caused concentration-dependent relaxation of the thoracic aorta (precontracted with endothelin), ileum (precontracted with potassium) and tracheal smooth muscle (spontaneously contracted). The potency (pD_2) of cromakalim ranged from $\approx$6.2 in the trachea to $\approx$5.6 in the thoracic aorta (see Table 1). The maximal relaxation produced by cromakalim was more variable (Table 1). Contractions in the thoracic aorta could be reversed an average of 50% while in the trachea the value was 80%. The concentration-effect curves for cromakalim were steep in all tissues (slope factors greater than unity in all cases; see Table 1).

Glibenclamide, phentolamine and alinidine were able to antagonize the actions of cromakalim in all preparations. Figures 2–4 show the mean data obtained in guinea-pig thoracic aorta, ileum and trachea respectively. In the ileal preparation phentolamine and alinidine (100 μM) caused a direct relaxation of the tone induced by K^+. Results where this concentration of the antagonists was used could therefore not be calculated.

Due to the limited concentration-range over which the cromakalim curve could be constructed, it was not possible to quantitate fully the interaction between cromakalim and the antagonists. However, it was apparent from the studies that higher concentrations of antagonists were required to block the actions of cromakalim in the thoracic aorta than in the trachea or ileum. This is more clearly shown in Figure 5 which shows the response to cromakalim (10 μM) in the absence and presence of increasing concentrations of antagonists (data extracted from Figures 2–4). In the trachea and ileum the response to cromakalim (10 μM) was significantly blocked by glibenclamide at concentrations in excess of 1 μM and phentolamine and alinidine in excess of 10 μM (Bonferroni's test, $P < 0.05$). However, in the thoracic aorta significant blockade of the response to this concentration of cromakalim required 10 μM glibenclamide and 30 μM phentolamine and alinidine. In all cases however glibenclamide was the most potent antagonist being approximately 3–10 times more active than phentolamine and alinidine.

Table 1 Effects of cromakalim on thoracic aorta, ileum and trachea from the guinea-pig

Tissue	n	pD_2	*Slope*	*% maximum relaxation*
Thoracic aorta	11	5.58[1]*	1.55[1]	50[1]
		(0.07)	(0.14)	(3)
Ileum	12	5.78[1]	1.44[1]	68[2]
		(0.06)	(0.14)	(4)
Trachea	13	6.18[2]	2.16[1]	80[3]
		(0.07)	(0.21)	(2)

* Values marked with different numeral were significantly different from others in the same group ($P < 0.05$, Scheffe's t test). pD_2 values are the $-\log EC_{50}$ value. The slope is the Hill coefficient calculated by the non-linear curve fitting technique described in the methods. The values in parentheses are the s.e.mean from the specified number of experiments (n).

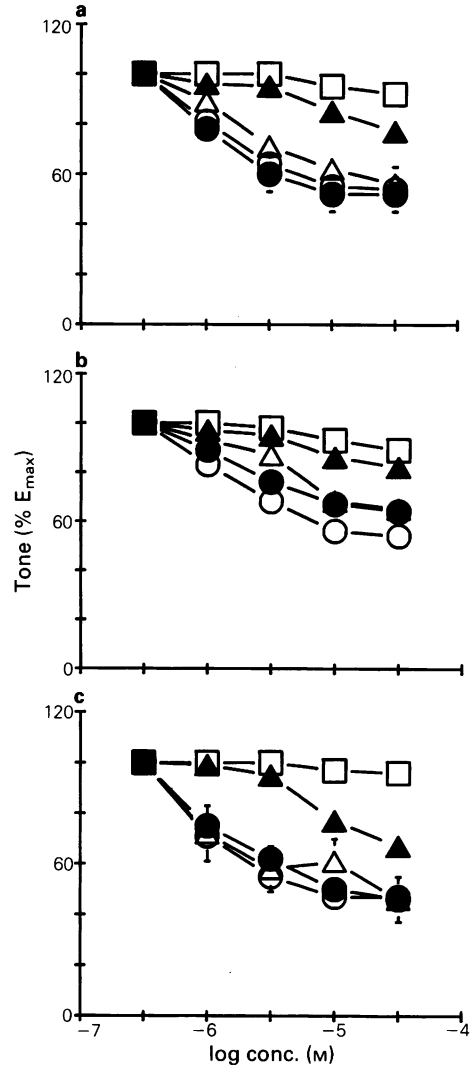

Figure 2 Mean concentration-effect curves for cromakalim obtained in guinea-pig thoracic aorta. Results are expressed as a percentage of the maximum contraction induced by endothelin (10 nM, 100%). The maximal possible tissue relaxant response was defined by use of papaverine (0.15 mM). Curves for cromakalim were constructed in the absence (○) and in the presence of increasing concentrations of: (a) glibenclamide 0.1 μM (●), 1 μM (△), 10 μM (▲) and 30 μM (□); (b) phentolamine 1 μM (●), 10 μM (△), 30 μM (▲) and 100 μM (□); (c) alinidine 1 μM (●), 10 μM (△), 30 μM (▲) and 100 μM (□). Results are the mean from 3 to 4 separate experiments; s.e.mean shown by vertical bars.

Effects of cromakalim on the rat right atria and right ventricle strips

Cromakalim, in concentrations up to 30 μM, had no significant effect on the rat spontaneously beating right atrial preparation (Figure 6a). In addition, it did not affect the concentration-effect curve for isoprenaline-induced tachycardia ($pD_2 \approx$ 10 nM). The three antagonists studied had variable effects.

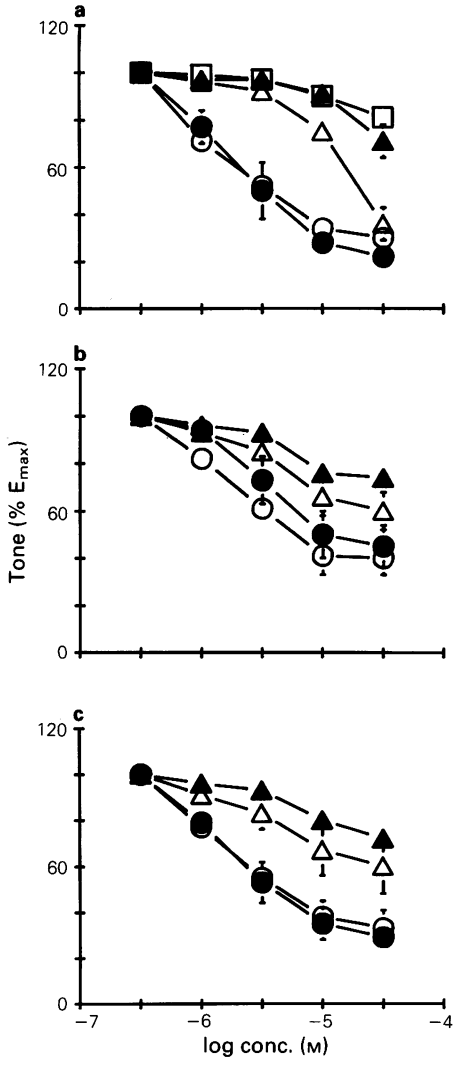

Figure 3 Mean concentration-effect curves for cromakalim obtained in guinea-pig ileum. Results are expressed as a percentage of the maximum contraction induced by K^+ (25 mM, 100%). The maximal possible tissue relaxant response was defined by use of papaverine (0.15 mM). Curves for cromakalim were constructed in the absence (○) and the in the presence of increasing concentrations of: (a) glibenclamide; (b) phentolamine; (c) alinidine. The concentrations used are identical to those described in Figure 2. The results are the mean from 4 separate experiments; s.e.mean shown by vertical bars.

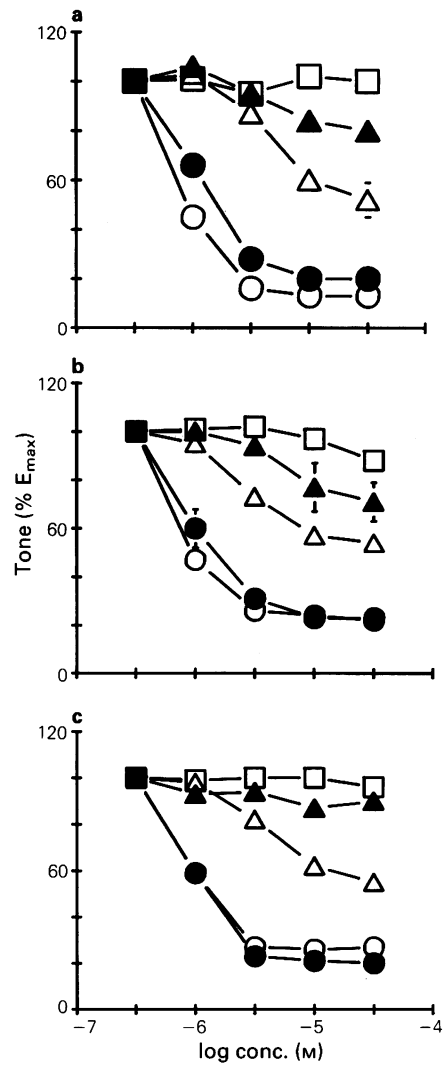

Figure 4 Mean concentration-effect curves for cromakalim obtained in guinea-pig tracheal rings. Results are expressed as a percentage of the maximum tone which developed spontaneously (100%). The maximal possible tissue relaxant response was defined by use of papaverine (0.15 mM). Curves for cromakalim were constructed in the absence (○) and the presence of increasing concentrations of: (a) glibenclamide; (b) phentolamine; (c) alinidine. The concentrations used are identical to those described in Figure 2. The results are the mean from 4–5 separate experiments; s.e.mean shown by vertical bars.

Alinidine and phentolamine produced a concentration-dependent bradycardia (Figure 6b). In this series of experiments alinidine was approximately 10 times more potent than phentolamine. Glibenclamide did not effect the rate of beating of the right atria at concentrations up to 3 μM (Figure 6b).

In the driven right ventricle strip preparation of the rat cromakalim was generally inactive at concentrations up to 30 μM. At 100 μM, the highest concentration tested, a small negative inotropic effect (<20%) was observed in 2 out of 4 preparations studied. Due to the variability in the response to cromakalim, and the high concentrations required, it was not possible to study the interaction with the K^+ channel blockers. Glibenclamide (30 μM), phentolamine and alinidine (both 100 μM) did not affect the force of contraction in this preparation.

Discussion

The major finding from this study was that cromakalim does not appear to show selectivity in its potency in relaxing vascular and non-vascular smooth muscle. In addition, three structurally different antagonists displayed the same relative ability to antagonize the actions of cromakalim in all tissues. Thus the mechanism underlying the activity of cromakalim (i.e. K^+ channel opening) is apparently ubiquitous in smooth muscle.

On the basis of the similar degree of antagonism by glibenclamide, phentolamine and alinidine of the relaxation response to pinacidil and cromakalim, it would appear that both compounds are acting through a similar mechanism to cause relaxation in dog isolated coronary artery. However, given that the antagonism produced by the three inhibitors is non-competitive in nature with cromakalim as the agonist (i.e. the maximum vasorelaxant response was diminished) but apparently competitive with pinacidil (i.e. the curves were shifted in a parallel fashion) it is possible that some qualitative differences exist. In the guinea-pig pulmonary artery, Eltze (1988) obtained identical pA_2 values for glibenclamide, independent of whether pinacidil or cromakalim were used as the agonist. The results indicate that the interaction between K^+ channel openers and the antagonists are complex, given that in the dog coronary artery the interaction is non-competitive (present study, McPherson & Angus, 1989) but competitive in

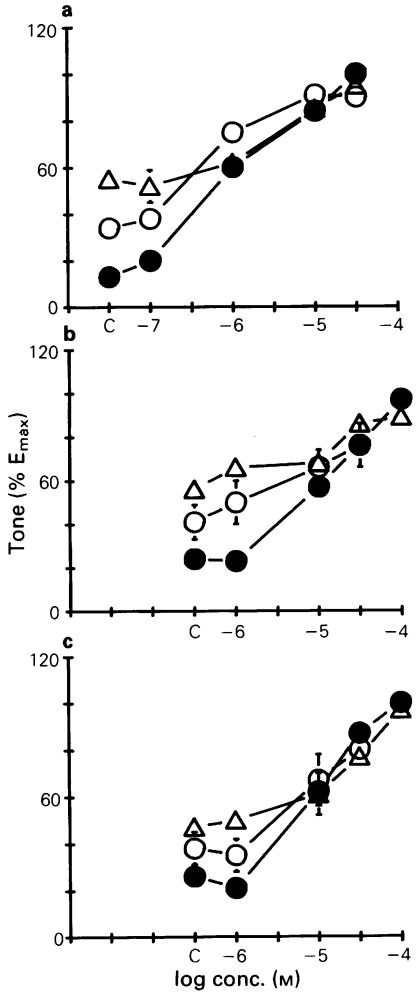

Figure 5 Mean concentration-effect curves of increasing concentrations of glibenclamide (a), phentolamine (b) and alinidine (c) in reducing the relaxation response to cromakalim (10 μM) in preparations from the guinea-pig (△) thoracic aorta, (○) ileum and (●) trachea. Results (derived from Figures 2–4) are expressed as a percentage of the maximum tone present in each preparation before the addition of cromakalim. The maximal possible tissue relaxant response was defined by use of papaverine (0.15 mM). In each panel (C) represents the amount of tone in the presence of cromakalim (10 μM) but in the absence of antagonist. Results are mean from 3–5 separate experiments; s.e.mean shown by vertical bars.

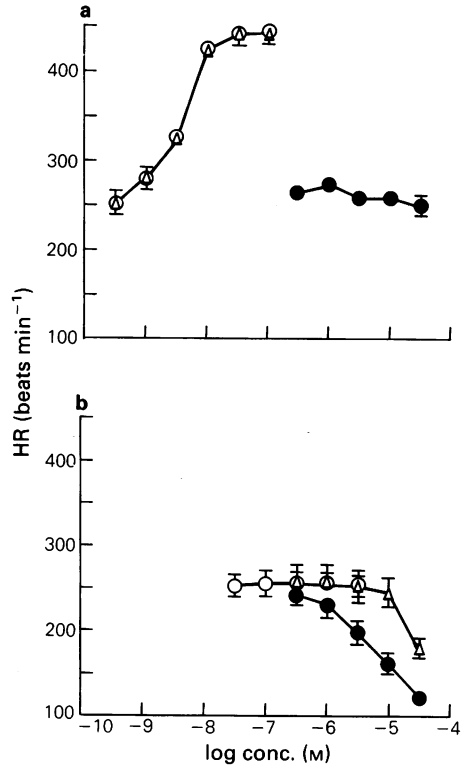

Figure 6 (a) Mean concentration-effect curves for cromakalim (●) and isoprenaline in the rat spontaneously beating right atrial preparation. Curves for isoprenaline were constructed in the absence (○) and in the presence (△) of cromakalim (30 μM). Results are expressed in absolute heart rate responses (HR) and are the mean from 4–5 separate experiments. (b) Mean concentration-effect curves for glibenclamide (○), phentolamine (△) and alinidine (●) obtained in the rat right atria. Results are expressed in absolute heart rate responses (HR) and are the mean from 3–4 separate experiments. Vertical bars show s.e.mean.

the guinea-pig pulmonary artery (Eltze, 1988). Clearly much more work is required in this area.

The results also highlight the differences in the mechanism of action of cromakalim and pinacidil compared with nicorandil. Previous studies (Eltze, 1988; McPherson & Angus, 1989) have shown that the effects of another purported K^+ channel opener, nicorandil, were insensitive to glibenclamide, phentolamine and alinidine. However, it is known that nicorandil can cause smooth muscle relaxation by a mechanism other than K^+ channel opening (Wier & Weston, 1986; Coldwell & Howlett, 1987).

The effects of cromakalim are not restricted to vascular smooth muscle since relaxant responses to this compound have also been reported in a number of non-vascular smooth muscle preparations (present study; see Allen *et al.*, 1986; Weir & Weston, 1986; Arch *et al.*, 1988; Buchheit & Bertholet, 1988; Eltze, 1989). The results from this study indicate that the effect of cromakalim on smooth muscle (vascular and non-vascular) is very similar. Thus cromakalim produced relaxant responses over a similar concentration-range in a number of guinea-pig smooth muscle preparations. The response to cromakalim in the thoracic aorta however appeared to be less sensitive to the effects of the three antagonists than those in the trachea or ileum. This result may indicate some subtle differences in the process activated by cromakalim in vascular compared with non-vascular smooth muscle. However, the exact mechanism underlying the interaction between the antagonists and cromakalim is unknown at present. It is possible for example that the potency of the antagonists is partially dependent on the nature of the agent used to produce tone. We are currently investigating this possibility. Despite these reservations, the finding that the response to cromakalim can be blocked by all three antagonists with the same rank order of potency (i.e. glibenclamide > phentolamine = alinidine) would suggest that the process activated by cromakalim is similar in all preparations studied. However, it is possible that the agents that are currently available to block the action of cromakalim are non-selective. Consequently any conclusion regarding the homogeneity of the K^+ channel opened by cromakalim should be viewed with some caution.

We also examined the cardiac actions of cromakalim. Previous studies have shown that cromakalim reduces action potential duration in guinea-pig papillary muscle (Scholtysik, 1987) and myocytes (Osterrieder, 1988). However, these cardiac effects of cromakalim occur at concentrations (10–300 μM) 10–100 fold greater than those causing vascular and non-vascular smooth muscle relaxation (0.1–1 μM). We found the cardiac effects of cromakalim to be very poor. It had no effect on the spontaneously beating right atria in concentrations up to 30 μM. In the electrically driven left ventricle strip, cromakalim caused a small and inconsistent negative inotropic action. On the basis of these results it would appear that there is minimal functional importance for the K^+ channel

opened by cromakalim in these preparations or, alternatively, that the channel functions but is poorly coupled to any cromakalim-modulated mechanism.

One reason for examining cardiac preparations was that previous studies have shown that phentolamine (Angus, unpublished observations) and particularly alinidine (see Heinzow *et al.*, 1982) cause a marked bradycardia in spontaneously beating isolated atria and in the conscious rabbit. Given that both compounds interacted with the K^+ channel activated by cromakalim, we suggested (McPherson & Angus, 1989) that the cardiac effects of these compounds may, in some way, be related to this channel. From the present studies however, this would appear to be incorrect. We showed that glibenclamide was approximately 10 times more potent than alinidine and phentolamine in antagonizing the effects of cromakalim and pinacidil. However, in the spontaneously beating right atrial preparation, glibenclamide was inactive while alinidine was approximately 10 times more potent than phentolamine. In addition, in the right ventricle strip all antagonists were inactive despite cromakalim displaying some negative inotropic effects. Both these findings would indicate lack of involvement of the K^+ channel activated by cromakalim in regulating cardiac function in the rat.

The K^+ channel opened by compounds like cromakalim and pinacidil are widespread and involved in a number of processes. They regulate tone in vascular and non-vascular smooth muscle (see above). In addition, recent studies (Ferrier *et al.*, 1989) have shown that cromakalim can directly release renin from rat isolated juxtaglomerular cells in addition to raising renin levels in man. Cromakalim has also been shown to modulate behavioural responses (Tricklebank *et al.*, 1988). This lack of specificity may limit usefulness of these compounds in conditions requiring chronic administration. However, recent studies have shown that diazoxide, a drug used for acute hypertensive crisis, may act through the same mechanism as cromakalim (Winquist *et al.*, 1989). Given that cromakalim is 100 times more potent than diazoxide in relaxing smooth muscle, it is possible that cromakalim would prove a better alternative to diazoxide in this acute condition.

In conclusion, the results from this study indicate that the mechanism by which K^+ channels are opened by cromakalim in both vascular and non-vascular smooth muscle is effectively homogeneous with respect to the actions of the three antagonists, glibenclamide, phentolamine and alinidine. There appears to be no importance of this potassium channel in regulating cardiac function in the rat under the conditions studied.

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Effects of inhibitors of protein kinase C and Na^+/H^+ exchange on α_1-adrenoceptor-mediated inotropic responses in the rat left ventricular papillary muscle

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1 α_1-Adrenoceptor stimulation of rat left ventricular papillary muscle produced a triphasic inotropic response: an initial transient positive inotropic effect (PIE) followed by a transient negative inotropic effect (NIE) and a sustained PIE.

2 The protein kinase C inhibitor, staurosporine, at concentrations ranging from 30 nM to 100 nM inhibited the sustained PIE, but had no significant effect on the transient PIE and NIE.

3 H-7, 1-(5-isoquinoline sulphonyl)-2-methylpiperazine, a less specific inhibitor of protein kinase C than staurosporine, at a concentration of 100 μM inhibited both the transient NIE and the sustained PIE without affecting the transient PIE.

4 Amiloride, an inhibitor of Na^+/H^+ exchange, at concentrations ranging from 0.1 mM to 1 mM inhibited the sustained PIE and, at higher concentrations, also inhibited the transient NIE.

5 An amiloride analogue, 5-(N-methyl-N-isobutyl)amiloride (MIBA), inhibited only the sustained PIE with an IC_{50} of 0.3 μM which is approximately two orders of magnitude lower than amiloride.

6 The receptor-linked stimulation of Na^+/H^+ exchange through protein kinase C activation may be a mechanism for α_1-adrenoceptor-mediated sustained PIE.

Introduction

The mechanism of α_1-adrenoceptor-mediated inotropic responses has been a matter of debate for many years (Ahlquist, 1948; Endoh *et al.*, 1982). A growing body of evidence indicates that an α_1-adrenoceptor-mediated positive inotropic effect (PIE) is associated with the receptor-linked degradation of phosphatidyl-inositol 4,5-bisphosphate and the resulting generation of inositol trisphosphate (IP_3) and diacylglycerol (Otani *et al.*, 1988; Scholz *et al.*, 1988). IP_3 is known to mobilize Ca^{2+} from intracellular compartments and is thought to participate as a transient component of the cellular response, whereas diacylglycerol may produce a sustained phase of the cellular response via the activation of protein kinase C (Berridge, 1984; Williamson *et al.*, 1985; Howe *et al.*, 1986). α_1-Adrenoceptor stimulation with phenylephrine in rat isolated left ventricular papillary muscle produces a triphasic inotropic response, in which IP_3-induced Ca^{2+}-mobilization has been shown to be involved in the initial transient PIE and the following transient negative inotropic effect (NIE) (Otani *et al.*, 1988). However, the mechanism involved in the phenylephrine-induced sustained PIE has not been well analysed. In this study, we have examined the role of protein kinase C activation in the sustained PIE by use of inhibitors of the kinase: staurosporine and 1-(5-isoquinoline sulphonyl)-2-methylpiperazine (H-7). The effects of these two protein kinase C inhibitors were tested because the mechanism of action and potency of protein kinase C inhibition are different (Nakadate *et al.*, 1988). Further, participation of the Na^+/H^+ exchange system in the sustained PIE was also examined by use of inhibitors of the Na^+/H^+ exchange system: amiloride and 5-(N-methyl-N-isobutyl)amiloride (MIBA). Activation of protein kinase C is known to stimulate a Na^+/H^+ exchange system in a number of different cell types including cardiac cells (Vigne *et al.*, 1985; Griendling *et al.*, 1988; Siffert & Akkerman, 1988). In addition, this antiporter could increase contractility in cardiac muscle by increasing Ca^{2+} influx through Na^+/Ca^{2+} exchange, following an accumulation of intracellular Na^+ (Ikeda *et al.*, 1988), or by increasing the sensitivity of the contractile protein for Ca^{2+} through intracellular alkalinization (Fabiato & Fabiato, 1978).

Methods

Male Sprague-Dawley rats weighing 250–300 g were anaesthetized with an intra-peritoneal injection of sodium pentobarbitone, and the heart was quickly removed. Anterior left ventricular papillary muscles of diameter 0.8–1.0 mm were suspended in organ baths containing Tyrode solution of the following composition (mM): NaCl 122.5, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.1, $NaHCO_3$ 24 and glucose 10 (pH 7.4) aerated with 95% O_2 and 5% CO_2 at 32°C. The muscle was initially loaded with 500 mg and driven electrically by rectangular pulses with a frequency of 1 Hz, a duration of 10 ms and a voltage ranging from 2–4 V which was twice threshold. The isometric tension was measured by a force displacement transducer (Shinko-Tsusin, Japan. UL-2) and recorded on a pen-writing recorder (Seconic, Japan. SS-250F) through an amplifier (Shinko-Tsusin, DS-601B). After 50 min equilibration, experimentation was started. The preparation was treated with 0.3 μM propranolol for 10 min and exposed to 10 μM phenylephrine. This concentration of phenylephrine was shown to produce a submaximum inotropic response, and propranolol was required to inhibit β-adrenoceptor stimulation at this phenylephrine concentration (Otani *et al.*, 1986). A single dose of H-7, staurosporine, amiloride or its analogue was added to the buffer 30 min before the addition of phenylephrine, and only a single response to phenylephrine was tested in each tissue to avoid the possible occurrence of tachyphylaxis. Changes in contractility following the administration of phenylephrine were measured when the peak responses appeared, respectively, and expressed as a % of that obtained just before the addition of phenylephrine.

1-(5-Isoquinoline sulphonyl)-2-methylpiperazine (H-7) and staurosporine were obtained from Seikagaku-Kogyo Co., Ltd.,

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Japan and Kyowa-medex Co., Ltd., Japan, respectively. The amiloride analogue, 5-(N-methyl-N-isobutyl)amiloride (MIBA) was synthesized by a previously described method (Cragoe *et al.*, 1967). Prazosin was a gift from Pfizer Inc., New York. Phenylephrine and amiloride were purchased from Sigma Chemical Co., St. Louis, Mo. Staurosporine and MIBA were prepared as stock solutions in DMSO (dimethyl sulphoxide). Final DMSO concentrations in bath media did not exceed 0.01%, and this concentration of DMSO was used as a vehicle control.

Statistical analysis

The results are expressed as mean ± s.e.mean. Student's t test was used for statistical analysis. The differences with $P < 0.05$ were considered to be significant.

Results

Treatment of rat left ventricular papillary muscle with 10 μM phenylephrine in the presence of 0.3 μM propranolol produced a triphasic inotropic response: a transient PIE (phase I) followed by a transient NIE (phase II) and a sustained PIE (phase III) (Figure 1a). An α_1-adrenoceptor antagonist, prazosin, at concentrations ranging from 0.01 to 1 μM concentration-dependently inhibited all the phases of the inotropic response (Figure 1b).

As shown in Figure 2, the protein kinase C inhibitors staurosporine (100 nM) and H-7 (100 μM) reduced the sustained PIE. Treatment with staurosporine (100 nM) or H-7 (100 μM) for 30 min reduced the basal contractility to 76.3 ± 4.5% (mean ± s.e.mean; $n = 6$) or 89.2 ± 2.0% (mean ± s.e.mean; $n = 6$), respectively, of that observed before the treatment. The effects of different concentrations of these reagents on each phase of the triphasic inotropic response are summarized in Figure 3. Staurosporine at concentrations ranging from 30 to 100 nM inhibited the sustained PIE in a concentration-dependent manner, but had no effect on the transient PIE. The transient NIE tended to be slightly augmented by 100 nM staurosporine (Figure 3a). H-7 at a concentration of 100 μM also inhibited the sustained PIE, though less potently than staurosporine, with no effect on the transient PIE. However, this protein kinase C inhibitor reduced the transient NIE in a concentration-dependent manner (Figure 3b).

The effects of Na^+/H^+ exchange inhibitors, amiloride (1 mM) and its analogue MIBA (100 nM), on the phenylephrine-induced inotropic responses are shown in representative traces (Figure 4). Both inhibitors markedly reduced the sustained PIE. Basal contractility did not change significantly on treatment with 1 mM amiloride, but it was reduced by 100 μM

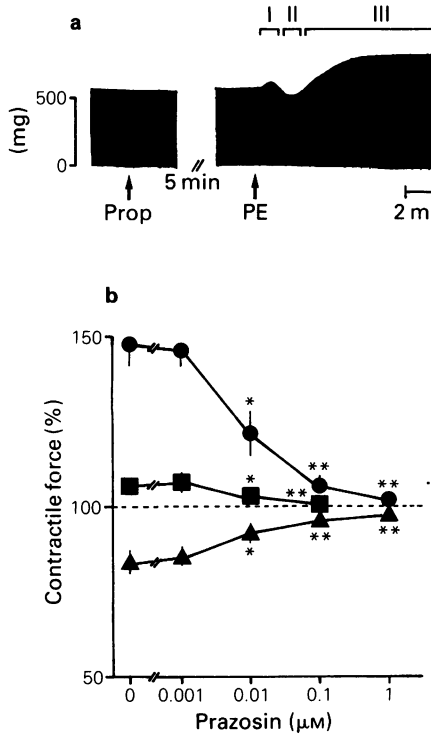

Figure 1 Effects of prazosin on α_1-adrenoceptor-mediated triphasic inotropic response in rat left ventricular papillary muscle. (a) Inotropic response induced by 10 μM phenylephrine (PE) in the presence of 0.3 μM propranolol (Prop). The scale shown in the graph indicates the active contractile force. I, transient positive inotropic response; II, transient negative inotropic response; III, sustained positive inotropic response. (b) Effect of pretreatment with 0.001–1 μM prazosin for 30 min on PE-induced triphasic inotropic responses in the presence of 0.3 μM propranolol. The change in the contractile force was expressed as a % of that obtained just before the addition of PE. (■) Transient positive inotropic response, (▲) transient negative inotropic response, (●) sustained positive inotropic response. Each symbol represents the mean of 6 preparations; vertical lines show s.e.mean. $*P < 0.05$, $**P < 0.01$ compared to the value without drug treatment.

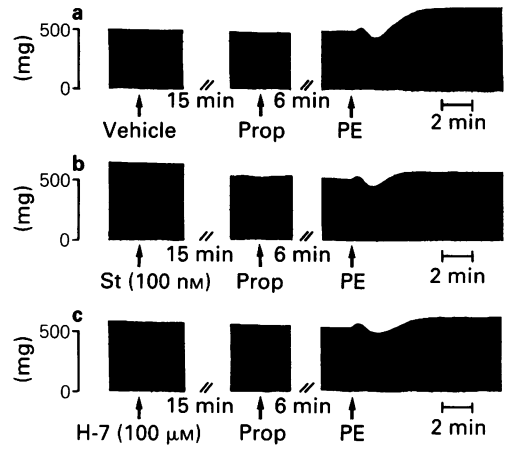

Figure 2 Representative traces of effects of staurosporine and H-7 on α_1-adrenoceptor-mediated inotropic responses. Rat left ventricular papillary muscle treated with vehicle (0.01% dimethyl sulphoxide, a), 100 nM staurosporine (St, b), or 100 μM H-7 (c) for 30 min was exposed to 10 μM phenylephrine (PE) in the presence of 0.3 μM propranolol (Prop).

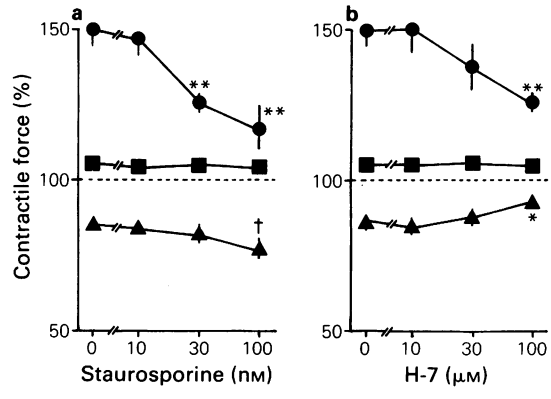

Figure 3 Effects of staurosporine and H-7 on α_1-adrenoceptor-mediated inotropic response in rat left ventricular papillary muscle. (a) Concentration-response effect of staurosporine on the 10 μM phenylephrine (PE)-induced inotropic response. (b) Concentration-response effect of H-7 on the 10 μM PE-induced inotropic response. In each figure, the maximum change in contractile force in each inotropic phase was expressed as a % of that obtained just before the addition of PE. (■) Transient positive inotropic response, (▲) transient negative inotropic response, (●) sustained positive inotropic response. Each symbol represent the mean of 6 preparations; vertical lines show s.e.mean. $\dagger P < 0.1$, $*P < 0.05$, $**P < 0.01$ compared to the value without drug treatment.

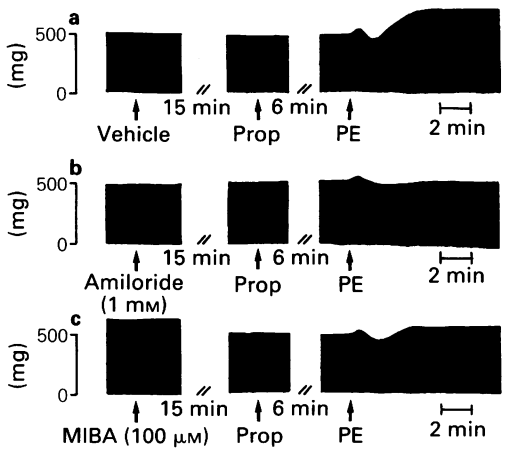

Figure 4 Representative traces of the effects of amiloride and an amiloride analogue, 5-(N-methyl-N-isobutyl)amiloride (MIBA), on the α_1-adrenoceptor-mediated inotropic response. Rat left ventricular papillary muscle treated with vehicle (0.01% dimethyl sulphoxide, a), 1 mM amiloride (b), or 100 μM MIBA (c) for 30 min was exposed to 10 μM phenylephrine (PE) in the presence of 0.3 μM propranolol (Prop).

MIBA to 72.5 ± 5.8% (mean ± s.e.mean; $n = 6$) of that observed before the treatment with MIBA. The effects of different concentrations of these inhibitors on each contractile phase are summarized in Figure 5. Amiloride at concentrations ranging from 0.1 to 1 mM inhibited the sustained PIE, and also inhibited the transient NIE at and above 0.3 mM (Figure 5a). A more specific inhibitor for Na^+/H^+ exchange, MIBA, inhibited only the sustained PIE with a potency approximately two orders of magnitude greater than amiloride (Figure 5b).

Discussion

The present study evaluated the concentration-response effects of an α_1-adrenoceptor blocker and inhibitors of protein kinase C and Na^+/H^+ exchange, to determine whether protein kinase C and Na^+/H^+ exchange activity are involved in the sustained PIE mediated by α_1-adrenoceptor stimulation. α_1-Adrenoceptor stimulation provoked a triphasic inotropic response, as has been observed in a previous study (Otani *et al.*, 1988). Prazosin showed a similar concentration-dependent

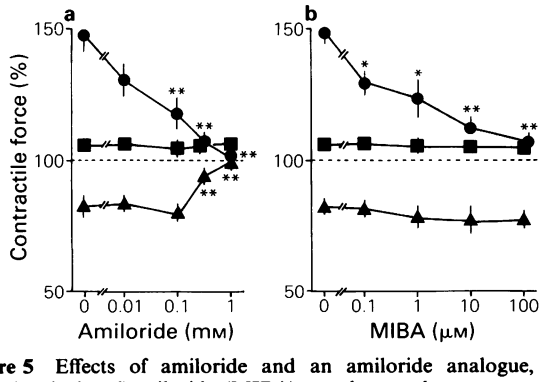

Figure 5 Effects of amiloride and an amiloride analogue, 5-(N-methyl-N-isobutyl)amiloride (MIBA), on the α_1-adrenoceptor- mediated inotropic response in rat left ventricular papillary muscle. (a) Concentration-response effects of amiloride on 10 μM phenylephrine (PE)-induced inotropic responses. (b) Concentration-response effects of MIBA on the 10 μM PE-induced inotropic response. In (a) and (b), the maximum change in contractile force in each inotropic phase was expressed as a % of that obtained just before the addition of PE. (■) Transient positive inotropic response, (▲) transient negative inotropic response, (●) sustained positive inotropic response. Each symbol represents the mean of 6 preparations and vertical lines indicate s.e.mean. *$P < 0.05$, **$P < 0.01$ compared to the value without drug treatment.

inhibition of the triphasic inotropic response, suggesting that all of these inotropic responses are mediated through α_1-adrenoceptor stimulation.

Both staurosporine and H-7 inhibited the sustained PIE without affecting the transient PIE, but the effect of these protein kinase C inhibitors on the transient NIE was opposite: staurosporine augmented the NIE, and H-7 inhibited it. A common inhibitory effect of staurosporine and H-7 on the sustained PIE and opposite effects of the transient NIE support the contention that protein kinase C is involved in the phenylephrine-induced sustained PIE, but not in the transient inotropic responses. Although the reason for the differential effect of H-7 on transient NIE from that observed with staurosporine is currently unknown, it may be related to their diverse action on some other cellular mechanisms.

Activation of protein kinase C could lead to a sustained PIE in cardiac muscle via Ca^{2+} channel activation and/or via stimulation of Na^+/H^+ exchange (Dösemeci *et al.*, 1988; Ikeda *et al.*, 1988). However, the role of the slow inward current in producing PIE is still controversial (Dösemeci *et al.*, 1988). On the other hand, the present study showed that the Na^+/H^+ exchange inhibitors amiloride and MIBA inhibited the α_1-adrenoceptor-mediated inotropic response. The amiloride analogue, MIBA, was used as a specific inhibitor of Na^+/H^+ exchange, with a lower K_i of 0.44 μM compared with the K_i of 84 μM for amiloride (Kleyman & Cragoe, 1988). Since these two Na^+/H^+ exchange inhibitors inhibited the sustained PIE but had no or opposite effects on the transient PIE or NIE, it is reasonable to assume that stimulation of Na^+/H^+ exchange is involved in the sustained PIE, but not in the transient inotropic responses. Although the mechanism by which Na^+/H^+ exchange induced the sustained PIE was not determined in this study, at least two mechanisms are envisaged. Stimulation of Na^+/H^+ exchange operates toward the stimulation of Na^+ influx, which may in turn increase intracellular Ca^{2+} via Na^+/Ca^{2+} exchange (Frelin *et al.*, 1984; Ikeda *et al.*, 1988). Further, intracellular alkalinization provoked by Na^+/H^+ exchange may increase the sensitivity of myofilaments for Ca^{2+} (Fabiato & Fabiato, 1978). Either mechanism could result in an increase in the force of contraction.

The inhibition of the transient NIE by amiloride at higher concentrations may be accounted for by the inhibition of Ca^{2+} extrusion via Na^+/Ca^{2+} exchange, since amiloride acts as an inhibitor of Na^+/Ca^{2+} exchange with a K_i of 1 mM in the cardiac sarcolemmal vesicle (Siegel *et al.*, 1984), and the transient NIE is thought to be at least in part due to stimulated Ca^{2+} extrusion through Na^+/Ca^{2+} exchange. Furthermore, this assumption raises the possibility that H-7 and amiloride, both of which inhibited the transient NIE, share a common mechanism of action in preventing the loss of intracellular Ca^{2+} during a period of transient NIE.

Amiloride is also known to act as an α_1-adrenoceptor antagonist at a concentration range similar to that inhibiting Na^+/H^+ exchange in rat perfused liver (Haüssinger *et al.*, 1987). α_1-Adrenoceptor antagonism by amiloride, however, is unlikely to provide an explanation for the inhibition of the transient NIE and the sustained PIE, because amiloride failed to inhibit the transient PIE, which has been shown to correlate closely with an α_1-adrenoceptor-mediated increase in IP_3 (Otani *et al.*, 1988). Prazosin inhibition of the transient PIE is consistent with this notion.

In summary, this paper provides evidence for the first time that α_1-adrenoceptor-mediated sustained PIE is sensitive to inhibitors of Na^+/H^+ exchange as well as those of protein kinase C. Receptor-linked stimulation of Na^+/H^+ exchange through protein kinase C activation is strongly suggested as a mechanism for the sustained PIE in the α_1-adrenoceptor-mediated inotropic response.

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Selective antagonism of calcium channel activators by fluspirilene

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1 Fluspirilene has been claimed to bind to a high affinity site in the calcium channel in skeletal muscle. We have investigated its calcium-antagonistic effects in smooth muscle and affinity for the channel in radioligand binding assays.

2 Fluspirilene was weakly active as an antagonist of Ca^{2+}-induced contractions in K^+-depolarized taenia preparations from the guinea-pig caecum, with threshold antagonism starting from concentrations of 30 nM. Nitrendipine, nicardipine and nimodipine were very potent antagonists in this model (threshold antagonism, > 1 nM).

3 In contrast, fluspirilene (10–1000 nM) was a potent non-competitive antagonist of the effects of Bay K 8644 (1–3000 nM) on Ca^{2+}-induced contractions and, at 10 nM, selectively antagonised the effects of Bay K 8644, abolished the Ca^{2+}-channel activator effects of CGP 28392, without changing the calcium antagonist effects of nitrendipine, or modifying the sensitivity of the tissues to Ca^{2+}. In contrast, the dihydropyridines were more effective as antagonists of Ca^{2+} than of Bay K 8644. Fluspirilene therefore selectively antagonised the effects of dihydropyridine Ca^{2+} channel activators without affecting the antagonist potency.

4 In radioligand binding experiments, fluspirilene was a potent displacer of [^{3}H]-PN-200-110 binding to rat cerebral cortical membranes (EC_{50} 30 nM), albeit with a low Hill slope (0.66), and was more potent than other lipophilic diphenylalkylamines such as flunarizine and lidoflazine. Fluspirilene interacted non-competitively with [^{3}H]-PN-200-110 and increased dissociation of the radioligand.

Introduction

It is now clear that there are three distinct binding sites for selective calcium-antagonists on the alpha$_1$ protein of the L-type calcium channel: a dihydropyridine site, a site for phenylalkylamines such as verapamil and a site of benzothiazepines such as diltiazem (Glossmann *et al.*, 1985; Mir & Spedding, 1987). However, although the lipophilic diphenylalkylamines, fendiline, prenylamine, flunarizine, cinnarizine, lidoflazine and pimozide (class III calcium antagonists, Spedding, 1985a,b) have been claimed to be calcium antagonists since the initiation of the concept of this class of drugs (e.g. Godfraind & Kaba, 1969; Fleckenstein, 1983), their mode of action has been much disputed, on the basis that the drugs have little selectivity for Ca^{2+} channels compared with Na^+ channels (Grima *et al.*, 1986), may inhibit the contractile proteins directly (Spedding, 1983) and may interfere with calmodulin-dependent processes (see Spedding, 1985b for a review). Thus it is not clear if these drugs act directly at the calcium channel, or at other sites. In this respect, the lipophilic nature of these agents (Spedding, 1985a) and their low affinity for the Ca^{2+} channel has precluded their being used in the tritiated form to define their site of action.

We were therefore interested by the finding that fluspirilene, a lipophilic diphenylalkylamine used as a neuroleptic, had very high affinity for the Ca^{2+} channels in skeletal muscle (Galizzi *et al.*, 1986; Qar *et al.*, 1987), so that [^{3}H]-fluspirilene could be used as a radioligand. We have tested fluspirilene in a well characterized model for studying the functional effects of calcium antagonists in K^+-depolarized smooth muscle: the taenia from the guinea-pig caecum. Interactions with Ca^{2+} channel activators such as Bay K 8644 (Schramm *et al.*, 1983) may be used to define the site of action of calcium antagonists. Dihydropyridine activators and antagonists interact competitively (Spedding & Berg, 1984; Su *et al.*, 1984; Spedding, 1985b), verapamil and diltiazem interact non-competitively with Bay K 8644 (Su *et al.*, 1984; Spedding & Berg, 1984; Spedding, 1985a,b), whereas the effects of the lipophilic diphenylalkylamines are not reversed by Bay K 8644 (Spedding & Berg, 1984; Spedding, 1985a; Boddeke *et al.*, 1988; Patmore & Duncan, 1988). These functional interactions reflect the allosteric interactions of the drugs at their respective binding sites (Glossmann *et al.*, 1985; Janis *et al.*, 1987) and are considered to be criteria for the classification of calcium antagonists (Vanhoutte & Paoletti, 1987). Using these criteria to define the site of action of fluspirilene, we found that fluspirilene selectively antagonised Bay K 8644 without modifying the effects of dihydropyridine calcium antagonists.

The potency of fluspirilene in displacing tritiated ligands from the Ca^{2+} channels in rat cerebral cortex membranes was also investigated; binding of dihydropyridines to cortical membranes is similar to that in smooth muscle membranes (Janis *et al.*, 1987). Our data confirm and extend previous results indicating high affinity for an undefined site in the Ca^{2+} channel in skeletal muscle, with slightly lower affinity in brain tissue (Quirion *et al.*, 1985; Galizzi *et al.*, 1986; Qar *et al.*, 1987). Also, they show that fluspirilene interacts non-competitively with the dihydropyridine site, but do not fully explain the selectivity against the calcium channel activators which we find in functional experiments.

Methods

Strips of taenia (1–2 mm diameter, 1.5–2 cm relaxed length) from the caecum of female guinea-pigs (200–350 g) were set up in 30 ml isolated organ baths containing K^+-Tyrode solution which was gassed with 95% O_2 : 5% CO_2 and maintained at 35°C (Spedding, 1982). The K^+-Tyrode solution had the following composition (mmol l^{-1}): NaCl 97, KCl 40, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, glucose 5.5. The preparations were washed in the Ca^{2+}-free, 40 mmol l^{-1} K^+-Tyrode solution for 30 min, by which time they were fully relaxed. Contractile responses were measured under isotonic conditions (1 g load) with Harvard or Bioscience isotonic transducers connected to BBC Goetz SE460 potentiometric recorders. Cumulative concentration-response curves to Ca^{2+} (10–3000 μmol l^{-1}) were obtained by increasing the Ca^{2+} concentration at 3–5 min intervals in logarithmic increments (Van Rossum, 1963). When concentration-response curves were repeated the curves were obtained at 30 min intervals. Antagonists were

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preincubated for 30 min. Concentration-ratios were calculated as the ratios of the concentrations of Ca^{2+} which produced a 50% maximal response (EC_{50}) in the presence and in the absence of the antagonists. In the experiments where Bay K 8644 was used the preparations were partially contracted with Ca^{2+} and Bay K 8644 added in a cumulative fashion, so that the increase in tone was taken as a percentage of the increase in contraction, as a fraction of the total increase possible (taken from the maximum response to Ca^{2+}).

Student's t test was used for comparisons of mean values and the Mann-Whitney U test for comparison of % changes. Concentration-ratios were calculated, and tested for statistical differences, in logarithmic units and the antilogarithms are quoted in the test. Standard errors of the mean are illustrated on percentage changes for representational purposes only. All concentrations refer to final bath concentrations of drugs.

[^3H]-(+)-PN 200-110 binding to rat cerebral cortex

Membranes were prepared from the cerebral cortex of male Sprague-Dawley rats by homogenization of the tissue in 30 volumes of ice-cold 50 mM Tris-HCl (pH 7.4 at 25°C) with a polytron tissue disruptor (setting 8, two 10 s bursts) followed by centrifugation at 48,000 $g_{av.}$ at 4°C for 15 min. The resulting pellet was washed twice by resuspension and centrifugation under the conditions described. The final pellet was resuspended in buffer and aliquots were stored under liquid nitrogen until required for assay.

Membrane aliquots (0.1–0.15 mg ml^{-1}) were incubated in 50 mM Tris-HCl (pH 7.4 at 25°C) with [^{3}H]-(+)-PN 200-110 (30 pM for competition studies, 0.005–1.0 nM for saturation studies) in a total volume of 2.0 ml. Incubations were carried out for 100 min at 25°C, after which the reaction was terminated by the addition of 5.0 ml ice-cold 50 mM Tris-HCl and rapid filtration over Whatman GF B filters by use of a Brandel cell harvester. The filters were washed twice with ice-cold buffer and the bound radioactivity determined by liquid scintillation counting. Non-specific binding was determined in the presence of 1.0 μM nitrendipine. For competition experiments, assays were carried out with at least 10 concentrations of competing drug and the resulting displacement curves were analysed by a non-linear least square parametric curve fitting program. Equilibrium binding parameters (K_d and B_{max}) were obtained by the non-linear least square fitting programme 'ligand' (Munson & Rodbard, 1980).

Drugs

The following drugs were used: Bay K 8644 (methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate), nifedipine, nisoldipine (Bayer AG, Leverkusen, FRG), nicardipine hydrochloride (Syntex), fluspirilene, lidoflazine and flunarizine tartrate (Jannssen), PN 200-110 (isopropyl-4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-methoxy-carbonylpyridine-3-carboxylate) and CGP 28392 (4-[2-(difluoromethoxy)-phenyl]1,4,5,7-tetrahydro-2-methyl-5-oxofluoro[3,4-b]pyridine-3-carboxylic acid ethylester).

Dihydropyridines were dissolved in ethanol (1 or 30 mmol l^{-1}), before being diluted in distilled water. Stock solutions were kept at 0°C for up to 12 h and aqueous dilutions prepared immediately before use. All solutions were shielded from light. The other drugs were dissolved in distilled water, with the exception of fluspiriline which has very low aqueous solubility (10 μg ml^{-1}) and was consequently dissolved in ethanol. The maximum concentration of ethanol in the bathing medium was <0.03%; this concentration did not affect responses to Bay K 8644.

Results

K^+-depolarised smooth muscle

Nitrendipine, nimodipine and nicardipine caused concentration-dependent inhibition of the contractions induced by cumulative addition of Ca^{2+} in K^+-depolarized taenia preparations from the guinea-pig caecum, with threshold antagonism occurring at concentrations of 1 nM (Figure 1). The antagonism by the dihydropyridines was non-competitive in these experiments. Fluspirilene was considerably less potent, in that threshold antagonism occurred at 100 nM (Figure 1d). An apparent pA_2 could be calculated for fluspirilene (7.5 ± 0.1, slope 1.1, n = 5), but not for the dihydropyridines.

Addition of Bay K 8644 (1–3000 nM) to an established submaximal contraction induced by Ca^{2+} (0.1 mM; 25–45% of the maximal contraction to Ca^{2+}) caused a concentration-

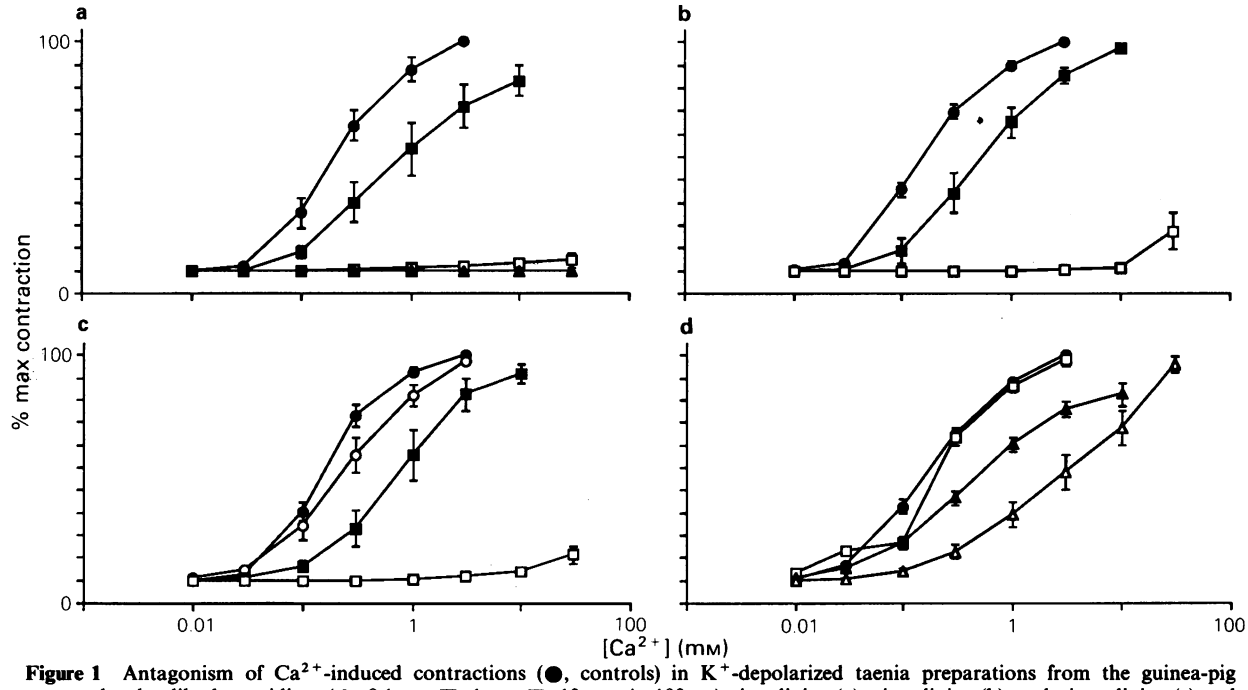

Figure 1 Antagonism of Ca^{2+}-induced contractions (●, controls) in K^+-depolarized taenia preparations from the guinea-pig caecum by the dihydropyridines (○, 0.1 nM; ■, 1 nM; □, 10 nM; ▲, 100 nM) nicardipine (a), nimodipine (b), and nitrendipine (c) and fluspirilene (□, 10 nM; ▲, 100 nM; △, 1000 nM; d). Vertical bars represent s.e.mean, n = 4–6.

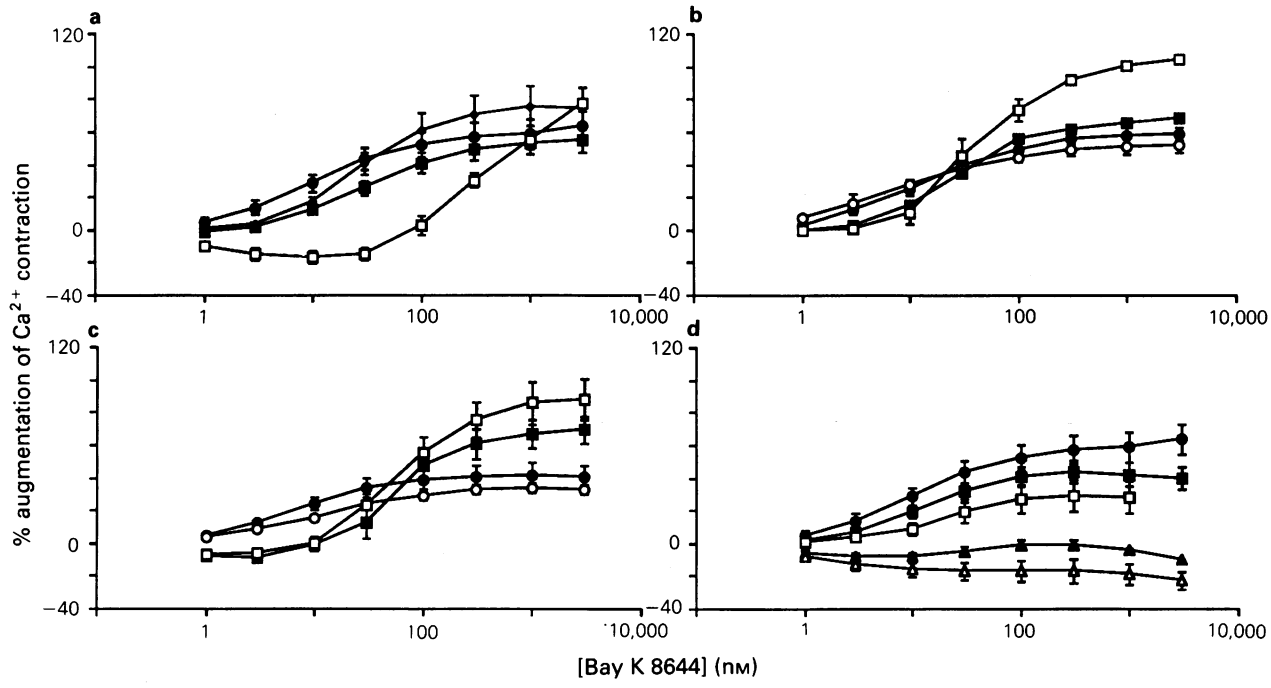

Figure 2 Cumulative concentration-response curves to Bay K 8644 in K^+-depolarised taenia preparations from the guinea-pig caecum (●, controls) which were contracted to 25–45% of maximum with Ca^{2+} (0.1–0.3 mM in control and fluspirilene experiments; 0.3–10 mM in dihydropyridine experiments) in the presence of nicardipine (◆, 0.1 nM; ■, 1 nM; □, 10 nM; a). nimodipine (○, 0.1 nM; ■, 1 nM; □, 10 nM; b), nitrendipine (○, 0.1 nM; ■, 1 nM; □, 10 nM; c) or fluspirilene (■, 1 nM; □, 10 nM; ▲, 100 nM; △, 1000 nM; d). Vertical bars represent s.e.mean, $n = 4$–8. The responses are expressed as % augmentation of the Ca^{2+}-induced contraction.

dependent augmentation of the contraction. Fluspirilene (1–1000 nM) antagonised the responses to Bay K 8644 in a concentration-dependent, but non-competitive, manner, reducing the maximum response, with a pD_2 of 8.3 (Figure 2d). In contrast, nicardipine (0.1, 1 nM) did not significantly affect the response to Bay K 8644 and at higher concentrations (10 nM) shifted the curve to the right, without depressing the maximum response (Figure 2). Similarly, nitrendipine and nimodipine did not depress the maximum response to Bay K 8644 and antagonism was seen only at low concentrations of Bay K 8644; indeed some augmentation of the response was apparent with high concentrations (Figure 2). In these experiments, progressively higher concentrations of Ca^{2+} (0.3, 1, 3, 10 mM) were used to recontract the tissues to 25–45% of the maximum contraction before the addition of Bay K 8644 when the dihydropyridines were used, but this was not necessary in the fluspirilene experiments as the sensitivity to Ca^{2+} was hardly impaired. This means that the apparently increased responses to Bay K 8644 seen after exposure to nimodipine and nitrendipine could be secondary to an increase in the driving force for Ca^{2+} entry, so that channels which were unblocked by Bay K 8644 would pass more Ca^{2+} than under control conditions.

In order to control for this difference, fluspirilene (100 nM) was added in the presence or absence of nimodipine (10 nM) and the effects of Bay K 8644 tested (Figure 3). Fluspirilene (100 nM for 25 min) did not antagonise the responses to Ca^{2+} in that 0.3 mM Ca^{2+} caused a large contraction (47 ± 5% max), but the activator effects of Bay K 8644 were almost abolished. Nimodipine (10 nM for 25 min) in these experiments abolished the contraction to 3 mM Ca^{2+}, yet this inhibition was reversed fully by Bay K 8644. A combination of fluspirilene (100 nM) and nimodipine (10 nM) also abolished the effects of 3 mM Ca^{2+}, but the sensitivity to Bay K 8644 was reduced 30 fold by fluspirilene. The slightly greater contraction induced by Bay K 8644 in the presence of fluspirilene and nimodipine, compared with that seen with fluspirilene alone, can probably be attributed to the increased Ca^{2+} concentration (3 mM compared with 0.3 mM) increasing Ca^{2+} flux through such channels as were activated by Bay K 8644.

In order to assess whether fluspirilene affected the potency of dihydropyridine calcium antagonists a series of experiments was performed in parallel (Figure 4a). Fluspirilene (10 nM) did not change the sensitivity of the taenia to Ca^{2+}, nor did it change the antagonistic effects of nitrendipine (1 nM; control mean Ca^{2+} concentration-ratio 4.4, $n = 10$; in the presence of fluspirilene 5.5, $n = 10$, $P > 0.5$). However, this concentration of fluspirilene markedly antagonised the effects of Bay K 8644 and completely antagonised the calcium channel activator effects of CGP 28392, revealing weak antagonistic effects (Figure 4b).

Fluspirilene is a potent dopamine antagonist, but this property does not relate directly to the antagonism of the effects of Bay K 8644 because sulpiride (10 μM) did not antagonise the effects of Bay K 8644 in four experiments (data not shown).

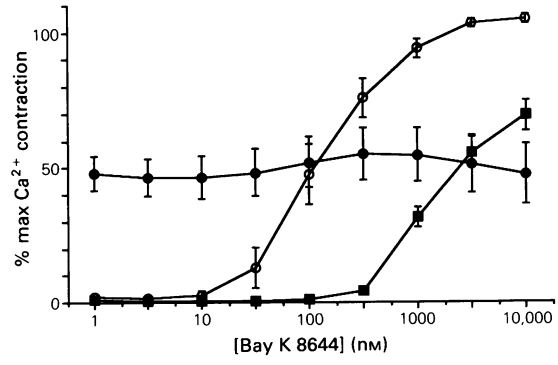

Figure 3 Effects of fluspirilene (●, 100 nM), nimodipine (○, 10 nM) or a combination of fluspirilene (100 nM) and nimodipine (10 nM) (■) on the sensitivity of taenia preparations to Bay K 8644. Ca^{2+} (0.3 nM) was used to contract the tissues in the presence of fluspirilene alone, otherwise 3 mM was added. The responses are expressed as the maximum response of the tissues to Ca^{2+} (3 mM). Vertical bars represent s.e.mean, $n = 5$.

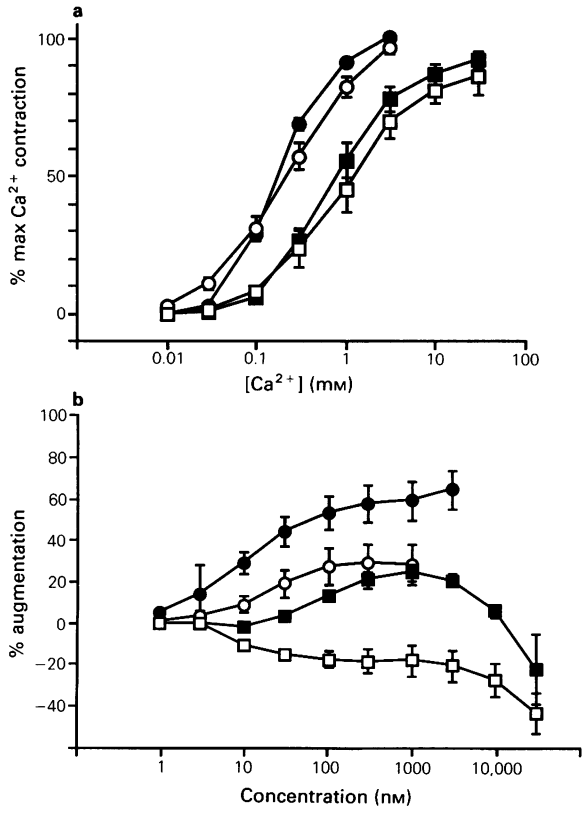

Figure 4 (a) Effects of fluspirilene (○, 10 nM) and nitrendipine (□, 1 nM) on cumulative concentration-response curves to Ca^{2+} (●, controls) in K^+-depolarised taenia preparations from the guinea-pig caecum. The effects of a combination of fluspirilene (10 nM) and nitrendipine (1 nM) are also shown (□). (b) Cumulative concentration-response curves to Bay K 8644 (●, ○) or CGP 28392 (■, □) in K^+-depolarised taenia preparations from the guinea-pig caecum which were contracted to 25–45% of maximum with Ca^{2+} in the presence of fluspirilene (○, □ 10 nM; ●, ■, controls). In (a) and (b) vertical bars represent s.e.mean, $n = 5$ (a) or 8 (b). (b) Note that fluspirilene abolishes the augmentation of the Ca^{2+}-induced contraction caused by CGP 28392.

Characteristics of [^{3}H]-PN 200-110 binding to rat cerebral cortex

Binding of [^{3}H]-(+)-PN 200-110 to rat cerebral cortex was saturable, reversible and of high affinity. Iterative non-linear analysis of the binding isotherms demonstrated a single class of high affinity sites with a K_d of 0.029 ± 0.008 nM and a B_{max} of 221 ± 20 fmol mg^{-1} protein. The affinity of [^{3}H]-PN 200-110 derived from transformations of saturation isotherms was similar to the value derived kinetically (0.026 ± 0.003 nM). Specific binding was linear between 20 and 270 μg ml^{-1} protein, and at a ligand concentration equivalent to K_d, represented 90% of the total binding ($n = 4$).

Modulation of [^{3}H]-PN 200-110 binding by calcium antagonists

Structurally-related dihydropyridines displaced [^{3}H]-PN 200-110 with high affinity and gave rise to 100% inhibition of specific binding (Table 1). Hill slopes were not significantly different from unity. Of the class III calcium antagonists (Spedding, 1985b), fluspirilene and pimozide displayed the highest affinity, although the former compound possessed a low Hill slope. This finding was also confirmed in competition experiments with another dihydropyridine ligand, [^{3}H]-nitrendipine (results not shown). Other class III agents displayed lower affinity.

The class II agents D-*cis*-diltiazem and verapamil inhibited binding of [^{3}H]-PN 200-110 but only displaced between 35 and 41% of the specific binding, respectively.

The effect of fluspirilene on [^{3}H]-PN 200-110 binding parameters and kinetics

Saturation isotherms of [^{3}H]-PN 200-110 binding to rat cerebral cortical membranes in the presence of fluspirilene indicated a non-competitive allosteric interaction, producing a decrease in K_d and B_{max} from control (K_d 0.03 ± 0.007 nM, B_{max} 224 ± 21 fmol mg^{-1} to 0.13 ± 0.03 nM, 138 ± 21 fmol mg^{-1} in the presence of 10^{-7} M fluspirilene, and 0.063 ± 0.01 nM, 211 ± 16 fmol mg^{-1} in the presence of 10^{-8} M fluspirilene, $n = 4$). In contrast, Scatchard transformations in the presence of nifedipine, a dihydropyridine antagonist, indicated a competitive interaction in that K_d was lowered without a significant change in the number of binding sites (control values: K_d 0.029 ± 0.008 nM, B_{max} 221 ± 20 fmol mg^{-1}; 0.17 ± 0.2 nM, 196 ± 6 fmol mg^{-1} in the presence of 0.5 nM nifedipine, $n = 4$).

The effect of fluspirilene on the dissociation rate of the PN 200–110: Ca channel complex is shown in Figure 5, which is a representative experiment from four similar experiments. Dissociation was induced at steady-state by the addition of 1 μM nitrendipine. The dissociation rate was increased from

Table 1 Binding affinities of calcium channel antagonists and activators at the [^{3}H]-PN 200-110 binding site in rat cerebral cortex at 25°C

Drug	*pK$_i$*	*% maximal inhibition of specific binding*	*nH*
Nifedipine	8.97 ± 0.25	100	0.88 ± 0.1
Nitrendipine	9.67 ± 0.15	100	0.94 ± 0.04
(+)-PN 200-110	10.1 ± 0.04	100	1.06 ± 0.1
(−)-PN 200-110	7.92 ± 0.02	110	1.14 ± 0.12
Bay K 8644	7.39 ± 0.12	100	0.96 ± 0.04
CGP 28392	6.65 ± 0.03	100	1.03 ± 0.11
Pimozide	7.72 ± 0.13	100	0.92 ± 0.02
	IC$_{50}$ (μM)		
Fluspirilene	0.03 ± 0.001	100	0.66 ± 0.09
Lidoflazine	0.54 ± 0.14	90	0.84 ± 0.02
Flunarizine	1.18 ± 0.24	92	1.28 ± 0.08
Verapamil	0.03 ± 0.009	41	0.92 ± 0.06
D-*cis*-Diltiazem	0.04 ± 0.013	35	1.00 ± 0.04
L-*cis*-Diltiazem		Not effective	

Values represent the means of 2–4 experiments performed in duplicate at a ligand concentration of 0.03–0.05 nM.
pKi values (where appropriate) were derived from the equation $K_i = IC_{50}/[1 + (L)/K_d]$ where L represents the free concentration of [^{3}H]-PN 200-110, and K_d represents the equilibrium dissociation constant. nH—Hill slope.

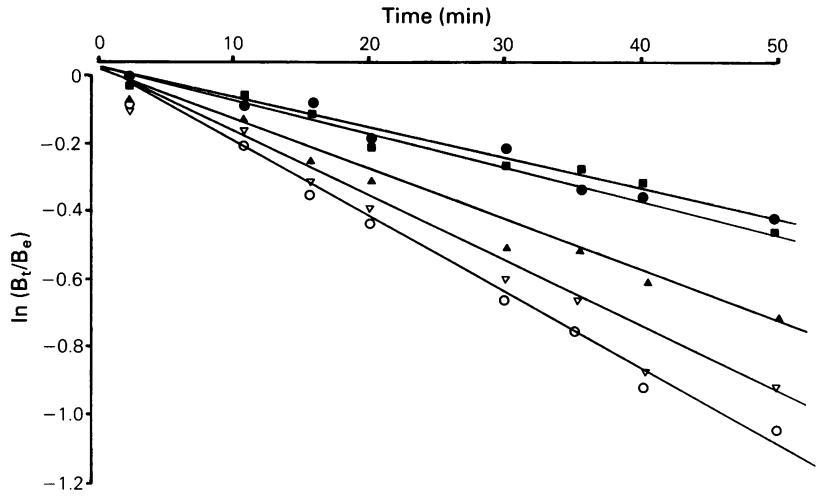

Figure 5 The effect of calcium antagonists on the dissociation rate of the [^{3}H]-PN 200-110–Ca^{2+}-channel complex. Data are shown as linear transformation, where B_e is specific [^{3}H]-PN 200-110 bound at equilibrium, and B_t is that at time t. Dissociation was initiated at steady-state by the addition of 1 μM nitrendipine in the absence, control (●), and presence of 1 μM fluspirilene (▲), 10 μM verapamil (○), 10 μM lidoflazine (▽) or 10 nM nifedipine (■).

$0.078 \pm 0.0009\,min^{-1}$ in the absence of fluspirilene to $0.014 \pm 0.001\,min^{-1}$ in the presence of 1 μM fluspirilene.

An increase in the dissociation rate was also observed with verapamil 10 μM ($0.017 \pm 0.002\,min^{-1}$), lidoflazine 10 μM ($0.016 \pm 0.003\,min^{-1}$) and flunarizine 10 μM (data not shown). No significant change was observed in the presence of nifedipine 10 nM ($0.0086\,min^{-1}$). These findings indicate that fluspirilene, by an allosteric interaction with the Ca^{2+} channel, increases the rate of dissociation of the dihydropyridine ligand.

Discussion

In this paper we have shown that fluspirilene has weak antagonistic effects on Ca^{2+}-induced contractions in K^+-depolarized smooth muscle, but only at much higher concentrations than are required to interact with binding sites in the putative Ca^{2+} channels in skeletal muscle (Galizzi *et al.*, 1986; Qar *et al.*, 1987). However, fluspirilene was much more potent (threshold 1 nM) in antagonising the effects of Bay K 8644 and at 10 nM abolished the augmentation of Ca^{2+}-induced contractions seen with CGP 28392. CGP 28392 is a less powerful activator of calcium channels than Bay K 8644 and these weak effects were more readily antagonised by fluspirilene. At concentrations which abolished the effects of CGP 28392, fluspirilene did not significantly affect the contractile responses to Ca^{2+} or the antagonistic effects of nitrendipine, indicating that the drug selectively antagonised the activator, but not the antagonistic effects of dihydropyridines.

How could this effect come about? A simple explanation would be that fluspirilene prevents the binding of Bay K 8644 but not of other dihydropyridines. However, it would appear likely that activator and antagonist dihydropyridines bind to a single site and can stabilize different states of the Ca^{2+} channel, with evidence from electrophysiological studies (e.g. Sanguinetti *et al.*, 1986) and from conformational energy calculations (Mahmoudian & Richards, 1986; Holtje & Marrer, 1987; see Janis *et al.*, 1987). The high affinity binding of [^{3}H]-Bay K 8644 to cell membranes that has been obtained by several groups (Bellemann, 1984; Lee *et al.*, 1987; Maan & Hosey, 1987; Sarmiento *et al.*, 1987) appears to correlate with 'antagonist' binding to a state of the channel corresponding to the inactivated state, i.e. there is competition for a single site between all the dihydropyridines tested. However, Maan & Hosey (1987) considered that activator binding was enthalpy driven and antagonist binding was entropy driven. Unfortunately, as yet, there is no clear method of investigating activated states of the channel in radioligand binding experiments and we do not consider that Bay K 8644 binding labels the activated form of the channel. We used [^{3}H]-PN-200-110 to label the channel, as it has high affinity and labels a large number of channels (high B_{max}) due to its high affinity for the inactivated state (Glossmann *et al.*, 1985). Saturation studies indicated a non-competitive interaction of fluspirilene with the [^{3}H]-PN-200-110 binding site. Fluspirilene increased the dissociation of the radioligand from its site, compatible with an allosteric interaction between the site for fluspirilene and the dihydropyridine binding site. Further studies indicated similar interactions if [^{3}H]-nitrendipine was used as the radioligand (data not shown). Fluspirilene may therefore resemble other diphenylalkylamines such as flunarizine and lidoflazine, but not verapamil on the grounds that verapamil does not fully displace dihydropyridine binding (Glossmann *et al.*, 1985; Janis *et al.*, 1987) and the effects of verapamil are readily reversed by Bay K 8644 (Spedding & Berg, 1984; Su *et al.*, 1984; Spedding, 1985a). Thus, fluspirilene has affinity for a distinct site on the calcium channel, as assessed by dihydropyridine binding, but we have no evidence for a selective inhibition of 'activator' binding by the drug. Indeed the weak inhibitory effects of CGP 28392 in the presence of fluspirilene may point to binding being unaffected, but the activator effects being selectively antagonised.

The conclusion from these results is that the site for fluspirilene is only weakly linked to the dihydropyridine site, but that binding of fluspirilene changes the channel such that the ability of activators to be effective is suppressed, whereas the effects of antagonists are less affected.

The low Hill slope observed in the [^{3}H]-PN-200-110 displacement studies indicates that the EC_{50} for fluspirilene can only be used as an empirical estimate of potency, even though fluspirilene was considerably more potent than the other diphenylalkylamines tested. In this respect, it may be considered as a prototypical agent for this class of drugs. Thus, the selective antagonism of Bay K 8644 does indicate that the site of these drugs is, if not at the dihydropyridine site, at least tightly coupled to the calcium channel. Flunarizine and lidoflazine have also been shown to be effective antagonists of the effects of Bay K 8644 (Spedding & Berg, 1984; Boddeke *et al.*, 1988; Patmore & Duncan, 1988), but the present study is the first to show that certain concentrations may selectively antagonise the effects of calcium channel activators.

However, despite the high affinity of fluspirilene for a site in the Ca^{2+} channel, in preliminary experiments we did not observe specific binding of [^{3}H]-fluspirilene to rat cerebral cortex membranes under a range of experimental conditions,

presumably because of high non-specific binding as demonstrated by Galizzi et al. (1986). These authors only found specific binding in skeletal muscle, where the high density of sites increases the signal to noise ratio. Further progress in this field with radioligand binding techniques will require conditions that allow stabilization of the activated state of the channels and demonstrable high affinity binding by [^{3}H]-fluspirilene in all relevant tissues.

In conclusion, we have shown that fluspirilene, at concentrations which non-competitively modify dihydropyridine binding, selectively antagonises the effects of calcium-channel activators and that this drug may be considered as a useful experimental tool to define the effects of the lipophilic diphenylalkylamines classified as class III calcium antagonists (Spedding, 1985a,b).

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The booster injection of antigen during active sensitization of guinea-pig modifies the anti-anaphylactic activity of the PAF antagonist WEB 2086

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1 Serum IgG levels of sensitized guinea-pigs bled at various times after the booster injection were evaluated and its capacity to sensitize passively lung strips from normal guinea-pigs assessed. Following the booster injection, both serum IgG and the ability to sensitize passively lung strips increased during the first week and decreased slowly thereafter.

2 The PAF antagonist WEB 2086 (3 mg kg^{-1}, i.v.) blocked the anaphylactic bronchoconstriction induced by intravenous administration of ovalbumin (1 mg kg^{-1}) when guinea-pigs were challenged 2 and 4 days after the booster injection, but became ineffective when tested in guinea-pigs challenged 7, 28 and 56 days after the booster injection.

3 The ability of WEB 2086 to reduce anaphylactic bronchoconstriction of guinea-pigs challenged 2 and 4 days after the booster injection was unrelated to either the selective involvement of one type of immunoglobulin, low IgG titres in sera or a reduced sensitizing capacity.

4 The booster injection, which accounts for the loss of efficacy of WEB 2086 from the fourth day thereafter, probably operates as a PAF-independent inflammatory challenge.

5 The protocol for immunisation and the day of experiment after the booster injection determines the sensitivity of the anaphylactic bronchoconstriction to inhibition by PAF antagonists.

Introduction

It has been suggested that platelet-activating factor (PAF) is involved in the pathogenesis of anaphylaxis in several species, including the guinea-pig (Vargaftig *et al.*, 1980; Basran *et al.*, 1984). In the past, we have used passive sensitization to study the ability of PAF antagonists to interfere with anaphylactic shock, because we thought that the transfer of antibodies to a non-sensitized animal, which is shocked within 10 days, favours IgE-dependent mechanisms, involved in human asthma. We later demonstrated that in fact serum from actively sensitized animals transfers a mixture of IgE and IgG antibodies, which are relevant for anaphylactic bronchoconstriction (S. Desquand, B. Rothhut & B.B. Vargaftig, unpublished). Since asthma involves active human sensitization and multiple rather than single exposures to antigen, we became interested in the potential antagonism by a standard PAF antagonist of anaphylactic bronchoconstriction in this model.

The effectiveness of the triazolothienodiazepine WEB 2086 (Casals-Stenzel., 1986) against active anaphylaxis was previously reported but in fact, different experimental procedures of sensitization may lead to differences in the effectiveness of this PAF antagonist against active anaphylaxis. Thus, Casals-Stenzel (1987) found that WEB 2086 blocks anaphylactic bronchoconstriction, whereas Pretolani *et al.*, (1987) reported that the same antagonist was ineffective. Since these differences might be pertinent to the sensitization regimen or to other factors, we have now studied the correlation between the time-course of the anaphylactic responses, the titres of circulating IgG and the ability of serum collected from actively sensitized guinea-pigs at different intervals after the sensitizing injection to transfer antigen responsiveness to isolated tissues. The possible relationship between the presence of different subclasses of immunoglobulins involved and particularly the role of the booster injection were correlated with the ability of WEB 2086 to interfere with anaphylaxis.

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Methods

Procedures of active sensitization

Guinea-pigs (300–400 g) were actively sensitized at day 0 by the s.c. injection of 0.5 ml of 0.9% w/v NaCl solution (saline) containing ovalbumin (OA) 10 μg dispersed in 1 mg $Al(OH)_3$ (modified from Andersson, 1980; 1981). When appropriate, guinea-pigs were boosted with a similar injection of antigen at day 14. They were used 2, 4, 7, 28, 56 or 84 days after the booster injection (groups called respectively 14+2, 14+4, 14+7, 14+28, 14+56 or 14+84). In some experiments, the booster injection was omitted and the animals were used 14, 21, 42, 70 or 98 days after the first injection (groups called respectively 0–14, 0–21, 0–42, 0–70 or 0–98).

Determination of ovalbumin-specific IgG by ELISA

Blood (0.5 ml) was collected from the carotid artery of pentobarbitone-anaesthetized sensitized guinea-pigs of the different groups described below, for the determination of IgG titres by enzyme-linked immunosorbant assay (ELISA). Flat-bottomed polystyrene microtitre plates (Nunc) were coated overnight at 4°C with 100 μl of OA 1 μg ml^{-1} in 0.05 M sodium carbonate buffer, pH 9.5. The plates were washed three times in 0.15 M PBS (phosphate buffer solution), pH 7.5 containing 0.1% Tween 20 (PBS-Tween) and drained. All washes were performed in this way, and subsequent incubations were for 2 h at 37°C. All dilutions of sera and antisera were prepared in PBS-Tween. Each well was incubated successively with the following solutions with washes between each step: 200 μl of 1 μg ml^{-1} of bovine serum albumin (BSA); 100 μl of labelled goat anti-guinea-pig IgG antiserum diluted 1 to 2000; 100 μl of the substrate solution at pH 4.0 containing 0.1 M citrate, 3% hydrogen peroxide (30% w/v) and 250 μg ml^{-1} of 2,2'-azino-*bis*(3-ethyl benzothiazoline sulphonic acid) di-ammonium salt, $0.5H_2O$ (ABTS). The enzyme-substrate reaction was stopped after 30 min by adding to each well 50 μl of a 10% sodium dodecyl sulphate (SDS) solution in distilled water. Absorbances were measured at 450 nm with an automated

microplate-reader (MR 700, Dynatech Laboratories Inc., Marnes La Coquette, France). Titres of OA-specific IgG were expressed as the maximal dilution giving 50% of the maximum net absorbance after subtraction of the values obtained with control sera from non-immunized guinea-pigs.

Preparation of the isolated lung strips

Non-immunized guinea-pigs were anaesthetized and ventilated. A mid-thoracotomy was performed, the lungs were then removed and rinsed with 20 ml of Krebs solution injected through the pulmonary artery. As described by Lulich *et al.*, (1976), peripheral sub-pleural strips (2.5–3 cm long; 3–4 mm width) were dissected and mounted under 2 g tension in an isolated organ bath containing 16 ml Krebs solution at 37°C and aerated with 95% O_2 plus 5% CO_2. Each strip was connected to a Gould-Statham force displacement transducer. After approximately 1 h for equilibration, histamine was added cumulatively (1–10–100 μM) at 3 min intervals. After three rinses, 30 min later, the same cycle was repeated; the maximal contraction induced by cumulative concentrations of histamine was taken as a reference.

Lung strips were incubated with 0.1, 0.3, 1, 3 or 30 $\mu l\,ml^{-1}$ of serum provided from actively sensitized guinea-pigs of the group 14+7 and with 30 $\mu l\,ml^{-1}$ of serum prepared with guinea-pigs from the other groups described below.

After 1 h of incubation, the Krebs solution was removed and a series of three rinses was performed. Twenty minutes later, the lung strips were stimulated with OA 10 $\mu g\,ml^{-1}$, added to the organ bath. The intensities of the contractions were expressed as a percentage of the maximal contraction induced by histamine. Aliquots of 0.2 and 1 ml of the Krebs solution were removed from the organ bath before and 20 min after stimulation by OA, to evaluate thromboxane B_2 (TxB_2) formation and histamine release respectively.

In vivo *preparation*

Guinea-pigs of groups 0–14, 14+2, 14+4, 14+28 or 14+56 were anaesthetized with sodium pentobarbitone (40 $mg\,kg^{-1}$, i.p.) and prepared for the recording of bronchial resistance to inflation on a Beckman Dynograph R 511 (Schiller Park, II, U.S.A) as described by Lefort & Vargaftig (1978).

Spontaneous breathing was suppressed with pancuronium (2 mg, i.v.). A jugular vein was catheterized for drug injection and both carotid arteries were cannulated, one to measure arterial blood pressure with a Statham-Gould tranducer P23Db and the other one for blood sampling. The bronchial reactivity was assessed with 5-hydroxytryptamine (5-HT, 3 μg, i.v.) and experiments were initiated when reproducible responses were obtained. According to Casals-Stenzel (1987) and Pretolani *et al.* (1987), guinea-pigs were treated 5 min before challenge with 5 $\mu g\,kg^{-1}$ of the histamine H_1-receptor blocker, mepyramine, to prevent the sudden death following antigen challenge but neither bronchoconstriction nor the arterial hypotension of acute anaphylaxis (Brocklehurst, 1960). Guinea-pigs were then challenged with a bolus injection of OA 1 $mg\,kg^{-1}$, i.v. Treated animals were injected with WEB 2086 3 $mg\,kg^{-1}$, i.v., 10 min before OA, control animals being injected with the solvent as previously described (Pretolani *et al.*, 1987). The bronchopulmonary resistance to inflation following administration of the antigen was monitored and bronchoconstriction was expressed in cm^2 of the area of the tracing over the base line for 20 min.

Blood cell counts

Blood samples (200 μl) were collected from the carotid artery for the assessment of total leucocyte and platelet counts with a Coulter Counter ZBI. Blood samples were obtained before then 1, 3 and 6 min after the injection of OA.

Radioimmunoassay of thromboxane B_2

TxB_2 was assayed according to the method of Sors *et al.* (1978). Briefly, aliquots (200 μl from the organ bath) were left at room temperature for 60 min (to allow conversion of TxA_2 into its stable metabolite TxB_2) and were stored at −20°C. For the radioimunoassay, the samples were thawed and incubated overnight at 4°C with ^{125}iodine-labelled TxB_2 and anti-TxB_2 antiserum in a phosphate buffer (10 mM, pH 7.4) containing bovine gamma globulin (0.3% w/w). The next day, bound and free fractions were separated by the addition of a solution of polyethyleneglycol 6000 (30% in distilled water) followed by centrifugation at 2000 *g* and 4°C for 10 min. The supernatant was decanted and the radioactivity present in the pellet, corresponding to the bound fraction was counted for 1 min in a Kontron Anlytical gamma counter (model MDA 312).

Spectrofluorimetric assay of histamine

One ml of the collected fractions was mixed with 1 ml of perchloric acid (0.8 N). After centrifugation for 30 min at 3500 r.p.m. and at 4°C, the supernatants were stored at 4°C. An automatic fluorimetric assay for histamine was performed according to Shore *et al.* (1959), as modified by Lebel (1983).

Data analysis

The results are expressed as the means ± s.e.mean. Statistical differences between two means of data were evaluated by the unpaired Student's *t* test.

Materials

The Krebs solution was composed of mM: NaCl 118, KCl 4.7, $CaCl_2 \cdot 2H_2O$ 2.5, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 5.6.

The PAF antagonist WEB 2086 (3-(4-(2-chlorophenyl)-9-methyl-6H-thieno (3,2-*f*) (1,2,4)-triazolo-(4,3-a)-(1,4)- diazepine-2-yl)-1-(4-morpholinyl)-1-propanone; Boerhinger-Ingelheim, F.R.G.) was initially dissolved in HCl 50 mM in distilled water and further diluted to the appropriate concentrations in distilled water. The other drugs were dissolved in saline and were obtained as indicated: sodium pentobarbitone (Nembutal, Sanofi, France); pancuronium bromide (Pavulon, Organon, France); mepyramine maleate (Rhône-Poulenc Santé, Vitry/Seine, France); 5-hydroxytryptamine (serotonin, 5-HT), histamine, ABTS, SDS (Sigma Chemical Co., St. Louis, MO, U.S.A.); chicken ovalbumin (OA, Miles, Naperville, IL, U.S.A.); $Al(OH)_3$, perchloric acid, polyethyleneglycol 6000 (Merck, Darmstadt, FRG); peroxidase-labelled goat anti-guinea-pig IgG (Biosis, Compiègne, France). The antibody radiolabelled ligands for the radioimmunoassay of TxB_2 were from the URIA (Unité Associée Institut Pasteur-INSERM n° 207).

Results

Time-course of the IgG production and of the ability of sera from actively sensitized guinea-pigs to sensitize lung strips in vitro

Sera of guinea-pigs collected up to 14 days after the sensitizing injection of OA contained neither IgG (Figure 1) nor transferable sensitizing activities as measured by parenchyma lung strip contraction and release of TxB_2 and histamine following OA challenge (Figures 2, 3, 4). When the animals were not boosted and were bled 21, 42, 70 or 96 days after the sensitizing injection of OA, their sera contained very low amounts of IgG (Figure 1) and also failed to sensitize passively lung strips from normal guinea-pigs (Figures 2, 3, 4).

In contrast, when a booster injection of OA was administered at day 14, the IgG titres as well as the intensity of the contractions of passively sensitized lung strips, accompanying

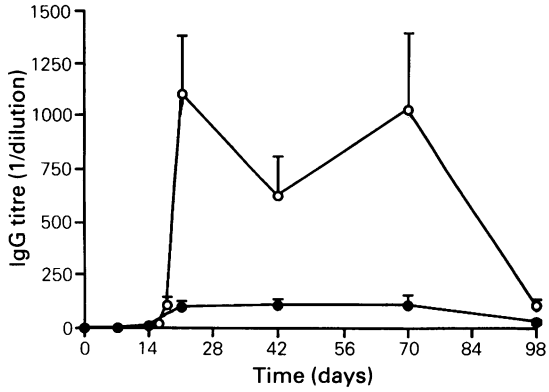

Figure 1 Titres of IgG in sera of guinea-pigs bled at different times during sensitization. The titres of IgG of the sera of guinea-pigs bled 2, 4, 7, 28, 56 or 84 days after the booster injection at day 14 (○) were compared to those of sera of non-boosted guinea pigs bled at days 14, 21, 70 or 98 (●). Titres are expressed according to the reciprocal of the maximal dilution giving 50% of the absorbance by ELISA. Bars = s.e.mean. Experiments with sera of guinea-pigs of group 14 + 2: $n = 10$; group 14 + 4: $n = 15$; group 14 + 7: $n = 34$; group 14 + 28: $n = 20$; group 14 + 56: $n = 10$; group 14 + 84: $n = 3$; group 0–14: $n = 7$; group 0–21: $n = 15$; group 0–42: $n = 7$; group 0–70: $n = 8$; group 0–98: $n = 6$.

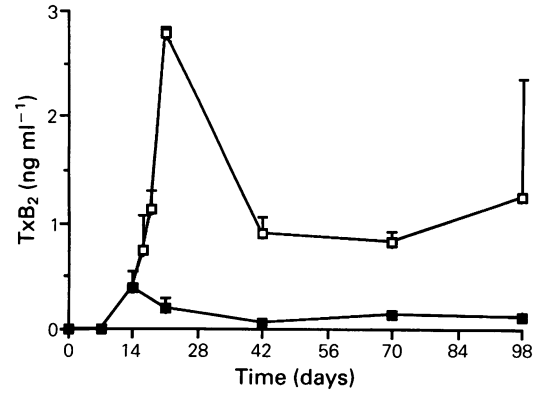

Figure 3 Ovalbumin-induced formation of thromboxane B_2 (TxB_2) by lung strips sensitized *in vitro* for 1 h with sera from actively sensitized guinea-pigs and bled as described in legend to Figure 1. The vertical scale shows the amounts of TxB_2 ($ng\,ml^{-1}$) formed following the addition of ovalbumin $10\,\mu g\,ml^{-1}$, to the organ bath after a 1 h tissue sensitization with $30\,\mu g\,ml^{-1}$ of sera from guinea-pigs sensitized and boosted at day 14 and bled at the indicated intervals (□) or non-boosted (■). Bars = s.e.mean. Experiments with sera of guinea-pigs of group 14+2: $n = 10$; group 14+4: $n = 12$; group 14+7: $n = 4$; group 14+28: $n = 14$; group 14+56: $n = 13$; group 14+84: $n = 3$; group 0–14: $n = 9$; group 0–21: $n = 14$; group 0–42: $n = 4$; group 0–70: $n = 4$; group 0–98: $n = 4$.

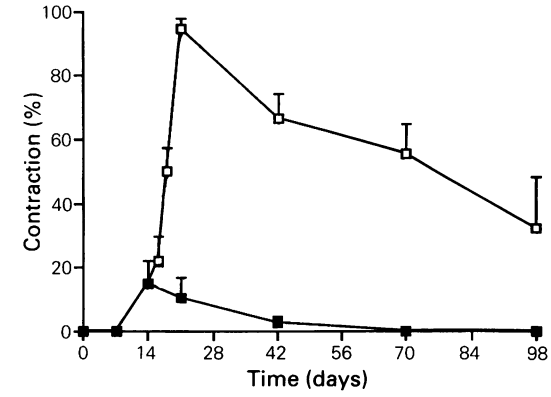

Figure 2 Ovalbumin-induced contractions of lung strips sensitized *in vitro* for 1 h with sera from actively sensitized guinea-pigs and bled as described in legend to Figure 1. The vertical scale shows the intensity (in % of the maximal effect of histamine) of the contractions induced by ovalbumin $10\,\mu g\,ml^{-1}$ added to the organ bath after a 1 h tissue sensitization with $30\,\mu g\,ml^{-1}$ of sera from guinea-pigs sensitized and boosted at day 14 and bled at the indicated intervals (□) or non-boosted (■). Bars = s.e.mean. Experiments with sera of guinea-pigs of group 14 + 2: $n = 10$; group 14 + 4: $n = 12$; group 14 + 7: $n = 4$; group 14 + 28: $n = 14$; group 14 + 56: $n = 13$; group 14 + 84: $n = 3$; group 0–14: $n = 9$; group 0–21: $n = 14$; group 0–42: $n = 4$; group 0–70: $n = 4$; group 0–98: $n = 4$.

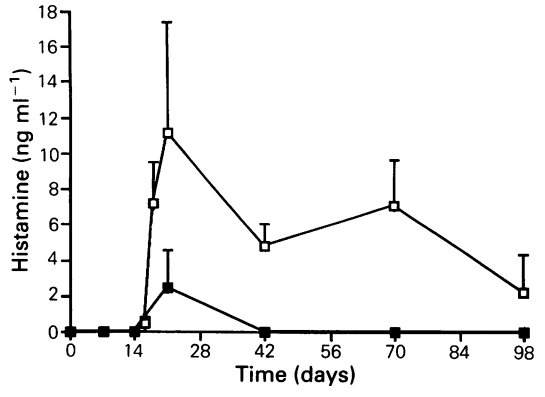

Figure 4 Ovalbumin-induced release of histamine by lung strips sensitized *in vitro* for 1 h with sera from actively sensitized guinea-pigs and bled as described in legend to Figure 1. The vertical scale shows the amounts of histamine ($ng\,ml^{-1}$) released following the addition of ovalbumin $10\,\mu g\,ml^{-1}$, to the organ bath after a 1 h tissue sensitization with $30\,\mu g\,ml^{-1}$ of sera from guinea-pigs sensitized and boosted at day 14 and bled at the indicated intervals (□) or non-boosted (■). Bars = s.e.mean. Experiments with sera of guinea-pigs of group 14+2: $n = 10$; group 14+4: $n = 12$; group 14+7: $n = 4$; group 14+28: $n = 14$; group 14+56: $n = 13$; group 14+84: $n = 3$; group 0–14: $n = 9$; group 0–21: $n = 14$; group 0–42: $n = 4$; group 0–70: $n = 4$; group 0–98: $n = 4$.

formation of TxB_2 and release of histamine rapidly increased to a maximum at day 21, to decrease slowly thereafter (Figures 1, 2, 3, 4).

It is noteworthy that the IgG titres followed the same time-course as the sensitizing capacity of the sera, with the exception that sera from guinea-pigs of group 14+4 showed a remarkable ability to sensitize isolated lung strips even though they contained low IgG titres (Figures 1, 2, 3, 4). To verify if the increase of the sensitizing activity involved antibodies of the IgE sub-class, strips were sensitized with serum from guinea-pigs of group 14+4, which had been previously heated at 56°C for 4 h to inactivate IgE (Parish, 1970). The OA-induced tissue contractions, the TxB_2 formation and histamine release from lung strips sensitized with heated sera were reduced, as compared to experiments with paired un-heated sera, by 41.4 ± 17.6%, 22.4 ± 13.3% and 25.7 ± 14.9% ($n = 4$), respectively. These reductions were not statistically significant.

Dose-dependence of the contraction, formation of thromboxane B_2 and release of histamine of lung strips sensitized in vitro

Lung strips were incubated with 30, 3, 1, 0.3, $0.1\,\mu l\,ml^{-1}$ of serum from actively sensitized guinea-pigs of group 14+7. The OA-induced responses were proportional to the concentration of sera used to sensitize the lung strips (Figure 5). Similar experiments were performed with sera from the other groups of sensitized guinea-pigs (boosted or non-boosted) and displayed the same patterns of response (data not shown).

*Effect of WEB 2086 on bronchoconstriction induced by the injection of ovalbumin to guinea-pigs actively sensitized with a booster injection (*groups 14+2, 14+4, 14+28 and 14+56)

Administration of OA $1\,mg\,kg^{-1}$, to non-boosted guinea-pigs

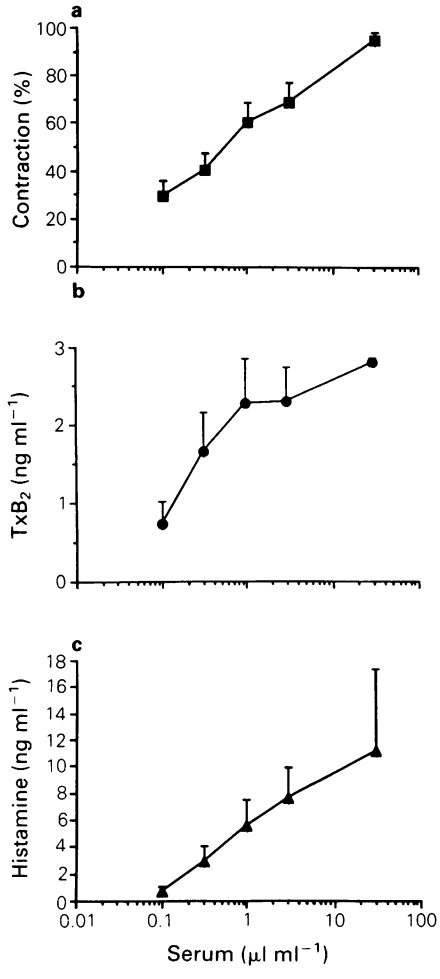

Figure 5 Intensity of contractions (in % of the maximal effect of histamine, a) and release of thromboxane B_2 (TxB_2 ng ml^{-1}, b) and histamine (ng ml^{-1}, c) following the stimulation with ovalbumin (10 µg ml^{-1}) of lung strips sensitized by different concentrations of serum provided from guinea-pigs of group 14+7. Bars = s.e.mean. Experiments with 0.1 µl ml^{-1}: $n = 8$; 0.3 µl ml^{-1}: $n = 10$; 1 µl ml^{-1}: $n = 9$; 3 µl ml^{-1}: $n = 12$; 30 µl ml^{-1}: $n = 4$.

7 days after the initial sensitizing injection failed to induce bronchoconstriction. In addition, erratic responses following provocation with OA were observed at day 14. In contrast, the administration of OA, 2 days after the booster injection, induced bronchoconstriction. The intensity of the response observed in guinea-pigs used 2, 4, 28 and 56 days after the booster increased gradually, although this was not significant. As shown in Figure 6, intravenous administration of WEB 2086, 3 mg kg^{-1}, blocked significantly the anaphylactic bronchoconstriction when OA was administered 2 and 4 days after the booster injection. In contrast, WEB 2086 failed to modify the anaphylactic bronchoconstriction when OA was administered 28 and 56 days after the booster injection (Figure 6). It is noteworthy that sera of both groups of guinea-pigs had a capacity to passively sensitize the isolated lung strips from non-immunized guinea-pigs similar to that from actively sensitized guinea-pigs of group 14+4 (Figures 2, 3, 4).

Failure of WEB 2086 to prevent the decrease of blood cell counts induced by ovalbumin

Anaphylactic bronchoconstriction in the different groups of guinea-pigs was accompanied by thrombocytopenia and leucopenia during the 6 min following the challenge (Table 1). These parameters were not modified by pretreatment with WEB 2086, even when bronchoconstriction was blocked (Table 1).

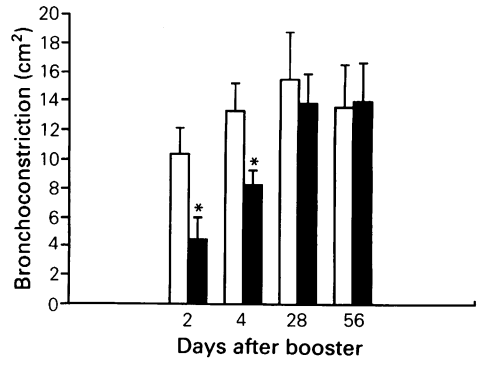

Figure 6 Interference of WEB 2086 (3 mg kg^{-1}) with the intensity of bronchoconstriction (cm^2) induced by an i.v. injection of ovalbumin 1 mg kg^{-1} to actively sensitized and mepyramine-treated (5 µg kg^{-1}) guinea-pigs, challenged 2, 4, 28 or 56 days after the booster injection at day 14 (groups 14+2, 14+4, 14+28 or 14+56 days respectively). Open columns represent the responses of control guinea-pigs and closed columns those of treated animals. Bars = s.e.mean. *$P < 0.05$. Groups 14+2 and 14+4, control guinea-pigs: $n = 5$ for each; treated guinea-pigs, $n = 6$ for each. Group 14+28, control guinea-pigs: $n = 6$; treated guinea-pigs: $n = 6$. Group 14+56, control guinea-pigs: $n = 6$; treated guinea-pigs: $n = 5$.

Discussion

In this study, the temporal development of guinea-pig sensitization, as evaluated by the IgG titres and by the appearance of homocytotropic antibodies in serum, i.e., antibodies which sensitize passively isolated tissues from the same animal species, was investigated by a procedure modified from that of Andersson (1980; 1981), which induces the formation of a mixture of IgE and IgG (S. Desquand, B. Rothhut & B.B. Vargaftig, unpublished).

Neither IgG nor transferable sensitizing activities were detected in serum up to 14 days after the first injection of antigen. In addition, sera from non-boosted animals bled 21, 42, 70 or 96 days later contained very low amounts of IgG and failed to sensitize passively lung strips from control guinea-pigs. In contrast, when a booster injection of antigen was administered at day 14, the IgG titres and the capacity of serum to sensitize lung strips increased rapidly, peaked at day 21, decreasing very slowly thereafter.

It is noteworthy that the IgG titres and the capacity of serum to sensitize lung tissues passively and to induce passive cutaneous anaphylaxis follow a similar time-course (Pretolani *et al.* 1989). We recently demonstrated that IgG of guinea-pigs of group 14+7 account more for the transferable activity of sera than does IgE, since they are thermo-resistant, whereas IgE are thermo-labile (S. Desquand, B. Rothhut & B.B. Vargaftig, unpublished). However, it should be noted that the serum of guinea-pigs of group 14 + 4, which contained low IgG titres, displayed a high *in vitro* sensitizing capacity, which was also not accounted for by IgE, since it was thermo-resistant (Parish, 1970).

The time-dependent profile of immunisation was used to understand the differential effectiveness of PAF antagonists. Indeed, Casals-Stenzel (1987) showed that WEB 2086 blocks the antigen-induced anaphylactic bronchoconstriction in guinea-pigs actively sensitized by a single injection of OA and used 28 days later in the presence of mepyramine. In contrast, Pretolani *et al.* (1987) reported that WEB 2086 fails to inhibit the anaphylactic bronchoconstriction when OA is administered to mepyramine-treated actively sensitized guinea-pigs of group 14+7. The differences in the ability of WEB 2086 to block antigen-induced bronchoconstriction reported by Casals-Stenzel (1987) and Pretolani *et al.* (1987), might have resulted from the different types and amounts of immunoglobulins involved or from a role of the booster injection. We have recently shown that anaphylactic bronchoconstriction induced by the intratracheal administration of antigen to

Table 1 Interference of WEB 2086 with the decrease of blood cell counts induced by ovalbumin (OA)

Cell count decrease (%)	min	14 + 2 *Control*	14 + 2 *WEB 2086*	14 + 4 *Control*	14 + 4 *WEB 2086*
Platelets	1	29.0 ± 16.7	28.7 ± 8.9	68.6 ± 12.9	71.8 ± 13.2
	3	46.2 ± 16.9	39.0 ± 11.4	88.8 ± 4.2	84.0 ± 8.2
	6	56.7 ± 20.5	52.7 ± 11.1	93.6 ± 1.6	9.11 ± 3.4
Leucocytes	1	18.5 ± 9.2	12.8 ± 3.7	31.2 ± 15.2	34.2 ± 8.4
	3	33.5 ± 8.6	36.5 ± 10.6	41.6 ± 20.4	22.0 ± 10.9
	6	38.7 ± 7.2	40.8 ± 11.1	33.2 ± 17.5	23.8 ± 11.9
		14 + 28		14 + 56	
	min	*Control*	*WEB 2086*	*Control*	*WEB 2086*
Platelets	1	39.8 ± 13.5	51.8 ± 16.3	2.7 ± 1.7	6.8 ± 2.2
	3	64.5 ± 18.4	77.0 ± 10.8	12.0 ± 6.5	10.6 ± 3.8
	6	67.5 ± 16.9	85.7 ± 4.7	35.8 ± 10.5	36.6 ± 12.3
Leucocytes	1	16.3 ± 10.4	24.3 ± 9.3	21.3 ± 7.3	26.6 ± 3.3
	3	34.8 ± 6.7	51.7 ± 6.1	33.8 ± 9.1	35.2 ± 12.5
	6	59.2 ± 10.0	51.4 ± 9.7	35.7 ± 12.2	28.8 ± 14.8

Platelet and leucocyte count decreases were evaluated 1, 3 and 6 min after the challenge by an i.v. injection of OA to 1 mg kg^{-1}, to mepyramine-treated guinea-pigs actively sensitized and used 2, 4, 28 or 56 days after the booster injection (groups 14+2, 14+4, 14+28, or 14+56 respectively). Treated animals were injected with WEB 2086 3 mg kg^{-1}, 10 min before OA. Figures are the % reduction in counts.
Groups 14 + 2 and 14 + 4, control guinea-pigs: $n = 5$ for each; treated guinea-pigs: $n = 6$ for each.
Group 14 + 28, control guinea-pigs: $n = 6$; treated guinea-pigs: $n = 6$.
Group 14 + 56, control guinea-pigs: $n = 6$; treated guinea-pigs: $n = 5$.

actively sensitized guinea-pigs without a booster injection is suppressed by another PAF antagonist, BN 52021, whereas bronchoconstriction triggered in boosted guinea-pigs becomes refractory to this PAF antagonist (S. Desquand, J. Lefort, C. Dumarey & B.B. Vargaftig, unpublished). For this reason, we studied the effects of WEB 2086 against the anaphylactic bronchoconstriction induced by the i.v. administration of OA to actively sensitized guinea-pigs at different time intervals after the booster injection. Since anaphylactic bronchoconstriction in actively sensitized and boosted guinea-pigs (group 14 + 7) is largely mediated by histamine (Detsouli *et al.*, 1985), the animals were pretreated with mepyramine to avoid sudden death (Brocklehurst, 1960), and thus allow the study of the supposedly PAF-dependent component of the anaphylactic reaction. Bronchoconstriction was always present, irrespective of mepyramine and of the group of boosted guinea-pigs concerned. These responses were of comparable intensity, even if a slight increase was noted with the increasing intervals between the booster injection and the day of the experiment.

It should be noted that guinea-pigs of group 14 + 2, which have low amounts of circulating immunoglobulins in the serum, also developed anaphylactic bronchoconstriction. This indicates that immunoglobulins are associated with the targeted inflammatory cells before their levels become detectable in the serum. Surprisingly, although anaphylactic bronchoconstrictions were of the same magnitude, WEB 2086 only antagonized bronchoconstriction when it was administered 2 and 4 days after the booster, but not later. This pattern of activity may result from a shift from a PAF-dependent early phase of sensitization to a less PAF-dependent delayed phase. The booster is the essential factor determining this shift, which accounts for the effectiveness of WEB 2086 in non-boosted animals (Casals-Stenzel, 1987) and for its failure 7 days after the booster (Pretolani *et al.*, 1987). In addition, since the titres of homocytotropic antibodies in the serum of the animals of groups 14 + 2, 14 + 28 and 14 + 56 had a time-course similar to that of IgG, the sensitivity of anaphylactic bronchoconstriction to WEB 2086 does not result from a selective involvement of one type of immunoglobulin. Furthermore, interference of WEB 2086 with anaphylactic bronchoconstriction in guinea-pigs of group 14 + 2 and 14 + 4 is probably not due to reduced IgG titres and/or capacity to induced passive sensitization, since sera of animals of group 14 + 4 were as able to cause passive sensitization of isolated tissues as those from guinea-pigs of groups 14 + 28 and 14 + 56. A final argument in favour of the dissociation between the sub-class and amount of immunoglobulins formed on one hand, and the failure of PAF antagonists to block bronchoconstriction on the other, is provided by our results (S. Desquand, J. Lefort, C. Dumarey & B.B. Vargaftig, unpublished) showing that guinea-pigs of the group 14 + 84, with low titres of circulating immunoglobulins were still at this late stage, as refractory to inhibition by BN 52021 as those of groups 14 + 7, 14 + 28, 14 + 56, which had elevated IgG titres and which were effective in passively sensitizing lung parenchyma strips. The failure of WEB 2086 to inhibit anaphylactic bronchoconstriction is thus accounted for by the same booster which triggers the development of lung hyperresponsiveness (Pretolani *et al.*, 1988). In addition, it is clear that the subtle differences in the effectiveness of PAF antagonists against anaphylaxis are neither specific for WEB 2086 nor result from different sub-classes or amounts of immunoglobulins.

Our claim that the booster injection determines whether the anaphylactic bronchoconstriction will become refractory to WEB 2086 four days later explains the initial disappointing results obtained with PAF antagonists used against active anaphylactic bronchoconstriction and negates previous conclusions that PAF is not important for acute anaphylaxis in actively sensitized guinea-pigs (Detsouli *et al.*, 1985; Lagente *et al.*, 1985; Danko *et al.*, 1988). The pharmacological modulation of the bronchopulmonary and secretory effect of PAF changes between the fourth and the seventh day following the booster injection, since the latter triggers important modifications of the lung reactivity to mediators including PAF probably due to its invasion by inflammatory cells (Pretolani *et al.*, 1988).

In conclusion, our results suggest that the booster injection to a pre-sensitized animal will determine the relative efficacy of a PAF antagonist against bronchoconstriction. As a corollary, potential allergic drugs should be tested in guinea-pigs sensitized according to very precise protocols, including boosted guinea-pigs, particularly since in clinical situations, atopic patients are repeatedly exposed to the allergen.

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NaF and guanine nucleotides modulate adenylate cyclase activity in NG108-15 cells by interacting with both G_s and G_i

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1 NaF (10 mM) produced a 2–3 fold increase in adenylate cyclase activity in homogenates of NG108-15 cells incubated in the presence of 1 μM GTP. Higher concentrations of NaF suppressed adenylate cyclase activity.

2 In the presence of the adenosine receptor agonist 5'-(N-ethyl)-carboxamidoadenosine (NECA; 100 μM) or the prostacyclin receptor agonist iloprost (10 nM), NaF produced a much smaller increase in adenylate cyclase activity, whereas in the presence of a saturating concentration of iloprost (1 μM), NaF only inhibited adenylate cyclase activity.

3 Similarly, Gpp(NH)p activated basal adenylate cyclase activity, and inhibited 1 μM iloprost-activated enzyme activity. In the presence of 10 μM forskolin, NaF or Gpp(NH)p increased adenylate cyclase activity synergistically. Analysis of concentration-effect curves indicated that NaF (2 mM) or Gpp(NH)p (100 μM) increased the potency with which forskolin activated adenylate cyclase, whilst reducing the maximum activation of adenylate cyclase by iloprost.

4 Opiate receptors mediate inhibition of adenylate cyclase, and the opiate agonist morphine (100 μM) reduced the capacity of NaF or Gpp(NH)p to inhibit iloprost-activated adenylate cyclase. Unexpectedly, pertussis toxin treatment enhanced the ability of NaF or Gpp(NH)p to inhibit iloprost-activated adenylate cyclase.

5 In the absence of GTP, NaF and Gpp(NH)p remained able both to activate basal adenylate cyclase and to be synergistic with forskolin in activating the enzyme. In contrast the ability of NaF and Gpp(NH)p to inhibit iloprost-activated adenylate cyclase was substantially lost in the absence of added GTP.

6 These results suggest that NaF modulates adenylate cyclase activity in NG108-15 cell membranes by interacting with the α subunits of both G_s and G_i regulatory proteins. The effects of NaF and Gpp(NH)p are critically dependent on the prior mode and extent of activation or inhibition of this transmembrane signalling pathway. This simple system may be of use in assessing alterations in G_s-G_i interaction following manipulations such as hormone receptor desensitization.

Introduction

An understanding of the mechanisms underlying the regulation of adenylate cyclase (ATP: pyrophosphate-lyase (cyclising); EC 4.6.1.1) activity has been greatly increased by the identification of two guanine nucleotide binding regulatory proteins, or G-proteins, which couple hormone receptors to the adenylate cyclase enzyme (Levitzki, 1987; Casey & Gilman, 1988). These proteins are named G_s and G_i and the former mediates stimulation of adenylate cyclase, while the latter mediates suppression of enzyme activity. Both G_s and G_i are heterotrimers with α, β and γ subunits. $G_{s\alpha}$ has a molecular weight of ~45 kDa and $G_{i\alpha}$ ~40 kDa, although α subunits of other molecular weights have been documented (see Gilman, 1987; Spiegel, 1987; Neer & Clapham, 1988). In the presence of a stimulatory agonist, the binding of guanosine 5'-triphosphate (GTP) to $G_{s\alpha}$ leads to its dissociation from $G_{s\beta\gamma}$. The $G_{s\alpha}$·GTP complex then binds to, and activates the adenylate cyclase enzyme. The GTP bound to $G_{s\alpha}$ is then hydrolysed to GDP, and $G_{s\alpha}$ and $G_{s\beta\gamma}$ reassociate to terminate the activation of adenylate cyclase. For adenylate cyclase inhibition, the binding of GTP to $G_{i\alpha}$ again leads to subunit dissociation, but what then follows is less clear. Either $G_{i\alpha}$ inhibits adenylate cyclase directly, or the βγ subunit liberated from G_i binds to $G_{s\alpha}$ and reduces its ability to activate adenylate cyclase. Both processes may occur simultaneously, and at the present time the exact mechanisms are the subject of some controversy (see Birnbaumer, 1987).

Apart from receptor-mediated hormonal activation of adenylate cyclase, a number of non-hormonal agents are able to activate this enzyme. Forskolin can activate directly the catalytic subunit of adenylate cyclase (Seamon & Daly, 1986), while the fluoroaluminate ion AlF_4^- and GTP analogues such as Gpp(NH)p (5'-guanylimidodiphosphate) interact with G-proteins directly, which then leads to effector modulation (Londos *et al.*, 1974; Howlett *et al.*, 1979; Ross & Gilman, 1980; Sternweis *et al.*, 1981). The fluoroaluminate ion (normally formed by combination of fluoride ions from NaF with trace quantities of aluminium ions present in buffers) is thought to activate G-proteins by mimicking the γ-phosphate of GTP when GDP is bound to the α-subunit of the G-protein (Bigay *et al.*, 1985), but this remains to be proven for G-proteins other than transducin (see Chabre, 1989; Stadel & Crooke, 1989). Nevertheless, NaF has been very useful in detecting changes in adenylate cyclase function, such as those that may occur following heterologous desensitization of this effector system (Clark, 1986). In various tissues, heterologous desensitization of adenylate cyclase is commonly seen as a reduction in NaF-activated adenylate cyclase activity following prolonged hormone treatment (for reviews see Harden, 1983 and Clark, 1986). In some cases this may be due to an agonist-induced functional loss of $G_{s\alpha}$ protein from the cell membrane, apparently confirmed by a reduction in the cholera toxin-induced [^{32}P]-ADP-ribosylation of $G_{s\alpha}$ observed under these conditions (Garrity *et al.*, 1983;

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Edwards et al., 1987; Kelly et al., 1990). However, it should be noted that other mechanisms may underlie some forms of heterologous desensitization (Rich et al., 1984). It is known that NaF will activate all G-proteins and not just G_s, implying that changes in NaF-activated adenylate cyclase activity may not simply reflect changes in G_s function (Katada et al., 1984a,b). Furthermore, we have shown in a number of tissues, including the NG108-15 hybrid cell line (Kelly et al., 1990) and human platelets (Edwards et al., 1987), that NaF produces a bell-shaped concentration-effect curve for adenylate cyclase activation, stimulating at lower concentrations, and inhibiting enzyme activity at higher concentrations. In the present study, we have investigated the effects of NaF and guanine nucleotides on adenylate cyclase activity in homogenates of NG108-15 cells under different conditions, in an attempt to determine whether the effects of these agents on adenylate cyclase were due to activation of different G-proteins. Our results indicate that Gpp(NH)p and NaF both activate via G_s and inhibit via G_i. In addition NaF appears to have an inhibitory effect on adenylate cyclase that is unrelated to G-protein activity.

Methods

Cell culture

Cells of the NG108-15 and NCB-20 neuroblastoma somatic hybrid cell lines were both derived by fusions of the 6-thioguanine-resistant clone of a mouse neuroblastoma (N18TG2). Fusions were made with (a) C6BU-1, a 5-bromodeoxyuridine-resistant clone of rat glioma to yield NG108-15, or (b) foetal Chinese hamster brain cells to yield NCB-20 (further details and references to these cells are given in MacDermot et al., 1979). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) foetal calf serum, and supplemented with 1 μM aminopterin, 100 μM hypoxanthine and 16 μM thymidine. Culture flasks (80 cm^2) were maintained at 37°C in a humidified atmosphere of 10% CO_2 and 90% air. Cells were harvested by agitation in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate buffered saline, and the resultant pellets frozen at −80°C until required.

Adenylate cyclase

Adenylate cyclase activity was measured as described previously (Salomon et al., 1974) with some modifications (Edwards et al., 1987). Cell pellets were thawed and disrupted in a glass Dounce homogeniser using a homogenisation buffer (25 mM Tris-HCl, 0.29 M sucrose, pH 7.4). Reaction mixtures of 100 μl contained 50 mM Tris-HCl pH 7.4, 5 mM magnesium chloride, 20 mM creatine phosphate disodium salt, 10 iu of creatine kinase, 1 mM adenosine 3':5'-cyclic monophosphate (cyclic AMP) sodium salt, 0.25 mM Ro20-1724 as a phosphodiesterase inhibitor, 1 mM [α-^{32}P]-ATP (2 μCi), 1 μM GTP and 200–400 μg of membrane protein. Reactions were incubated at 37°C for 15 min and then terminated by the addition of 800 μl of 6.25% (w/v) trichloroacetic acid. To each tube was added 100 μl of [8-^{3}H]-cyclic AMP (about 20,000 c.p.m.) and the reaction mixtures centrifuged at 4°C for 20 min at 800 g. The [^{32}P]-ATP and [^{32}P]-cyclic AMP were separated by a two step chromatographic procedure (Salomon et al., 1974) and the yield of [^{32}P]-cyclic AMP was corrected for losses on the columns by measurement of the recovery of [^{3}H]-cyclic AMP. Unless otherwise stated, each measurement within an assay was performed in duplicate.

Where applicable, the dose-response data for adenylate cyclase assays were analysed according to a logistic equation describing a single site and giving V_{max}, EC_{50} and n, the Hill coefficient. Statistical significance, where applicable, was determined by two-way analysis of variance or paired t tests. All experiments were performed on at least two separate occasions.

Materials

[8-^{3}H]-adenosine 3':5'-cyclic monophosphate (23.6 Ci $mmol^{-1}$) and [α-^{32}P]-ATP (40–50 Ci $mmol^{-1}$) were obtained from Amersham International. Ro20-1724 (4-(3-butoxy-4-methoxybenzyl)- 2-imidazolidinone) was a kind gift from Roche Products, and iloprost a generous gift from Schering AG Berlin. All other chemicals and drugs were obtained from Sigma Chemical Co. Ltd. or BDH Chemicals Ltd.

Results

Effect of NaF on adenylate cyclase activity

Increasing concentrations of NaF had a biphasic effect on adenylate cyclase activity in homogenates of NG108-15 cells incubated in the presence of 1 μM GTP (Figure 1). Lower concentrations of NaF (1–10 mM) activated adenylate cyclase with a maximum 2–3 fold increase in activity, whilst higher concentrations suppressed adenylate cyclase activity with a near complete abolition of activity at 100 mM NaF. The effect of NaF on hormone receptor-activated adenylate cyclase was then investigated. The prostacyclin receptor agonist iloprost and the adenosine receptor agonist 5'(N-ethyl)-carboxamidoadenosine (NECA) both activate adenylate cyclase activity in NG108-15 cells (Kelly et al., 1990). Saturating concentrations of these agonists (100 μM NECA and 1 μM iloprost) produce around 2–4 fold and 5–10 fold increases in adenylate cyclase activity, respectively (Figure 1a). In the presence of 100 μM NECA, NaF produced a smaller increase in adenylate cyclase activity than observed with NaF alone. Furthermore, in the presence of 1 μM iloprost, NaF only inhibited adenylate cyclase activity, albeit with a 'shoulder' in the inhibition curve (Figure 1a). Figure 1b shows the effect of NaF in the presence of selected concentrations of iloprost. In contrast to the NaF-dependent inhibition of adenylate cyclase in the presence of a saturating concentration of iloprost, in the presence of a low (10 nM) concentration of iloprost, NaF produced only a small increase in enzyme activity. In all cases, very high concentrations of NaF inhibited adenylate cyclase activity below basal levels. The data in Figure 1a were submitted to 2-way analysis of variance, which showed that the three

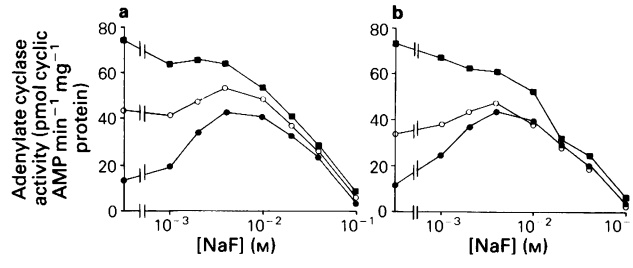

Figure 1 Effect of increasing concentrations of NaF on adenylate cyclase activation in NG108-15 cell homogenates under different conditions of enzyme activation. (a) NaF was added alone (●), in the presence of 100 μM 5'-(N-ethyl)- carboxamidoadenosine (○), or in the presence of 1 μM iloprost (■). (b) NaF was added alone (●), or in the presence of 10 nM (○) or 10 μM (■) iloprost. GTP (1 μM) was present throughout. The full experiment was repeated on one further occasion with the same result. In six separate experiments, enzyme activity measured in the presence of 1 μM iloprost but in the absence of NaF was 75.2 ± 5.4 pmol cyclic AMP $min^{-1} mg^{-1}$ protein (mean ± s.e.mean). This result was significantly different ($P < 0.05$, paired t test) from activities measured in the presence of NaF at the following concentrations: 1 mM NaF (66.8 ± 6.2), 2 mM NaF (63.8 ± 5.6), 4 mM NaF (62.3 ± 5.9), 10 mM NaF (50.8 ± 5.1) and 20 mM NaF (37.0 ± 4.2). It should be noted that activities at 1 and 2 mM NaF were not significantly different from each other, suggesting a complex or biphasic inhibitory curve.

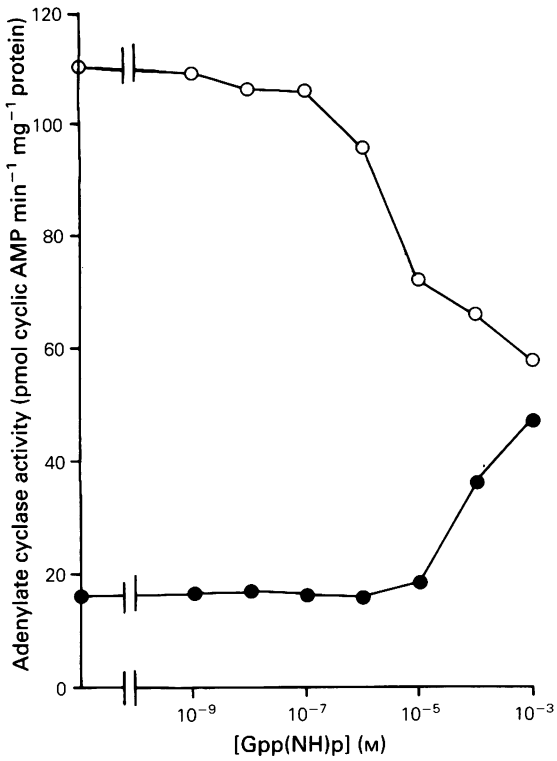

Figure 2 Effect of increasing concentrations of Gpp(NH)p on basal (●) or 1 μM iloprost (○)-activated adenylate cyclase activity in an NG108-15 cell homogenate. GTP (1 μM) was present throughout. This experiment was repeated on one further occasion with similar results.

curves were significantly different from each other ($P < 0.05$). Details of the t tests relating to individual points from six separate experiments are given in the figure legend.

Effect of Gpp(NH)p on adenylate cyclase activity

Gpp(NH)p produced a concentration-dependent increase in basal activity, and inhibited adenylate cyclase activity in the presence of 1 μM iloprost (Figure 2). The results in Figure 2 were analysed according to a logistic equation describing a single site. The EC_{50} for Gpp(NH)p activation of basal adenylate cyclase was 67.4 ± 7.6 μM, and the IC_{50} for Gpp(NH)p inhibition of iloprost-stimulated enzyme activity was 3.1 ± 1.1 μM (means ± s.e. of calculated parameters). A similar estimation for NaF was difficult due to the 'shoulder' observed for inhibition of iloprost-activated adenylate cyclase (Figure 1).

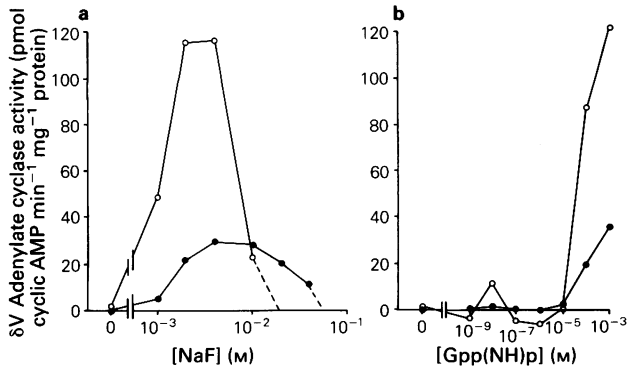

Figure 3 Effect of increasing concentrations of (a) NaF or (b) Gpp(NH)p on basal (●) or 10 μM forskolin (○)-activated adenylate cyclase activity in NG108-15 cell homogenates. Enzyme activation is represented as the increase in adenylate cyclase activity over levels in the absence of NaF or Gpp(NH)p (δV). In experiment (a), adenylate cyclase activity in the absence of NaF or Gpp(NH)p was 13.4 and 243.6 pmol cyclic AMP min^{-1} mg^{-1} protein under basal or 10 μM forskolin-activated conditions respectively. The dashed lines indicate where δV had become negative at high concentrations of NaF. These are not shown in full for clarity. In experiment (b), activity in the absence of NaF or Gpp(NH)p was 16.4 and 310.1 pmol cyclic AMP min^{-1} mg^{-1} protein under basal or 10 μM forskolin-activated conditions. GTP (1 μM) was present throughout. This experiment was repeated on one further occasion with similar results.

Effect of NaF and Gpp(NH)p on forskolin- and iloprost-stimulated adenylate cyclase activity

The diterpene compound forskolin (10 μM) increased adenylate cyclase activity by around 20 fold. In the presence of 10 μM forskolin, the increase in adenylate cyclase activity due to NaF or Gpp(NH)p was much greater than that observed in the absence of forskolin (Figure 3). In other words, forskolin and NaF or Gpp(NH)p activated adenylate cyclase synergistically. However, as observed previously, high concentrations of NaF virtually abolished forskolin-activated adenylate cyclase activity. In further experiments, the effect of single concentrations of NaF and Gpp(NH)p on the concentration-effect curves for adenylate cyclase activation by forskolin and iloprost were examined (Figure 4). The concentrations of NaF (2 mM) and Gpp(NH)p (100 μM) were chosen because these concentrations are able to activate or inhibit adenylate cyclase under different conditions (see Figures 1 and 2). These experiments revealed the contrasting effects of NaF or Gpp(NH)p on adenylate cyclase activation by forskolin and iloprost. Thus 2 mM NaF or 100 μM Gpp(NH)p appeared to enhance the potency with which forskolin activated adenylate cyclase, but markedly inhibited the maximum increase in iloprost-stimulated activity without changing iloprost potency (Figure 4). Also NaF, but not Gpp(NH)p, inhibited the response to high concentrations of forskolin (Figure 4a). The Hill coefficients for forskolin activation of adenylate cyclase activity were less than unity, consistent with the known effects of forskolin in this system, which involves interaction with both G_s and the adenylate cyclase molecule.

In order to assess whether NaF exerted a non-specific ionic action on the adenylate cyclase system, the effects of NaCl on basal, iloprost, and forskolin-activated adenylate cyclase were investigated. In each case, NaCl did not mimic the effects of NaF, although high concentrations of the salt slightly increased enzyme activity under the three different conditions (data not shown).

Inhibition of adenylate cyclase activity by NaF and Gpp(NH)p in the presence of morphine

Activation of opiate receptors inhibits enzyme activity in NG108-15 cells by 30–40% (Kelly *et al.*, 1990; also Figure 5). In the presence of iloprost and morphine, the inhibitory effects of NaF, or Gpp(NH)p were not additive (Figure 5), suggesting that morphine, NaF and Gpp(NH)p utilise at least partially the same mechanism to effect adenylate cyclase inhibition. It should be noted, however, that morphine did not prevent the inhibition of adenylate cyclase by high concentrations of NaF (Figure 5a). To investigate further the interaction of morphine and NaF, we examined the ability of a single concentration of morphine (100 μM) to inhibit adenylate cyclase activity due to increasing concentrations of iloprost or NaF. The results (Figure 6) indicated that morphine-dependent inhibition of iloprost-activated adenylate cyclase is constant irrespective of iloprost concentration, whereas morphine-dependent inhibition of NaF-activated adenylate cyclase decreases with increasing NaF concentration. We also found that morphine-dependent inhibition of Gpp(NH)p-activated adenylate cyclase decreases with increasing Gpp(NH)p concentration (data not shown). These results support the

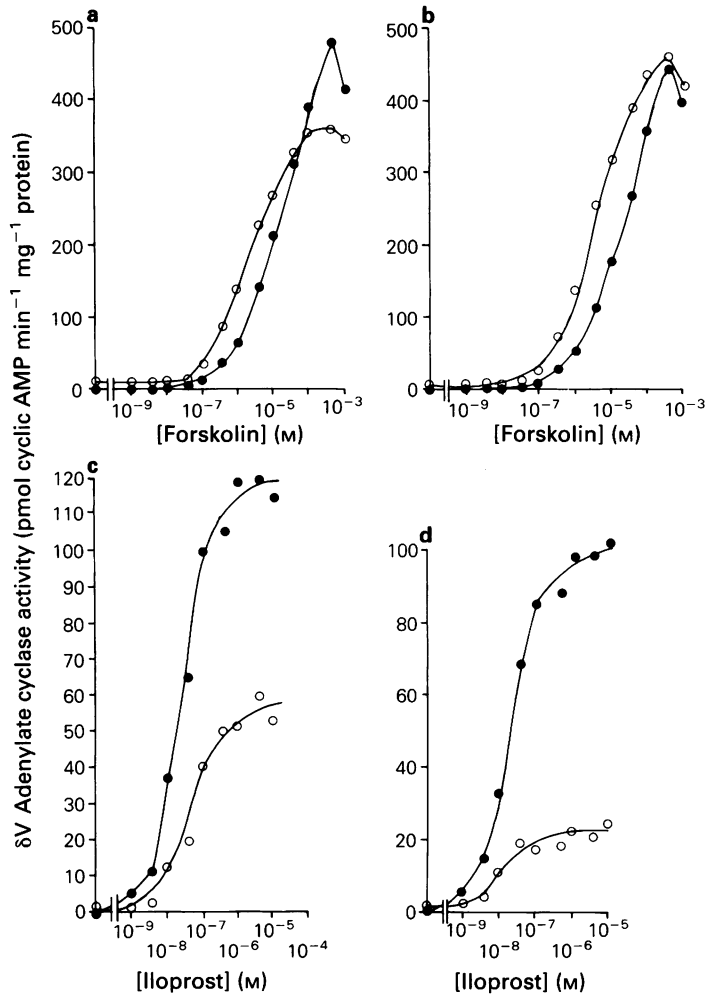

Figure 4 Effect of increasing concentrations of forskolin (a and b) or iloprost (c and d) on adenylate cyclase activity in homogenates of NG108-15 cells measured in the absence (●) or presence (○) of 2 mM NaF (a and c), or in the absence (●) or presence (○) of 100 μM Gpp(NH)p (b and d). GTP (1 μM) was present throughout. Enzyme activation is represented as the increase in adenylate cyclase activity over levels in the presence or absence of NaF or Gpp(NH)p alone (δV). In general 2 mM NaF or 100 μM Gpp(NH)p alone produced a 2–4 fold increase in basal adenylate cyclase activity. Curve parameters for this experiment were estimated as follows: (a) forskolin $EC_{50} = 13 \pm 3$ μM and n (Hill coefficient) $= 0.74 \pm 0.09$, forskolin + NaF $EC_{50} = 2 \pm 1$ μM and $n = 0.75 \pm 0.03$; (b) forskolin $EC_{50} = 18 \pm 4$ μM and $n = 0.73 \pm 0.08$, forskolin + Gpp(NH)p $EC_{50} = 3 \pm 1$ μM and $n = 0.79 \pm 0.06$; (c) iloprost $EC_{50} = 27 \pm 4$ nM and $n = 1.02 \pm 0.13$, iloprost + NaF $EC_{50} = 52 \pm 11$ nM and $n = 1.03 \pm 0.19$; (d) iloprost $EC_{50} = 19 \pm 2$ nM and $n = 1.06 \pm 0.10$, iloprost + Gpp(NH)p $EC_{50} = 11 \pm 4$ nM and $n = 1.00 \pm 0.31$ (all values are means ± s.e. of parameters calculated). The data in (c) and (d) were also analysed without prior subtraction of 'basal' enzyme activity. In these circumstances, the maximum enzyme activity in the presence of saturating iloprost concentrations was still reduced as shown. In (c), maximum enzyme activity was 134 ± 10 and 103 ± 5 pmol cyclic AMP $min^{-1} mg^{-1}$ protein in the absence or presence respectively of 2 mM NaF. In (d), maximum enzyme activity was 115 ± 6 and 65 ± 3 pmol cyclic AMP $min^{-1} mg^{-1}$ protein in the absence or presence of 100 μM Gpp(NH)p. Similar values were obtained in a repeat experiment.

suggestion that NaF and morphine activate a common inhibitory pathway in the regulation of adenylate cyclase activity.

Effects of pertussis toxin

The effects of pertussis toxin treatment of the NaF- and Gpp(NH)p-mediated inhibition of iloprost-activated adenylate cyclase were examined. These results are displayed both as absolute inhibition and % inhibition of iloprost activation (Figure 7). First, pertussis toxin treatment of NG108-15 cells (50 ng ml^{-1} medium; 17 h) markedly enhanced iloprost-activated adenylate cyclase by up to 3 fold (basal enzyme activity in the presence of 1 μM GTP was similarly increased). Secondly, pertussis toxin treatment magnified the inhibitory capacity of NaF and Gpp(NH)p, as seen both with absolute levels of adenylate cyclase activity and with % inhibition of iloprost-stimulated adenylate cyclase activity (Figure 7). The kinetics of this effect were also examined in homogenates of control and pertussis toxin-treated cells. The accumulation of cyclic AMP was linear in the presence of iloprost (1 μM) between 0 and 15 min, in both control and toxin-treated cells (Figure 8). The rate of increase was much greater following toxin treatment. Addition of 100 μM Gpp(NH)p inhibited iloprost-activated adenylate cyclase to a much greater extent in toxin-treated cells. There was also a lag observed before the onset of Gpp(NH)p-mediated inhibition of about 2 min in control cells, which was apparently unchanged following pertussis toxin treatment (Figure 8).

Finally, we examined the effect of NaF and Gpp(NH)p on adenylate cyclase activity in NG108-15 membranes in the absence of GTP (Figure 9). NaF and Gpp(NH)p increased basal and forskolin-activated enzyme activity irrespective of whether GTP was present or not (compare Figure 9 with Figures 1 and 3). Conversely, Gpp(NH)p-mediated inhibition of adenylate cyclase activity in the presence of 1 μM iloprost was virtually abolished in the absence of GTP, as was inhibition of this activity by low concentrations of NaF (up to

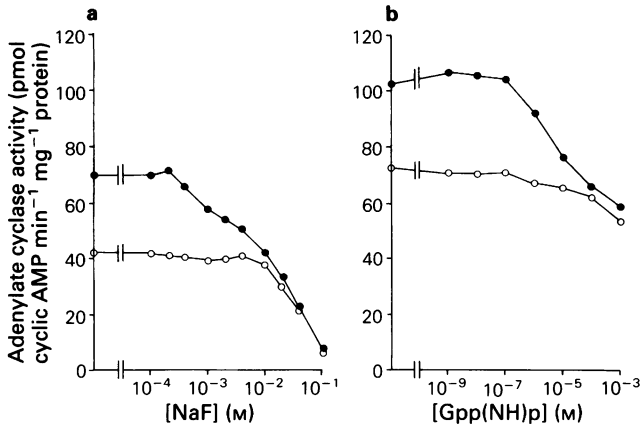

Figure 5 Effect of increasing concentrations of (a) NaF or (b) Gpp(NH)p on either 1 μM iloprost-activated adenylate cyclase activity (●) or 1 μM iloprost plus 100 μM morphine-activated adenylate cyclase activity (○) in homogenates of NG108-15 cells. GTP (1 μM) was present throughout. This experiment was repeated on one further occasion with similar results.

10 mM). However, very high concentrations of NaF were still unable to inhibit adenylate cyclase activity under these conditions.

Discussion

In NG108-15 cell homogenates, NaF produced a bell-shaped concentration-effect curve with relation to adenylate cyclase activity. This characteristic response was modified when stimulatory hormone receptors were co-activated, and in a manner dependent upon the extent of hormonal activation. In the presence of saturating concentrations of NECA or iloprost, which produced about 3 fold and 7 fold increases in adenylate cyclase activity, NaF now produced either a much smaller increase in, or inhibition of enzyme activity respectively. However, these divergent responses observed with different agonists were not due to a qualitative difference in

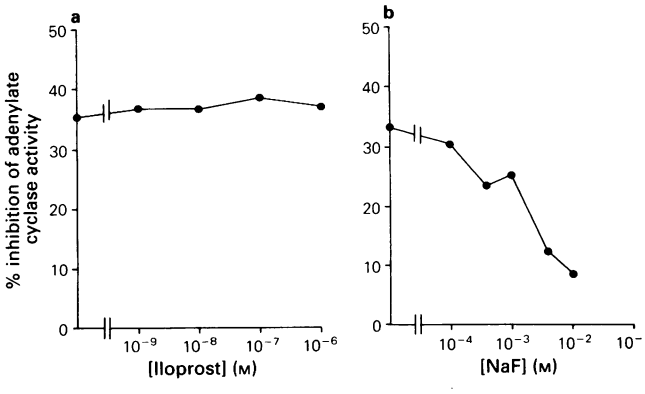

Figure 6 The inhibitory effect of 100 μM morphine on adenylate cyclase activity activated by inreasing concentrations of (a) iloprost or (b) NaF. The effect of morphine is represented as % inhibition of total adenylate cyclase activity in the presence of a particular concentration of iloprost or NaF. GTP (1 μM) was present throughout. In (a), actual enzyme activities due to selected iloprost concentrations in the absence or presence of 100 μM morphine respectively were as follows: no iloprost (basal) 23.9 and 15.3, 1 nM iloprost 27.4 and 17.2, 10 nM iloprost 50.4 and 31.5, 100 nM iloprost 132.9 and 81.8, 1 μM iloprost 163.3 and 102.7 pmol cyclic AMP min^{-1} mg^{-1} protein. Corresponding values for (b) in the presence of increasing NaF concentrations were: no NaF (basal) 12.8 and 8.6, 0.1 mM NaF 12.2 and 8.5, 0.4 mM NaF 12.4 and 9.5, 1 mM NaF 19.1 and 14.4, 4 mM NaF 45.0 and 39.5, and 10 mM NaF 42.9 and 38.1 pmol cyclic AMP min^{-1} mg^{-1} protein. This experiment was repeated with similar results.

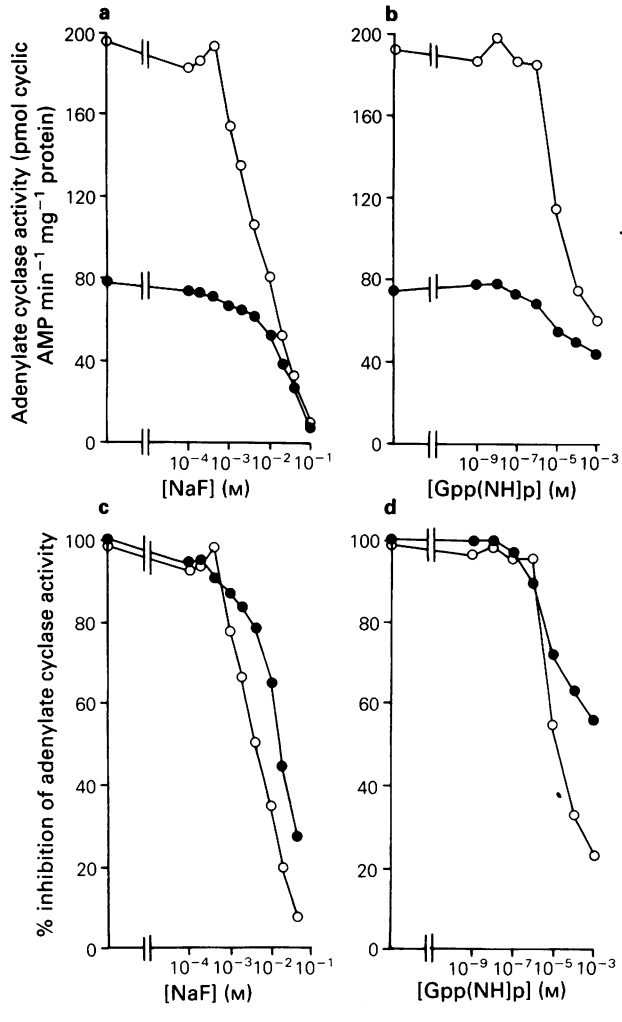

Figure 7 Effect of increasing concentrations of NaF (a and c) or Gpp(NH)p (b and d) on 1 μM iloprost-activated adenylate cyclase activity in homogenates of NG108-15 cells that had been preincubated in the absence (●) or presence (○) of pertussis toxin (50 ng ml^{-1}) for 17 h. GTP 1 μM was present throughout. In (a) and (b) absolute levels of adenylate cyclase activity are shown whereas (b) and (d) show % inhibition of activity in the presence of iloprost, using data transformed from (a) and (b). This experiment was repeated with similar results.

receptor type, but rather to a quantitative difference in the extent of adenylate cyclase activation, since in the presence of a low concentration of iloprost, having an equivalent effect on enzyme activity as the saturating concentration of NECA, an equivalent small increase in adenylate cyclase activity was observed when NaF was included. This indicates that NaF and hormonal activation of adenylate cyclase are not additive, and apparently utilise the same G_s-adenylate cyclase pool in the membrane. When hormonal activation produced high levels of adenylate cyclase activity, as with saturating iloprost concentrations, NaF inhibited enzyme activity. Such an inhibitory effect has been detected in other tissues (Manganiello & Vaughan, 1976; Katada *et al.*, 1984a,b). If NaF is modulating adenylate cyclase by interacting with both G_s and G_i, then under conditions where G_s activity is already high, the more pronounced effect of NaF would be G_i activation with consequent enzyme inhibition. Clearly, this interpretation does not exclude the possibility that the inhibitory effect of NaF might be mediated by protein(s) other than G_i. NaF-dependent inhibition of adenylate cyclase is not observed under basal conditions since NaF would also be interacting with a large and relatively inactive pool of G_s, and consequently stimulation is observed. Interestingly, the inhibition of adenylate cyclase by NaF appeared to be biphasic (Figure 1). This could indicate

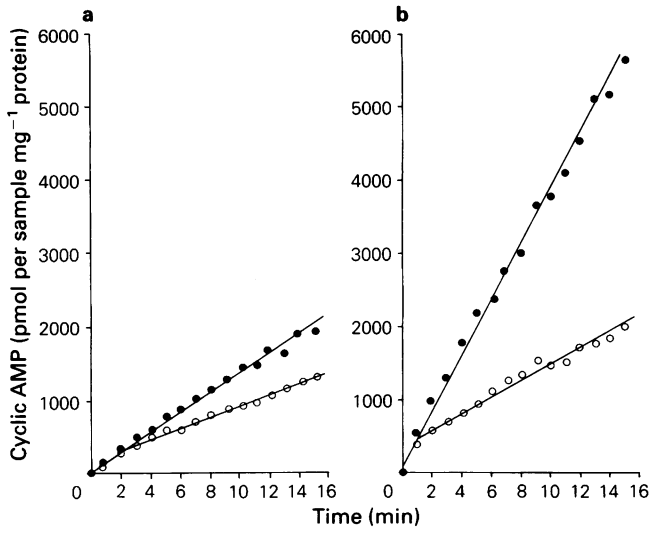

Figure 8 Time course of 1 μM iloprost-activated increase in [^{32}P]-cyclic AMP formation in (a) control and (b) pertussis toxin-treated NG108-15 cells. Incubations were performed in the absence (●) or presence (○) of 100 μM Gpp(NH)p. Each point represents a single determination and 1 μM GTP was present throughout. Two further repeats of this experiment produced similar results.

the involvement of two different mechanisms, or more simply that even in the presence of a saturating concentration of iloprost, NaF remains able to interact with a small fraction of G_s that has remained inactive, thus explaining the apparent 'shoulder' in the inhibition curve.

NaF probably interacts with G-proteins by mimicking the γ-phosphate of GTP when GDP is bound to the α-subunit (Bigay *et al.*, 1985). Poorly hydrolysed guanine nucleotides such as Gpp(NH)p also interact with the α-subunits of G-proteins in an essentially irreversible manner and lead to subunit dissociation (Gilman, 1987). Therefore we postulated

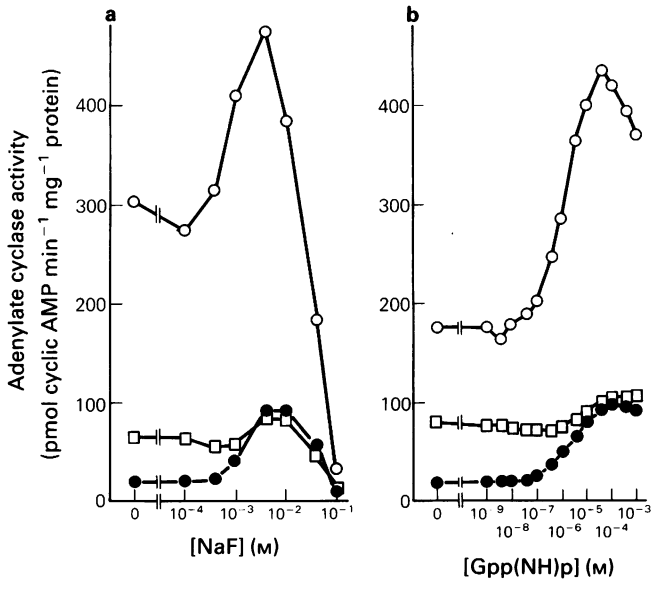

Figure 9 Effect of increasing concentrations of NaF (a) or Gpp(NH)p (b) on basal (●), 1 μM iloprost-activated (□) or 10 μM forskolin-activated (○) adenylate cyclase activity in NG108-15 cell membranes incubated in the absence of GTP. To remove endogenous GTP, membranes were washed three times by spinning at 80,000 *g* for 20 min and resuspended in 50 mM Tris-HCl pH 7.4. This experiment was repeated with the same result. Reference to Figures 1 and 2 indicates the differing effects of NaF and Gpp(NH)p on iloprost-activated enzyme activity in the presence (Figures 1 and 2) or absence (Figure 9) of GTP.

that if both NaF and Gpp(NH)p activated G-proteins by interacting with the α-subunits, then they should have the same effect on adenylate cyclase activity in cell membranes. This was largely confirmed by our finding that Gpp(NH)p activated basal and inhibited iloprost-activated adenylate cyclase, indicating the likelihood that NaF, like Gpp(NH)p, can activate G_s and G_i in this system. However, some differences were apparent in the characteristics of adenylate cyclase modulation by these two agents. Firstly, Gpp(NH)p did not produce a bell-shaped concentration-effect curve for adenylate cyclase activation. This could be because high enough concentrations of the GTP analogue were not employed, or more probably that the inhibition of adenylate cyclase seen at high NaF concentrations is unrelated to G_i function. Secondly, inhibition of adenylate cyclase by Gpp(NH)p occurred at concentrations around 10 fold lower than activation, and no 'shoulder' was observed in the inhibition curve. This latter may again relate to factors other than G-protein activation, whilst the former may reflect the different affinities of G_s and G_i for guanine nucleotide (Jakobs *et al.*, 1985). Interestingly, no obvious difference was observed in the concentrations of NaF that activated or inhibited adenylate cyclase (see Figure 1). However, if the fluoraluminate ion mimics the γ-phosphate of GTP, then its interaction with $G_{s\alpha}$ and $G_{i\alpha}$ would depend upon GDP which is already bound, thus masking any differences in the relative affinities of these proteins for guanine nucleotides.

The interaction of NaF and Gpp(NH)p with forskolin-activated adenylate cyclase indicated a synergistic interaction between these non-hormonal agents. Forskolin is a direct activator of the adenylate cyclase enzyme, but also potentiates hormonal activation of the enzyme by a mechanism involving the G_s protein (Seamon & Daly, 1986). In a number of tissues, Gpp(NH)p inhibits forskolin-activated adenylate cyclase activity (Seamon & Daly, 1982; Jakobs *et al.*, 1983). However, in NG108-15 cell membranes, both NaF and Gpp(NH)p were clearly synergistic with forskolin in activating adenylate cyclase. This was confirmed by constructing full concentration-effect curves for forskolin-activation of adenylate cyclase in the presence or absence of NaF or Gpp(NH)p, revealing that both agents enhanced the potency of forskolin. This effect contrasted markedly with the interaction between NaF or Gpp(NH)p and the iloprost concentration-effect curve. Both NaF and Gpp(NH)p reduced the maximal activation by iloprost, whilst having no apparent effect on the potency of iloprost. These differences may relate to the different sites of action of iloprost and forskolin, that is, hormone receptor and G_s-adenylate cyclase. It is of interest that NaF and Gpp(NH)p increase high affinity [^{3}H]-forskolin binding in rat brain membranes (Seamon & Daly, 1985), thus providing a possible explanation for the functional synergism we have observed. NaF and Gpp(NH)p were synergistic with forskolin, but inhibitory with saturating iloprost concentrations. This is of interest, since both iloprost and forskolin increase adenylate cyclase activity. The most straightforward rationalization of this result would be that iloprost activates the enzyme by promoting the coupling of $G_{s\alpha}$ to adenylate cyclase, whereas forskolin, in the absence of hormone, probably activates adenylate cyclase almost exclusively by a direct interaction with the enzyme, thus leaving open the possibility for activation of G_s by NaF or Gpp(NH)p.

In further attempts to confirm the specificity of action of NaF and Gpp(NH)p on G-proteins, we reasoned that in the presence of an inhibitory receptor agonist such as morphine, the extent of NaF- and Gpp(NH)p-mediated inhibition of adenylate cyclase activity should be attenuated, if all are ultimately utilising the G_i protein. This was found to be the case, suggesting that morphine and the two non-hormonal agents do indeed inhibit adenylate cyclase by a common mechanism involving G_i. Interestingly, morphine did not prevent the inhibition seen at high NaF concentrations (above 10 mM), indicating again the likelihood that this phase of inhibition relates neither to G_i nor to a non-specific ionic effect. The involve-

ment of G_i in NaF-mediated inhibition was further investigated by examining the % inhibition of adenylate cyclase by morphine under conditions of increasing NaF concentration. This revealed a reciprocal relationship between NaF concentration and adenylate cyclase inhibition by morphine, again implicating a common G_i pathway. In contrast to this, the % inhibition of adenylate cyclase by morphine in the presence of increasing iloprost concentrations was constant, which might be expected since iloprost activates the G_s and not the G_i pathway.

Pertussis toxin blocks receptor- and GTP-mediated inhibition of adenylate cyclase activity by ADP-ribosylating the $G_{i\alpha}$ protein (Ui, 1984). If NaF and Gpp(NH)p are inhibiting adenylate cyclase via G_i, then pertussis toxin might be expected to alter their ability to do so by covalently modifying $G_{i\alpha}$. Pertussis toxin treatment markedly increased basal and iloprost-activated adenylate cyclase activity in NG108-15 cell homogenates, consistent with a functional loss of G_i activity (Katada *et al.*, 1982). However, unexpectedly, pertussis toxin treatment greatly enhanced the capacity of NaF and Gpp(NH)p to inhibit iloprost-activated adenylate cyclase activity. It is not easy to assess how covalent modification of $G_{i\alpha}$, which blocks receptor-mediated inhibition of adenylate cyclase by over 70% (data not shown), could lead to an increase in the inhibitory capacity of a non-hydrolysable GTP analogue. A similar effect has been observed previously in C6 glioma cells (Katada *et al.*, 1982). However, the change may relate to the functional loss of GTP activity at the ADP-ribosylated G-protein rather than a real increase in Gpp(NH)p activity. In other systems, pertussis toxin treatment blocks receptor- and GTP-mediated inhibition of adenylate cyclase, but inhibition by non-hydrolysable GTP analogues remains intact (Jakobs *et al.*, 1983). In our control membranes GTP tonically activates G_i, but is unable to do so after toxin treatment, which leads to enhanced basal and iloprost-activated adenylate cyclase activity. Since Gpp(NH)p appears to activate G_i irrespective of whether or not the α-subunit is ADP-ribosylated, the greater inhibition mediated by Gpp(NH)p after pertussis toxin (Figure 7b and d) probably only reflects that the GTP in the assay system is unable to do so. These results suggest that in the whole cell, where $G_{i\alpha}$ has access to GTP, G_i may tonically inhibit adenylate cyclase. In these circumstances, the significance of relatively small receptor-mediated inhibition of adenylate cyclase exerted by some hormones is not easily explained. Although in some other tissues, pertussis toxin pretreatment does not prevent adenylate cyclase inhibition by stable GTP analogues, it does markedly increase the lag phase before the onset of inhibition (Jakobs *et al.*, 1983). However, we were unable to observe any increase in the lag phase before the onset of Gpp(NH)p-mediated inhibition in the presence of pertussis toxin; both before and after toxin, the lag was around 2 min. This indicates the likelihood of differences in the kinetics of G-protein activation between different tissues.

Finally, the effect of GTP itself on the modulation of adenylate cyclase by NaF and Gpp(NH)p was examined. These agents activated adenylate cyclase under basal conditions or in the presence of forskolin irrespective of whether GTP was present or not. Inspection of Figure 9 shows that Gpp(NH)p and low concentrations of NaF have little or no capacity to inhibit adenylate cyclase activity in the absence of GTP. A possible explanation is that the activity of G_i is being masked by the greater effect of G_s. In the presence of iloprost and GTP, NaF or Gpp(NH)p have little further effect on G_s-stimulation of adenylate cyclase activity and, consequently, inhibition is observed.

In conclusion, we have demonstrated that NaF and Gpp(NH)p have multiple effects on adenylate cyclase activity in NG108-15 homogenates. Both agents can activate or inhibit enzyme activity depending upon the assay conditions employed, and these effects appear to relate to interactions with G_s and G_i respectively. A further inhibition of adenylate cyclase activity occurred with concentrations of NaF greater than 10 mM, but this is most probably unrelated to G_i activity. In a situation where NaF or GTP analogues are employed to assess the integrity of a G-protein-effector system, caution should be observed since these agents will in fact reflect the integrity of all G-proteins functionally linked to a particular effector. For instance, a reduction in NaF- or Gpp(NH)p-activated adenylate cyclase activity in a tissue following prolonged agonist treatment may reflect a functional loss of G_s, or a functional increase in G_i (Rich *et al.*, 1984), or both together. Under carefully defined conditions, the use of non-hormonal G-protein activators could be important in assessing G_i function more directly than by using hormone agonists, or to investigate possible 'cross-talk' between G-proteins reciprocally coupled to a common effector. Furthermore, these agents could be used to detect novel G-proteins that inhibit a particular effector, as appears to be the case for phospholipase C (Godfrey & Watson, 1988; Whitworth & Kendall, 1989). Apart from this, NaF and GTP analogues will remain vital tools in any attempt to understand the regulation of G-proteins coupled processes.

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Co-release of PHI and VIP in dog stomach by peripheral and central vagal stimulation

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1 The purpose of this investigation was to examine the outputs of peptide histidine isoleucine (PHI) and vasoactive intestinal peptide (VIP) from the gastric corpus in response to stimulation of the peripheral (PVS) and central (CVS) ends of the vagus nerve and to compare the effects of PHI on gastric motility and blood flow with those of VIP in atropine-treated dogs.

2 PVS and CVS caused parallel increases in gastric venous plasma concentrations of VIP and PHI. The molar ratios of the peptides (VIP/PHI) appearing in the gastric venous plasma were about 1.5 for PVS at 10 Hz, 0.65 for PVS at 40 Hz and about 0.7 for CVS at 10 Hz.

3 The molar ratio of peptide concentration extracted from the gastric corpus was about 1.5 in the muscle layer and 2.7 in the mucosal layer.

4 Intra-arterially-injected PHI was about 5 fold and 50 fold less effective in producing gastric relaxation and vasodilatation, respectively, than VIP.

5 These results indicate that PHI is co-released with VIP by peripheral vagal stimulation and by initiation of the vago-vagal reflex, and that PHI seems to have an important role in the regulation of gastric motility but not gastric blood flow in the dog.

Introduction

Peptide histidine isoleucine (PHI) has a marked sequence homology with vasoactive intestinal peptide (VIP) (Tatemoto & Mutt, 1981). Also, it has been demonstrated that a prepro VIP, the VIP precursor, contains a PHI-related peptide (peptide histidine methionine) in its sequence, suggesting that VIP and PHI are co-synthesized in the same precursor molecule (Itoh *et al.*, 1983). Recent immunohistochemical studies have also provided evidence that the two peptides coexist in neurones of various tissues (Bishop *et al.*, 1984; Lundberg *et al.*, 1984a; Ekblad *et al.*, 1985; Holst *et al.*, 1987).

PHI has been shown to be co-released with VIP in response to parasympathetic nerve stimulation (Lundberg *et al.*, 1984b; Holst *et al.*, 1987; Yasui *et al.*, 1987). In particular, vagal stimulation releases PHI with VIP into the portal plasma of dogs (Yasui *et al.*, 1987) and into the perfusate from the pig pancreas (Holst *et al.*, 1987). PHI and VIP share several biological activities, such as stimulation of pancreatic secretion (Dimaline & Dockray, 1980; Holst *et al.*, 1987), smooth muscle relaxation (Lundberg *et al.*, 1984a; Bardrum *et al.*, 1986) and vasodilatation (Lundberg & Tatemoto, 1982; Lundberg *et al.*, 1984a; Suzuki *et al.*, 1984; Bardrum *et al.*, 1986). However, their relative potencies vary in different tissues.

We have recently shown that peripheral or central (vago-vagal reflex) vagal stimulation releases VIP into the venous effluent from the dog gastric corpus. We suggested that VIP output evoked by peripheral vagal stimulation mainly originated from peptidergic (VIP) vasodilator nerves in the submucous plexus (Ito *et al.*, 1988a), whereas that released by reflex vagal stimulation originated from peptidergic (VIP) neurones in the myenteric plexus (Ito *et al.*, 1988b). The aim of the present study was to investigate whether or not PHI is relased with VIP from the gastric corpus of the dog by peripheral and central vagal stimulation, and to compare the effects of PHI and VIP on the gastric motility and blood flow in atropine-treated dogs.

Methods

Experimental procedure

Twenty-three adult dogs of either sex weighing between 8 and 13 kg were used after overnight fasting. Dogs were anaesthetized with chloralose (50 mg kg^{-1}, i.v.) and urethane (100 mg kg^{-1}, i.v.) after induction with pentobarbitone (30 mg kg^{-1}, i.v.). Operative and experimental procedures were as previously described in detail (Ito *et al.*, 1988a). In brief, a tracheal cannula was inserted and polyethylene cannulae were inserted into the cephalic vein and the right femoral artery for systemic administration of drugs and for measurement of systemic blood pressure through a pressure transducer (Gold Stantham Inc., P23 IC), respectively. After the spleen and greater omentum had been removed, a thin polyethylene cannula was inserted retrogradely into a branch of the splenic artery for close arterial injection of drugs into the stomach. A 0.1 ml bolus of drugs dissolved in sodium phosphate buffered saline (PBS; sodium phosphate 10 mM, NaCl 150 mM, pH 7.4) was injected and then flushed in with 0.15 ml PBS. After heparinization (1,000 u kg^{-1}, i.v.), the left gastroepiploic-splenic vein was cannulated for measurement of gastric blood flow by a photo-electric drop counter (Sanei, 1321) and for collection of gastric venous blood. Venous flow was expressed as drops min^{-1} (55 μl per drop). The venous effluent was continuously returned to the external jugular vein by a peristaltic pump after warming at 37°C. To measure the arterial peptide concentration, arterial blood was collected from a branch of the left femoral artery. Lateral thoracotomy was performed by removing the 8th and 11th ribs and the animals were maintained with artificial ventilation. The peripheral and central cut ends of a communicating branch of the left vagus nerve to the dorsal vagus trunk in the thorax were stimulated electrically with bipolar silver ring electrodes. To record gastric motility, a balloon was introduced into the stomach via the mouth and was connected to a water reservoir mounted on a force displacement transducer. The intraballoon pressure was set at 5–10 cmH_2O. Rectal temperature was maintained at 37°C by a heating table. Experiments were performed 30–60 min after the preparatory surgery and 20–30 min after the intra-venous injection of atropine (1 mg kg^{-1}) or hexamethonium (15 mg kg^{-1}).

Measurement of plasma VIP and PHI

The concentrations of VIP and PHI in venous and arterial plasma were determined by radioimmunoassay. When the blood was collected, a corresponding amount of PBS was infused from the jugular vein to compensate for blood loss. In each animal, two or three tests were performed at about

30 min intervals and the sequence of the tests was randomized. The blood samples were collected for 1 min in ice-cold tubes containing aprotinin (Sigma, 800 u) and EDTA (Dojin, 12.5 μmol) and haematocrit was measured. Blood samples were centrifuged at 4°C and the plasma was extracted with a double amount of absolute ethanol. Following centrifugation (10,000 g) for 10 min, the extract was dried under vacuum and kept frozen at −20°C until assayed. Each sample was reconstituted in 40 mM sodium phosphate buffer (pH 7.4) at the original volume. Over 90% of the immunoreactive peptide in plasma was extracted, so that data were not corrected for recovery.

VIP (Protein Inc., Osaka, 0.6 nmol) was iodinated in $Na^{125}I$ (Amersham, 1 mCi) by the cholramine T method and the iodinated product was purified by ion exchange chromatography as described previously (Ito *et al.*, 1988a). PHI (Sigma, 3 nmol) was also iodinated in the same manner as VIP. The reaction products were applied to a CM Sephadex C25 column (1 × 10 cm) equilibrated with ammonium acetate (50 mM, pH 6.6) and then eluted by a linear gradient of equal volume (30 ml) of 50 mM and 500 mM ammonium acetate at a flow rate of 0.3 ml min^{-1}. The radioactive peak fraction was collected in a tube containing 500 u aprotinin. The iodinated peptides were then aliquoted and lyophilized.

Radioimmunoassay procedures were conducted by the method described previously (Ito *et al.*, 1986). The VIP antiserum (VK208) used recognized the middle and carboxyl terminal regions of a VIP molecule (Ito *et al.*, 1988a). The PHI antiserum (AE104, Cambridge Research Biochemicals) reacted with the C-terminal but not with the N-terminal. The VIP antiserum did not cross-react with PHI and vice versa. Standards contained 1.56 fmol synthetic porcine VIP or PHI at the lowest level and the concentration was doubled to 100 fmol at the highest level. The detection limit was 0.8 fmol 0.8 ml^{-1} for VIP and 2.0 fmol 0.8 ml^{-1} for PHI with 95% confidence. The respective inter- and intra-assay ($n = 5$) coefficients of variation were between 5% and 14% for VIP radioimmunoassay and 8% and 15% for PHI radioimmunoassay.

Tissue extraction and gel filtration

Tissue extraction was performed as described previously (Ito *et al.*, 1988c). In brief, dogs not used for nerve stimulation were killed by a rapid injection of pentobarbitone and then the gastric corpus was isolated and separated into mucosal and muscle layers. The isolated tissues were frozen with liquid nitrogen and weighed. Following 5 min of boiling in 0.5 M acetic acid, the sample was homogenized for 20 s with Physicotron (Nition, NS310E) and centrifuged for 3 min (10,000 *g*). The supernatant was aliquoted, lyophilized and stored at −20°C until assayed. The concentrations of immunoreactive peptide in the extracts were determined at 3 or 4 different dilutions. Venous plasma extracts were fractionated by chromatography on a Sephadex G50 superfine column (1 × 90 cm) eluted with 0.5 N acetic acid at a flow rate of 2.7 ml h^{-1}. The effluent collected for 20 min was divided into two and each elution position of immunoreactive VIP or PHI was determined.

Statistical analysis

The results are expressed as means ± s.e.mean. Regression analysis was carried out by the method of least squares. Statistical significance was assessed by Student's *t* test and *P* values less than 0.05 were considered to be statistically significant.

Results

PHI and VIP outputs evoked by peripheral vagal stimulation

The resting concentrations of gastric venous and arterial peptides were 57.0 ± 6.7 and 40.8 ± 6.0 pM ($n = 12$) for PHI and 29.2 ± 2.6 and 23.6 ± 2.8 pM ($n = 12$) for VIP, respectively, in atropine-treated dogs (1 mg kg^{-1}, i.v.). The mean resting VIP and PHI levels in gastric venous plasma were slightly higher than those in arterial plasma, although these values were not statistically different.

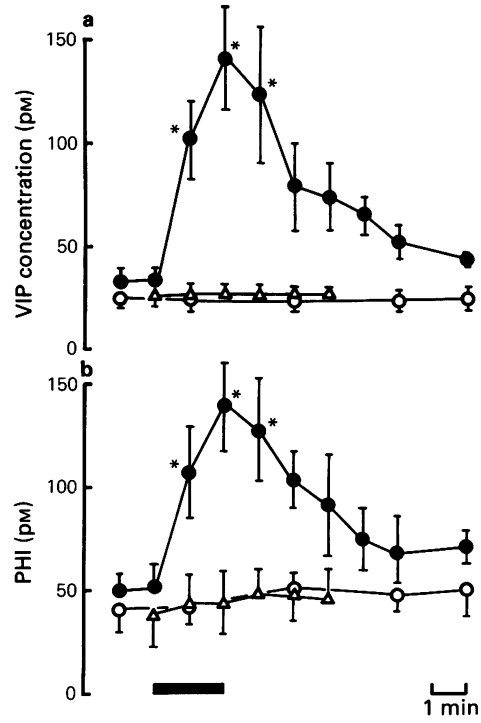

Figure 1 The effect of peripheral vagal stimulation on vasoactive intestinal peptide (VIP) (a) and peptide histidine isoleucine (PHI) (b) concentration in the plasma of the gastric vein (●) and femoral artery (○). (△) Gastric venous concentration of peptides after treatment with hexamethonium (15 mg kg^{-1}, i.v.). Peripheral vagal stimulation ($n = 6$, 10 Hz, 40 V, 0.5 ms) for 2 min (solid bar). Each point represents the mean and vertical lines show s.e.mean. $*P < 0.05$ when compared with the resting level.

The vagus nerve was stimulated for 2 min (10 Hz, 40 V, 0.5 ms) and gastric venous blood was collected continuously before and after the start of stimulation. Peripheral vagal stimulation elicited an increase in gastric blood flow and evoked gastric relaxation. As shown in Figure 1, it evoked a prominent increase in the venous plasma PHI as well as in VIP without any significant changes in the arterial concentration of either peptide. The concentrations of PHI and VIP in venous plasma increased within the first 1 min period of stimulation, attained a maximum in the next 1 min and returned to basal within 7 min after the end of stimulation. There was a statistically significant difference between the peak and resting concentrations of each peptide. These responses were inhibited by pretreatment with hexamethonium (15 mg kg^{-1}, i.v.). There was no statistically significant difference between the amounts of VIP and PHI released from the stomach in response to peripheral vagal stimulation at 10 Hz (Table 1). A positive correlation was obtained between the outputs of both peptides (Figure 3a), and the mean molar ratio of net VIP to PHI output was 1.5. Stimulation at 40 Hz evoked a gastric relaxation of similar magnitude to stimulation at 10 Hz, but it produced a less pronounced gastric vasodilatation and a smaller increase in the outputs of VIP and PHI than stimulation at 10 Hz. The mean molar ratio of VIP to PHI output decreased to 0.65 with stimulation at 40 Hz (Table 1).

PHI and VIP outputs evoked by central vagal stimulation

Central vagal stimulation for 2 min (10 Hz, 40 V, 0.5 ms) evoked slight vasodilatation and prominent gastric relaxation.

Table 1 The outputs of vasoactive intestinal peptide (VIP) and peptide histidine isoleucine (PHI) in response to peripheral (PVS) and central (CVS) vagal stimulation and the concentrations of peptides in the gastric corpus of the dog

			Peptide output (fmol for 7 min)		
		n	*VIP*	*PHI*	*VIP/PHI*
PVS	10 Hz	6	679.8 ± 133.1	536.0 ± 139.3	1.47 ± 0.20
	40 Hz	3	161.7 ± 27.2	247.0 ± 24.9	0.65 ± 0.05
CVS	10 Hz	6	146.7 ± 56.6	183.0 ± 43.0	0.71 ± 0.61
			Peptide concentration (pmol g^{-1} wet weight)		
		n	*VIP*	*PHI*	*VIP/PHI*
Muscle layer		5	300.9 ± 47.7	255.6 ± 60.4	1.51 ± 0.44
Mucosal layer		5	326.8 ± 94.7	122.9 ± 15.8	2.70 ± 0.61

The total peptide output from the gastric corpus was calculated as the veno-arterial difference in plasma peptide concentration multiplied by the gastric plasma flow, which was determined from a knowledge of blood flow and haematocrit. To estimate the amounts of vagal stimulation-induced release of the peptides into the venous effluent from the stomach, resting peptide outputs for 7 min were subtracted from the total peptide outputs for 2 min during stimulation and for 5 min after stimulation. We calculated the mean molar ratios (VIP/PHI) after determining the molar ratio of each individual dog. *n* = number of experiments.

PHI was released into the gastric venous plasma together with VIP in response to reflex vagal stimulation without any significant changes in arterial peptide levels. The time course of PHI output was similar to that of VIP (Figure 2). The concentrations of both peptides in the plasma in response to central vagal stimulation were significantly less than those evoked by peripheral vagal stimulation. These responses were inhibited by hexamethonium. There was a statistically significant difference between the resting and peak venous concentration for VIP but not PHI. Table 1 shows the output of both peptides from the stomach. A positive correlation was obtained between them (Figure 3b) and the mean molar ratio of VIP to PHI output was 0.71 (Table 1).

Metabolism of exogenous PHI in the gastric circulation

To examine the metabolism of PHI in the gastric circulation, porcine PHI at doses of 1 and 2 pmol was injected intra-arterially into the stomach. The amount of PHI in the venous plasma was measured continuously for 5 min after injection and resting PHI output for 5 min was subtracted from this. The amount of PHI recovered from the venous plasma was

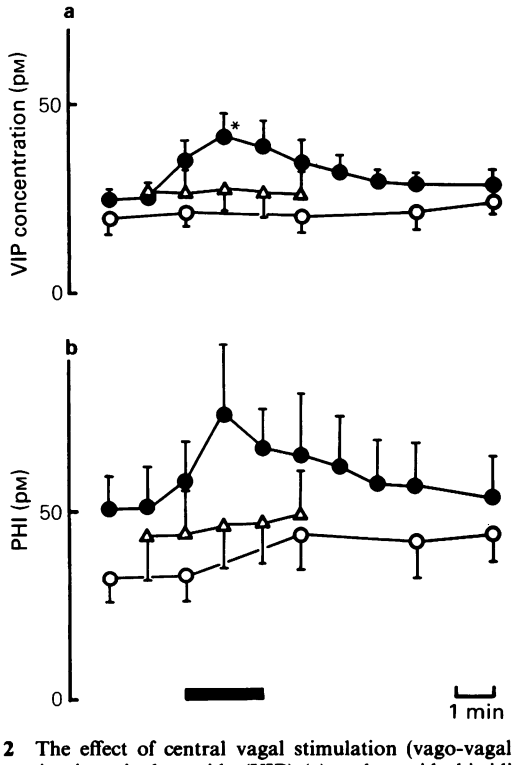

Figure 2 The effect of central vagal stimulation (vago-vagal reflex) on vasoactive intestinal peptide (VIP) (a) and peptide histidine isoleucine (PHI) (b) concentration in the plasma of the gastric vein (●) and femoral artery (○). (△) Gastric venous concentration of peptides after treatment with hexamethonium (15 mg kg^{-1}, i.v.). Central vagal stimulation (n = 6, 10 Hz, 40 V, 0.5 ms) for 2 min (solid bar). Each point represents the mean and vertical bars show s.e.mean. *$P < 0.05$ when compared with the resting level.

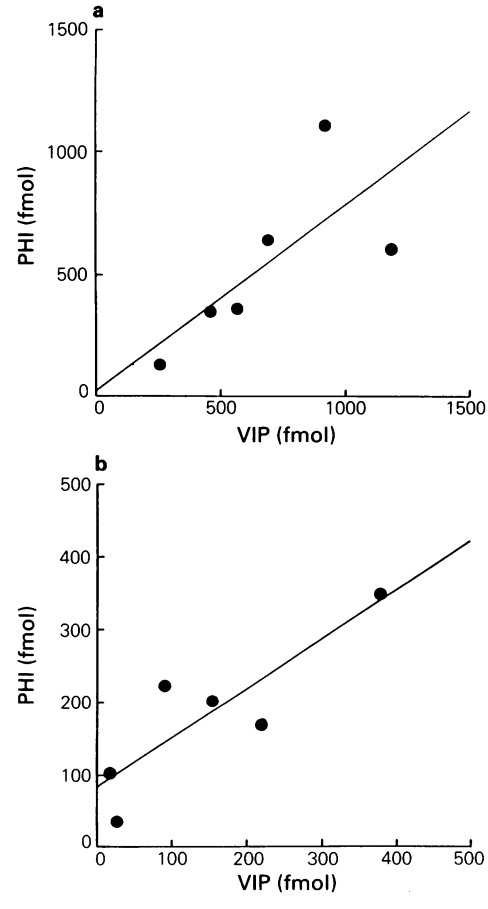

Figure 3 Correlation between peptide histidine isoleucine (PHI) and vasoactive intestinal peptide (VIP) released into the gastric venous plasma for a 7 min period in response to peripheral (a) and central (b) vagal stimulation at 10 Hz for 2 min. The regression line indicated in each figure was computer fitted (a, $Y = 0.75X + 28.0$, $r = 0.71$, $P < 0.05$; b, $Y = 0.67X + 85.8$, $r = 0.86$, $P < 0.05$)

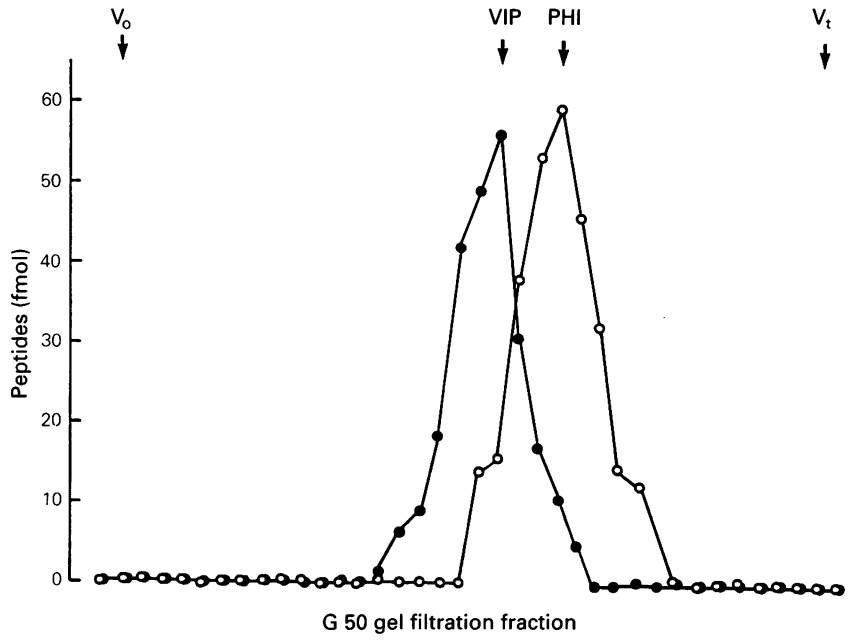

Figure 4 The profile of gel chromatography of vasoactive intestinal peptide (VIP) (●) and peptide histidine isoleucine (PHI) (○) in plasma extract from gastric venous effluent collected during peripheral vagal stimulation. The elution positions for porcine VIP and PHI are indicated by arrows. Vo; void volume, Vt; total mobile phase.

435.0 ± 68.4 fmol after an injection of 1 pmol ($n = 6$) and 977.5 ± 101.2 fmol after 2 pmol ($n = 4$), indicating that the mean recovery rate of exogenous PHI was 45.1 ± 4.7%. This rate was significantly higher than that of porcine VIP (28.5 ± 0.02%; $P < 0.05$) as determined previously with the same techniques (Ito *et al.*, 1988a).

Gel filtration and tissue concentration

Figure 4 shows the gel filtration profile of venous plasma extract collected during peripheral vagal stimulation. PHI and VIP were eluted in the same position as the respective authentic porcine peptides and no extra peaks appeared. The plasma extract obtained during central vagal stimulation showed a similar result (not shown).

The concentrations of VIP and PHI in the muscle and mucosal layers of the gastric corpus were measured in dogs which were not used for nerve stimulation (Table 1). The concentration of VIP in the stomach was somewhat higher than that of PHI. The mean molar ratios of VIP to PHI concentration were 1.5 in the muscle layer and 2.7 in the mucosal layer. Dilution curves for plasma and gastric extracts were parallel to standard curves for porcine VIP and PHI.

The effects of VIP and PHI on gastric motility and blood flow

Representative gastric and vascular responses to PHI and VIP are shown in Figure 5. Close intra-arterial bolus injection of PHI or VIP caused a dose-dependent gastric relaxation and an increase in gastric blood flow without any significant effects on arterial blood pressure. Although the gastric relaxation induced by these peptides had a very slow onset and was long lasting, gastric vasodilatation developed quickly. The peak vasodilator responses to peptides increased with increasing doses, but a further increase in dose prolonged the duration of the responses without increasing the peak values. The dose-response curves for the effect of PHI and VIP on gastric relaxation and vasodilatation are shown in Figure 6. The gastric relaxation induced by both peptides attained a maximum, but PHI-induced vasodilatation did not reach a maximum at 1 nmol. The threshold doses required to elicit gastric relaxation and vasodilatation were 50 and 100 pmol for PHI and 10 and 2 pmol for VIP, respectively. Simultaneous injection of PHI (20–500 pmol) and VIP (10–50 pmol) produced gastric relaxation and vasodilatation which equalled the sum of the responses to the two peptides injected separately.

Discussion

The present experiments indicate that PHI is released with VIP by peripheral and central vagal stimulation in the dog

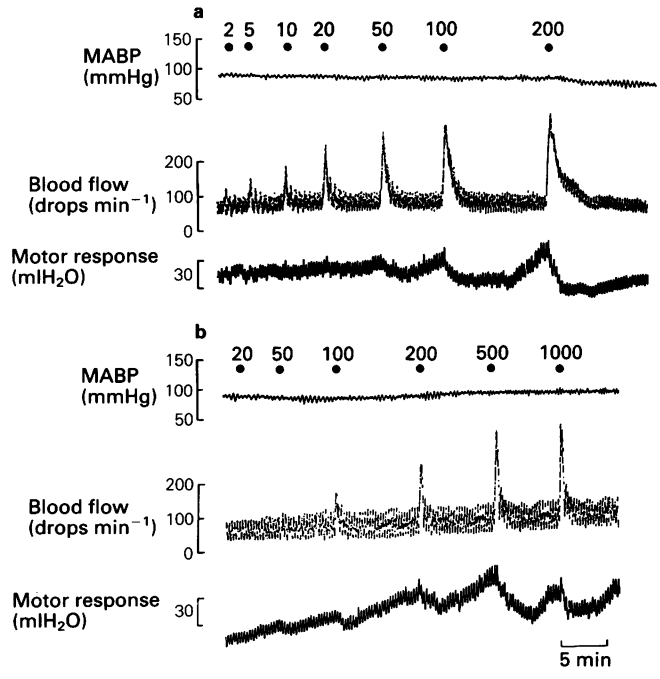

Figure 5 Effects on mean arterial blood pressure (MABP), gastric blood flow and relaxant responses to intra-arterial injection (●) of vasoactive intestinal peptide (VIP) (a) and peptide histidine isoleucine (PHI) (b) at various doses. The data were obtained from the same animal. Numbers above the dots show the doses (pmol) of peptides.

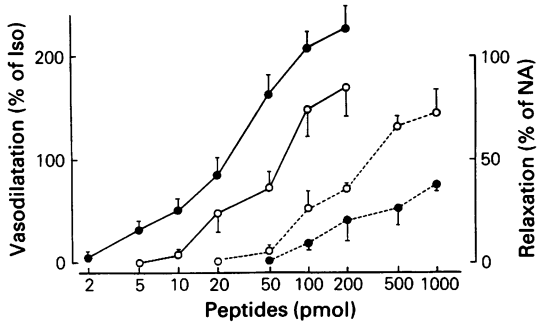

Figure 6 Dose-response curves showing gastric vasodilator (●) and relaxant (○) responses to intra-arterial injection of vasoactive intestinal peptide (VIP) and peptide histidine isoleucine (PHI). The increment of the vasodilator response above the resting level (total change in blood flow) and the peak value of the relaxant response are expressed as a percentage of those of responses to isoprenaline (Iso, 0.1 nmol) and noradrenaline (NA, 5 nmol), respectively ($n = 5$). Continuous and dashed lines respectively indicate the effects of VIP and PHI. Abscissa scale, doses of VIP and PHI (pmol) on a log scale. Each point represents the mean and vertical bars show s.e.mean.

stomach. The VIP concentration in the dog gastric corpus is higher than that of PHI as is the case in many tissues (Bishop *et al.*, 1984; Christofides *et al.*, 1984; Lundberg *et al.*, 1984a; Fahrenkrug *et al.*, 1985; Bardrum *et al.*, 1986). These differences may be attributable to differences in the processing scheme of the VIP precursor.

Dog VIP has the same amino acid sequence as porcine VIP (Eng *et al.*, 1986). Although the primary structure of dog PHI has not yet been elucidated, dog PHI is considered to have a similar amino acid composition to porcine PHI (Yiangou *et al.*, 1983). However, a large form of dog PHI has been found by using N-terminal region specific PHI antiserum (Yasui *et al.*, 1987). In our experiments, C-terminal specific antiserum was used so that gel chromatography of plasma extracts from the dog stomach showed a single peak of immunoreactive PHI in the same position as porcine PHI.

Although co-release of PHI and VIP from the stomach occurred with both methods of stimulation, a molar ratio of VIP to PHI output was different (VIP/PHI; peripheral vagal stimulation, 1.5; central vagal stimulation, 0.7). It is unlikely that the ratio of VIP/PHI evoked by peripheral vagal stimulation reflects a physiological abnormality of the stimulus because vagal stimulation elicits frequency-dependent gastric relaxation and vasodilatation up to 10 Hz. Central vagal stimulation at 10 Hz elicits gastric relaxation of similar magnitude to peripheral vagal stimulation at 10 Hz, but is less effective in producing gastric vasodilatation. We previously suggested that VIP output evoked by peripheral vagal stimulation mainly originates from peptidergic (VIP) vasodilator neurones (Ito *et al.*, 1988a) and that evoked by reflex vagal stimulation from peptidergic (VIP) myenteric neurones (Ito *et al.*, 1988b). Therefore, the difference in the ratio of VIP/PHI could be due to the difference in their origins. The mucosal and submucosal layers of the guinea-pig are rich in peptidergic (VIP) neurones innervating the blood vessels (Costa & Furness, 1983) and circular smooth muscles are exclusively innervated by peptidergic (VIP) fibres coming from the myenteric plexus but not from the submucous plexus (Furness *et al.*, 1988). It is, therefore, suggested that the molar ratio of peptide concentration (VIP/PHI) is higher in the peptidergic (VIP) vasodilator neurones (2.7) in mucosal layers than peptidergic (VIP) myenteric neurones (1.5) in muscle layers. If this is the case, the molar ratio in the tissue is somewhat higher than that appearing in the plasma. However, the ratio decreases if both peptides are released into the plasma, because VIP is metabolized more easily than PHI in the gastric circulation.

Peripheral vagal stimulation at 40 Hz caused less pronounced vasodilatation and VIP output than stimulation at 10 Hz, although both frequencies elicited a similar magnitude of gastric relaxation, as in our previous experiments (Ito *et al.*, 1988a). We have proposed that the gastric peptidergic (VIP) vasodilator neurones are less effective during stimulation at such a high frequency, but peptidergic (VIP) myenteric neurones are not influenced by stimulation frequency (Ito *et al.*, 1988a,b). This contention is supported by the fact that the ratio of peptides appearing in the plasma in response to peripheral vagal stimulation at 40 Hz is almost the same as that in response to central vagal stimulation.

PHI was a much less potent gastric vasodilator than VIP. However, in regard to gastric relaxation, PHI was only slightly less effective than VIP. PHI has been shown to be a less potent vasodilator in submandibular gland (Lundberg & Tatemoto, 1982) and brain (Suzuki *et al.*, 1984). These results therefore suggest that PHI released by vagal nerve activation is able to affect gastric motility but not vascular tone. Simultaneous injections of both peptides cause only additive effects, suggesting that PHI does not modify the actions of VIP.

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Local formation of angiotensin II in the rat aorta: effect of endothelium

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1 The local formation of angiotensin II (AII) from its precursors, angiotensin I (AI) and tetradecapeptide (TDP) renin substrate, was studied in intact (with endothelium) and rubbed (without endothelium) aortic rings of the rat.

2 AI and TDP renin substrate maximally contracted intact tissues in a similar way to AII. The same observations were made in rubbed tissues.

3 The maximal response and the sensitivity of the aorta to these agonists were greater in rubbed than in intact tissues.

4 In intact preparations, methylene blue increased the contractile response to AII and TDP to the same extent as endothelium removal.

5 In intact preparations, AII receptor blockade completely suppressed all contractile responses, converting enzyme inhibition completely blocked the responses to AI and TDP, and renin inhibition partially blocked the responses to TDP.

6 In rubbed preparations, AII receptor blockade completely inhibited all contractile responses, converting enzyme inhibition completely suppressed the responses to AI but only partially inhibited those to TDP, and renin inhibition partially blocked the responses to TDP.

7 In conclusion, the formation of AII from TDP and its blockade by a converting enzyme inhibitor and a renin inhibitor shows that converting enzyme and a renin-like aspartic proteinase are present in the aortic wall. Furthermore, the results show that the endothelium is not essential for the conversion of the TDP to AII, but modulates the responses to locally formed AII through the release of endothelium-derived relaxing factor (EDRF).

Introduction

There is substantial evidence that the essential components of the renin-angiotensin system (RAS) (angiotensinogen, renin, converting enzyme, angiotensin II (AII) receptors) are present in a variety of tissues including blood vessels and the heart (Desjardin-Giasson *et al.*, 1981; Re *et al.*, 1982; Campbell & Habener, 1986; Dzau, 1986; Campbell, 1987; Samani *et al.*, 1987). In particular, the endothelium of blood vessels has been shown to be a major site of conversion of AI to AII by the converting enzyme located on its luminal surface (Caldwell *et al.*, 1976; Ryan *et al.*, 1976). Moreover, bovine aortic endothelial cells have been shown to contain renin and angiotensinogen and to be capable of synthesizing and secreting angiotensins (Lilly *et al.*, 1985; Kifor & Dzau, 1987). Also AII receptors have recently been described in porcine aortic endothelial cells (Patel *et al.*, 1989). These findings indicate that endothelial cells may be an important site for the synthesis of AII in vascular tissues. AII produced locally in vascular tissues may be involved in the regulation of vascular tone, through a direct vasoconstrictor action, an ability to increase noradrenaline neuroeffector function and/or through endothelial stimulation of prostacyclin (PGI_2) and, possibly, endothelium-derived relaxing factor (EDRF) biosynthesis (Zimmerman *et al.*, 1984; Peach, 1988). Locally synthesized AII may also play an important role in the development of vascular hypertrophy (Geisterfer *et al.*, 1988).

Local formation of AII from AI and the synthetic tetradecapeptide (TDP) renin substrate has been shown in different vascular tissues (Malik & Nasjletti, 1976; Oliver & Sciacca, 1984; Ziogas *et al.*, 1986; Juul *et al.*, 1987; Eglème *et al.*, 1989a). In the rat aorta, we showed that TDP was metabolized to AII via AI (Eglème *et al.*, 1989a).

The present experiments were performed to study the role of the endothelium in the formation of AII from AI and TDP renin substrate in the rat isolated aorta. The contraction of aortic rings with and without endothelium to AI and TDP was therefore studied in the presence and absence of inhibitors of the RAS and compared with the contraction induced by AII. A preliminary communication of these results has been presented to the British Pharmacological Society (Eglème *et al.*, 1989b).

Methods

Animals

Male Sprague-Dawley rats (RA 25, Tif, Sisseln, Switzerland) weighing 280–350 g were used. The rats were fed a regular diet and allowed free access to tap water.

Experimental protocol

The rats were killed by decapitation, and the thoracic aorta was removed and cleaned. Four rings (2 mm wide) were cut from the aorta and were left intact or mechanically rubbed to remove endothelial cells. The rings were then carefully suspended under a tension of 2 g in 20 ml organ baths filled with a physiological salt solution (mM: NaCl 112, KCl 5, $NaHCO_3$ 25, KH_2PO_4 1.0, $MgSO_4$ 1.2, $CaCl_2$ 2.5 and glucose 11.2) at 37°C gassed with 95% O_2:5% CO_2 to obtain a pH of 7.4. Contractile responses were measured with an isometric strain gauge (Biegestab K30, Hugo Sachs Elektronik, FGR) coupled to a potentiometric pen recorder (Lineacorder Mark VII WR3101, Hugo Sachs Elektronik, FGR). After an equilibration period of 60 min, tissues were exposed to a depolarizing solution containing 100 mM KCl (mM: NaCl 17, KCl 100, $NaHCO_3$ 25, KH_2PO_4 1.0, $MgSO_4$ 1.2, $CaCl_2$ 2.5 and glucose 11.2), which induced a maximal contraction. After 15 min of contraction, acetylcholine 1 μM was added which induced a relaxation in intact rings and no relaxation in rubbed rings. Preparations were then washed and left for a further 60 min equilibration.

Single responses to AII, AI or TDP were obtained by adding the appropriate concentration to the bath and only

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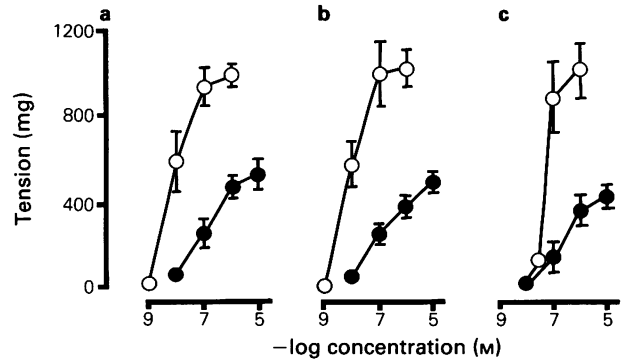

Figure 1 Concentration-effect curves for (a) angiotensin II (AII), (b) AI and (c) tetradecapeptide in rat aortic rings with (●) and without (○) endothelium. Responses are expressed in tension (mg). Each point is the mean of at least 4 observations and vertical lines show s.e.mean.

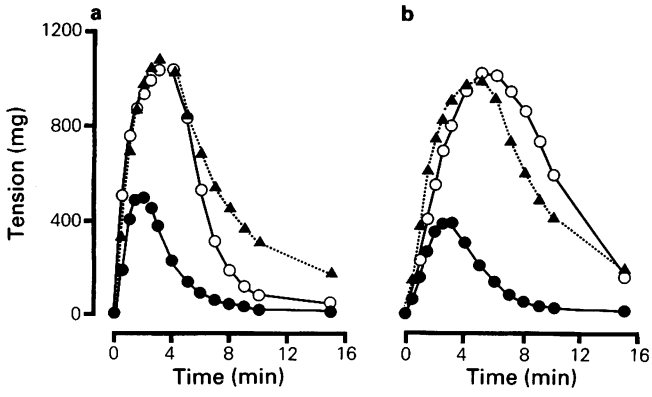

Figure 2 Time-course of the contraction induced by (a) angiotensin II (1 μM) and (b) tetradecapeptide (1 μM) in rat aortic rings without endothelium (○) and with endothelium untreated (●) or pretreated (▲) for 30 min with methylene blue 3 μM. Responses are expressed in tension (mg). Each point is the mean of at least 4 observations.

Table 1 Effect of pharmacological inhibitors of the renin-angiotensin system on the responses induced by submaximal concentrations of angiotensin II (AII), AI and tetradecapeptide (TDP) in rat aortic rings with and without endothelium

	Responses (mg)	
	Endothelium present	*Endothelium absent*
AII	1 μM	100 nM
Control	424 ± 39 (14)	1016 ± 83 (5)
[Sar¹, Ile⁸]-AII (18 nM)	0 (8)***	0 (5)***
Enalaprilat (6.1 μM)	430 ± 71 (5)	1014 ± 83 (5)
CGP 44099 (4 μM)	408 ± 52 (9)	1084 ± 99 (5)
AI	1 μM	100 nM
Control	449 ± 44 (15)	940 ± 57 (5)
[Sar¹, Ile⁸]-AII (18 nM)	0 (8)***	0 (5)***
Enalaprilat (6.1 μM)	0 (8)***	24 ± 24 (5)***
CGP 44099A (4 μM)	387 ± 20 (7)	903 ± 88 (5)
TDP	1 μM	100 nM
Control	343 ± 18 (17)	940 ± 65 (5)
[Sar¹, Ile⁸]-AII (18 nM)	0 (8)***	0 (5)***
Enalaprilat (6.1 μM)	18 ± 12 (6)***	198 ± 87 (5)***
CGP 44099A (4 μM)	175 ± 36 (9)***	280 ± 55 (5)***

Values shown are means ± s.e.mean and numbers in parentheses represent number of observations.
Significant differences between control and treated preparations were determined by unpaired Student's *t* test: ***$P < 0.001$.

one contraction per ring was performed, to avoid the problems of tachyphylaxis observed with these components of the RAS. When concentration-effect curves of an agonist were constructed, each ring of the same aorta was exposed to a different concentration of the agonist. When the effect of methylene blue or of a pharmacological inhibitor of the RAS was studied, the substance was preincubated with the ring for 30 min before the addition of one of the components of the RAS, and only one agonist per aorta was studied. Appropriate controls were run at the same time.

Drugs

AII (Hypertensin, CIBA), AI (Bachem), [Sar¹, Ile⁸]-AII (Bachem), enalaprilat (Merck) and acetylcholine HCl (Roche) were dissolved in bidistilled water as 1 mg ml^{-1} stock solutions and stored at −20°C. Equine TDP (Bachem) was dissolved in a slightly acidic solution as a 1 mM stock solution and stored at −20°C. CGP 44099A (Z-Arg-Arg-Pro-Phe-Val-Cha ψ [$CHOHCH_2$] Val-Val-Tyr-OMe · 2HCl, Ciba-Geigy) was dissolved in an aqueous solution containing 5% glucose to a 1 mg ml^{-1} stock solution and stored at −20°C. Methylene blue (Fluka) was prepared as a 10 mM stock solution in bidistilled water.

Statistical analysis

The maximal amplitudes of the contractions induced by each agonist were measured. Results are expressed as means ± s.e.mean. Significance of differences was evaluated by use of Student's *t* test, *P* values less than 0.05 being considered significant.

Results

AII, AI and TDP produced concentration-dependent increases in tension of rat aortic rings with and without endothelium, when each drug was added to the bath in single concentrations (Figure 1). In the presence of endothelium, the maximal responses induced by AII, AI and TDP were reached with a concentration of 10 μM and were of similar magnitude (AII: 496 ± 46 mg, $n = 5$; AI: 530 ± 73 mg, $n = 5$; TDP: 429 ± 49 mg, $n = 4$). They represented about 50% of the maximal response induced by a depolarizing solution (1079 ± 30 mg, $n = 23$). The sensitivity of the aorta to TDP ($EC_{50} = 0.25 \pm 0.09$ μM, $n = 4$) was about half the sensitivity to AI and AII (0.14 ± 0.05 μM and 0.13 ± 0.03 μM, $n = 5$), but this difference was not statistically significant. In the absence of the endothelium, the maximal responses to TDP, AI and AII were obtained at a concentration of 1 μM and were twice those recorded in the presence of endothelium. They were comparable to the maximal contraction induced by the depolarizing solution (1198 ± 43 mg, $n = 16$) (Figure 1). The sensitivity of the aorta to TDP, AI and AII was also increased. This effect was, however, less pronounced for TDP (EC_{50} values were decreased by about 5 fold) than for AII and AI (EC_{50} values were decreased by about 15 and 11 fold, respectively).

To determine whether EDRF was involved in the endothelium-dependent inhibition of the contractile responses induced by TDP and AII, we examined the effects of methylene blue, an inhibitor of soluble guanylate cyclase which inhibits the action of EDRF at the level of vascular smooth muscle cells (Ignarro & Kadowitz, 1985; Al Osachie & Godfraind, 1986), on contractile responses to AII and TDP. In intact preparations, methylene blue (3 μM) increased the contractile response to TDP and AII (1 μM) to a similar extent as the endothelium removal (Figure 2). The inhibition by the endothelium of the contractile response to AII in the rat aorta could be due to either the basal release of EDRF, or stimulated release of EDRF by AII. AII did not appear to liberate EDRF since it did not relax an intact preparation of rat aorta which had been precontracted with prostaglandin $F_{2\alpha}$ (3 μM), but induced a further contraction (unpublished observation). Such conclusions confirm those of Bullock *et al.* (1986).

To ascertain whether removal of the endothelium modified the pathways of AII formation from AI and TDP in the rat aorta, specific pharmacological inhibitors of the RAS were used, and their effects were evaluated on contractions induced by a submaximal concentration of AII, AI or TDP in intact and rubbed aortic rings. This concentration was estimated to be about 1 μM in intact preparations, and 100 nM in denuded preparations. In both intact and rubbed preparations, none of the inhibitors studied had a significant effect on baseline tone when added alone. In the presence of endothelium, AII receptor blockade (with [Sar^1, Ile^8]-AII, 18 nM) completely inhibited the responses to AII, AI and TDP. Converting enzyme inhibition (enalaprilat, 6.1 μM) had no effect on the response to AII but completely blocked the responses to AI and TDP. Renin inhibition (CGP 44099A, 4 μM) had no effect on the responses to AII and AI but inhibited the responses to TDP by about 49% (Table 1). Endothelium removal did not seem to modify the main pathway of AII formation from AI and TDP. In summary, AII receptor blockade completely blocked all contractile responses, converting enzyme inhibition completely suppressed the responses to AI but only partially inhibited those to TDP, and renin inhibition blocked by about 70% the responses to TDP (Table 1).

Discussion

The present results show that removal of the endothelium in the rat aorta did not prevent the local conversion of AI and TDP renin substrate to AII. Moreover, they demonstrate that the maximal response and the sensitivity of the tissue to AII, whether given exogenously or locally formed from AI and TDP renin substrate, were increased by removal of the endothelium. Our observations concerning the influence of the endothelium on the response to exogenous AII confirm previous studies performed in the rat aorta and in some other vascular tissues, such as bovine coronary artery, rabbit mesenteric and coeliac arteries (Oshiro *et al.*, 1985; Bullock *et al.*, 1986; Gruetter *et al.*, 1988). However, in other vessels such as rabbit aorta or canine carotid artery, the endothelium has no effect on the contractile response to AII (Saye *et al.*, 1984; D'Orléans-Juste *et al.*, 1985). In the present study, methylene blue, an inhibitor of EDRF, produced the same potentiation of the contractile response to AII as endothelium removal. This indicates that, in the rat aorta, the inhibitory effect of the endothelium on the contraction induced by AII may be attributed to the release of EDRF, as has been proposed by Bullock *et al.* (1986) and Gruetter *et al.* (1988).

When the endothelium was removed, the contractile responses of the rat aorta to AI and AII were potentiated to a similar extent. Contractions induced by a submaximal concentration of AI were still completely blocked by the converting enzyme inhibitor, enalaprilat, and by the receptor antagonist, [Sar^1, Ile^8]-AII. These observations show that, in the rat aorta without endothelium, AI was still converted to AII by converting enzyme. This indicates that the converting enzyme was present at sites other than the endothelium, and in amounts sufficient to allow the local conversion of AI to AII. These findings do not bear out previous data indicating that the converting enzyme is mainly localized in vascular endothelium (Caldwell *et al.*, 1976; Ryan *et al.*, 1976). However, they are in good agreement with the results of more recent pharmacological and biochemical studies. In different vessels, such as the rabbit aorta, the rat femoral artery, the rat caudal artery and the guinea-pig pulmonary artery the absence of endothelium does not alter the functional response to AI (Saye *et al.*, 1984; Story & Ziogas, 1986; Schölkens *et al.*, 1987; Urabe *et al.*, 1987). Moreover, it has been suggested that angiotensin-converting enzyme activity is negligible in the vascular endothelium of rat and rabbit aortae, and of the guinea-pig pulmonary artery, since it did not differ in preparations with and without endothelium (Velletri & Bean, 1982; Schölkens *et al.*, 1987).

When the endothelium was removed, or when the action of EDRF was chemically inhibited by methylene blue, the responses induced by TDP and AII (1 μM) were increased to a similar extent. These observations demonstrate that the endothelium was not the major site of TDP metabolism but modulated the action of AII formed locally from TDP. However, a minor role of the endothelium in the conversion of TDP to AI cannot be ruled out: although endothelial removal increased the maximal contractile responses to TDP and AII to a similar extent, the responses induced by the lowest concentrations were potentiated less for TDP than for AII. The results of the present experiments also indicate that the main pathway of AII formation from TDP in the rat aorta, i.e. a two-step metabolism through a renin-like enzyme and converting enzyme (Eglème *et al.*, 1989a), is not modified by removal of endothelium. In rubbed preparations, the contraction induced by a submaximal concentration of TDP was still inhibited by AII receptor antagonism, converting enzyme inhibition and renin inhibition. However, some small differences in the inhibitory effects of enalaprilat and CGP 44099A were observed when the endothelium was removed. The renin inhibitor, CGP 44099A, inhibited the response to TDP to a greater extent when the endothelium was removed (70% compared to 49% with endothelium). Removal of the endothelium may facilitate the access of this compound to its critical sites of action. In contrast, the converting enzyme inhibitor, enalaprilat, was less effective in blocking the response to TDP when the endothelium was removed. This might indicate that, when the endothelium is removed, a small amount of TDP is converted directly to AII without first being metabolized to AI. Another explanation may be that the preparation of TDP was contaminated with small amounts of AII barely detectable in the less sensitive intact aortic rings but detectable in the more sensitive rubbed rings.

In summary, we showed that removal of the endothelium potentiated the maximal contractile responses to AII, AI and synthetic TDP renin substrate to a similar extent, and did not modify the main pathway of AII formation from TDP i.e. a two-step pathway formation through a renin-like enzyme and converting enzyme. Such observations indicate that the endothelium of the rat aorta is not essential for the vascular conversion of AI and TDP to AII, but may modulate the responses of locally formed AII through EDRF release.

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Interactions of palmitoyl carnitine with the endothelium in rat aorta

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1 Palmitoyl carnitine (10–1000 μM) resembled Bay K 8644 (10–1000 nM) in that it directly contracted rat aortic rings which were partially depolarized with K^+ (12 mM). However, the effects of Bay K 8644 were reduced in the presence of endothelium whereas the presence of the endothelium hardly affected the palmitoyl carnitine-induced contractions, which occurred at high concentrations (> 10 μM).

2 Lower concentrations of palmitoyl carnitine (0.3–30 μM; EC_{50} 1.1 μM), but not Bay K 8644, carnitine or palmitic acid, antagonized the relaxant effects of acetylcholine in rat aorta. The antagonism was specific for endothelium-dependent relaxations, in that the relaxations to ATP and the calcium ionophore A23187 were also non-competitively antagonized, albeit at slightly higher concentrations, whereas the direct relaxant effects of sodium nitroprusside were unaffected. Palmitoyl carnitine therefore antagonizes the effects or the release of endothelial-derived relaxant factor (EDRF). The inhibitory effects were reversed on prolonged washout, indicating that the effects were not due to destruction of the endothelial cells.

3 In superfusion experiments, palmitoyl carnitine inhibited the release of EDRF from rat aorta but did not affect the responsiveness to exogenous EDRF, indicating a site of action at the endothelial cell. In superfusion experiments, palmitoyl carnitine, and lysophosphatidyl choline, caused direct relaxations of the aorta, indicating EDRF release, prior to inhibition of release evoked by receptor stimulation. These substances may modulate vascular responsiveness under certain conditions.

Introduction

Acyl carnitines are key intermediates in lipid metabolism, effecting the transfer of lipids into mitochondria. During ischaemic episodes β-oxidation of fatty acids in mitochondria is inhibited, resulting in large increases in the acyl carnitine content of the cytoplasm (Idell-Wenger *et al.*, 1978; Liedtke *et al.*, 1978; Neely & Feuvray, 1981; Corr *et al.*, 1984) and associated cellular membranes (Knabb *et al.*, 1986). The accumulation of acyl carnitines in myocardial sarcolemma may be responsible for some of the electrophysiological aberrations caused by ischaemia (Knabb *et al.*, 1986). Spedding & Mir (1987) have advanced the hypothesis that palmitoyl carnitine may be an endogenous modulator of Ca^{2+} mobilisation, on the basis that the effects of palmitoyl carnitine in K^+-depolarized smooth muscle resemble those of the dihydropyridine Ca^{2+} channel activator, Bay K 8644. Inoue & Pappano (1983) had previously shown that palmitoyl carnitine increases Ca^{2+} current in avian ventricular muscle. Furthermore, low concentrations (0.5–5 μM) of palmitoyl carnitine cause positive inotropic effects in chick myocytes, and higher concentrations reverse the inhibitory effects of verapamil and nisoldipine (Duncan *et al.*, 1986; 1987; Patmore *et al.*, 1989). However, more recent evidence points to a site of action at the sarcoplasmic reticulum in the heart, tightly linked to the Ca^{2+} channel (Spedding *et al.*, 1989). It is therefore possible that acyl carnitines, produced in high concentrations during ischaemia, might mediate some or all of their deleterious effects via an interaction with the Ca^{2+} channel or a tightly coupled structure associated with it.

Endothelial cells have key interactions with lipids, being involved in the transport of free fatty acids from the circulation to muscle. Endothelial cells also can rapidly metabolise palmitate, which can be used as an energy source; oxidation is increased in the presence of carnitine (Hulsmann *et al.*, 1988), although it is not known to what extent acyl carnitines accumulate in endothelial cells during ischaemia.

Endothelial cells release a relaxant factor (endothelial-derived relaxant factor, EDRF) which may be nitric oxide (Furchgott & Zawadzki, 1980; Rubanyi & Vanhoutte, 1985; Gryglewski *et al.*, 1986; Palmer *et al.*, 1987; Furchgott, 1988). EDRF may have a critical role in inhibiting vascular spasm during or after ischaemic episodes.

The question whether Ca^{2+} channel activators cause EDRF release is controversial. Rubanyi *et al.* (1985) found that very low concentrations of Bay K 8644 released EDRF in dog femoral arteries and concluded that Ca^{2+} channels were involved. In contrast, Spedding *et al.* (1986) concluded that Bay K 8644 did not release EDRF from rat aorta, but that the contractile effects of Bay K 8644 in smooth muscle could be countered by a tonic liberation of EDRF. A thorough electrophysiological study found no evidence for the presence of L channels in endothelial cells (Takeda *et al.*, 1987). We have tested the effects of the putative Ca^{2+} channel activator palmitoyl carnitine in rat aorta, in the presence and absence of endothelium. A preliminary account of these findings has been published in abstract form (Bigaud & Spedding, 1986; Dainty *et al.*, 1987).

Methods

Rings of rat aorta, taken from male Sprague-Dawley rats (200–340 g), were set up between two horizontally arranged steel wires in 10 ml isolated baths, filled with Krebs solution (composition, mM: NaCl 137, KCl 2.7, $MgCl_2$ 1.1, $CaCl_2$ 1.8, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, glucose 5.5) which was maintained at 35°C and gassed with 95% O_2:5% CO_2. Where depolarizing solutions were used, 12 mM K^+ was substituted for the equivalent concentration of Na^+. In some experiments a slightly different solution was used (composition, mM: NaCl 118.4, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.1; Figure 4) which had no effect on the sensitivity to acetylcholine. Isometric developed tension was recorded after resting tension had been set to 1 g. After a 60 min equilibration period, the rings were contracted with phenylephrine, 1 μM, and then challenged with acetylcholine, 1 μM, to verify the presence or absence of endothelium. Strips which did not relax by more than 40% were not used. Strips which were denuded of endothelium were gently rubbed 4–5

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times over the steel wires. The presence of the endothelium was verified histologically in some preparations by staining with silver nitrate (12.5% for 20 min). More than 90% of the interior surface of the aorta was covered with endothelial cells in control preparations, and <10% was covered in the rubbed preparations.

Superfusion experiments

In experiments where EDRF release from one aorta to a recipient, endothelium-denuded, preparation was assessed, 4 mm lengths of rat aorta were opened by a single lengthways cut, denuded of endothelium, and tensioned between two pins, one of which was connected to an isometric force transducer, in a plastic gutter; the preparations were perfused with Krebs solution (6 ml min^{-1}). Resting tension was adjusted to 1 g. A 4 mm strip of aorta, with endothelium present, was placed transversely over the intimal surface of the endothelium-denuded aorta, which was contracted with phenylephrine (0.1 μM). EDRF was liberated from the upper preparation by superfusion with carbachol (10 μM). The lower concentration of phenylephrine and the higher concentration of carbachol were used in order to increase the sensitivity of the superfused preparation.

In experiments where palmitoyl carnitine was shown to release EDRF directly, rings of rat aorta were used and the substance injected as a bolus into the perfusate.

Drugs

The following drugs were used: adenosine 5'-triphosphate (Sigma), A23187 (Sigma), acetylcholine hydrochloride (Sigma), Bay K 8644 (methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethyl-phenyl)pyridine-5-carboxylate, a gift from Dr M. Schramm, Bayer AG), L-carnitine hydrochloride (Sigma), palmitoyl-DL-carnitine (Sigma), sodium nitroprusside (Sigma), palmitic acid (Sigma), lysophosphatidyl choline (Sigma). The drugs were dissolved in distilled water and all solutions prepared fresh daily.

Statistics

Student's t test was used for comparison of mean values. Mann-Whitney U-test was used for comparison of % changes. All concentrations refer to final bath concentrations of drugs.

Results

Bay K 8644, in the presence of a threshold depolarization (12 mM K^+), caused a concentration-dependent contraction of the rat aorta. The magnitude of the contractions was greater in preparations which had been rubbed to destroy the endothelial cells (Figure 1; maximum tension rubbed 1.25 ± 0.15 g, unrubbed 0.75 ± 0.15, $P < 0.05$). In the presence of 12 mM K^+, palmitoyl carnitine caused a concentration-dependent contraction of the rat aorta, but there was no significant difference between the maximum effects of palmitoyl carnitine in the presence or absence of endothelium (Figure 1; rubbed 1.3 ± 0.3 g, unrubbed 1.1 ± 0.2 g, $P > 0.1$). In these experiments the drugs were administered cumulatively. Thus, although palmitoyl carnitine resembled Bay K 8644 in its ability to contract the aorta, the effects were unchanged in the presence of the endothelium. Palmitoyl carnitine in concentrations up to 1 mM did not contract the aorta directly in non-depolarizing conditions (data not shown, $n = >12$) and did not significantly modify submaximal contractions to phenylephrine ($n = 6$, Figure 2).

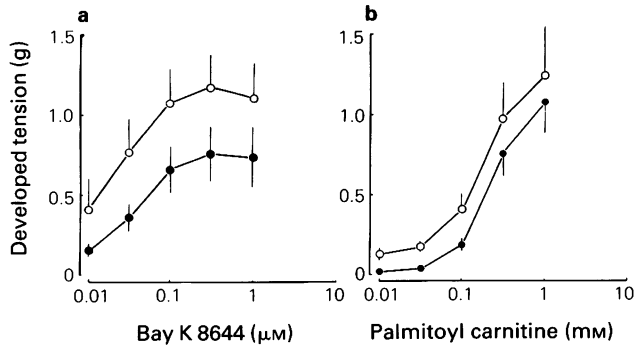

Figure 1 Contraction of rat aorta by cumulative addition of (a) Bay K 8644 or (b) palmitoyl carnitine in the presence (●) or absence (○) of endothelium. The preparations were partially depolarized with 12 mM K^+. Vertical bars represent s.e.mean; $n = 5$.

Bay K 8644 (1 μM for 20 min) did not significantly affect the relaxant effects of acetylcholine (1 μM) in tissues contracted by phenylephrine (1 μM), in that acetylcholine relaxed the tissues by 54 ± 10% in the presence of Bay K 8644 and by 59 ± 9% in control conditions ($n = 5$). In contrast, palmitoyl carnitine (100 μM for 20 min) reduced acetylcholine-induced relaxations from 68 ± 10% to 3 ± 3% ($n = 5$). Palmitoyl carnitine (10 μM) caused an immediate reversal of acetylcholine-induced relaxations (Figure 2), without affecting tone in rubbed preparations. The effects of palmitoyl carnitine did not washout immediately (Figure 2) and were not mimicked by palmitic acid (10 μM, Figure 2) or L-carnitine (10 μM; data not shown).

Because palmitoyl carnitine has surface-active properties we assessed whether the endothelium was being damaged by incubation with high concentrations of the substance. Prolonged wash out (60–120 min) fully reversed the inhibitory effects of palmitoyl carnitine (30 μM) in three experiments, indicating that the endothelial cells were not destroyed by the procedure. This washout period did not significantly modify the relaxant effects of acetylcholine, in that three successive cumulative concentration-response curves were unchanged (concentration ratio <2, $n = 28$).

The concentration-dependency of the ability of palmitoyl carnitine to reverse carbachol-induced relaxations is shown in

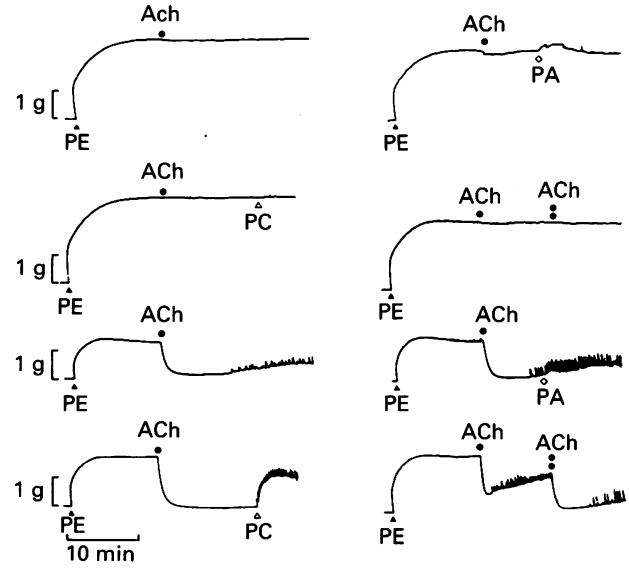

Figure 2 Effects of acetylcholine (●, ACh, 1 μM) on phenylephrine (▲, PE, 1 μM)-induced contractions of rat aorta rings in the absence (two upper preparations) or presence (two lower preparations) of endothelium. There is a 20 min time interval between left and right panels during which period the preparations were washed four times. Palmitoyl carnitine (△, PC, 10 μM) partially reversed the effects of acetylcholine (lower panel) but had no effect in the absence of endothelium. The effects of palmitoyl carnitine were not immediately reversed by washing because acetylcholine-induced relaxation of a subsequent contraction to phenylephrine was also attenuated (lower right panel) although further challenge with acetylcholine (:, 10 μM) relaxed the tissue fully. Palmitic acid (◇, PA, 10 μM) did not affect acetylcholine-induced relaxations. This experiment is a representative example of 4 similar experiments.

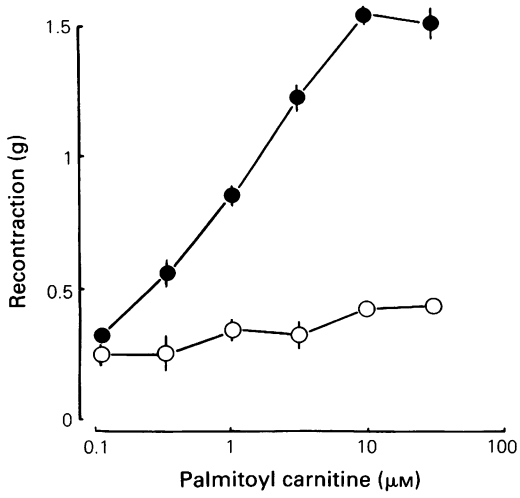

Figure 3 Concentration-dependent reversal by palmitoyl carnitine of the relaxant effects of carbachol (1 μM) in rat aorta precontracted by phenylephrine (1 μM). Palmitoyl carnitine was added in increasing concentrations at 5 min intervals (●); the spontaneous recontraction in parallel control preparations is also shown (○). The initial phenylephrine contraction was 1.5 ± 0.3 g. Vertical bars represent s.e.mean, $n = 5$–6.

Figure 3. In these experiments palmitoyl carnitine was added cumulatively to the organ baths when the carbachol-induced relaxation was maximal; the threshold for the effect was 0.3 μM and the EC_{50} 1.1 μM. With a different protocol, the effects of pretreatment with palmitoyl carnitine (10–100 μM for 20 min) on cumulative concentration-response curves to acetylcholine were obtained (Figure 4); the antagonism was non-competitive.

Adenosine 5'-triphosphate (ATP) and the Ca^{2+} ionophore A23187 relax vascular smooth muscle by an endothelium-dependent mechanism. Relaxant responses to ATP and A-23187 were antagonized by palmitoyl carnitine (Figure 4). In contrast, the relaxant responses to sodium nitroprusside, a vasodilator with direct effects on vascular smooth muscle cells, were unaffected by palmitoyl carnitine (30 μM for 20 min; Figure 4). Thus, although palmitoyl carnitine causes a non-specific antagonism of the effects of a variety of agents which stimulate EDRF release, it does not appear to affect relaxant processes in smooth muscle cells.

Superfusion experiments

A simple bioassay of EDRF release from rat aorta was developed (Figure 5). Rubbed rat aorta preparations were used to bioassay EDRF released from the endothelial cells of an aortic preparation placed above them. The assay preparations, once stabilized, were used to test a series of endothelial preparations; in 20 preparations, from 10 rats, phenylephrine-induced contractions were relaxed by 41.8 ± 3.7%. The relaxation was rapidly reversed by lifting off the 'donor' preparation (Figure 5). There was no relaxation if the 'donor' preparations were rubbed of endothelium (4 preparations from 2 rats). Pretreatment of the 'donor' preparations with palmitoyl carnitine (30 μM for 20 min, 12 preparations from 6 rats) reduced EDRF-induced relaxation to 11.7 ± 2.9%, in comparison with a 35.7 ± 5.3 relaxation observed in parallel control preparations (10 preparations from 6 rats; $P < 0.005$). Thus pretreatment with palmitoyl carnitine inhibited EDRF release. The assay preparations did not lose their sensitivity to EDRF, compared to untreated preparations, if they had previously been perfused with palmitoyl carnitine (30 μM for 20 min; 35.3 ± 8.7% relaxation; 4 preparations from 2 rats), indicating that the site of action of palmitoyl carnitine was at the endothelial cell.

In static isolated organ bath experiments palmitoyl carnitine occasionally caused relaxations when added directly to preparations with endothelium, but not in preparations devoid of endothelium. However, these effects were not consistently reproducible. We therefore tested the effects of palmitoyl carnitine in superfused ring preparations. The preparations responded with marked relaxations to doses of carbachol added to the superfusate. Palmitoyl carnitine

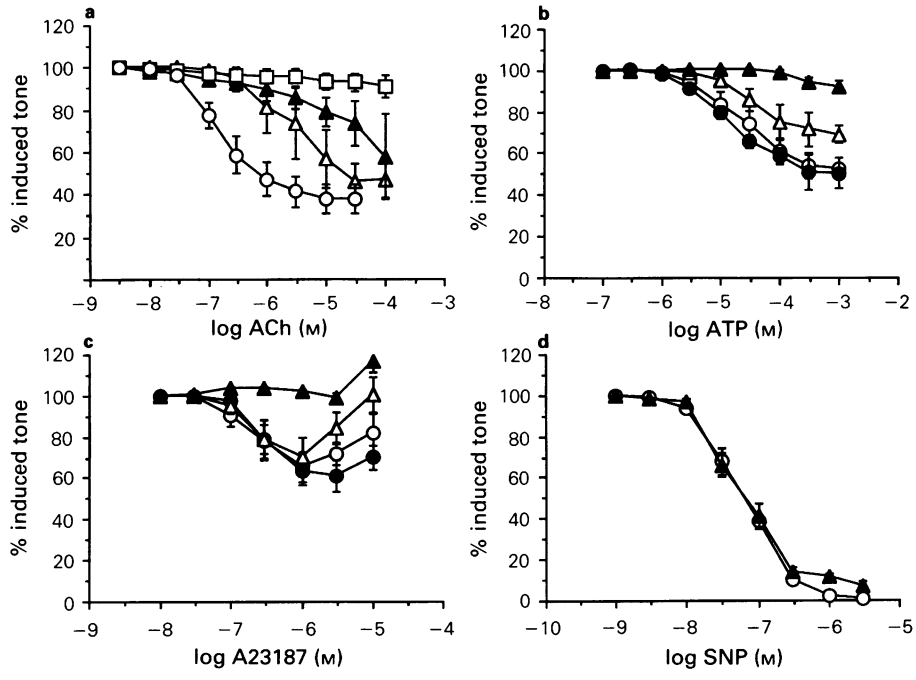

Figure 4 (a) The effect of palmitoyl carnitine (△, 10; ▲, 30; □, 100 μM) on acetylcholine (ACh)-induced relaxations of rat aortic rings precontracted with phenylephrine (1 μM). (b) The effect of palmitoyl carnitine (●, 3; △, 10; ▲, 30 μM) on ATP-induced relaxations of rat aortic rings precontracted with phenylephrine (1 μM). (c) The effect of palmitoyl carnitine (●, 3; △, 10; ▲, 30 μM) on A23187-induced relaxations of rat aortic rings precontracted with phenylephrine (1 μM). (d) The effect of palmitoyl carnitine (▲, 30 μM) on sodium nitroprusside-induced relaxations of rat aortic rings precontracted with phenylephrine (1 μM). All relaxations (○, control) are expressed as % of the phenylephrine-induced contraction. Vertical bars represent s.e.mean, $n = 4$–6.

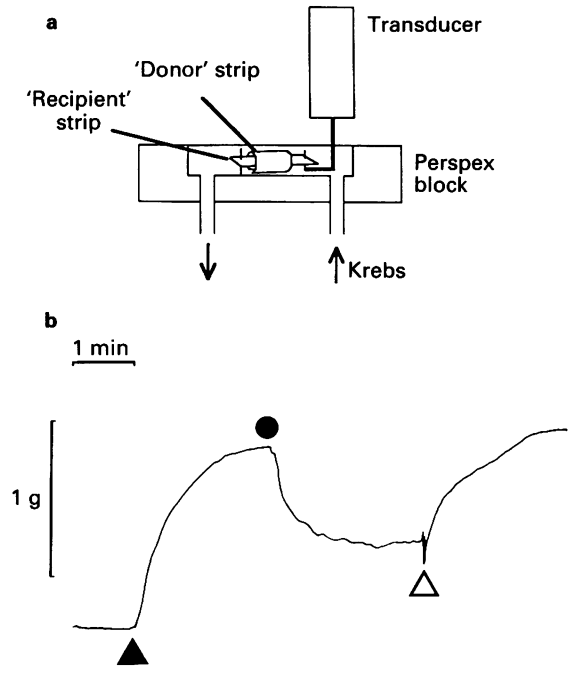

Figure 5 (a) Diagram of the superfusion apparatus used to ascertain effects on endothelial cells. Contractile responses were obtained from the lower rubbed strip, in the presence or absence of a strip of aorta with the endothelium located over the rubbed intimal surface of the lower preparation. (b) Effects of inclusion of phenylephrine (▲, 0.1 μM) and carbachol (●, 10 μM) in the superfusion solution; at (△) the donor strip was lifted from the lower rubbed strip, which subsequently recontracted to the level prior to addition of carbachol.

caused dose-dependent relaxations of phenylephrine-induced contractions under these conditions, although the relaxations were smaller than those induced by carbachol (Figure 6). In these experiments palmitoyl carnitine was added in a single dose and the effect allowed to wane before the next administration of compound. Subsequent carbachol-induced relaxations were attenuated after exposure to the last dose of palmitoyl carnitine, indicating that the compound caused a certain degree of release of EDRF before preventing further release; responses to carbachol did not change with time in 4 control experiments.

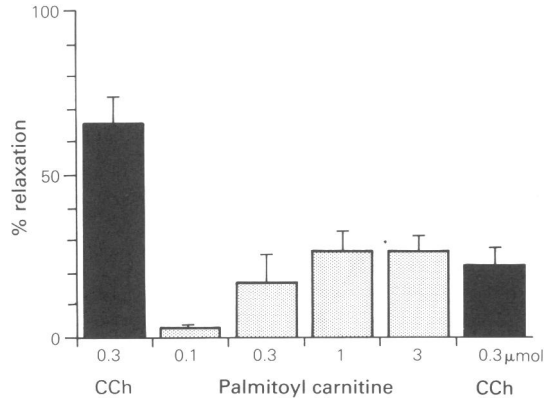

Figure 6 Relaxant effects of carbachol (CCh, 0.3 μmol) and of single doses of palmitoyl carnitine (0.1, 0.3, 1, 3 μmol) on rat superfused aorta preparations, with intact endothelium. Immediately after the relaxant effects of palmitoyl carnitine had washed out, the preparations were rechallenged with carbachol (0.3 μmol), and the relaxant effects were significantly attenuated ($P < 0.005$) compared with the initial response to carbachol. All drugs were added to the superfusate as a bolus. Vertical bars represent s.e.mean of the % maximum relaxation of the phenylephrine-induced contraction, $n = 6$.

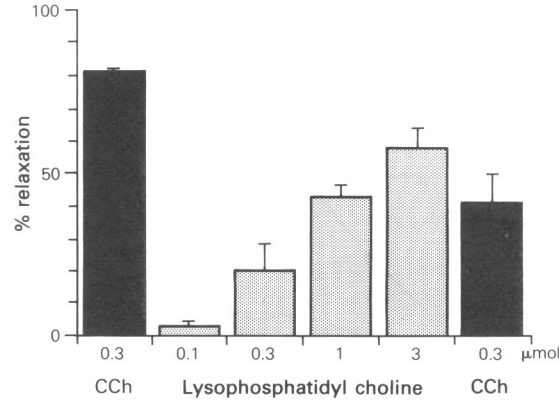

Figure 7 Relaxant effects of carbachol (CCh, 0.3 μmol) and of lysophosphatidyl choline (0.1, 0.3, 1, 3 μmol) on rat superfused aorta preparations, with intact endothelium. Immediately after the relaxant effects of lysophosphatidyl choline had washed out, the preparations were rechallenged with carbachol (0.3 μmol), and the relaxant effects were significantly attenuated ($P < 0.005$) compared with the initial response to carbachol. Drugs were added to the superfusate as a bolus. Vertical bars represent s.e.mean of % maximum relaxation of the phenylephrine-induced contraction, $n = 6$.

Lysophosphatidyl choline, another amphiphile which accumulates during ischaemia, has also been shown to release EDRF and claimed to be an endogenous factor modifying release (Saito *et al.*, 1988). Lysophosphatidyl choline resembled palmitoyl carnitine in its ability to relax phenylephrine-induced contractions and subsequently to attenuate the effects of carbachol (Figure 7).

Discussion

Endothelial cells appear to exert a tonic inhibition of vascular smooth muscle by release of EDRF, which may be nitric oxide (Furchgott & Zawadzki, 1980; Gryglewski *et al.*, 1986; Rubanyi & Vanhoutte, 1986; Palmer *et al.*, 1987). Endothelial cells also metabolise and transport lipids. In addition, endothelial damage may be a key factor in atherogenesis and other circulatory disorders and is regularly seen after ischaemic episodes (Heber *et al.*, 1987). However, most pharmacological experiments investigating the role of EDRF have been conducted in physiological salt solutions without exogenous lipids. The finding that palmitoyl carnitine may inhibit the release of EDRF at concentrations which are similar to those found to occur in ischaemic myocardial cells (Knabb *et al.*, 1986) may therefore be of importance, especially as acyl carnitines will be critical for mitochondrial lipid oxidation in endothelial cells (Hulsmann *et al.*, 1988).

Palmitoyl carnitine resembled Bay K 8644 in causing contractions of the aorta in the presence of a depolarizing stimulus. However, unlike Bay K 8644, the maximal contractile effects of palmitoyl carnitine were not attenuated by the presence of the endothelium. This is presumably because palmitoyl carnitine inhibits EDRF release, thereby inhibiting the influence of the endothelium. In the case of Bay K 8644, baseline levels of guanosine 3':5'-cyclic monophosphate (cyclic GMP) in the aorta are increased by spontaneously released EDRF, which can reduce the contractile effects of Bay K 8644 (Spedding *et al.*, 1986). Only high (> 10 μM) concentrations of palmitoyl carnitine caused a direct contraction of the aorta, which was evident only under depolarizing conditions (data not shown). These direct effects on the smooth muscle cells occurred at higher concentrations than those on the endothelium (threshold 0.3 μM).

The site of action of palmitoyl carnitine was clearly dependent on the endothelial cells, because the superfusion experiments indicated that the responses of smooth muscle to EDRF were unaffected. Furthermore, the effects of sodium

nitroprusside, which, like EDRF, relaxes smooth muscle by a cyclic GMP-dependent mechanism, were not modified by palmitoyl carnitine. This amphiphilic molecule did not cause irreversible endothelial disruption under the present experimental conditions, although such amphiphiles may be expected to modify membrane characteristics, and ultimately membrane permeability, in concentrations similar to those used in this study (Busselen *et al.*, 1988).

Palmitoyl carnitine inhibited EDRF release induced by both muscarinic agonists and ATP, indicating that the site of action was not as a receptor antagonist at the endothelial cell. Thus, responses to the calcium ionophore A23187 were also inhibited, although responses to ATP and A23187 were slightly more resistant to palmitoyl carnitine than were those to acetylcholine. Palmitoyl carnitine, at the concentrations used in the present study, does inhibit receptor activation in gastrointestinal smooth muscle (Bigaud & Spedding, 1986; Spedding *et al.*, unpublished observations) and receptor-mediated EDRF release is associated with increased levels of inositol 1,4,5-trisphosphate (Loeb *et al.*, 1988). The site of action of palmitoyl carnitine remains to be defined.

In this paper we have concentrated on inhibition of release of EDRF. However, Criddle *et al.* (1987) showed that palmitoyl carnitine caused relaxation of rat aorta by an endothelial-dependent mechanism. In our hands, this aspect appeared less marked than the subsequent impairment of endothelium-dependent relaxations. Interestingly, the relaxation observed by Criddle *et al.* (1987) may be due to the formation of an ethyl carnitate ester which is selectively vasodilator (B. Woodward, personal communication). Such an ester may be important if it occurs physiologically. In our experiments, relaxant effects to palmitoyl carnitine occurred only in superfusion experiments. It is not known whether metabolism of palmitoyl carnitine would be different under the two experimental procedures, but the relaxant effects were only apparent following administration of bolus doses in the superfusate. The final effect of palmitoyl carnitine in a given tissue, i.e. increased or decreased release of EDRF, will presumably be dependent on the tissues involved and the degree of ongoing receptor activation. This is being investigated currently.

Lysophosphatidyl choline, which also accumulates during ischaemia (Corr *et al.*, 1981; 1982; 1984) following activation of phospholipases, resembled palmitoyl carnitine in its ability to relax phenylephrine-induced contractions of rat aorta and to block the subsequent effects of carbachol. This finding indicates that, even if the membrane is not irreversibly disrupted, the effects of both palmitoyl carnitine and lysophosphatidyl choline may be secondary to changes in membrane fluidity, as membrane fluidity increases following incorporation of the amphiphiles in sarcolemmal membranes (Fink & Gross, 1984). Such an effect may explain the rather non-specific inhibition of EDRF release following a number of different stimuli. It remains to be seen whether these agents modulate the inhibition of EDRF release which follows inhibition of oxidative phosphorylation (Griffith *et al.*, 1986), but both palmitoyl carnitine and lysophosphatidyl choline can inhibit EDRF release under our experimental conditions. As endothelial cells metabolise lipids to a considerable extent, then acyl carnitines may be a critical factor in endothelial mitochondrial function. Our data indicate that they may be expected to exert a variety of effects on endothelial function as they accumulate during ischaemia. Further studies are required to define the extent of accumulation and consequent effects in ischaemia.

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Nedocromil sodium inhibits antigen-induced contraction of human lung parenchymal and bronchial strips, and the release of sulphidopeptide-leukotrienes and histamine from human lung fragments

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1 The effects of nedocromil sodium on antigen-induced release of sulphidopeptide-leukotrienes and histamine from passively sensitized fragments of human lung, and on antigen-induced contraction of sensitized strips of human lung parenchyma and bronchus, have been studied.

2 Nedocromil sodium 0.1 and 1 μM inhibited leukotriene release from fragments of human lung by 30% and 38% respectively, and histamine release by 43% for both concentrations, but 10 μM was ineffective. The lung fragments, which were passively sensitized to house dust mite, *Dermataphagoides pteronyssinus*, in control experiments released leukotrienes (6.58 $\pm$ 0.12 nmol equiv. leukotriene C_4 per g, $n = 6$) and histamine (10.3% $\pm$ 1.8 of total tissue histamine, $n = 5$) when challenged with house dust mite extract.

3 Isolated strips of human lung parenchyma, passively sensitized to *D. pteronyssinus*, contracted when treated with house dust mite extract to a mean value of 40% of the maximal histamine response for each strip. Nedocromil sodium 0.1 and 1 μM inhibited these contractions by 50% and 70% of the control response, but 10 μM had no inhibitory effect.

4 Isolated rings from human bronchus, also passively sensitized to *D. pteronyssinus*, contracted when treated with house dust mite extract to a mean value of 86% of the maximal histamine response. Nedocromil sodium 1 μM, but not 0.1 or 10 μM, inhibited contractions by 48% of the control response.

5 The therapeutic effects of nedocromil sodium in allergic asthma may depend, partly, on its inhibition of antigen-induced release of leukotrienes and histamine in human lung and its consequent inhibition of antigen-induced contractions of parenchymal and bronchial tissue.

Introduction

Nedocromil sodium is an anti-inflammatory drug used in the treatment of asthma. It protects atopic asthmatic subjects against antigen-induced bronchospasm and prevents the late response to allergen which occurs in some subjects (Dahl & Pedersen, 1986). It also protects against bronchoconstriction induced by exercise (Konig *et al.*, 1987), cold air (del Bono *et al.*, 1986), sulphur dioxide (Altounyan *et al.*, 1986), adenosine (Crimi *et al.*, 1988) and neurokinin A (Joos *et al.*, 1989).

Nedocromil sodium is related to the 'mast cell protecting' drug sodium cromoglycate, which has been shown to inhibit the antigen-induced release from human lung fragments of sulphidopeptide-leukotrienes, previously known as slow-reacting substance of anaphylaxis or SRS-A, and histamine (Sheard & Blair, 1970). Butchers *et al.* (1979), also demonstrated inhibition by sodium cromoglycate of antigen-induced SRS-A and histamine release, which was maximal at drug concentrations of 3 and 14 μM respectively.

The IgE-mediated release of histamine from isolated mast cells from monkey lung is inhibited by nedocromil sodium, with an IC_{30} of 5.2 μM (Eady, 1986). Similar inhibition of histamine release was shown with mast cells from human lung (Leung *et al.*, 1986).

Recently Kumlin *et al.* (1989), demonstrated that a high concentration of nedocromil sodium (100 μM) causes a rightward shift in the contractile dose-response relation of bronchial strips cumulatively challenged with anti-IgE, and inhibits anti-IgE-induced release of histamine and leukotrienes from human lung tissue by 40 and 50%, respectively.

In the present study, we have measured the effects of nedocromil sodium on antigen-induced release of leukotrienes and histamine from human passively sensitized lung fragments, and antigen-induced contractions of isolated parenchymal strips and bronchial rings from human sensitized lung. A preliminary account of some of the results has been presented previously (Temple *et al.*, 1988).

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Methods

Release of mediators from lung fragments

The method used (Hughes *et al.*, 1983) is described briefly. Small samples of macroscopically normal lung obtained from lobectomy for carcinoma were immersed in oxygenated Tyrode solution (composition, mM: NaCl 137, KCl 2.7, $MgSO_4$ 1.1, NaH_2PO_4 0.4, glucose 5.6, $NaHCO_3$ 11.9, $CaCl_2$ 1.8) at 37°C, cut into 2 mm^3 fragments, washed repeatedly with Tyrode solution and passively sensitized by incubating for 3 h at 37°C in serum from an atopic subject with a Rast titre for IgE of 4+ to house dust mite, *Dermataphogoides pteronyssinus*. Aliquots (250 mg) of lung fragments in Tyrode solution, with or without drug, at 37°C were challenged with an extract of *D. pteronyssinus*. Nedocromil sodium, 0.1, 1 and 10 μM, was added to some replicates 15 min before challenge, and appropriate controls for drug-induced or spontaneous release of mediators were included. The supernatant solutions were assayed for leukotrienes and histamine.

On one occasion, a small sample of lung from a subject atopic to *D. farinae* became available; this was treated as above, omitting passive sensitization, and challenged with a *D. farinae* extract. Nedocromil sodium 1.0 μM and 10 μM was used in this experiment.

Leukotrienes were bioassayed (Hughes *et al.*, 1983) with leukotriene C_4 (LTC_4) standards and the leukotriene antagonist

FPL 55712. Histamine was assayed with an automated fluorimetric method, modified from Evans et al. (1973), and released histamine expressed as a proportion of total tissue histamine.

The effect of each concentration of nedocromil sodium on leukotriene and histamine release from each of 6 lung specimens was expressed as a percentage of control, after correction for any drug-induced or spontaneous release. The statistical significance of the drug treatments on mediator release compared with control release was determined by Student's t test for paired data.

Antigen-induced contraction of parenchymal strips and bronchial rings

Samples of human lungs were cut into strips of parenchyma apparently free of small vessels and bronchi, as described by Creese & Temple (1986). From each lung sample, four such parenchymal strips were sensitized as for lung fragments, and suspended in 20 ml organ baths in Krebs-Henseleit solution (composition, mM: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11.7, $NaHCO_3$ 25, $CaCl_2$ 2.5) at 37°C gassed with 5% CO_2 in O_2. Each strip was attached to an isometric force transducer (Grass FT03) with an initial load of 0.5 g. Histamine, 300 μM, was added initially to provide a maximal contractile response as a reference value for the contractility of each strip and the tissue washed before antigen treatment.

From other lung samples, bronchi with diameter 1–2 mm were dissected, cut into four rings and set up as described by Black et al. (1988).

For both parenchyma and bronchus, three of the tissue samples were treated with nedocromil sodium 0.1, 1 and 10 μM, while the fourth was used as a drug-free control. After 15 min, house dust mite extract was added to each organ bath to give a final concentration of 1 protein nitrogen unit ml^{-1} (Creese & Temple, 1986), and the resulting contractions were recorded.

Antigen-induced contractile responses were expressed relative to the maximal histamine response of each tissue. The response in each drug-treated strip was then compared with its control and the significance of the drug-induced differences in response determined by Student's t test for paired data.

Materials

D. pteronyssinus extract was a gift from Dr E. Tovey, Department of Medicine, University of Sydney, Australia; LTC_4 was a gift from Merck Frosst Canada, Ponte-Claire – Dorval, Quebec, and nedocromil sodium and 7-[3-(acetyl-3-hydroxy-2-propylphenoxy)-2-hydroxypropoxy]-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylic acid (FPL 55712) were donated by Fisons Pharmaceutical Division, Loughborough, Leicestershire.

Results

Release of mediators from lung fragments

The control release of leukotrienes from 6 lung samples was 6.58 ± 0.12 nmol equivalents of $LTC_4\,g^{-1}$ lung (mean ± s.e.mean). Another 5 lung samples failed to release a measurable amount of leukotrienes after antigen challenge and were discarded. The leukotriene antagonist FPL 55712 2 μM inhibited the contractions of the bioassay tissue to released leukotrienes to the same extent as for the LTC_4 standard, indicating that sulphidopeptide-leukotrienes were released. Histamine release was 10.3 ± 1.8% of total tissue histamine ($n = 5$), or 7.84 ± 1.89 nmol histamine g^{-1} lung tissue.

The antigen-induced release of leukotrienes from human lung was significantly inhibited by nedocromil sodium 0.1 and 1 μM, by 30% and 38% respectively, but the highest concentration used, 10 μM, caused no inhibition (Table 1). Histamine release was also significantly inhibited by 0.1 and 1 μM nedocromil sodium, by 43% in each case (Table 1). Nedocromil sodium at the concentrations used did not affect the bioassay for leukotrienes or the fluorometric assay.

Table 1 The effect of nedocromil sodium on antigen-induced release of mediators from sensitized fragments of human lung

Nedocromil (μM)	*Percentage of control release* *Leukotrienes (SRS-A)*	*Histamine*
0	100	100
0.1	70.3 ± 8.4 (4)*	57.5 ± 12.8 (4)*
1.0	62.3 ± 13.6 (6)*	57.3 ± 12.4 (5)*
10.0	101.8 ± 15.8 (5)	86.8 ± 14.8 (5)

Data shown are means ± s.e.mean of number of samples in parentheses. The mean release of leukotrienes was equivalent to 6.58 ± 0.12 nmol $LTC_4\,g^{-1}$ lung from 6 lung samples, and of histamine 7.84 ± 1.89 nmol g^{-1}, from 5 samples. Antigen-induced release of each of these mediators in the presence of each concentration of nedocromil sodium was calculated as a percentage of the control antigen-induced release in the absence of drug, after correcting for spontaneous release. *$P < 0.05$, paired t test.

The results from the inherently atopic lung sample are included in those presented in Table 1. Leukotriene release from this lung, 3.6 nmol equivalents of $LTC_4\,g^{-1}$, was reduced to 84% of control by nedocromil sodium 1 μM and increased by 10 μM to 114% of control. Histamine release, determined only for nedocromil sodium 1 μM, was reduced to 81% of control.

Antigen-induced contraction of human lung strips

Parenchymal strips Contractile responses to histamine of parenchymal strips from 6 separate lung specimens ranged from 20 to 130 mg tension (mean ± s.e.mean 45.5 ± 5.3 mg, $n = 24$). The addition of *D. pteronyssinus* extract caused contraction of the parenchymal strips to 40.0 ± 10.9% of the maximal histamine response.

At concentrations from 0.1 to 10 μM, nedocromil sodium did not affect the tone of parenchymal strips or their contractile responses to histamine or LTC_4. The effects of nedocromil sodium 0.1, 1 and 10 μM, on antigen-induced contraction are summarized in Table 2. There was a significant inhibition of contractions, 50% and 70% compared with control values, by nedocromil sodium 0.1 and 1 μM respectively. This inhibition

Table 2 The effect of nedocromil sodium on antigen-induced contractions of sensitized strips of human lung parenchyma

Nedocromil sodium (μM)	*Response* (% of histamine maximal contraction)	*Effect of drug* (% control)
0 (control)	40.0 ± 10.9	100
0.1	14.3 ± 4.9	49.7 ± 16.8*
1.0	11.2 ± 4.9	30.2 ± 11.2**
10.0	27.7 ± 18.1	120.2 ± 89.3

Data shown are means ± s.e.mean. Antigen-induced contractions were standardized in terms of the maximal contractile response to histamine of each lung strip. The effect of nedocromil sodium was determined by comparing the antigen-induced contraction of the lung strip in the presence of each concentration of drug with the paired control response in the absence of drug. The right hand column shows the mean effects of nedocromil determined from 6 lung samples, each of which provided 4 strips. Significantly different from paired control, *$P < 0.05$, **$P < 0.005$.

Table 3 The effect of nedocromil sodium on antigen-induced contractions of sensitized rings of human bronchi

Nedocromil sodium (μM)	*Response* (% of histamine maximal contraction)	*Effect of drug* (% control)
0 (control)	86.4 ± 13.8 (7)	100
0.1	93.5 ± 31.7 (6)	112.9 ± 11.0
1.0	47.5 ± 16.9 (7)	52.1 ± 19.7*
10.0	86.9 ± 19.6 (7)	99.9 ± 24.3

Data shown are means ± s.e.mean of (n) samples. Antigen-induced contractions of rings of bronchus were standardized by expressing each in terms of its maximal contractile response to histamine. The effect of each concentration of drug was determined by expressing the antigen-induced contraction of each preparation in the presence of drug as a percentage of the contractile response of its paired control to which no drug was added. Seven bronchial samples were used, each cut into 4, or in one case 3, rings. *$P = 0.051$, paired t test.

was not concentration-dependent since the antigen-induced contractions were either unaffected by nedocromil sodium 10 μM, or tended to increase.

Bronchial rings Contractile responses to histamine of bronchial rings from 7 operative specimens ranged from 28 to 1225 mg tension (mean, 423.5 ± 63.7 mg, $n = 27$). Antigen-induced contraction was 86.4 ± 13.8% of the histamine maximal response. Nedocromil sodium at concentrations from 0.1 to 10 μM did not affect the tone of the preparations or their contractility. The effect of the drug on antigen-induced contractions was less than on the parenchymal strips, as seen in Table 3. Neither the lowest nor the highest concentration modified the effect of antigen, but 1 μM nedocromil sodium inhibited antigen-induced contractions by 48%, although this just failed to reach statistical significance ($P = 0.051$).

Discussion

Nedocromil sodium inhibited the antigen-induced release of leukotrienes and histamine from human sensitized lung *in vitro*. These results agree with those of Leung *et al.* (1986), who showed that nedocromil sodium, 0.1 to 100 μM, inhibits anti-IgE-induced histamine release from human isolated lung mast cells. Nedocromil sodium is more potent than sodium cromoglycate, used by Sheard & Blair (1970) and Butchers *et al.* (1979), in this *in vitro* model of the anaphylactic release of mediators in lung. This high potency relative to cromoglycate has been shown previously (Leung *et al.*, 1986; Eady, 1986; Altounyan *et al.*, 1986; del Bono *et al.*, 1986; Crimi *et al.*, 1986; Konig *et al.*, 1987).

Inhibition of antigen-induced contractions of isolated preparations of human respiratory tissue showed quantitative differences in the effects of nedocromil in each type of preparation. The inhibition by 0.1 and 1 μM nedocromil sodium of antigen-induced contraction of human lung parenchymal strips was greater than its inhibition of corresponding contractions of rings of human bronchus. However, antigen-induced contractions of bronchus were much stronger than those of parenchyma in terms of maximal tension developed. Parenchymal and bronchial contractions induced by antigen are mediated by leukotrienes and histamine, and by other spasmogens released additionally, since contractions are only partly blocked by drugs which inhibit the effects or production of leukotrienes and histamine (Creese & Temple, 1986). The 70% inhibition of antigen-induced contractions by nedocromil sodium 1 μM contrasts with the 39% inhibition by sodium cromoglycate 20 μM in similar experiments (Creese & Temple, 1986). Probably nedocromil sodium at the concentration used is inhibiting the release of additional contractile mediators from the lung.

These results suggest a different contribution of contractile mediators between parenchymal and bronchial tissue, while bronchial rings may possess a greater proportion of contractile tissue such as smooth muscle.

The degree of inhibition of leukotriene and histamine release shown in the present results for nedocromil sodium 1 μM is similar to that obtained by Kumlin *et al.* (1989), for inhibition of anti-IgE-induced release of leukotrienes and histamine by nedocromil sodium 100 μM. It is possible that tolerance may have developed to nedocromil sodium at the high concentration used by Kumlin *et al.* (1989), since tolerance to sodium cromoglycate incubated with human lung fragments has been demonstrated (Church & Young, 1983). The concentrations used by us (0.1–10 μM) were in the same range as those used with monkey tissues (Eady, 1986) and are likely to be clinically relevant.

The inhibition shown for nedocromil sodium was not concentration-dependent, since nedocromil 1 μM was inhibitory but 10 μM was without effect in experiments in which leukotriene and histamine release and also antigen-induced contractility were measured. This suggests that this drug has a biphasic effect in each of these preparations. Previous findings of bell-shaped or non-dose-related inhibitory dose-response curves particularly involve sodium cromoglycate. Concentration-independent inhibition of mediator release from human lung by cromoglycate was demonstrated by Assem & Mongar (1970), Butchers *et al.* (1979) and Church & Young (1983). Both nedocromil sodium and sodium cromoglycate inhibited degranulation of rat skin mast cells by 48/80 with bell-shaped dose-response curves (Riley *et al.*, 1987). Desensitization of the tissue, described by Church & Young (1983) for sodium cromoglycate, may also be caused by nedocromil sodium 10 μM and may reduce its effect after a 15 min incubation.

Under some conditions, concentration-dependent inhibition of anaphylactic responses by sodium cromoglycate and nedocromil sodium may occur, as demonstrated by Butchers *et al.* (1979), Eady (1986) and Leung *et al.* (1986). It seems possible that in human sensitized tissue inhibition unrelated to concentration may depend on the type of immunological challenge used. Church (1978) showed that sodium cromoglycate inhibited histamine release from human lung in an apparently biphasic manner when pollen extract was used, in contrast to its dose-related inhibition after anti-IgE challenge. The concentration-independence of the inhibitory action of nedocromil sodium, in the three *in vitro* models described here, may reflect the use of an extract of antigen to challenge the sensitized preparations.

The present results suggest that the useful therapeutic effects of nedocromil in the treatment of allergic asthma depend, partly, upon its ability to inhibit the immunological release of leukotrienes and histamine and consequently of antigen-induced contraction of airways.

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2-Chloroadenosine induction of vagally-mediated and atropine-resistant bronchomotor responses in anaesthetized guinea-pigs

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1 The bronchoconstrictor effects of intravenous administration of adenosine derivatives in anaesthetized non-curarized guinea-pigs have been studied.

2 2-Chloroadenosine (2-Cl-Ade), 5'-N-ethylcarboxamideadenosine (NECA) and L-N^6-phenylisopropyladenosine (L-PIA) all produced dose-dependent, transient increases in tracheal insufflation pressure, with an order of potency (NECA $\geqslant$ 2-Cl-Ade $\gg$ L-PIA) typical of A_2-receptor mediated biological responses.

3 2-Chloroadenosine-induced bronchoconstrictor responses disappeared after vagotomy or topical application of tetrodotoxin (TTX) on cervical vagal trunks.

4 2-Chloradenosine-induced bronchospasm was unaffected by atropine (1 mg kg^{-1} i.v.), physostigmine (50 μg kg^{-1} i.v.) and hexamethonium (30 mg kg^{-1} i.v.) but was significantly reduced by theophylline (25 mg kg^{-1} i.v.).

5 The magnitude of 2-Cl-Ade-induced bronchospasm was significantly reduced by acute (10 μg kg^{-1} i.v.) or chronic (55 mg kg^{-1} s.c. four days before the experiment) pretreatment with capsaicin.

6 Guanethidine (20 mg kg^{-1} s.c. on two consecutive days), prazosin (10 μg kg^{-1} i.v.), diphenhydramine (1 mg kg^{-1} i.v.) and indomethacin (1 mg kg^{-1} i.v.) failed to block the bronchomotor response to 2-Cl-Ade. In contrast, cyproheptadine (1–5 mg kg^{-1} i.v.) markedly reduced, but did not abolish the bronchospasm elicited by the purine derivative.

7 We conclude that in anaesthetized non-curarized guinea-pigs, a transient vagally-mediated bronchospasm can be induced by stimulation of A_2-purinoceptors. This effect is complex and involves, at least in part, stimulation of capsaicin-sensitive sensory nerves and 5-hydroxytryptamine release. This experimental model might be useful for the further study of the potential role of adenosine in asthma, and for the evaluation of new antiasthma drugs.

Introduction

The purine nucleoside, adenosine, has been proposed as a possible mediator of asthma (Holgate *et al.*, 1984; Church & Holgate, 1986). In man, it is released by allergen challenge (Mann *et al.*, 1985) and it induces prompt bronchoconstriction in asthmatic, but not in normal subjects (Cushley *et al.*, 1983; Holgate *et al.*, 1984). However, experimental evidence for a bronchoconstrictor role of adenosine is still scanty. On human isolated bronchi, adenosine has very little motor effect (Finney *et al.*, 1985) and in various animal studies this purine has been shown to possess bronchodilator rather than bronchospastic properties (Farmer & Farrar, 1976; Karlsson *et al.*, 1982; Caparrotta *et al.*, 1984). Very recently it has been shown that in one particular strain of rat (BDE), intravenous injections of adenosine evoke an increase in lung resistance and a decrease in dynamic compliance (Pauwels & Van Der Straeten, 1987). However, unlike the adenosine-induced bronchoconstriction in man (Cushley *et al.*, 1984; Mann *et al.*, 1985; Mann & Holgate, 1985; Holgate *et al.*, 1985), the adenosine-induced bronchospastic response in BDE rats was atropine-sensitive and largely theophylline-resistant (Pauwels & Van Der Straeten, 1987).

We now show that in anaesthetized, non-curarized guinea-pigs, 2-chloro-adenosine (2-Cl-Ade) induces bronchoconstrictor responses whose physiological and pharmacological profile resembles the response of human asthmatic lungs more than the responses obtained in rats. We present evidence that the effect of this adenosine agonist in guinea-pig lung is due to stimulation of A_2-purinoceptors, is vagally-mediated and, at least partly, due to the release of 5-hydroxytryptamine (5-HT) and activation of capsaicin-sensitive sensory nerves.

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Methods

In vivo *studies*

Urethane-anaesthetized (1.5 g kg^{-1} i.p.) fasted (18 h) male albino guinea-pigs (Savo-Charles River, Calco Bergamo), weighing 300–350 g, were used. Body temperature was controlled electronically (Marb 8511) and maintained at 34.5°C.

The animals were ventilated mechanically through a tracheal cannula. The ventilation pump (Basile 7025) was adjusted at a rate of 60 strokes min^{-1}. Respiration volume was kept constant by means of a water valve providing an initial basal pressure of 1.2–1.7 kPa. Insufflation pressure was measured by attaching a pressure transducer (Bentley Trantec) to a side-arm of the tracheal cannula. Changes in insufflation pressure were monitored on a Basile Unirecord 7050 polygraph. A polyethylene catheter was inserted in the left jugular vein for drug injection. Drugs were administered in a volume less than 0.5 ml kg^{-1}. At the end of the stabilization period (30 min), additional urethane (250 mg kg^{-1} s.c.) was administered. The basal value of insufflation pressure remained stable for at least 2 h and no significant changes were produced by i.v. saline administration.

Preliminary experiments showed that at least three reproducible responses to 2-Cl-Ade (20 μg kg^{-1}) could be evoked at intervals of 30 min. Dose-response curves to 2-Cl-Ade, 5'-N-ethylcarboxamide adenosine (NECA) and L-N^6-phenylisopropyladenosine (L-PIA) were obtained by administration of increasing concentrations of the agonist in a non-cumulative manner at 30 min intervals.

The effect of potential antagonists on 2-Cl-Ade bronchomotor responses was evaluated by comparing, in the same animal, responses to 2-Cl-Ade before and 15 or 30 min (capsaicin) after drug administration.

Some experiments were performed in guinea-pigs pretreated (four days before the experiments) with capsaicin. Capsaicin (10 mg ml^{-1}) was dissolved in ethyl alcohol (10%) and Tween 80 (10%), and diluted in saline. Multiple s.c. injections (total dose 55 mg kg^{-1}) were given over two days (four administrations per day) starting with a dose of 0.3 mg kg^{-1}. To counteract capsaicin-induced respiratory impairment, the animals were pretreated i.p. with aminophylline (4 mg kg^{-1}), diphenydramine (2.5 mg kg^{-1}), atropine sulphate (1.5 mg kg^{-1}) and terbutaline (0.1 mg kg^{-1}) twenty min before each capsaicin injection.

In some animals chemical sympathectomy was produced by two s.c. injections of guanethidine (10 mg kg^{-1}) at a 16 h interval. Experiments were performed one h after the second administration. Effectiveness of this procedure was confirmed by the disappearance of tyramine (5 mg kg^{-1} i.v.)-induced positive chronotropic action, the maximal increase in heart rate being 62.5 ± 13 and 5 ± 5 beats min^{-1} in control and guanethidine-pretreated guinea-pigs, respectively ($n = 5$, $P < 0.01$).

Drugs

The following drugs were used: acetylcholine bromide (Carlo Erba); adenosine (Janssen); 2-chloroadenosine (Sigma); arachidonic acid (Sigma); atropine sulphate (Merck); capsaicin (Sigma); cyproheptadine (Sigma); decamethonium bromide (Sigma); diphenhydramine (Gianni); 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) (Sigma); guanethidine sulphate (Gianni); hexamethonium (Aldrich); histamine (Merck); 5-hydroxytryptamine (Merck); indomethacin (Merk); inosine (Janssen); NECA (RBI); neurokinin A (Peninsula); physostigmine (Merck); phenylephrine (Janssen); L-PIA (RBI); prazosin (Ricerchimica); succinylcholine chloride (Serva); theophylline ethylenediamine (Malesci); terbutaline (Terbasmin, Farmitalia); tetrodotoxin (TTX, Serva); tyramine chloride (Fluka); (+)-tubocurarine (Fluka).

Statistical analysis

All data in the text are means ± s.e. Statistical analysis was performed by means of Student's t test for paired or unpaired data when applicable.

Results

Effects of 2-chloroadenosine, NECA, L-PIA and inosine on insufflation pressure in urethane-anaesthetized guinea-pigs

Intravenous administration of 2-Cl-Ade (0.5–20 μg kg^{-1}), NECA (0.5–10 μg kg^{-1}) and L-PIA (1–20 μg kg^{-1}) produced a prompt (within 10–15 s), dose-related increase in tracheal insufflation pressure with a rank order of potency of NECA ≥ 2-Cl-Ade ≫ L-PIA (Figure 1). NECA was slightly more effective than 2-Cl-Ade at the doses of 0.5 and 1 μg kg^{-1}, and in addition, both NECA and 2-Cl-Ade were significantly ($P < 0.01$) more potent than L-PIA at all doses tested. At the dose of 10 μg kg^{-1}, the increases in insufflation pressure elicited by 2-Cl-Ade, NECA and L-PIA were 0.80 ± 0.09 kPa ($n = 5$), 0.90 ± 0.08 kPa ($n = 5$) and 0.52 ± 0.10 kPa ($n = 5$), respectively. As shown in Figure 2, the response to 2-Cl-Ade or L-PIA returned to basal values quite rapidly (3–5 min), while NECA-induced increase in insufflation pressure was long-lasting (longer than 15 min). With this dose, reproducible responses (at 30 min intervals) could be obtained with 2-Cl-Ade, but not with NECA.

Adenosine (20 μg kg^{-1} i.v.), but not its final metabolite, inosine (20 μg kg^{-1} i.v., Figure 2), also elicited an increase in insufflation pressure. Amplitude (0.33 ± 0.09 kPa; $n = 5$) and duration (about 1 min) of the adenosine effect were significantly lower than those observed with the same dose of 2-Cl-Ade. These findings are probably linked to the greater metabolic stability of 2-Cl-Ade (Daly *et al.*, 1987) and therefore in the following sets of experiments we chose to use the chloride derivative of adenosine. Administration of theophylline (25 mg kg^{-1} i.v.) significantly reduced the response to 2-Cl-Ade (20 μg kg^{-1} i.v.) from 0.85 ± 0.04 to 0.52 ± 0.03 kPa ($n = 5$, $P < 0.05$) and also that to acetylcholine (25 μg kg^{-1} i.v.) from 1.16 ± 0.2 to 0.56 ± 0.1 kPa ($n = 8$; $P < 0.05$). As the animals were not pretreated with (+)-tubocurarine, higher doses of theophylline could not be used in view of the occurrence of excessive voluntary respiratory movements.

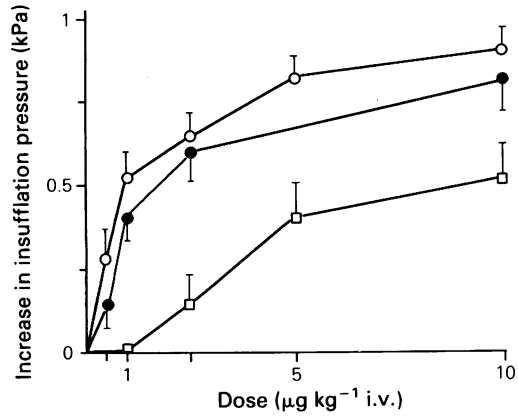

Figure 1 Increase in tracheal insufflation pressure elicited by 2-chloroadenosine (●), 5'-N-ethylcarboxamideadenosine (○) and L-N^6-phenylisopropyladenosine (□) in urethane-anaesthetized non-curarized guinea-pigs. Each value is the mean of at least 5 experiments; vertical lines show s.e.

Effect of vagotomy or topical application of TTX on cervical vagal trunks on 2-chloroadenosine-induced increase in insufflation pressure

Thirty minutes after bilateral cervical vagotomy, the bronchospastic response to 2-Cl-Ade (20 μg kg^{-1} i.v.) was reduced

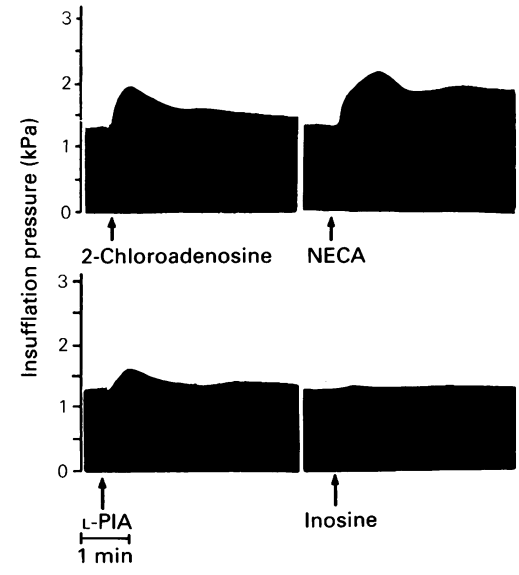

Figure 2 Typical tracings showing the bronchomotor response induced by the intravenous administration of 2-chloroadenosine, 5'-N-ethylcarboxamideadenosine (NECA), L-N^6-phenylisopropyladenosine (L-PIA) (10 μg kg^{-1}) and inosine (20 μg kg^{-1}) in urethane-anaesthetized non-curarized guinea-pigs. Arrows indicate agonist administration.

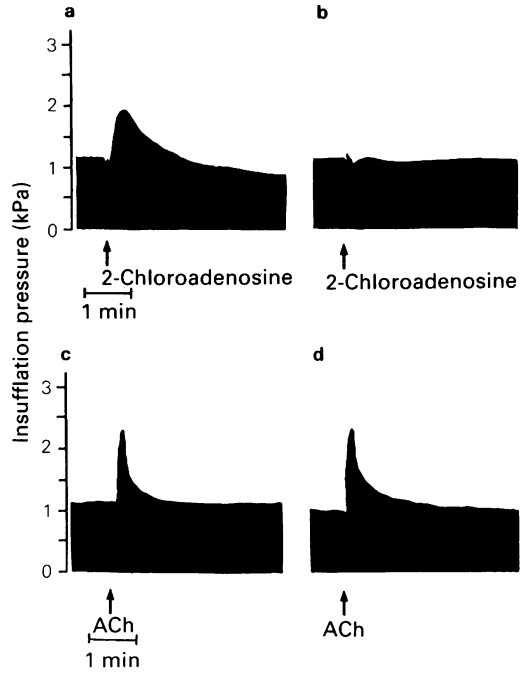

Figure 3 Typical tracings showing the effect of bilateral vagotomy on the increase in insufflation pressure elicited by 2-chloroadenosine ($20\,\mu g\,kg^{-1}$) (a and b) or by acetylcholine (ACh, $50\,\mu g\,kg^{-1}$) (c and d) in urethane-anaesthetized non-curarized guinea-pigs. Arrows indicate agonist administration. (a and c) Control responses; (b and d) responses 30 min after bilateral vagotomy.

from 0.74 ± 0.09 to 0.08 ± 0.03 kPa ($n = 12$, $P < 0.001$). In 9 out of 12 animals the response to 2-Cl-Ade was abolished by vagal resection (Figure 3). Vagotomy had no inhibitory effect on acetylcholine ($50\,\mu g\,kg^{-1}$ i.v.)-induced increase in insufflation pressure, which amounted to 1.51 ± 0.19 and 2.16 ± 0.22 kPa before and 30 min after vagotomy, respectively ($n = 10$, $P < 0.01$). Interestingly enough, the inhibitory effect of vagal resection on 2-Cl-Ade-induced bronchospasm was time-dependent, being significantly greater at 30 ($91.2 \pm 5\%$, $n = 12$) than at 5 min after vagotomy ($62.6 \pm 5\%$, $n = 10$, $P < 0.01$) (Figure 4).

In a further set of experiments, tetrodotoxin (TTX) was applied locally ($20\,\mu l$ of a $100\,\mu g\,ml^{-1}$ solution) on both vagal nerves at the cervical level. Thirty minutes after TTX-treatment, 2-Cl-Ade bronchospastic responses were reduced from 1.08 ± 0.2 to 0.34 ± 0.16 kPa ($n = 4$; $P < 0.01$) with an inhibition of $73.9 \pm 11\%$. Topical application of TTX also

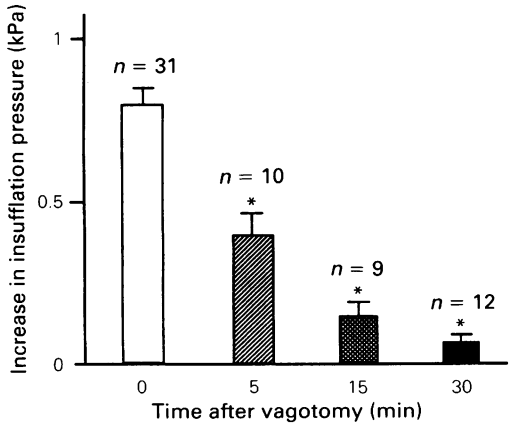

Figure 4 Time-dependency of the inhibition of 2-chloroadenosine ($20\,\mu g\,kg^{-1}$)-induced increase in insufflation pressure produced by the bilateral resection of vagal nerves. Each column shows the mean of n determinations; vertical bars represent s.e. The open column refers to the responses obtained before vagotomy. * $P < 0.001$.

abolished bronchospastic responses elicited by electrical stimulation (20 Hz, 1 ms, supramaximal voltage for 20 s) of the vagal nerve ($n = 4$).

Effect of atropine, physostigmine, hexamethonium, or curarizing agents on 2-chloroadenosine-induced increase in insufflation pressure

In this set of experiments we investigated whether or not responses to 2-Cl-Ade ($20\,\mu g\,kg^{-1}$ i.v.) might be modulated by pharmacological agents acting on cholinergic neuroeffector junction.

As shown in Table 1, atropine, at a dose ($1\,mg\,kg^{-1}$ i.v.) which abolished bronchospasm elicited by acetylcholine, had no inhibitory effect on 2-Cl-Ade-induced increase in insufflation pressure. On the other hand, inhibition of acetylcholinesterases by physostigmine ($50\,\mu g\,kg^{-1}$ i.v.) resulted in a significant enhancement of acetylcholine- but not of 2-Cl-Ade-induced bronchospasm (Table 1).

The intravenous administration of the ganglionic blocking agent hexamethonium ($30\,mg\,kg^{-1}$ i.v.) markedly diminished the transient increase in insufflation pressure produced by the nicotinic stimulant DMPP ($0.2\,mg\,kg^{-1}$ i.v.) (amplitude of responses was reduced from 0.82 ± 0.18 to 0.15 ± 0.06 kPa, $n = 5$, $P < 0.01$), but was without appreciable effect on 2-Cl-Ade-induced bronchomotor responses (being respectively 0.77 ± 0.1 and 0.66 ± 0.07 kPa before and after hexamethonium administration, $n = 5$, NS). Further experiments indicated that 2-Cl-Ade ($20\,\mu g\,kg^{-1}$)-induced increase in insufflation pressure could not be elicited in (+)-tubocurarine ($3\,mg\,kg^{-1}$ i.v.)-pretreated guinea pigs ($n = 6$). A marked inhibition could be observed even with doses of (+)-tubocurarine as low as $0.25\,mg\,kg^{-1}$ (bronchomotor responses to 2-Cl-Ade was reduced from 0.57 ± 0.07 to 0.17 ± 0.07 kPa, $n = 6$, $P < 0.01$). Interestingly, the depolarizing agents decamethonium ($1\,mg\,kg^{-1}$ i.v.) and succinylcholine ($3\,mg\,kg^{-1}$ i.v.) also abolished the bronchomotor response to 2-Cl-Ade ($n = 5$ for each antagonist).

Effect of capsaicin-desensitization on 2-chloroadenosine-induced increase in insufflation pressure

The aim of these experiments was to evaluate the potential role of capsaicin-sensitive structures (non-adrenergic, non-cholinergic vagal endings) in the bronchomotor response to 2-Cl-Ade.

Intravenous capsaicin ($10\,\mu g\,kg^{-1}$) resulted in a prompt increase in insufflation pressure amounting to 1.85 ± 0.18 kPa ($n = 7$). A second administration of capsaicin did not evoke any bronchospastic response, indicating complete desensitization. When the challenge with 2-Cl-Ade ($20\,\mu g\,kg^{-1}$) was carried out 30 min after capsaicin exposure, amplitude of its bronchospastic response was reduced from 0.69 ± 0.06 kPa to 0.48 ± 0.09 kPa ($n = 7$; $P < 0.01$). Capsaicin administration had no inhibitory effect on the bronchomotor response elicited by intravenously administered neurokinin A ($0.5\,\mu g\,kg^{-1}$) (increase in insufflation pressure were respectively 0.52 ± 0.07 and 0.51 ± 0.06 kPa before and after capsaicin administration, $n = 4$, NS).

In a further set of experiments, the bronchomotor response to 2-Cl-Ade was evaluated in guinea-pigs systemically pretreated with a high dose of capsaicin ($55\,mg\,kg^{-1}$ s.c. four days before the experiments—see Methods section), a procedure which has been demonstrated to reduce markedly sensory neuropeptide content in guinea-pig respiratory tract (Manzini *et al.*, 1987). The amplitude of the bronchomotor response to 2-Cl-Ade ($20\,\mu g\,kg^{-1}$) was significantly ($P < 0.05$) lower in capsaicin-pretreated (0.51 ± 0.04 kPa, $n = 8$) as compared to control animals (0.75 ± 0.09 kPa, $n = 12$). In capsaicin-pretreated guinea-pigs, the amplitude of capsaicin ($10\,\mu g\,kg^{-1}$ i.v.)-induced bronchospasm was markedly reduced ($79 \pm 2.3\%$ inhibition, $n = 9$).

Table 1 Effect of atropine and physostigmine on the amplitude of bronchospasm elicited by 2-chloroadenosine and acetylcholine in anaesthetized non-curarized guinea-pigs

Drugs	Dose ($\mu g\,kg^{-1}$ i.v.)	Increase in insufflation pressure (kPa) Control	Atropine ($1\,mg\,kg^{-1}$ i.v.)	Physostigmine ($50\,\mu g\,kg^{-1}$ i.v.)
2-Chloroadenosine	20	0.63 ± 0.09 ($n = 10$)	0.64 ± 0.1[c] ($n = 5$)	0.55 ± 0.04[c] ($n = 5$)
ACh	12.5	0.74 ± 0.20 ($n = 5$)	0[b] ($n = 5$)	2.83 ± 0.28[a] ($n = 5$)

Atropine and physostigmine were administered 15 min before the challenge with the agonists. Each value is the mean ± s.e. of n determinations.
[a] $P < 0.01$; [b] $P < 0.001$; [c] $P > 0.05$ as compared to respective controls.

Effect of guanethidine, diphenhydramine, indomethacin, prazosin and cyproheptadine on 2-chloroadenosine (20 $\mu g\,kg^{-1}$ i.v.)-induced increase in insufflation pressure

In these experiments we investigated whether or not the response to 2-Cl-Ade might involve the excitation of sympathetic nerve fibres (guanethidine pretreatment), α_1-adrenoceptors (prazosin), H_1-histamine receptors (diphenhydramine), 5-HT-receptors (cyproheptadine) and/or products of the cyclo-oxygenase metabolic pathway (indomethacin).

Chemical sympathectomy was obtained by pretreatment of the animals with guanethidine ($20\,mg\,kg^{-1}$ s.c.). No significant difference was detected in the bronchomotor responses to 2-Cl-Ade in guanethidine-pretreated (0.69 ± 0.1 kPa, $n = 5$) as compared to control animals (0.70 ± 0.08, $n = 10$, NS).

Prazosin, at a dose ($10\,\mu g\,kg^{-1}$ i.v.) with substantial α-adrenoceptor blocking activity (Bonacchi *et al.*, 1988), had no inhibitory effect on 2-Cl-Ade-induced increase in insufflation pressure ($n = 4$). Furthermore, the α-adrenoceptor agonist, phenylephrine, up to a dose of $100\,\mu g\,kg^{-1}$ i.v. did not produce any detectable increase in insufflation pressure ($n = 4$). Also diphenhydramine, at a dose ($1\,mg\,kg^{-1}$ i.v.) which abolished the bronchoconstriction elicited by histamine ($2.5\,\mu g\,kg^{-1}$ i.v.) (1.32 ± 0.11 kPa, $n = 4$), exerted no antagonistic effect on the 2-Cl-Ade-induced bronchomotor response ($n = 4$).

In contrast, cyproheptadine ($1\,mg\,kg^{-1}$ i.v.) significantly reduced bronchomotor responses to both 2-Cl-Ade (from 0.82 ± 0.11 to 0.38 ± 0.11 kPa, $55.9 \pm 8.1\%$ of inhibition, $n = 5$, $P < 0.01$) and 5-HT ($5\,\mu g\,kg^{-1}$ i.v.) (from 1.85 ± 0.26 to 0.34 ± 0.10 kPa, $n = 5$, $P < 0.01$). At the higher dose of $5\,mg\,kg^{-1}$ i.v. cyproheptadine had no further inhibitory effect on responses to 2-Cl-Ade ($45.5 \pm 9.9\%$ of inhibition, $n = 5$), but abolished responses to 5-HT ($n = 4$) (Figure 5).

Finally, the cyclo-oxygenase inhibitor indomethacin ($1\,mg\,kg^{-1}$ i.v.) had no effect on the bronchomotor response to 2-Cl-Ade (from 1.20 ± 0.12 to 1.21 ± 0.1 kPa, $n = 3$, NS), but significantly enhanced the increase in insufflation pressure elicited by the exogenous administration of arachidonic acid ($0.25\,mg\,kg^{-1}$ i.v.) (from 0.72 ± 0.15 to 0.94 ± 0.16 kPa, $n = 5$, $P < 0.01$).

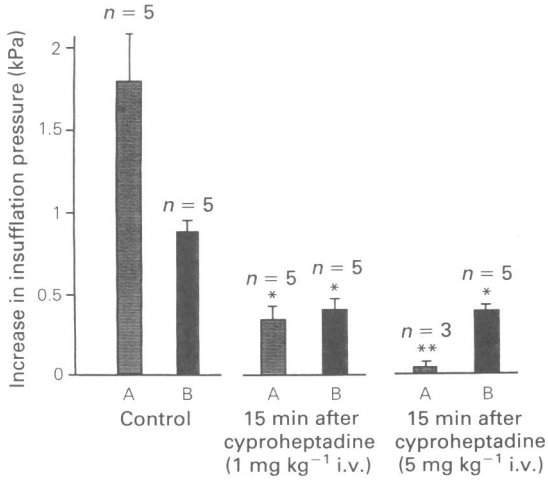

Figure 5 Inhibitory effects of cyproheptadine (at doses of 1 and $5\,mg\,kg^{-1}$ i.v.) on the increase in tracheal insufflation pressure elicited by 2-chloroadenosine ($20\,\mu g\,kg^{-1}$) (columns B) and 5-hydroxytryptamine (5-HT, $5\,\mu g\,kg^{-1}$) (columns A). Each column shows the mean of n determinations; vertical bars represent s.e. * $P < 0.01$, ** $P < 0.001$.

Discussion

In this study, we have attempted to characterize an *in vivo* model for studying adenosine-mediated bronchospasm in the guinea-pig. In our experimental conditions, 2-Cl-Ade evoked an increase in insufflation pressure which, like adenosine-induced bronchospasm in man (Cushley *et al.*, 1984; Mann & Holgate, 1985; Holgate *et al.*, 1985), was atropine-resistant and theophylline-sensitive. In addition, 2-Cl-Ade-induced bronchospasm was characterized by its dependency on vagal fibre integrity and its partial sensitivity to capsaicin desensitization and 5-HT-receptor blockade. The use of non-curarized guinea-pigs is mandatory for obtaining purine-induced bronchospasm. This methodological point may explain previous failures to obtain an *in vivo* bronchomotor response to adenosine. At the moment we have no satisfactory explanation for this finding, which has not been investigated further in this study.

Purine bronchomotor responses, as well as other adenosine-mediated biological actions are classically linked to the activation of two receptor subtypes named A_1 and A_2 (Daly *et al.*, 1987). Interestingly enough, 2 Cl-Ade-induced bronchospasm is antagonized by theophylline. However, theophylline also blocked bronchospasm induced by acetylcholine with similar potency, suggesting that, in our experimental conditions, it behaves as a bronchodilator rather than as a selective blocker of adenosine receptors.

In guinea-pig isolated trachea, Ghai *et al.* (1987) have recently presented evidence for the existence of both receptor subtypes, with A_1 mediating contraction and A_2 relaxation of smooth muscle. These findings are in contrast with *in vivo* studies which suggest that adenosine-induced bronchospasm is mediated by A_2-receptors (Pauwels & Van der Straeten, 1986; 1987). In our experimental conditions we obtained a rank order of potency for adenosine derivatives in eliciting bronchospasm (NECA ≥ 2-Cl-Ade ≫ L-PIA) which is typical of an A_2-mediated response. Also radioligand binding studies have confirmed the existence in guinea-pig lung of stimulating A_2-adenosine receptors coupled to activation of adenylate cyclase (Ukena *et al.*, 1985). A possible explanation of the discrepancy between *in vitro* and *in vivo* data might be that A_2-adenosine receptor-mediated bronchospasm is linked to a modification of the neurogenic control of respiratory function. Indeed, 2-Cl-Ade-induced bronchomotor responses disappeared after cervical vagotomy or topical application of tetrodotoxin on vagal fibres. Hexamethonium did not exhibit

any inhibitory effect, suggesting that a centrally-mediated reflex is not involved. Gustafsson *et al.* (1986) showed that in rabbit bronchi, cholinergic transmission is enhanced by adenosine A_2-receptor stimulation, but, in our experiments, atropine failed to inhibit and physostigmine to enhance the bronchomotor response to 2-Cl-Ade. Certainly our studies do not rule out the involvement of other excitatory cotransmitters putatively present in postganglionic cholinergic nerves. However, it is important to appreciate that cutting the vagi should have eliminated not only efferent cholinergic input but also non-adrenergic, non-cholinergic (NANC) excitatory fibres. Interestingly, the reduction in amplitude of 2-Cl-Ade-induced bronchospasm produced by vagal resection was time-dependent, became maximal only after 30 min. These findings could suggest that adenosine might act at some postganglionic vagal fibres, stimulating the release of NANC neurotransmitter(s) whose availability might be strictly dependent upon a rapid refilling process along the neural axis (typical of peptidergic fibres). In this context, the results obtained with capsaicin are interesting. Capsaicin, the hot ingredient of the red pepper, selectively stimulates a specific subset of sensory afferents leading to the release and subsequent depletion of sensory neuropeptides (Holzer, 1988; Maggi & Meli, 1988). These capsaicin-sensitive sensory fibres possess the peculiarity of a dual sensory-efferent function (Szolcsanyi, 1984). It is now well established that neurokinins locally released from capsaicin-sensitive sensory fibres are among the most potent endogenous substances in eliciting bronchospasm, neurogenic inflammation and secretion (Jancso *et al.*, 1967; Lundberg & Saria 1982a,b; Coles *et al.*, 1984; Manzini *et al.*, 1989; Rogers & Barnes, 1989) and their involvement in asthma pathogenesis has been proposed (Barnes, 1986). Following acute or chronic capsaicin desensitization, the bronchomotor response elicited by 2-Cl-Ade was partially reduced, suggesting that at least part of the adenosine action is mediated by the excitation of A_2-receptors prejunctionally located on sensory afferents. It has been shown that, in rabbit iris sphincter, adenosine can exert a dual modulation of the neural control by inhibiting the cholinergic component through stimulation of A_1-receptors and by enhancing the capsaicin-sensitive NANC component through stimulation of prejunctional A_2-receptors (Gustafsson & Wiklund, 1986). Very recently, Kamikawa & Shimo (1989) have shown that adenosine treatment antagonizes the motor response elicited by non-cholinergic neurotransmission in guinea-pig isolated bronchial chain preparations, but no attempt was made to identify the purinoceptor subtype mediating this response. Further studies will be necessary to clarify the possible existence of an A_2-excitatory purinoceptor on nerve endings of capsaicin-sensitive NANC fibres in the guinea-pig tracheobronchial tree.

As the motor response to 2-Cl-Ade was only partially reduced by capsaicin desensitization, other mechanism(s) also appear to be involved. Adenosine possesses various biological effects which might contribute to the production of its bronchospastic actions, such as inhibition of adrenergic neurotransmission (Su, 1978), release of histamine from mast cells and basophils (Welton & Simko, 1980; Church *et al.*, 1983), release of 5-HT from mast cells (Nordstrom & Delbro, 1986) and formation of cyclo-oxygenase products (Advenier *et al.*, 1982). Our findings with guanethidine, prazosin, diphenhydramine and indomethacin appear to rule out the possible involvement of adrenergic mechanisms, histamine release and local prostanoid generation. On the other hand, the observation that 2-Cl-Ade-induced bronchoconstrictor responses were significantly reduced (but not abolished) by cyproheptadine strongly suggests that 5-HT might play a role as mediator of this type of bronchoconstriction. It has already been demonstrated that other 5-HT antagonists, such as methysergide or ketanserin, inhibit the bronchomotor effects of adenosine in anaesthetized BDE-rats (Pauwels & Van der Straeten, 1987) and in rat trachea (Nordstrom & Delbro, 1986). Whether adenosine does release 5-HT derived from mast cells, platelets or other cells in guinea-pigs remains to be established. Interestingly, the inhibitory properties of cyproheptadine were not additive with those elicited by the capsaicin pretreatment (data not shown), and it is therefore possible that 5-HT released by adenosine might also act by stimulating sensory afferents. Indeed, prejunctional 5-HT_3-receptor binding sites on terminal endings of capsaicin-sensitive sensory nerves have recently been described (Hamon *et al.*, 1989).

In conclusion, we have presented evidence that in anaesthetized non-curarized guinea-pigs 2-Cl-Ade elicits transient, vagally-mediated bronchospasm through stimulation of A_2-purinoceptors. This effect is multifactorial and involves stimulation of capsaicin-sensitive nerves and 5-HT release. Since some pharmacological features of the bronchomotor response to adenosine observed in this experimental model resemble those obtained in human asthmatic subjects, we propose that this model might be suitable for the further study of the role of adenosine in the pathophysiology of asthma, and possibly for testing new antiasthma drugs.

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Responses of rat substantia nigra compacta neurones to L-DOPA

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1 The electrophysiological actions of L-DOPA were studied on substantia nigra dopamine-containing neurones by use of intracellular recordings, current and voltage clamp, *in vitro*.

2 L-DOPA (3–300 μM), applied by superfusion, decreased the spontaneous firing activity of the neurones, hyperpolarized the membrane potential and produced an outward current.

3 These effects of L-DOPA were graded and were antagonized by sulpiride, a D_2-receptor antagonist.

4 The L-DOPA activated membrane hyperpolarization persisted in slices exposed to tetrodotoxin while it was markedly reduced or abolished in solutions with no calcium plus cobalt (2 mM).

5 In slices treated with carbidopa, an inhibitor of the aromatic amino acid decarboxylase enzyme, the actions of L-DOPA were greatly reduced.

6 We propose that L-DOPA inhibits the firing of substantia nigra zona compacta neurones by being converted to dopamine which is then released by these cells.

Introduction

It is now more than 20 years since L-DOPA was shown to be the most suitable treatment for Parkinson's disease (Cotzias *et al.*, 1969; Barbeau, 1981; Juncos *et al.*, 1987). It is generally believed that the clinical benefits produced by the administration of L-DOPA are due to the drug-induced release of dopamine from the surviving nigro-striatal neurones (Hornykiewicz, 1966; 1973; Melamed *et al.*, 1980; 1984). Previous studies have shown that L-DOPA increases the efflux of dopamine in the striatum (Ng *et al.*, 1970; Hefti & Melamed, 1981; Ponzio *et al.*, 1983; Zetterstrom *et al.*, 1986; Snyder & Zigmond, 1987; Brannan *et al.*, 1989).

An increase in the intracellular and extracellular content of dopamine in the substantia nigra zona compacta has also been reported following the administration of L-DOPA in rats (Bunney *et al.*, 1973; Robertson & Robertson, 1988). It is well known that dopamine, released locally from cell dendrites (Geffen *et al.*, 1976; Korf *et al.*, 1976; Nieullon *et al.*, 1977; Cheramy *et al.*, 1981), interacts with receptors located on the soma and dendrites of dopamine-containing neurones of the substantia nigra zona compacta, decreasing firing rate by hyperpolarizing the cellular membrane (Aghajanian & Bunney, 1973; 1977; Pinnock, 1983; Lacey *et al.*, 1987).

Bunney *et al.* (1973) have reported that systemic application of L-DOPA reduced the extracellular unitary discharge of rat dopaminergic neurones. However, the cellular mechanisms underlying the neuronal inhibition induced by L-DOPA are still unclear. In this study we have therefore examined in isolation the actions of L-DOPA on dopaminergic neurones using an *in vitro* slice preparation from which we obtained intracellular recordings.

Methods

The methods used have been described previously by Lacey *et al.* (1987). Albino Wistar rats (150–350 g) of either sex were killed by a blow on the chest under ether anaesthesia. The brain was quickly removed and slices of the mesencephalon (300–350 μm thick) which contained the substantia nigra were cut by an Oxford vibratome. A single slice was then transferred to a recording chamber and continuously superfused at a rate of 2.5 ml min^{-1} with a solution maintained at 36°C and gassed with 95% O_2/5% CO_2. The standard solution contained (mM): NaCl 126, KCl 2.5, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.4, glucose 10, $NaHCO_3$ 26. When $CoCl_2$ (2 mM) was added to the solution NaH_2PO_4 was omitted.

Intracellular recording electrodes were filled with 2 M KCl and had a resistance of 30–100 MΩ. A single electrode voltage clamp amplifier was used (Axoclamp 2A) for both voltage and current recordings. Voltage clamp recordings were made using 2–3.5 Hz switching frequency, while the headstage signal was continuously monitored on a separate oscilloscope. Membrane potentials and currents were displayed on chart by a Gould RS 3400 chart recorder.

Drugs were applied by superfusion, substituting the control solution with one containing the drug at known concentration. Drug solutions entered the recording chamber no later than 20 s after turning a 3 way tap. Complete replacement of the medium in the chamber took 90 s. The following drugs were used: L-DOPA, 1–300 μM, (gift of Dr Amenta), carbidopa, 300 μM, (Merck Sharp & Dhome), dopamine hydrochloride, 10–30 μM, (Sigma), (−)-sulpiride, 1 μM, (Ravizza).

Numerical data were expressed as mean ± standard error of the mean (s.e.mean).

Results

Data were obtained by intracellular recording from a total of 41 neurones located in the substantia nigra zona compacta. The properties of these cells correspond to those previously described for the principal cells of the substantia nigra zona compacta (Lacey *et al.*, 1987; 1988; 1989a,b; Mercuri *et al.*, 1989a).

Actions of L-DOPA

In all neurones tested, application of L-DOPA (3–300 μM) resulted in a concentration-dependent depression of firing rate and in membrane hyperpolarization (Figure 1). The doses of 30 and 100 μM caused a hyperpolarization of 4 ± 0.4 mV, $n = 4$ and 7.1 ± 1.7 mV, $n = 7$ respectively. Under voltage clamp at −50 to −60 mV holding potential, L-DOPA caused an outward current (Figure 2a). This outward current was dose-dependent and the concentration that caused the half maximal effect (EC_{50}) was 48 μM, as determined by the dose-

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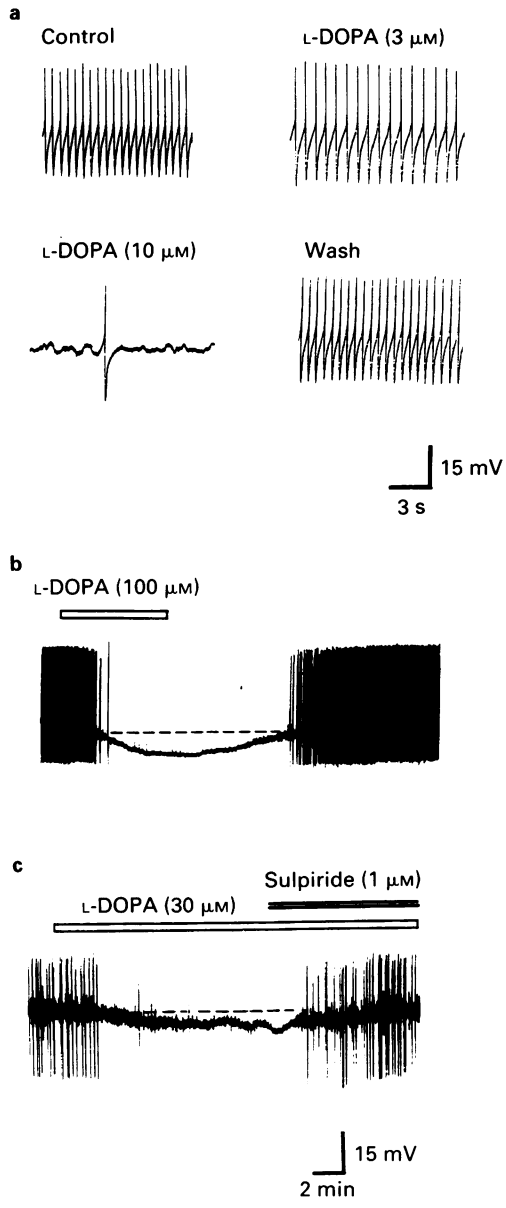

Figure 1 The firing of the substantia nigra dopamine-containing neurones is inhibited by L-DOPA and accompanied by a hyperpolarization. (a) Dose-related reduction of the firing rate produced by increasing doses (3 μM and 10 μM) of L-DOPA on a single cell. After 15 min of washing with control solution the firing rate recovered. (b) L-DOPA (100 μM), superfused for the period indicated by the bar; blocked the firing activity and hyperpolarized the membrane of another dopamine neurone. Broken line indicates −58 mV. (c) Sulpiride (1 μM) antagonized the membrane hyperpolarization and the block of the action potential discharge, induced by 30 μM L-DOPA in a different cell. Broken line indicates −57 mV. Full amplitude of the action potentials is not reproduced due to the low frequency response of the pen recorder. Note the different chart speeds in (a) with respect to (b) and (c).

response curve (Figure 2b). The minimal effective concentration was 3 μM while the maximal effect was obtained with 100 μM. The effects of L-DOPA took 2–3 min to appear and reached a steady state after superfusion for 4–7 min. Full recovery from the drug could generally be seen after 7–15 min wash out with L-DOPA-free solution. No desensitization was observed with prolonged (more than 20 min) or repeated applications of the drug on the same neurone.

Actions of dopamine

Ten neurones were also exposed to dopamine. This substance (10 and 30 μM) in the perfusion medium, hyperpolarized the

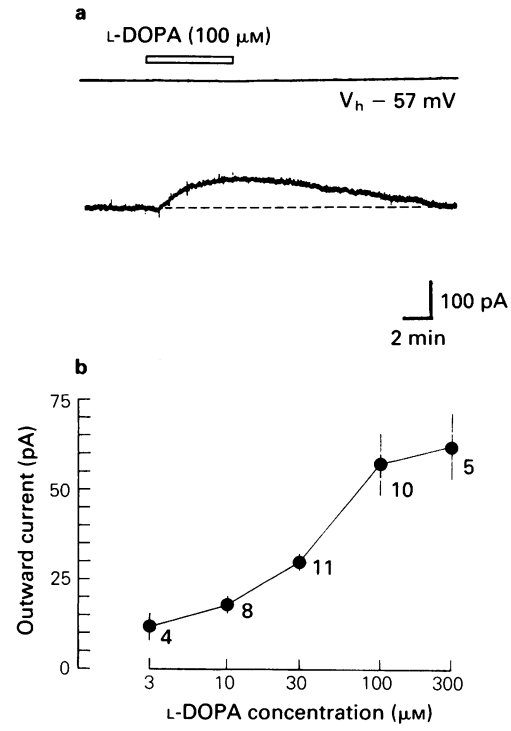

Figure 2 L-DOPA produces an outward current in the dopamine containing cells which is concentration-dependent. (a) Upper trace, voltage. Lower trace, current. L-DOPA (100 μM), applied by superfusion during the period indicated by the bar, produced an outward current. The broken line indicates 0 current. (b) Plot of the peak amplitude of the L-DOPA-induced outward current as a function of the concentration applied. Points show mean effect and vertical lines indicate s.e.mean for the number of observations indicated. (The holding potential was between −50 and −60 mV).

substantia nigra zona compacta cells by 5.4 ± 0.9 mV, $n = 5$ and 7 ± 0.8 mV, $n = 5$ respectively. Under voltage clamp these concentrations of dopamine produced an outward current of 45 ± 12 pA, $n = 3$ and 59 ± 10 pA, $n = 5$ (Figure 3).

Antagonism with sulpiride and effects of sodium and calcium blockade

Sulpiride (1 μM), a dopamine D_2 receptor antagonist, readily reversed the actions of L-DOPA (3 cells) (Figure 1c). Tetrodotoxin (TTX) (1 μM) which blocks the sodium action potentials, did not prevent the hyperpolarizing effect of L-DOPA (Figure 4a).

However, when L-DOPA (100 μM) was applied to neurones exposed to zero calcium plus cobalt (2 mM), a calcium channel blocker, the effects of the drug were markedly depressed (2 cells) or abolished (3 cells) (Figure 4b).

Pretreatment with carbidopa

Carbidopa (300 μM), which is a potent inhibitor of the enzyme dopa decarboxylase, was applied for 20–30 min. This agent produced no detectable effects on the properties of the cells but attenuated to 25% ± 5.9% of control the amplitude of the outward current caused by 100 μM L-DOPA ($n = 4$). The cellular responses to L-DOPA from such an experiment are illustrated in Figure 3 before and during the application of carbidopa. However, carbidopa did not prevent the outward current produced by 30 μM dopamine.

Discussion

The present study has shown that L-DOPA inhibits substantia nigra zona compacta neurones maintained *in vitro* by hyper-

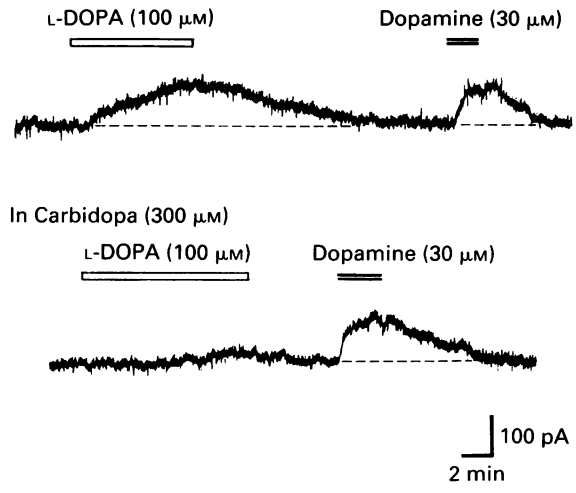

Figure 3 Action of carbidopa on the outward currents caused by L-DOPA and dopamine on a single cell. L-DOPA (100 μM) and dopamine (30 μM) produced an outward current in this neurone. After 25 min exposure to a solution containing carbidopa (300 μM) the effects of L-DOPA disappeared while dopamine was still effective. Note the prolonged application times of L-DOPA and dopamine in the presence of carbidopa. The cell was voltage clamped at −55 mV. Broken line 0 pA.

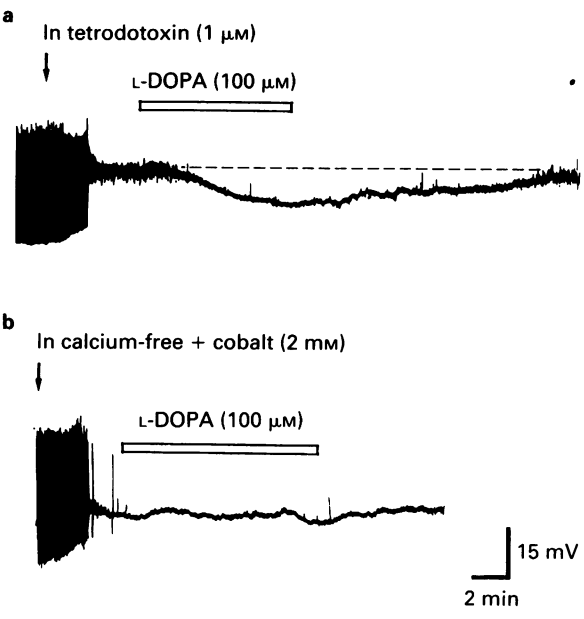

Figure 4 Effects of tetrodotoxin (TTX) and calcium-free/cobalt solutions on the L-DOPA-induced responses. (a) The membrane hyperpolarization, produced by L-DOPA (100 μM), persists in TTX (1 μM) applied at the point indicated by the arrow. Broken line indicates −56 mV. (b) Changing the control solution at the point indicated by the arrow to one containing zero calcium and 2 mM cobalt abolished the membrane effects of L-DOPA in another dopaminergic neurone. Note that the calcium-free, cobalt solution also blocked the spontaneous activity of the cell.

polarizing the membrane and that this hyperpolarization was related to an outward current. An outward current similar to the one produced by L-DOPA is mediated through D_2 receptor activation by dopamine and dopamine-like agents in the zona compacta principal neurones (Lacey *et al.*, 1987; 1988; 1989b; Mercuri *et al.*, 1989a).

The most probable interpretation of the present data is that the depression of substantia nigra zona compacta cell activity results from the stimulation of D_2 receptors by dopamine, which has been synthesized from L-DOPA by the endogenous aromatic amino acid decarboxylase enzyme. The evidence supporting such a hypothesis is as follows.

Firstly, sulpiride, a D_2-receptor antagonist, reversed the effects of L-DOPA as well as those produced by dopamine and dopamine receptor agonists (Pinnock, 1984; Lacey *et al.*, 1987; 1989b; Mercuri *et al.*, 1989a).

Secondly, carbidopa depressed the actions of L-DOPA, confirming the indirect nature of the interaction with dopamine receptors. The efficient and rapid conversion of L-DOPA to dopamine in the substantia nigra, is further supported by the steady state responses for a given concentration of L-DOPA which occurred within about a few minutes of application.

A difference between the effects of L-DOPA and dopamine was that dopamine produced larger hyperpolarizations and outward currents than L-DOPA (see also, Lacey *et al.*, 1987). In addition, the time to peak and the recovery time of the effects of L-DOPA were prolonged in comparison to those produced by dopamine.

The experiments with tetrodotoxin proved that functioning tetrodotoxin-sensitive sodium channels are not required for L-DOPA to act. Similar results were obtained with dopamine, which was not blocked by tetrodotoxin nor were the effects of cocaine and amphetamine on dopamine cells (Lacey *et al.*, 1987; 1989b; Mercuri *et al.*, 1989a).

Calcium-free solutions with added cobalt are capable of reducing or blocking L-DOPA effects, suggesting that the overflow of dopamine may occur from a catecholaminergic pool sensitive to the intracellular level of calcium (Leslie *et al.*, 1985; Woodward *et al.*, 1988). Amphetamine, unlike L-DOPA may induce a calcium independent efflux of dopamine in the substantia nigra (Mercuri *et al.*, 1989a).

Although the intracerebral concentration of L-DOPA reached during the treatment of Parkinsonian patients is unknown, it is worth noting that the plasma level of L-DOPA necessary to improve the motor functions is in the micromolar range (Nutt *et al.*, 1988). This is about the concentration observed to have effects in the substantia nigra.

Thus the treatment of Parkinson's disease with L-DOPA does not exclusively increase the dopaminergic activity in the striatum; it could also augment the effects of dopamine in the substantia nigra. The final outcome of the dopamine substitute therapy is a cellular inhibition both in the striatum (Calabresi *et al.*, 1988) and the substantia nigra pars compacta. The effects of L-DOPA at the compacta cell body level are clear and contrary to current opinion, which considers them as 'unwanted effects'. They could prove to be important in controlling the amount of dopamine released in the target striatal areas (Hoffman *et al.*, 1988). Indeed, newly synthesized dopamine, maintaining the firing rate of the dopamine-containing neurones in a critical range, might play a role in restoring the physiological activity of the residual nigro-striatal pathway in Parkinsonian patients (Mercuri *et al.*, 1989b).

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$GABA_A$ receptor-mediated increase in membrane chloride conductance in rat paratracheal neurones

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1 The actions of γ-aminobutyric acid (GABA) on the intramural neurones of 14–18 day old rats were studied *in situ* by use of intracellular current- and voltage-clamp techniques. The ionic conductance changes and the effects of various GABA-receptor agonists and antagonists on these neurones were also investigated.

2 Prolonged application of GABA either by ionophoresis (10 pC–10 nC) or superfusion (10–100 μM), evoked a biphasic membrance depolarization in over 90% of all paratracheal neurones studied. Typically, the response consisted of an initial rapid depolarization (18–45 ms) that subsequently faded over a period of 15–25 s to reveal a second smaller depolarization which was maintained for the duration of GABA application. Both components of the evoked response resulted in an increase in membrane conductance and an inward flow of current.

3 The amplitude of the transient inward current, recorded during the initial phase of the response, was linearly related to the membrane potential at which it was elicited and reversed symmetrically at a membrane potential of −32.7 mV. The underlying increase in conductance was largely independent of membrane potential. The equilibrium potential for the sustained inward current was −38.7 mV. Replacement of extracellular chloride with gluconate ions initially enhanced the GABA-evoked inward current. With successive applications of GABA in low chloride, the evoked current and conductance changes declined markedly.

4 Muscimol superfusion (1–10 μM) or ionophoresis (10 pC–10 nC) mimicked both the initial and late phases of the GABA-induced conductance change and inward current. Baclofen (1–100 μM) had no effect upon either resting membrane potential or conductance in any of the cells tested.

5 The large transient initial phase of the GABA-evoked inward current and depolarization were potently inhibited by picrotoxin (1–50 μM), whereas the smaller sustained inward current was largely resistant to picrotoxin.

6 All of the observed actions of GABA and muscimol were antagonized by bicuculline (0.1–10 μM) in an apparently competitive manner.

7 It is concluded that GABA acts via $GABA_A$ receptors present on the soma of paratracheal neurones to produce an increase in membrane chloride conductance. Prolonged application of GABA results in a decline in the observed current due to a combination of two processes: receptor desensitization and shifts in the chloride equilibrium potential. The possible roles for GABA in neural regulation of airway excitability are discussed.

Introduction

γ-Aminobutyric acid (GABA) is considered to be the principal inhibitory neurotransmitter in the mammalian central nervous system (CNS) (Kelly & Beart, 1975). The first evidence to suggest that there are GABAergic neurones in the vertebrate peripheral nervous system was the discovery that a population of intrinsic neurones in the guinea-pig myenteric plexus possessed a high-affinity uptake system for GABA, and were able to synthesize GABA from glutamic acid (Jessen *et al.*, 1979). Subsequent investigations now indicate a possible neurotransmitter role for GABA in a number of peripheral tissues (for review see Erdo & Bowery, 1986). In the periphery, as in the CNS, both $GABA_A$ and $GABA_B$ receptor subtypes are present (Dunlap, 1981; Cherubini & North, 1984a,b). Peripheral $GABA_A$ receptors are similar to those in the CNS, in that they have an integral chloride channel and are antagonized by bicuculline and/or picrotoxin (DeGroat, 1970; Adams & Brown, 1975; Bormann *et al.*, 1987). In contrast, peripheral $GABA_B$ receptors appear to differ from those found in the CNS, in that they are almost exclusively linked to calcium channels (Dunlap & Fischbach, 1981; Cherubini & North, 1984b; Deisz & Lux, 1985; Holz *et al.*, 1986), whereas central $GABA_B$ receptor activation involves an increase in potassium conductance (Newberry & Nicoll, 1984; Gähwiler & Brown, 1985). The main function of many of the GABA receptors, especially peripheral $GABA_B$ receptors, appears to be the modulation of release of other neurotransmitters such as acetylcholine, substance P and noradrenaline (Adams & Brown, 1975; Kato & Kuba, 1980; Cherubini & North, 1984b).

In the airways, GABA has been implicated in the regulation of tracheal smooth muscle tone through both central and peripheral mechanisms (Haxhui *et al.*, 1986; Tamaoki *et al.*, 1987). Results suggest that GABA decreases the contractile response of airway smooth muscle to cholinergic nerve stimulation by inhibiting the release of acetylcholine from postganglionic parasympathetic nerves in the guinea-pig trachea. This effect is thought to be mediated by chloride-dependent, bicuculline-sensitive receptors present on the postganglionic neurones (Tamaoki *et al.*, 1987). Furthermore, in an animal model of asthma, GABA and the $GABA_B$ agonist (−)-baclofen have been found to protect againt anaphylactic bronchospasm as well as spasm induced by aerosols of histamine and prostaglandins (Luzzi *et al.*, 1987). More recently, GABA has also been shown to inhibit bronchoconstriction, via the action of $GABA_B$ receptors that are thought to be present on sensory nerve terminals (Belvisi *et al.*, 1989).

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We have recently developed a preparation of rat trachea that permits us to visualize and record from intramural paratracheal neurones present in the trachealis muscle *in situ*. In a previous study we examined the basic electrophysiological characteristics of these cells and found that they were made up of a population of neurones showing two extreme types of firing behaviour (Allen & Burnstock, 1990). The majority of cells (65–75%) discharged short high-frequency bursts of action potentials at regular intervals in response to prolonged stimulation; these bursts of firing were followed by a slow calcium-dependent after-hyperpolarization. A further 10–15% of cells showed no burst firing activity, but fired tonically at low frequencies for the duration of current stimulation; the remaining cells displayed characteristics in between these two extremes. In this paper, we describe the effects of GABA and related substances upon these cells and discuss the possible implications of these actions on airway function.

Methods

All experiments were performed on 14–18 day old Sprague-Dawley rat pups. Animals were stunned then killed by cervical dislocation. The trachea between the base of the larynx and the bifurcation of the left and right bronchi was removed, cut midline along the length of its ventral surface and pinned out onto a small block of Sylgard (Dow Corning). The Sylgard block bearing the trachea was pre-bonded to a glass slide which formed the base of a Perspex recording chamber. The assembled chamber was then clamped to the stage of a Zeiss Ergoval microscope equipped with modulation contrast optics (Hoffman, 1977) giving 320 and 640 fold magnification. The preparation was perfused at a rate of 6 ml min^{-1} with Krebs solution maintained at 33–34°C by a remote thermostatically-controlled heating coil. The Krebs solution was of the following composition (mM): NaCl 117, KCl 4.7, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11, gassed with 95% O_2/5% $C0_2$.

Impalements were made by electrodes with d.c. resistances between 90 and 130 MΩ, containing 2 M potassium citrate solution (pH 7.2). Before impalement, electrode resistance and offset potentials were nulled to allow estimations of input resistance and membrane potential to be made during the recording. In order to aid the placment of electrodes and impalement of neurones, the final 3–6 mm of the electrode were bent through an angle of 60° before being filled. Single electrode current- and voltage-clamp recordings were made by an amplifier with a 30% current passing duty cycle by use of sampling rates of between 3 and 5 kHz (Axoclamp-2A).

Ionophoretic application of drugs was made from microelectrodes containing 0.1–0.5 M solutions (pH 3–4); the tips of the pipettes being placed within 1–5 μm of the cell soma. The recording and ionophoretic electrodes were connected to separate input stages of a recording system with active bridge and constant current circuits (Axoclamp-2A).

Fractional increases in conductance during drug responses were calculated as $(R/R^1) - 1$ where R is the input resistance at resting membrane potential and R^1 the input resistance at a given point during the response. Predicted equilibrium potentials were calculated from the observed current and conductance changes from the equation: $V_h - E_r = 1/(G^1 - G) \times I$ where V_h is the membrane potential at which the cell is held, E_r is the equilibrium potential for the response, G is the resting membrance conductance, G^1 is the membrane conductance at a given time (t) during the response and I the amplitude of the induced current at time (t).

Data were either stored on tape for future analysis (Racal store 4DS) or displayed using Tektronix storage oscilloscope (model D13) and a Gould pressure ink recorder (model 2200S).

Ionic substitution

Low chloride-containing solutions were made by directly substituting sodium gluconate for sodium chloride in the Krebs solution. Low calcium-containing solutions were made by direct substitution for magnesium.

Drugs

GABA, bicuculline methiodide, muscimol, picrotoxin, tetrodotoxin (Sigma), cadmium chloride (Fisons), baclofen (CIBA) and phaclofen (Tocris Neuramin) were used.

Results

Application of GABA, either by superfusion (1–100 μM) or ionophoresis (0.5 M, pH 4.0, 2–100 nA) produced a membrane depolarization in 156 out of a total of 171 (approximately 90%) rat paratracheal neurones studied. Typically, GABA evoked a two-component response, that consisted of a large initial depolarization which rapidly faded to reveal a second, smaller depolarization which was maintained for the duration of GABA application (see Figure 1a(i), b and c(i)). Both the initial transient and the slow sustained responses to GABA appeared to result from the direct action of GABA upon the impaled cell, since the responses were unaffected by superfusion with either calcium-free or tetrodotoxin-containing (0.3–1 μM) solutions.

The currents underlying the GABA-induced depolarizations were investigated under voltage-clamp. Brief ionophoretic application of GABA for a period of 1–100 ms (10 pc–10 nC) produced a large increase in membrane conductance that resulted in a transient inward flow of current (see Figure 1a(ii)). Under optimal conditions, where there was no connective tissue between the ionophoretic electrode and the impaled cell, the latency of response was between 18 and 45 ms. The magnitude of the inward current was dependent upon the total amount of ionophoretic charge ejected (see Figure 1a(ii)) and became larger at more negative potentials (see later). Prolonged application of GABA, either by superfusion or by ionophoresis (2–5 nA) for periods of up to 2 min, induced a similar rapid, transient inward flow of current, which subsequently declined over a period of 15–25 s to reveal a second smaller, sustained component to the inward current. This sustained inward current was also associated with an increase in membrane conductance and showed little or no rundown during prolonged GABA application (see Figure 1c(ii)). At the end of the period of stimulation, membrane conductance and current returned to control values within 10–20 s.

Conductance changes

Fast transient component For any given cell studied under current-clamp, the greater the amplitude of the fast depolarization, the larger the underlying conductance increase. However, as the total ionophoretic charge ejected was increased, the amplitude of response became maximal whereas the peak conductance continued to rise (see Figure 1a(i)). When ionophoretic currents sufficient to evoke a maximal depolarization were applied, the absolute amplitude of the response was almost entirely dependent upon the membrane potential of the cell. At any given membrane potential, the maximum size of the evoked depolarization occurred when the amplitude of the response became large enough to cause the cell to depolarize to a potential of approximately −35 mV. This potential was found to correspond quite closely to the equilibrium/reversal potential for the response (see later).

Examination of the observed increase in conductance under voltage-clamp revealed that the magnitude of the GABA-evoked current was directly related to the observed increase in conductance. The peak fractional increase in conductance (see Methods) varied considerably between cells, and ranged between 1.05 and 12.8 (mean 4.51 ± 0.336, $n = 83$). For any given cell, the amplitude of the inward current was dependent upon membrane potential (see later). However, the fractional

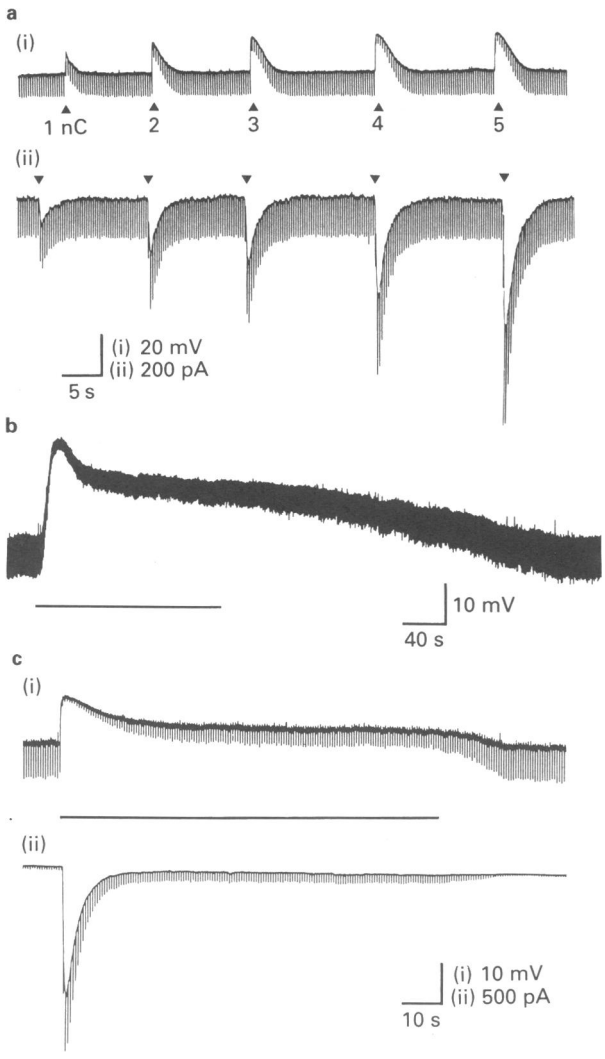

Figure 1 The effects of brief and prolonged GABA application upon rat paratracheal neurones. (a, i) Rapid depolarizations induced by brief (100 ms) ionophoretic applications of GABA, the total charge ejected in each case is indicated in nC below the responses. Downward deflections are the membrane voltage responses to constant hyperpolarizing current pulses (100 pA, 50 ms) used to monitor membrane resistance changes. (a, ii) Membrane currents for the same cell to similar GABA ionophoretic currents recorded under voltage-clamp. Downward deflections are membrane currents in response to −10 mV (50 ms duration) voltage steps. Membrane/holding potential in (a, i) and (a, ii) was −52 mV. (b) The effect of prolonged superfusion of GABA (100 μM) for the period indicated by the bar. The recording was made under current-clamp (membrane potential −60 mV), downward deflections are membrane voltage responses to constant current pulses (100 pA/50 ms). (c) The effects of prolonged ionophoretic application of GABA (8 nA) for the period indicated by the bar (1.5 min). At the onset of GABA ionophoresis there was a rapid depolarization, this response then subsided to reveal a sustained depolarization which persisted for the duration of GABA ionophoresis (see c, i). Downward deflections are the membrane voltage responses to passing brief (50 ms/80 pA) constant intrasomal current pulses to monitor input resistance. (c, ii) The effects of prolonged GABA ionophoresis upon membrane current in the same cell as in (c, i). Downward deflections are the membrane current in the same cell as in (c, i). Downward deflections are the membrane currents in response to −5 mV, 50 ms duration voltage commands. Resting/holding potential in (c, i and ii) was −50 mV.

increase in conductance underlying the response was largely independent of the membrane potential at which it was evoked (see Figure 2).

Slow sustained component The conductance change and the resulting sustained inward current flow during prolonged application of GABA were considerably smaller than those seen during the initial transient response. The amplitude of the inward current became larger with increasing concentrations of GABA and was generally maximal in the presence of GABA at concentrations greater than 100 μM. Over the normal range of resting membrane potentials (−50 to −65 mV), the maximum amplitude of the sustained inward current rarely exceeded 150 pA (mean 107 ± 6.7 pA, $n = 19$). When the response was evoked by prolonged ionophoretic application of GABA, the amplitude of the sustained inward current reached a maximum even with very low ejection currents (1–3 nA) and could rarely be increased by further raising the current. However, in the presence of agents which acted to inhibit the initial transient inward current, raising the ionophoretic ejection current increased the rate of rise to this plateau, maximum value of the sustained inward current. It is worth noting that care was needed when applying prolonged ionophoretic currents. If excessively high currents were applied (>10 nA), then the prolonged ionophoretic current itself could induce a sustained inward current flow similar to the GABA-induced current. In order to remove this source of error, the ionophoretic current was adjusted to a level where all of the inward current was antagonized by superfusion with bicuculline (see later). The observed increase in conductance during the slow response varied between cells, with the mean fractional increase in conductance ranging between 0.15 and 1.78 (mean 0.846 ± 0.061, $n = 42$).

Ionic-and voltage-dependence

Transient inward current The voltage-dependence of the fast GABA-evoked inward current was investigated in the presence of tetrodotoxin (0.3–1 μM; see Figure 2a). The amplitude of the inward current was linearly related to the membrane potential at which it was elicited (see Figure 2b). Whe potassium citrate-filled electrodes were used in the recording, the mean null/reversal potential for the response was −32.7 mV ± 1.66 ($n = 8$; see Figure 2). For membrane potentials between −20 and −80 mV, both the time-course and the peak conductance change underlying the depolarization were largely independent of membrane potential (see Figure 2a). At strongly depolarized potentials (< −20 mV), outward rectification of the response was observed in some cells. However, this phenomenon was not studied in detail as it was difficult to maintain impalements at such depolarized potentials. Altering the extracellular chloride concentration from 129.1 to 12.1 mM transiently increased the amplitude of the fast GABA-evoked current by approximately 150–180%. However, multiple exposures to GABA, in the presence of low chloride-containing solutions, results in a pronounced decline in both the inward current and the observed conductance increase, as a result of a fall in intracellular chloride concentration. No initial reversal in the polarity of the GABA-evoked response was observed at the start of low chloride superfusion. On returning to normal chloride-containing solutions, both the inward current and the conductance increase slowly returned to their control values over a period of 5–10 min.

Slow sustained inward current In order to determine the equilibrium potential for the sustained GABA-evoked response, current-voltage relationships were constructed before and during GABA ionophoresis (see Figure 3a). The point of intersection of the curves gave a mean equilibrium/null potential for the response of −38.7 ± 1.56 mV ($n = 12$; see Figure 3b). This value agrees well with the predicted equilibrium potential of −39.2 ± 1.1 mV ($n = 15$) calculated from the observed increase in conductance (see Methods).

GABA analogues

Muscimol, applied either by superfusion or ionophoretically, mimicked all the actions of GABA upon paratracheal neu-

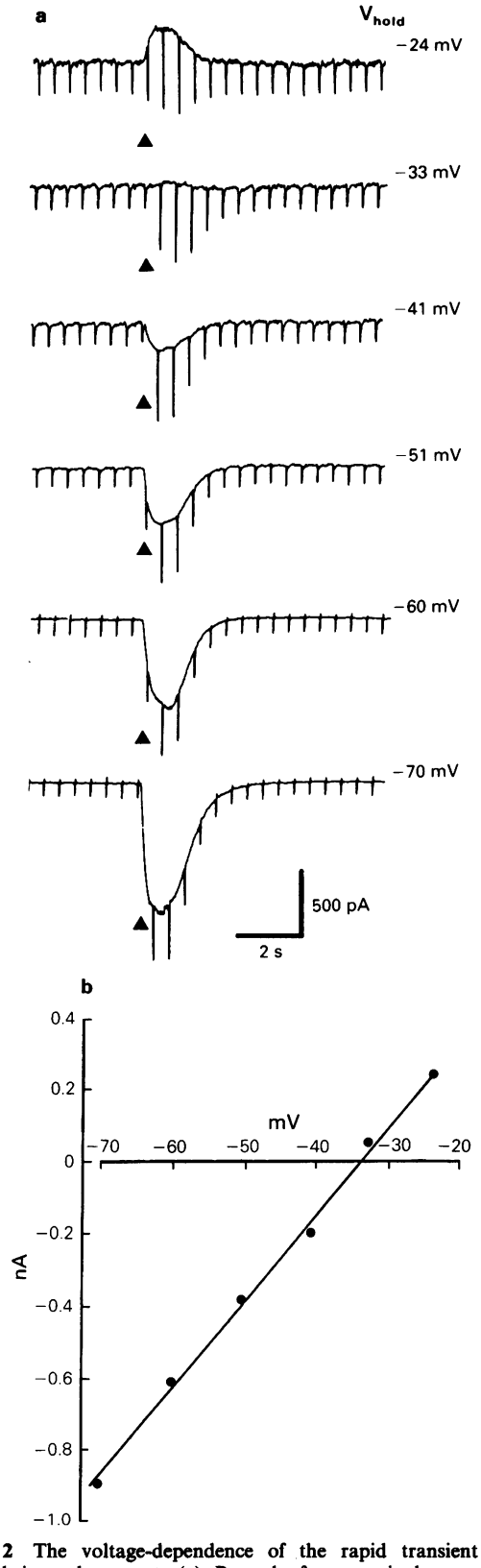

Figure 2 The voltage-dependence of the rapid transient GABA induced inward current. (a) Records from a single paratracheal neurone held at different membrane potentials. Downward deflections are the membrane current responses to hyperpolarizing voltage commands (10 mV, 50 ms) used to monitor changes in membrane conductance. Arrows beneath the records indicate the point at which GABA was applied (5 nA/100 ms). Note that the underlying fractional increase in membrane conductance was largely independent of the holding potential (V_h) at which the response was elicited. (b) The amplitude of the transient GABA-induced inward current as a function of holding potential. Line is least squares fit to raw data. The current was linearly related to membrane potential (correlation coefficient, $r = 0.998$) and reversed symmetrically at a membrane potential of −34.1 mV to become an outward current.

rones. When applied briefly by ionophoresis, it induced a similar rapid depolarization and an increase in conductance. Under voltage-clamp this could be seen to result from a transient inward flow of current (see Figure 4a). When muscimol was applied for prolonged periods, the initial current subsided in a similar way to the GABA-induced response to reveal a non-inactivating component to the inward current (see Figure 4b and c). The $GABA_B$ receptor agonist β-*p*-chlorophenyl-GABA (baclofen) at concentrations up to 100 μM had no effect upon either the resting membrane potential or conductance in any of the cells tested ($n = 11$).

Antagonist studies

Both the fast transient depolarization evoked by brief application of GABA and the initial transient depolarization/inward current seen at the start of prolonged GABA ionophoresis were reversibly inhibited by picrotoxin (1–50 μM) in a dose-related manner (see Figure 5a). However, picrotoxin at concentrations up to 500 μM was never observed to antagonize the sustained depolarization/inward current evoked by either superfusion or prolonged ionophoretic application of GABA or muscimol (see Figure 5b).

Bicuculline (0.1–10 μM) reversibly inhibited the fast GABA-evoked depolarization. Approximate dose-response curves were constructed by briefly applying GABA with increasing ionophoretic currents (see Figure 6a). Bicuculline produced a parallel shift to the right in the dose-response curve for GABA (see Figure 6b). The GABA-induced sustained depolarization/inward current was also potently antagonized by bicuculline. However, the sensitivity of this response to inhibition by bicuculline was slightly lower than that observed for the transient component and required concentrations of bicuculline of between 10 and 50 μM to antagonize fully the actions of GABA (Figure 7).

Phaclofen at concentrations up to 1 mM did not antagonize either of the GABA- or muscimol-induced inward currents in any of the cells tested ($n = 5$).

Other agents

Superfusion with nipecotic acid (1–10 μM; $n = 5$), a GABA uptake blocker, or inhibition of electrogenic pumping with ouabain (1–100 μM; $n = 4$) were never observed to alter either the amplitude or the time-course of the two components of the GABA-induced inward currents. Superfusion with either calcium-free, high magnesium-containing solutions, or blockade of calcium entry with $CdCl_2$ (100 μM), did not reduce the observed amplitude or the underlying increase in conductance during either phase of the GABA-induced response.

Discussion

The findings of the present study showed that GABA exerted a powerful and direct effect upon a large proportion of the parasympathetic intramural neurones of the rat trachea. This effect was mediated via bicuculline-sensitive receptors and resulted in a large increase in resting chloride conductance. In a typical cell, held near its resting membrane potential, application of GABA produced a biphasic response consisting of an initial, transient depolarization, which rapidly declined to reveal a smaller, sustained depolarization. The maximum amplitude of the initial depolarization under these conditions was usually between 15 and 25 mV and reflected the level of the chloride equilibrium potential, which was approximately 20 mV positive to the resting membrane potential in these cells. Substituting extracellular chloride ions for a less permeant anion, such as gluconate, produced a transient increase in the amplitude of the GABA-induced depolarization and inward current. However, with repeated exposure to GABA in the presence of low extracellular chloride, both the current and the underlying increase in conductance declined markedly. This rundown of the GABA-evoked response in low

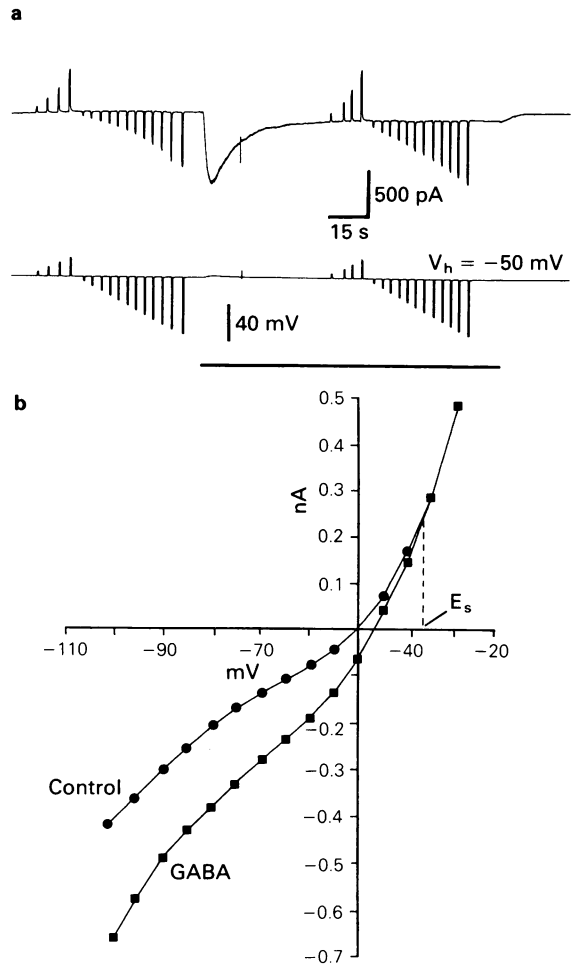

Figure 3 The voltage-dependence of the sustained GABA-induced inward current in a paratracheal neurone examined under voltage-clamp. (a) Under control conditions with the cell held at a membrane potential of −50 mV (the resting membrane potential for this cell), the cell was subjected to a series of 500 ms duration positive and negative voltage commands of increasing amplitude (5 mV increments). GABA was then applied ionophoretically (ejection current 5 nA) for the period indicated by the bar. This evoked the characteristic transient inward current which subsequently declined to reveal the sustained inward current. During the plateau phase of the sustained current, the cell was subjected to a second series of voltage commands identical to those applied under control conditions. At the end of this procedure the ionophoretic ejection current was switched off and the current returned to control levels. (b) The current-voltage relationships for the cell shown in (a) under control conditions and in the presence of GABA. The point of intersection of the two curves gives a value for the equilibrium potential (E_s) for sustained current response of approximately −38 mV.

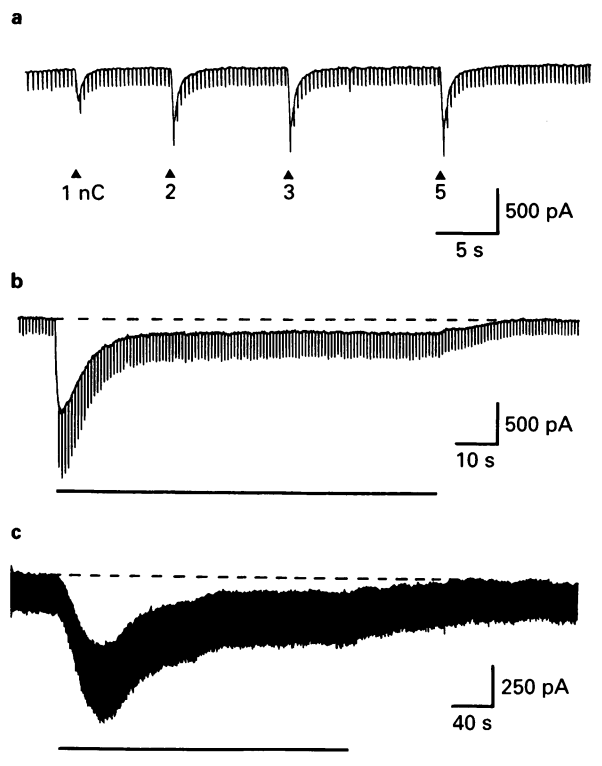

Figure 4 The actions of muscimol, a $GABA_A$ receptor agonist, upon rat paratracheal neurones. (a) Brief (100 ms) ionophoretic application of muscimol using increasing ejection currents (total ionophoretic charge ejected is given in nC below each record). Downward deflections are membrane current responses to −10 mV voltage steps used to monitor changes in membrane conductance. Holding potential for the cell shown in (a) was −62 mV. (b) Prolonged ionophoretic application of muscimol, for the period indicated by the bar (7 nA/1.5 min), produced a biphasic inward current similar to that produced by GABA (downward deflections are current responses to −10 mV voltage steps as in a). Holding potential −54 mV. (c) Superfusion with muscimol (10 μM) for the period indicated by the bar mimicked the effect of prolonged ionophoretic application, but the latency and rate of rise of the initial transient current were considerably slower. Downward deflections are current responses to 50 ms duration −10 mV voltage steps. Holding potential −53 mV.

chloride-containing solutions can best be explained by considering the underlying changes in the electrochemical gradient for chloride ions (see Adams & Brown, 1975). Initially, superfusion with low-chloride-containing solutions would be expected to increase the net outward electrochemical driving force on chloride ions by shifting E_{Cl} to more positive potentials. This would result in a large increase in the outward flow of chloride ions from the cell during the GABA-evoked response, and account for the large increase in the observed depolarization. Repeated application of GABA would, however, reduce the intracellular concentration of chloride ions causing a large negative shift in E_{Cl}, which would lead to a decline in the amplitude of subsequent responses. This reduction in the concentration of intracellular chloride ions, together with the low extracellular chloride concentration, would also explain the observed reduction in the underlying conductance that would be predicted to occur as a result of a deficiency in the total number of charge-carrying chloride ions. Interestingly, the voltage-dependence of the transient GABA-induced chloride current in rat paratracheal neurones displayed very little outward rectification over the membrane potential range −20 to −80 mV. This is contrary to what has been obtained in other central and peripheral neurones (Gray & Johnston, 1985; Ashwood *et al.*, 1987; Bormann *et al.*, 1987; Peters *et al.*, 1989) and also what might be expected from the asymmetrical distribution of chloride ions across the membrane. Outward rectification of the GABA response in paratracheal neurones was sometimes observed at very strongly depolarized potentials, but in the present study this phenomenon was not explored in detail as it was difficult to maintain impalements at such depolarized levels.

In addition to the initial transient response, all paratracheal neurones displayed a second, sustained depolarization which persisted for the duration of GABA application. Biphasic responses to prolonged application of GABA have been described in a variety of different cells (Deschenes *et al.*, 1976; Mayer *et al.*, 1981; Cherubini & North, 1984a; Yasui *et al.*, 1985). Superficially, the second depolarizing phase in many of these cells was similar to the sustained response seen in rat paratracheal neurones. However, in enteric AH type cells for example, the slow GABA response was mimicked by baclofen, had a low affinity for bicuculline and was attributed to activation of $GABA_B$ receptors (Cherubini & North, 1984a). In contrast, in rat paratracheal neurones, baclofen was never observed to produce membrane depolarization, whereas

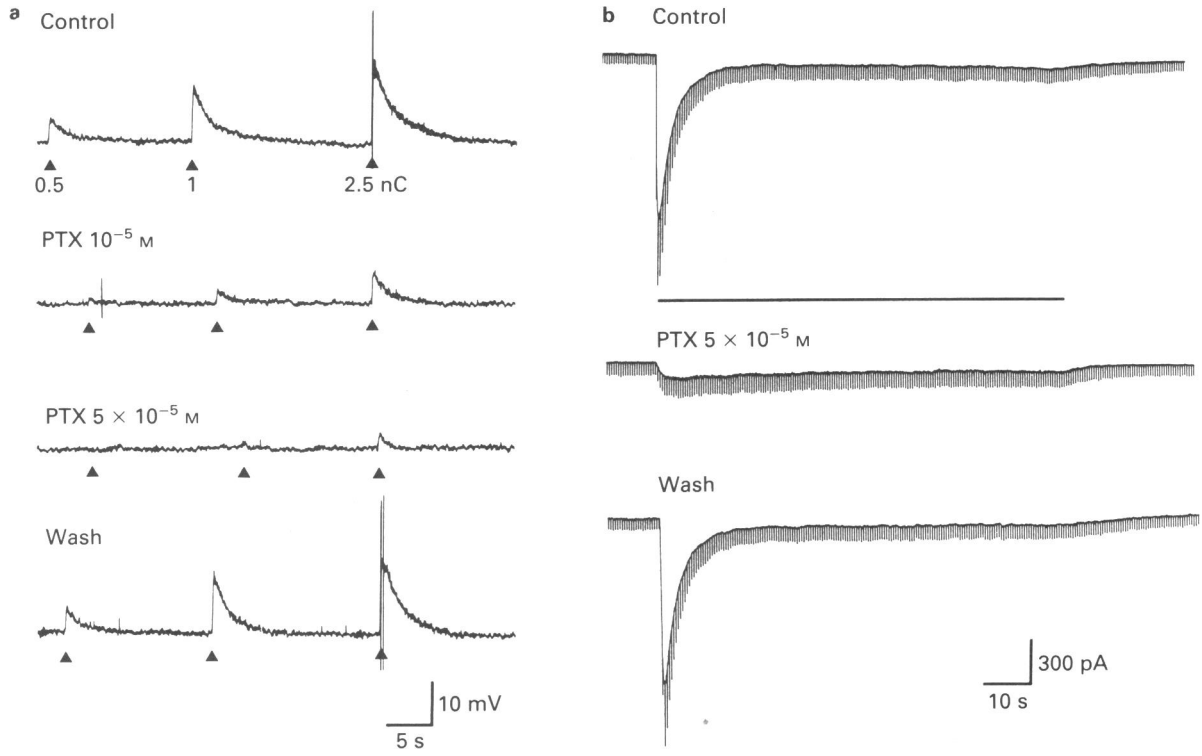

Figure 5 Inhibitory action of picrotoxin (PTX) upon the GABA-evoked response in a rat paratracheal neurone. (a) The fast GABA-evoked depolarization. Upper panel shows control depolarizations to GABA produced by increasing the total ionophoretic charge ejected (all pulses 50 ms duration; the amount of charge ejected in each case is indicated in nC below each response). In the presence of picrotoxin (10 and 50 μM; middle panels), the amplitude of the evoked depolarizations was reduced in a dose-related manner. Resting membrane potential −54 mV. (b) The rapid transient and sustained inward currents evoked by prolonged ionophoretic application of GABA (8 nA/1.5 min). In the presence of picrotoxin (50 μM), the initial rapid transient component of the response was almost totally abolished, whilst the slow sustained current and associated increase in conductance remained largely unaffected. Downward deflections are membrane currents in response to 10 mV/50 ms voltage steps. Holding potential −68 mV.

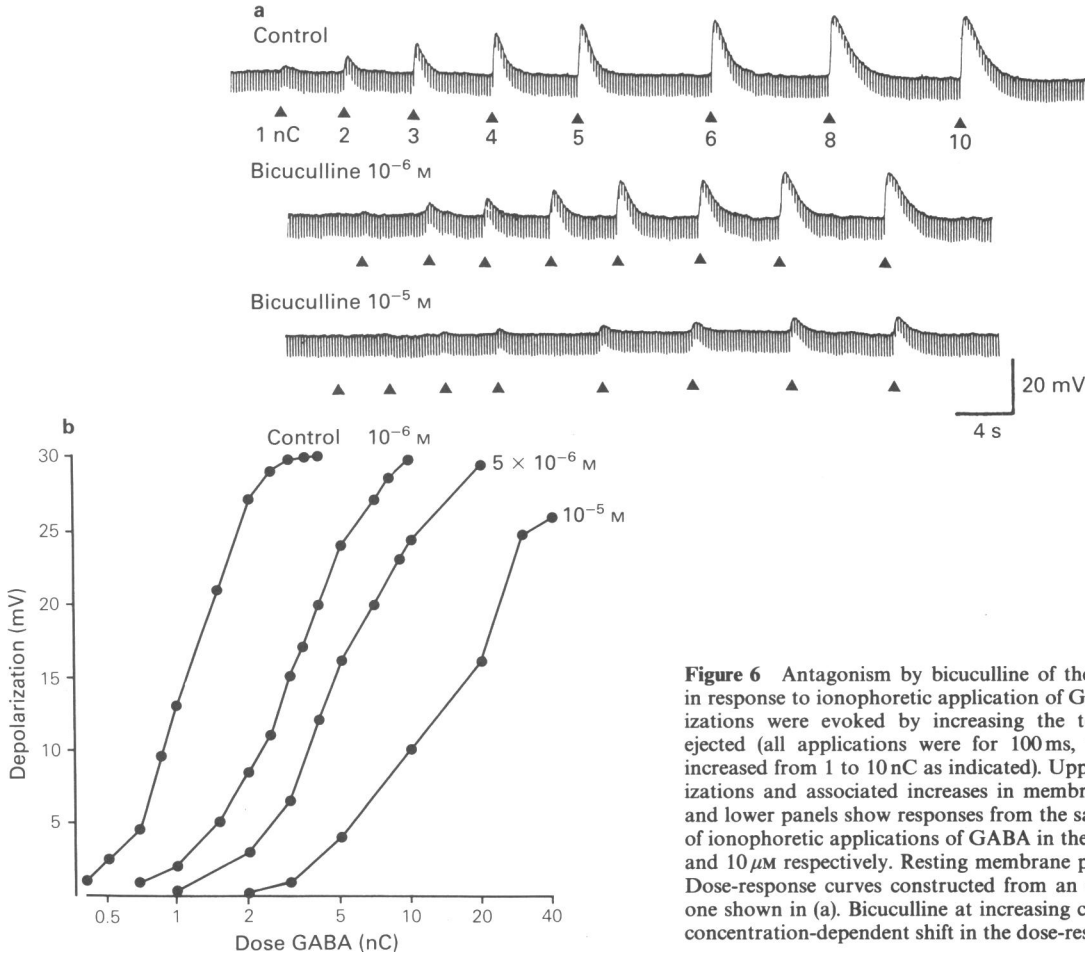

Figure 6 Antagonism by bicuculline of the transient depolarization in response to ionophoretic application of GABA. (a) Graded depolarizations were evoked by increasing the total ionophoretic charge ejected (all applications were for 100 ms, total charge ejected was increased from 1 to 10 nC as indicated). Upper panel, control depolarizations and associated increases in membrane conductance. Middle and lower panels show responses from the same cell to a similar series of ionophoretic applications of GABA in the presence of bicuculline, 1 and 10 μM respectively. Resting membrane potential was −54 mV. (b) Dose-response curves constructed from an experiment similar to the one shown in (a). Bicuculline at increasing concentrations produced a concentration-dependent shift in the dose-response curve to GABA.

muscimol mimicked all the actions of exogeneously applied GABA. This finding, together with the selective inhibition of all of the observed actions of GABA and muscimol by bicuculline, indicated that the sustained as well as the transient responses in rat paratracheal neurones, were mediated by $GABA_A$ receptors.

Multi-component, $GABA_A$ receptor-mediated chloride currents have been described in dorsal root ganglion neurones, for example (Akaike *et al.*, 1987). In these cells, the sustained inward current was similar to the one described here in rat paratracheal neurones, in that it was largely insensitive to picrotoxin (Yasui *et al.*, 1985). The equilibrium potential for the sustained inward current in paratracheal neurones was close to that of the transient component, indicating that, as in the dorsal root ganglion, it may also result from an increase in chloride conductance. The slightly more negative equilibrium potential of the sustained response probably reflected a small shift in E_{Cl}, as a result of the large efflux of chloride ions that occurred during the initial transient, response. Similar shifts of the GABA reversal potential (E_{GABA}) between the initial and late phases of the GABA-evoked response have been described in rat and cat ganglion cells (Adams & Brown, 1975; Mayer *et al.*, 1983).

In paratracheal neurones, the time-dependent decline of the GABA-evoked current, which occurred during continuous application of GABA, was similar to that described in, for example, hippocampal and dorsal root ganglion neurones. In these cells, the initial and late phases of the GABA current have been shown to result from a combination of two processes; receptor desensitization and shifts in E_{GABA} (Yasui *et al.*, 1985; Huguenard & Alger, 1986; Akaike *et al.*, 1987). It seems likely that similar mechanisms underlie the biphasic inward current in rat paratracheal neurones.

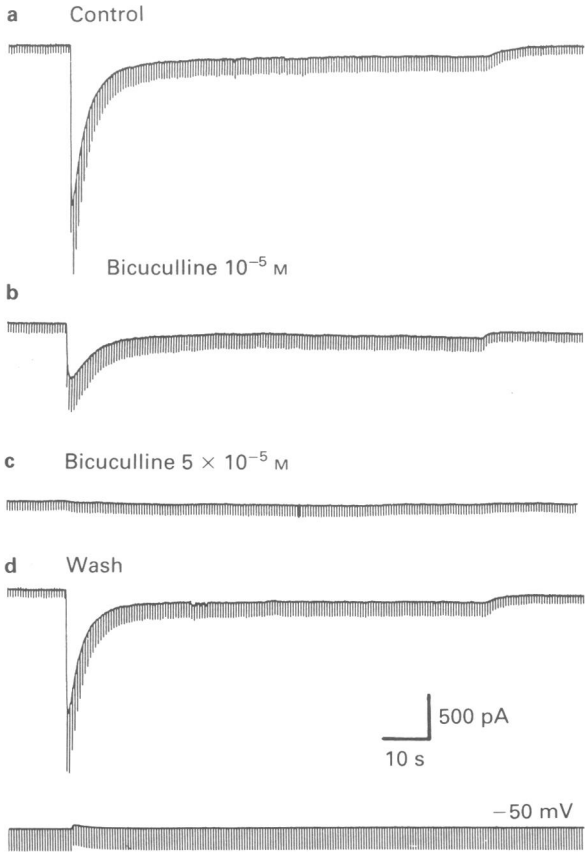

Figure 7 Antagonism of the transient and sustained inward currents by bicuculline in a rat paratracheal neurone voltage-clamped at −54 mV. (a) Control response to prolonged GABA ionophoresis (8 nA/1.5 min). (b) In the presence of 10 μM bicuculline, there was a pronounced decrease in the conductance change and inward current flowing at the start of GABA application, and a smaller reduction in the size of the sustained inward current. (c) In the presence of 50 μM bicuculline, both the initial transient and the slow sustained GABA-evoked currents were almost totally abolished. (d) Wash. Downward deflections are the induced membrane currents resulting from 10 mV negative commands (50 ms duration) used to monitor changes in membrane conductance.

Given the powerful effect that GABA exerted upon rat paratracheal neurones, it is obviously important to determine if there is any endogenous GABA within the airways. GABA has been localized in a population of neurones in the enteric nervous system (Jessen *et al.*, 1986) and it is possible, given the shared embryological origin of the gut and trachea, that there are also intrinsic GABAergic neurones in the airways. Alternatively, GABA may be released from extrinsic nerves to act upon the intramural ganglia. Another possible source of GABA could be blood, since levels of circulating GABA, at concentrations near to those required to depress ganglionic transmission, have been demonstrated in the bullfrog and cat (Crowcroft *et al.*, 1967; Kato *et al.*, 1980). In addition, it has been shown that glial cells in mammalian sympathetic ganglia are capable of taking up GABA from solutions containing concentrations of GABA as low as 1 μM (Bowery & Brown, 1972; Iversen & Kelly, 1975). Therefore, glial cells could regulate the extracellular GABA concentration and thereby modulate the excitability of neurones; it has been proposed that this type of mechanism may occur in sympathetic ganglia (Brown & Galvan, 1977).

If the excitability of paratracheal neurones were in some way modulated by either circulating or locally released GABA, then this could have wide-ranging consequences for airway function. It has previously been shown that rat paratracheal neurones display a high level of subthreshold, spontaneous synaptic activity (Allen & Burnstock, 1990). High levels of subthreshold synaptic activity have also been observed in cat tracheal ganglia *in vivo*, and it has been suggested that synchronization of multiple inputs are required to elicit action potential discharge in these cells (Mitchell *et al.*, 1987). One of the observed actions of GABA in the present study was a large increase in resting membrane conductance. This would act as a shunt across the membrane and result in a decrease in the size of the depolarization produced by individual synaptic events. Under such conditions, the overall intensity of vagal input required to elicit action potential discharge would be greatly increased by GABA and may explain the observed GABA-evoked reduction in acetylcholine release from postganglionic parasympathetic nerves in the guinea-pig trachea (Tamaoki *et al.*, 1987). Conversely, a fall in levels of GABA acting at the ganglion level, or a reduction in the sensitivity of these cells to GABA, could increase the excitability of the ganglion cells and partially explain the hyperreactivity of tracheobronchial smooth muscle observed in conditions such as asthma.

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The actions of adenosine 5′-triphosphate on guinea-pig intracardiac neurones in culture

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1 The actions of adenosine 5′-triphosphate (ATP) and related nucleotides and nucleosides on the membrane ion conductances of M and AH type intracardiac neurones cultured from ganglia within the atria and interatrial septum of newborn guinea-pig heart were studied with intracellular current- and voltage-clamp techniques.

2 Approximately 74% (120 out of 161) of AH type cells and 41% (5 out of 12) M cells responded to direct application of ATP (500 μM) onto their soma.

3 In 41% of M and 43% of AH type cells, focal application of ATP (500 μM) evoked rapid depolarization with an increase in conductance which frequently elicited action potential discharge. The underlying inward current had a null potential of −11.2 mV and was reduced in solutions containing low extracellular sodium and calcium but unaffected by reduced chloride-containing solutions.

4 In a further 31% of AH type cells, ATP evoked a multi-component response consisting of an initial depolarization followed by a hyperpolarization and a slow prolonged depolarization. The current underlying the initial depolarization resulted from an increase in conductance and had a null potential of −19.1 mV. The current was increased in low chloride-containing solutions and was only slightly reduced in low sodium- and calcium-containing solutions. The subsequent hyperpolarization and outward current resulted from an increase in membrane conductance and had a null potential of −88.5 mV, which was close to the potassium equilibrium potential in these cells. The slow depolarization and inward current was not associated with change in membrane conductance.

5 In less than 2% of AH cells, ATP evoked a second type of slow depolarization. This was associated with a fall in conductance and had a null potential of −90.7 mV.

6 In 40% of AH cells, adenosine (10–100 μM) inhibited the calcium-sensitive potassium current responsible for the after-hyperpolarization. The action of adenosine was antagonized by the P_1-purinoceptor antagonist 8-phenyltheophylline (1–10 μM).

7 The potency order of agonists for all of the ATP-evoked responses, except the slow depolarization associated with a fall in conductance was ATP > ADP with AMP and adenosine being ineffective.

8 Responses to ATP were only weakly desensitized by α,β-methylene ATP (3×10^{-6} M) and the potency order of analogues was 2-methylthio ATP $\geqslant$ ATP > α,β-methylene ATP, indicating the involvement of receptors similar to P_{2Y} purinoceptors.

Introduction

There is now considerable evidence to suggest that purine nucleotides and nucleosides act as neurotransmitters and neuromodulators in a variety of different tissues (Burnstock, 1972; 1985; 1986; Phillis & Wu, 1981; Stone, 1981; Su, 1983; Gordon, 1986). In addition, exogenously applied purines have also been shown to act directly on a number of peripheral and central neurones (for example see Jahr & Jessell, 1983; Krishtal *et al.*, 1983; Salt & Hill, 1983; Fyffe & Perl, 1984; Akasu & Koketsu, 1985; Dolphin *et al.*, 1986; Palmer *et al.*, 1987; Williams, 1987; Katayama & Morita, 1989).

The division of purinoceptors into two subtypes was first proposed by Burnstock (1978). According to this classification, P_1-purinoceptors, which are most sensitive to adenosine, produce changes in the levels of adenosine 3′:5′-cyclic-monophosphate (cyclic AMP) and are competitively inhibited by methylxanthines. In contrast P_2-purinoceptors recognize ATP, are not associated with changes in intracellular levels of cyclic AMP and are not antagonized by methylxanthines. Subdivisions of both these receptor subtypes have subsequently been proposed. P_1-purinoceptors consist of two subtypes; A_1/R_i and A_2/R_a (Van Calker *et al.*, 1979; Londos *et al.*, 1980), while P_2-purinoceptors have been subdivided into P_{2t}, P_{2X}, P_{2Y} and P_{2Z} receptors (Burnstock & Kennedy, 1985; Gordon, 1986).

For many years it has been known that exogenous ATP and adenosine exert a powerful influence upon the mammalian heart (Drury & Szent-Györgyi, 1929). Their effects include negative chronotrophic and dromotrophic actions upon the sino-atrial and atrio-ventricular nodes, as well as potent vasodilatation of coronary blood vessels (for review, see Burnstock, 1980). The possibility that there might be purinergic innervation of the heart is supported by the observation of nerve fibres and intramural neurones in guinea-pig and rabbit atria showing positive reactions to quinacrine, a fluorescent compound that binds strongly to ATP (Irvin & Irvin 1954; Da Prada *et al.*, 1978; Crowe & Burnstock, 1982). At present, the projections of the intramural ganglia are largely unknown; however, many of the ganglia are concentrated around the sino-atrial node and in the interatrial septum and, as such, are ideally placed to influence both nodal and conducting tissues (King & Coakley, 1958).

We have been utilizing a dissociated mixed cell culture preparation of newborn guinea-pig atria and interatrial septum (Hassall & Burnstock, 1986) to study the electrophysiological and neurochemical properties of intracardiac neurones (Allen & Burnstock, 1987; 1990). In a previous electrophysiological study of the properties of guinea-pig intracardiac neurones in culture, we distinguished two main cell types which were termed AH and M cells (Allen & Burnstock, 1987). M cells displayed non-accommodating tonic firing characteristics when stimulated by intrasomal current injection, whilst AH type neurones were highly refractory and displayed pronounced calcium-dependent after-

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hyperpolarizations. In the present study we have investigated the actions of exogenously applied ATP and related nucleotides and nucleosides on these neurones using single electrode current- and voltage-clamp techniques.

Methods

Experiments were carried out on intracardiac neurones, dissociated from the atria and interatrial septum of newborn guinea-pigs. The mixed cell cultures containing these neurones were prepared and maintained for between 5 and 14 days by use of the methods developed by Hassall & Burnstock (1986). Prior to starting experiments, the culture chamber was dismantled and the coverslip bearing the cultured cells was gently rinsed in oxygenated Krebs solution. The coverslip was then sealed to the underside of a Perspex recording bath with paraffin wax, so that it formed the base of the chamber. The combination of bath and coverslip were then clamped to the modified stage of an inverted microscope (Zeiss invertoscope D), equipped with conventional phase-contrast optics.

The preparation was perfused at a rate of $6\,ml\,min^{-1}$ with oxygenated Krebs solution, warmed to 36–37°C with a remote thermostatically controlled heating coil. The Krebs solution had the following composition (mM): NaCl 117, KCl 4.7, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11 and was gassed with 95% O_2/5% CO_2.

Impalements were made with electrodes having d.c. resistance between 90 and 130 MΩ, containing 2 M potassium citrate solution (pH 7.3). Sylgard (Dow Corning) was used to coat the outside of electrodes to within 100 μm of their tips in order to reduce capacitance and therefore increase the frequency response of the electrodes. Discontinuous single electrode voltage- and current-clamp recordings were made with an amplifier that had a 30% current duty cycle (Axoclamp-2A) with sampling frequencies of 3–5 kHz.

Prior to impalement, electrode resistance and tip offset potentials were nulled to permit estimations of input resistance and membrane potential to be made during the recording. These were checked at the end of each experiment by withdrawing the electrode and passing currents of similar magnitude to those used during the impalement. The fractional increase in input conductance during drug-induced responses was calculated as $(R/R^1) - 1$ where R was the input resistance at resting membrane potential and R^1 that during the drug-mediated response.

To study the actions of adenosine on the post-spike calcium-dependent potassium current, a 'hybrid' voltage-clamp technique was employed. This entailed switching the recording mode of the amplifier briefly from voltage-clamp into current-clamp. In current-clamp, a short train of action potentials was evoked with a train of intrasomal current pulses (30 Hz/1.5 s). At the end of this train the amplifier was then automatically switched back into the voltage-clamp recording mode to enable measurement of the evoked current.

The whole-cell patch-clamp recording technique (Hamill *et al.*, 1981) was used to make recordings from a small sample of M type intracardiac neurones, which due to their small size were otherwise difficult to record from by conventional intracellular microelectrodes. A few AH type cells were also studied by this technique, but the presence of glial cells over these neurones (see ultrastructural study by Kobayashi *et al.*, 1986) prevented the use of this technique to study the majority of cells. The patch electrode solution had the following composition (mM): KCl 110, HEPES 40, $MgCl_2$ 3 and EGTA 3; pH 7.2.

Drugs were applied either by local pressure ejection or by bath perfusion. Pressure application was carried out with blunt microelectrodes (3–10 μm diameter tip) containing drugs diluted in Krebs solution. Electrodes placed 100–500 μm away from the cell surface and the drugs ejected under pressures of 50–100 kPa.

Data were either stored on tape for future analysis (Racal store 4DS) or displayed using a Tektronix storage oscilloscope (model D13) and a Gould pressure ink recorder (model 2200S). Numerical data are expressed as mean ± s.e.mean.

Ionic substitution

Low chloride-containing solutions were made by substituting sodium chloride with sodium gluconate. Low sodium-containing solutions were made by substituting sodium chloride with choline chloride. Reduced calcium-containing solutions were made by substitution with magnesium.

Drugs

Adenosine, adenosine 5'monophosphate (AMP), adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), α,β-methylene ATP, 2-methylthio ATP, indomethacin, 8-phenyltheophylline, reactive blue 2 and tetrodotoxin were obtained from Sigma.

Results

The actions of exogenously applied ATP, adenosine and related purine nucleotides upon guinea-pig intracardiac neurones in culture were studied with single electrode current- and voltage-clamp techniques. The majority of recordings were made from the larger AH type cells, with a few additional studies being made on M type cells by use of the whole-cell patch-clamp technique.

With the exception of depolarization-evoked firing, all of the observed actions of ATP were unaffected by superfusion with tetrodotoxin (0.3 μM). Approximately 74% (120 out of 161) of AH type and 41% of M type cells (5 out of 12) responded to direct application of ATP on to their soma. Three different responses were observed. In 43% of AH type cells and all ATP-responsive M cells, ATP elicited a monophasic depolarization (amplitude 5–30 mV) of short latency (18–55 ms) which was associated with an increase in membrane conductance that frequently resulted in action potential discharge (see Figure 1a). In a further 31% of AH cells, ATP evoked a multi-component response. This second type of response consisted of an initial transient depolarization, followed by a small hyperpolarization and a slow prolonged depolarization (see Figure 1b). In a number of cells, the hyperpolarizing outward current phase of the response was observed to be very small or absent. The initial inward current in all cells resulted from an increase in membrane conductance (see Figure 1bii). The subsequent hyperpolarization and outward current also resulted from a small increase in membrane conductance (see Figure 1bii). However, the slow membrane depolarization/inward current following these initial components was never seen to be associated with any measurable change in input resistance. The third type of response to ATP, seen in only two of the cells studied (both AH type), consisted of a slow depolarization lasting for up to 2 min which was associated with a fall in resting membrane conductance (see Figure 1c).

The rapid depolarization and inward current evoked by ATP in neurones which displayed only a monophasic response differed in a number of respects from the initial depolarization seen in cells exhibiting multi-phase responses. Although both these events resulted in an increase in membrane conductance, the latency and rate of rise of the rapid monophasic depolarization, observed in the majority of cells, was considerably shorter than the initial depolarization seen in cells displaying multi-component responses. The latency of this response in 'monophasic' cells was between 18 and 55 ms (mean 39.4 ± 2.2 ms, $n = 20$) and the mean time to peak was 304 ± 22.4 ms ($n = 17$), compared with a latency of 90 to 210 ms (mean 155.4 ± 15.3 ms, $n = 11$) and a mean time to peak of 662 ± 35 ms ($n = 18$) for the response in cells displaying multi-component responses.

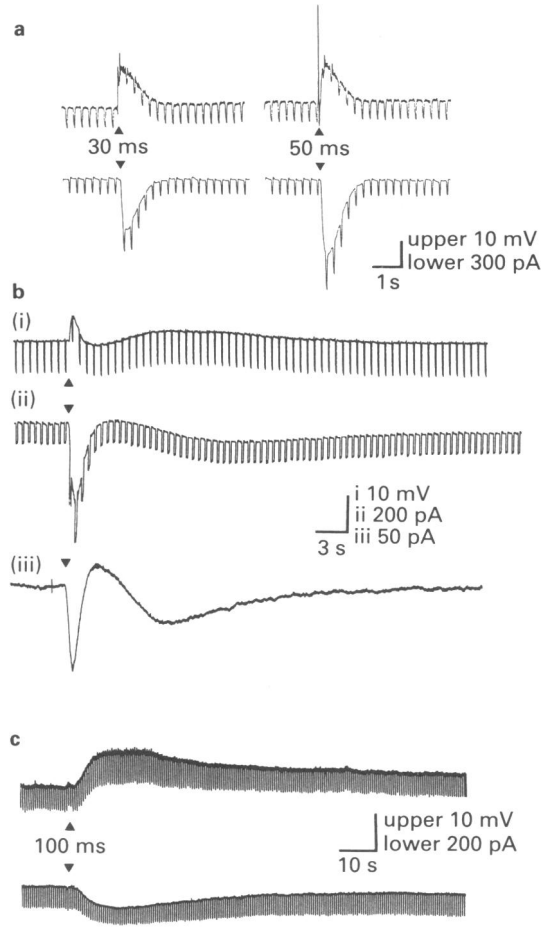

Figure 1 The actions of exogenously applied ATP on AH type intracardiac neurones cultured from guinea-pig heart. (a) Responses from an AH type cell to focally applied ATP (500 μM) for the period indicated by the arrows (membrane potential −64 mV). Upper records were obtained under current-clamp and the lower records with voltage-clamp. Downward deflections on current- and voltage-clamp records were responses to 110 pA/−10 mV pulses of 50 ms duration which were used to monitor changes in input resistance. (b) Multi-component responses to ATP evoked from AH type cells. Upper trace (i) a current-clamp recording showing a three component response to ATP consisting of an initial transient depolarization followed by a small hyperpolarization and a prolonged depolarization. Membrane −64 mV, downward deflections were membrane voltage changes to 150 pA/50 ms intrasomal current pulses. Traces (ii) and (iii), voltage-clamp records from two cells displaying multi-component responses to ATP (membrane potentials were −60 and −52 mV respectively). Downward deflections in (ii) were membrane currents evoked by passing −10 mV/100 ms duration voltage steps. (c) ATP-induced membrane depolarization and fall in membrane conductance in an AH type cell evoked by application of ATP (500 μM) for 100 ms at point indicated by arrows. Upper record: current-clamp. Lower record: voltage-clamp. Membrane potential was −53 mV, downward deflections were the result of 100 pA/50 ms and −9 mV/50 ms duration current/voltage steps used to monitor changes in input resistance.

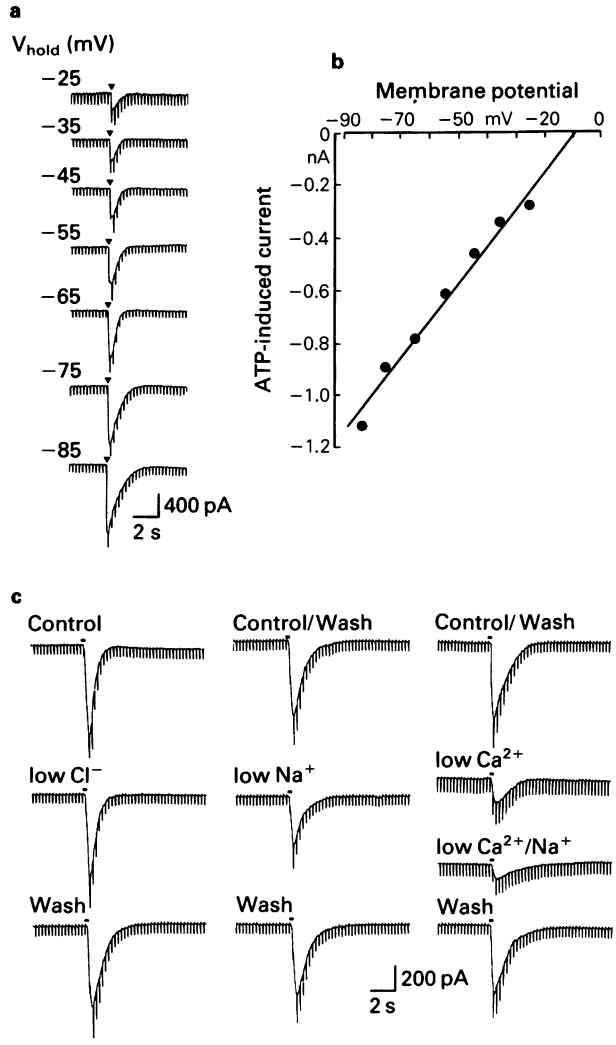

Figure 2 The ionic- and voltage-dependence of the ATP-induced rapid transient inward current and increase in conductance in an AH type intracardiac neurone. (a) The inward current evoked by focal application of ATP (500 μM/50 ms; as indicated by the arrows) at different holding potentials. Downward deflections were the currents evoked by −10 mV, 50 ms duration voltage steps used to monitor changes in membrane conductance. (b) A plot of evoked current against membrane potential for the cell shown in (a). The amplitude of the ATP-induced current was linearly related to membrane potential (correlation coefficient = 0.998) and had a null/equilibrium potential of −9.8 mV. (c) The ionic-dependence of the ATP-induced transient inward current in an AH type intracardiac neurone. ATP (500 μM) was applied by focal pressure ejection for the period indicated by the bar above each trace. Left panel, superfusion with low (9 mM) chloride-containing solution had no effect on the ATP-induced inward current or conductance change. Middle panel, superfusion with low (26.2 mM) sodium-containing solutions reduced the amplitude of the ATP-induced inward current by approximately 34%, but had little effect upon the time course of the underlying conductance change. Right panel, superfusion with low (0.25 mM) calcium-containing solutions increased resting membrane conductance and greatly reduced (approximately 68%) the amplitude of the inward current. Subsequent superfusion with a low calcium- and low sodium-containing solution (0.25 and 26.2 mM respectively) produced a further reduction in the amplitude of the inward current (approximately 82%). Holding potential throughout the recording was −70 mV.

Ionic- and voltage-dependence

ATP-indiced transient depolarizations The amplitude of the ATP-induced transient depolarization in monophasically responsive M and AH type cells at resting membrane potential was between 5 and 30 mV (mean 16.6 ± 1.27 mV, $n = 22$). Under voltage-clamp, the increase in input conductance calculated as a fractional increase (see methods) was 2.45 ± 0.28 ($n = 12$) and resulted in a large inward current, mean 788.8 ± 86.2 pA ($n = 21$). The inward current was linearly related to membrane potential and increased with hyperpolarization (see Figure 2a and b). Extrapolation of the plot of ATP-induced current against membrane potential, indicated a mean null/equilibrium potential for this current of −11.2 ± 1.45 mV ($n = 10$). The amplitude of the ATP-induced inward current was reduced when the cell was superfused with low sodium- (26.2 mM) and/or calcium- (0.25 mM) containing solutions, whereas reducing extracellular chloride concentration (9 mM) had no effect on either the evoked current or the underlying conductance change ($n = 3$; see Figure 2c).

The longer latency, initial transient depolarization in AH cells displaying multi-component responses to ATP was generally smaller than that seen in monophasically responsive M and AH cells and never elicited action potential discharge. The average amplitude of the response was 11.2 ± 1.15 mV ($n = 18$), whilst the underlying fractional increase in conductance and evoked currents were also smaller, having mean values of 0.46 ± 0.08 ($n = 15$) and 203.7 ± 22.4 pA ($n = 30$) respectively. The inward current increased with membrane hyperpolarization and was linearly related to membrane potential between -75 and -45 mV. The predicted null/equilibrium potential of this response, determined by extrapolation was -19.1 ± 1.1 mV ($n = 2$), which was slightly more negative than the value for the fast inward current in other M and AH cells (see Figure 3a and b). Low extracellular chloride-containing solutions (9 mM) increased the amplitude of the current (mean increase $58 \pm 8.1\%$, $n = 3$), whilst superfusion with low sodium- or low calcium-containing solutions only slightly reduced the current, mean reduction $15.7 \pm 1.45\%$ and $9.3 \pm 3.71\%$ ($n = 3$) respectively (see Figure 3d).

Slow outward current/hyperpolarization This response was generally small, range -1 to -3.5 mV (mean -2.0 ± 0.2 mV, $n = 10$) and resulted from a small increase in membrane conductance, mean fractional increase 0.11 ± 0.025 ($n = 6$). Under voltage clamp, the ATP-induced outward current at resting membrane potential was 41.1 ± 5.6 pA ($n = 13$). In all cells studied, the current decreased with membrane hyperpolarization (see Figure 3a and c). The current was linearly related to membrane potential and extrapolation of the plot of evoked current against membrane potential gave a predicted mean null/equilibrium potential of -88.5 mV ($n = 2$), indicating that the current may have resulted from an efflux of potassium ions.

Slow inward current with no associated change in conductance This current was the most commonly observed slow inward current in AH cells which displayed multi-component responses. The response had a slow rate of rise and the time to peak was 10.8 ± 0.37 s ($n = 23$) following focal application of ATP (500 μM). The mean inward current associated with this ATP-induced current was 107.1 ± 13 pA ($n = 28$), and the average observed depolarization was 4.5 ± 0.64 mV ($n = 9$). However, no significant change in membrane conductance was observed in any of the cells studied. Furthermore, it was not possible to determine ionic- or voltage-dependence of this current because of difficulties encountered in reproducibly eliciting the response. However, it was generally observed to increase with membrane hyperpolarization (see Figure 3a).

Slow inward current associated with a fall in conductance This ATP-induced slow inward current was only observed in two cells. It was distinct from the more commonly observed slow inward current that was seen in cells displaying multiphase responses, in that it resulted from a clear decrease in membrane conductance. In one experiment the outward current was reduced by membrane hyperpolarization, and was linearly related to membrane potential between -40 and -70 mV (correlation coefficient $r = 0.989$). Extrapolation of the least squares fit of the raw data gave an equilibrium potential for the response of -90.7 mV.

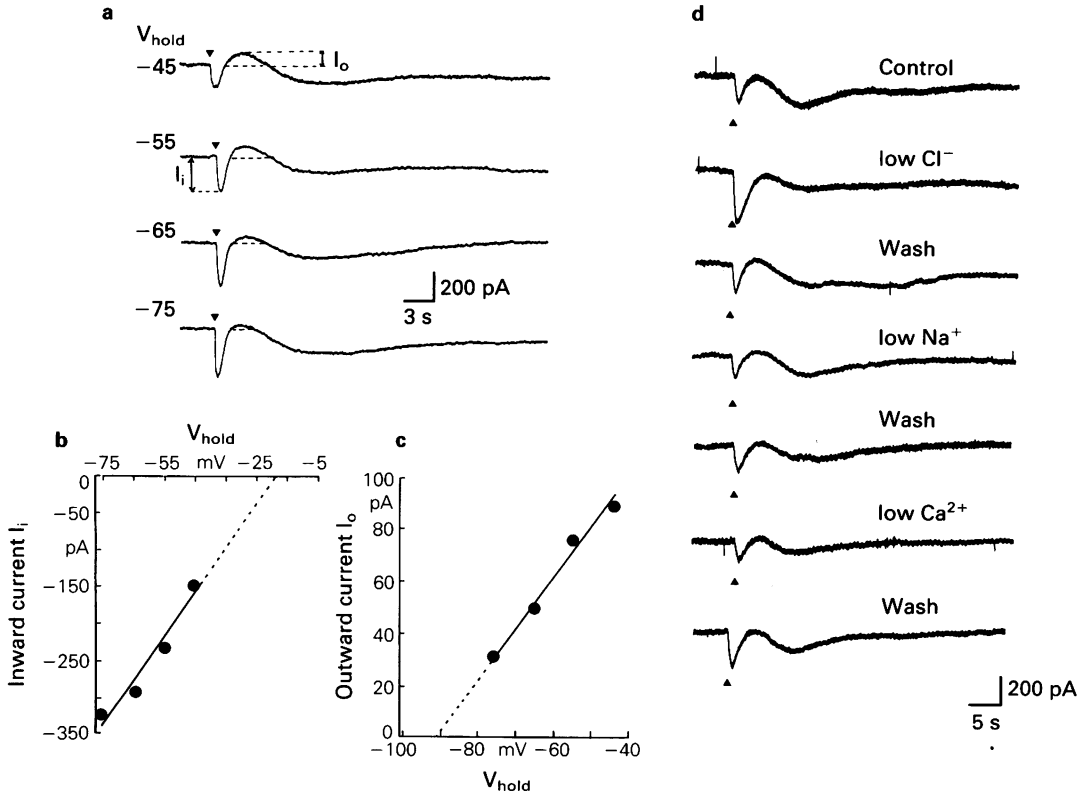

Figure 3 The ionic- and voltage-dependence of the initial transient inward and slow outward current in AH type neurones cultured from the guinea-pig heart. (a) The voltage-dependence of a typical three-component response to brief (50 ms) focal application of ATP (500 μM) to the soma of the cell (see arrows). (b and c) Plots of the amplitude of the initial inward (I_i) and subsequent outward currents (I_o) as a function of membrane potential. In both cases the evoked currents were linearly related to membrane potential between -45 and -75 mV (correlation coefficients, 0.987 and 0.978 respectively). Extrapolation from the obtained data gave a predicted null/equilibrium potential for the inward current of -18 mV and a value of -90.8 mV for the outward current. (d) The ionic-dependence of the transient inward current in an AH type cell displaying a multi-component response to ATP. In low extracellular chloride- (9 mM) containing solutions the current was enhanced by approximately 88%. Superfusion with low sodium- (26.2 mM) or low calcium- (0.25 mM) containing solutions produced only a small reduction in the amplitude of the evoked current. Holding potential -57 mV.

Other purine compounds

2-Methylthio ATP, which potently stimulates P_2-purinoceptors, mimicked all the observed actions of ATP and was generally found to be slightly more potent than ATP (see Figure 4a and b). In general α,β-methylene ATP, which has been shown to stimulate potently P_{2X}-purinoceptors, only poorly mimicked the ATP-evoked rapid transient depolarization (see Figure 4a). In most cells, α,β-methylene ATP evoked only a very small depolarization/current. In a few cells, however, ($n = 4$) it was seen to elicit a depolarization of similar amplitude to that produced by ATP. In these cells, the peak increase in conductance and current examined under voltage-clamp was always smaller than that evoked by ATP and the rate of rise of the depolarization produced was notably slower. When applied to cells that produced a multi-component response to ATP, α,β-methylene ATP weakly mimicked the initial transient inward current, but was never observed to evoke the subsequent slow outward and inward currents (see Figure 4b).

ADP only very weakly mimicked the actions of ATP, whilst AMP and adenosine were ineffective in mimicking any of the actions of ATP. However, adenosine (10–100 μM) was found to reduce the calcium-dependent after-hyperpolarization that followed a train of action potentials (see Figure 5). Both the amplitude and duration of the potassium current and the underlying conductance increase recorded by use of a hybrid voltage-clamp technique (see methods) were reduced by adenosine in a concentration-dependent manner. This effect was antagonized by 8-phenyltheophylline (10 μM; see Figure 5).

Antagonism of the actions of ATP

Superfusion with the putative P_{2Y} receptor antagonist, reactive blue 2, at concentrations up to 3×10^{-5} M initially enhanced the fast transient inward current evoked by ATP by up to 100% (mean 40.4 ± 15.1%, $n = 5$). With continuous application (>3 min) the duration of responses became considerably prolonged. The mean increase in duration was 203 ± 23% ($n = 7$). In addition, there was generally a fall in input resistance and an increase in the overall level of current noise, which reflected a general reactive blue 2-induced deterioration in the cell (see Figure 6c and d). With longer periods of application (up to 25 min) responses to ATP slowly declined in all cells studied ($n = 14$). Complete washout of the effects of reactive blue 2 were never achieved even after prolonged periods (up to 45 min). Reactive blue 2 also strongly depressed $GABA_A$ receptor-mediated chloride currents (mean reduction 78.7 ± 7.2%, $n = 3$) and reduced, though to a lesser extent, the amplitude of the acetylcholine-induced nicotinic responses (mean reduction 26 ± 2.6%, $n = 3$).

Superfusion with α,β-methylene ATP (3×10^{-6} M) only partially reduced the amplitude of the ATP-induced brief latency inward current observed in M and AH type cells (see Figure 6a). The maximum observed reduction in this inward current ranged between 24 and 60% (mean 44.8 ± 6.3%, $n = 6$). When applied to neurones exhibiting multi-component responses to ATP, a similar 19–59% reduction in the initial transient current (mean 35.6 ± 7.2%, $n = 5$) was observed (see Figure 6b).

Inhibition of prostaglandin synthesis with indomethacin (5×10^{-5} M) had no effect upon any of the ATP-induced currents ($n = 7$). Similarly the P_1-purinoceptor antagonist 8-phenyltheophylline (10 μM) was also without effect ($n = 11$).

Discussion

The present study shows that a large population of guinea-pig intracardiac neurones in culture responded to exogenous application of ATP. Three different responses to ATP were observed. The first was a rapid transient depolarization, exhibited by all ATP-responsive M cells (41%) and approximately 43% of AH type neurones. This response displayed a similar agonist potency (ATP > ADP with AMP and adenosine ineffective) and brief latency to the ATP-induced responses seen in mammalian dorsal horn and sensory ganglion neurones (Jahr & Jessell, 1983; Krishtal *et al.*, 1983). In all these cell types, ATP produced a transient increase in mem-

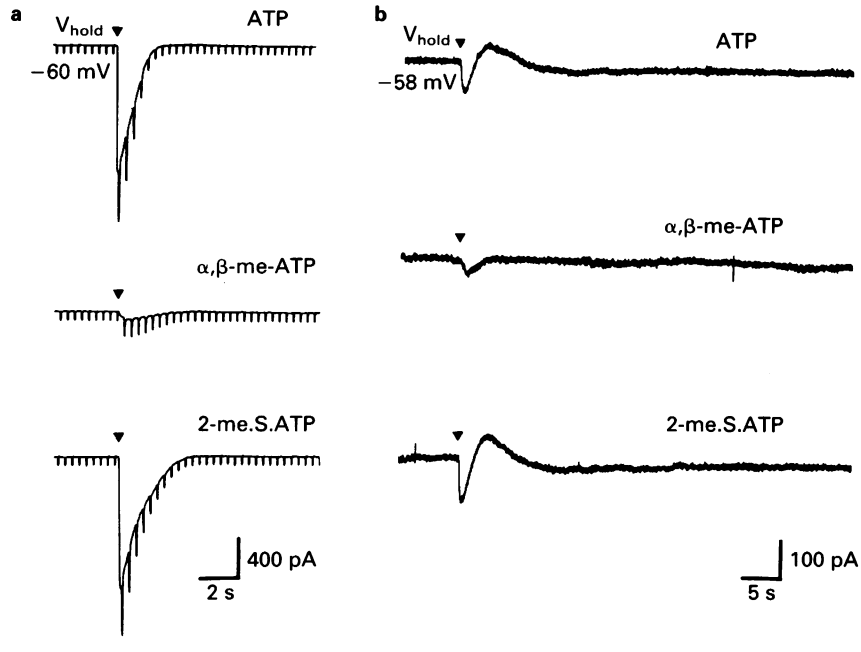

Figure 4 (a) and (b) The actions of ATP, α,β-methylene ATP (α,β-me-ATP) and 2-methylthio ATP (2-me.S.ATP) on intracardiac neurones cultured from the guinea-pig heart. (a) The rapid monophasic inward current evoked by focal pressure application of ATP (500 μM/50 ms). α,β-me-ATP (500 μM/50 ms) only weakly mimicked this response, whereas 2-me.S.ATP (500 μM/50 ms), potently mimicked this action of ATP. Downward deflections were evoked by −10 mV hyperpolarizing voltage steps of 50 ms duration used to monitor changes in input resistance. Holding potential −60 mV. (b) An AH type cell exhibiting a three-component response to exogenous application of ATP. α,β-me-ATP weakly mimicked the initial transient inward current but did not evoke the subsequent slow outward and inward components of the response. 2-me-S.ATP mimicked ATP and evoked a similar three-component response which was slightly larger than that produced by ATP. Holding potential −58 mV.

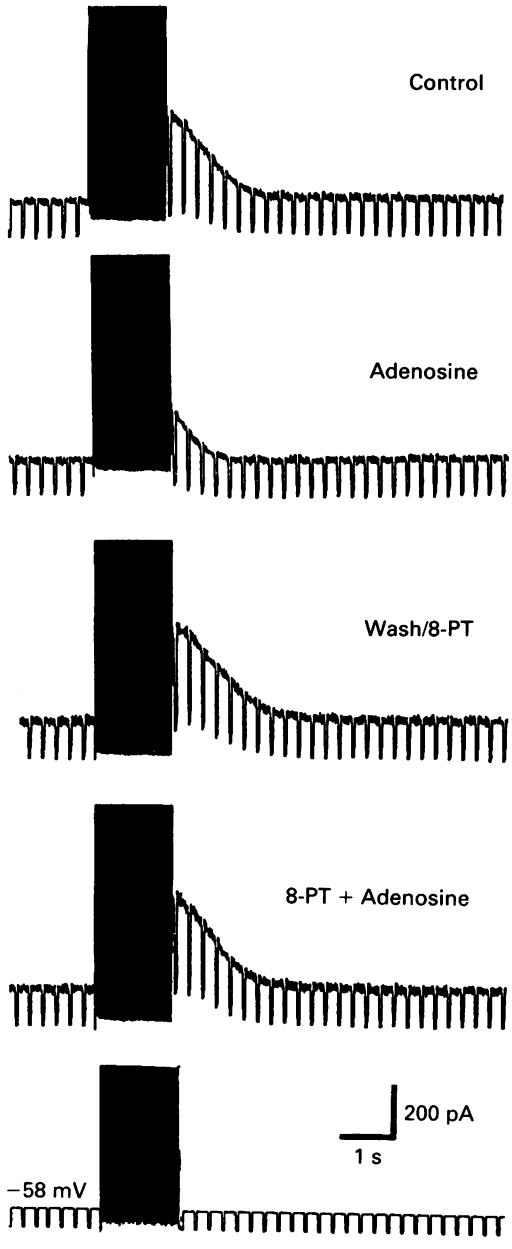

Figure 5 Inhibition of the post-spike calcium-activated potassium current in an AH type intracardiac neurone cultured from guinea-pig heart. The recording was carried out with a hybrid voltage-clamp technique (see methods). Under voltage-clamp at a membrane potential of −58 mV the cell was briefly switched into current-clamp and a train of action potentials was evoked by passing a train of intrasomal current pulses (30 Hz/1.5 s). At the end of this train, the cell was then switched back to voltage-clamp and the evoked outward current and conductance change monitored. Downward deflections were evoked by −10 mV negative command pulses of 50 ms duration used to monitor changes in membrane conductance. Adenosine (50 μM) reduced the outward current and conductance that underlies the after-hyperpolarization. In the presence of 8-phenyltheophylline (8-PT; 10 μM) this inhibitory action of adenosine was inhibited.

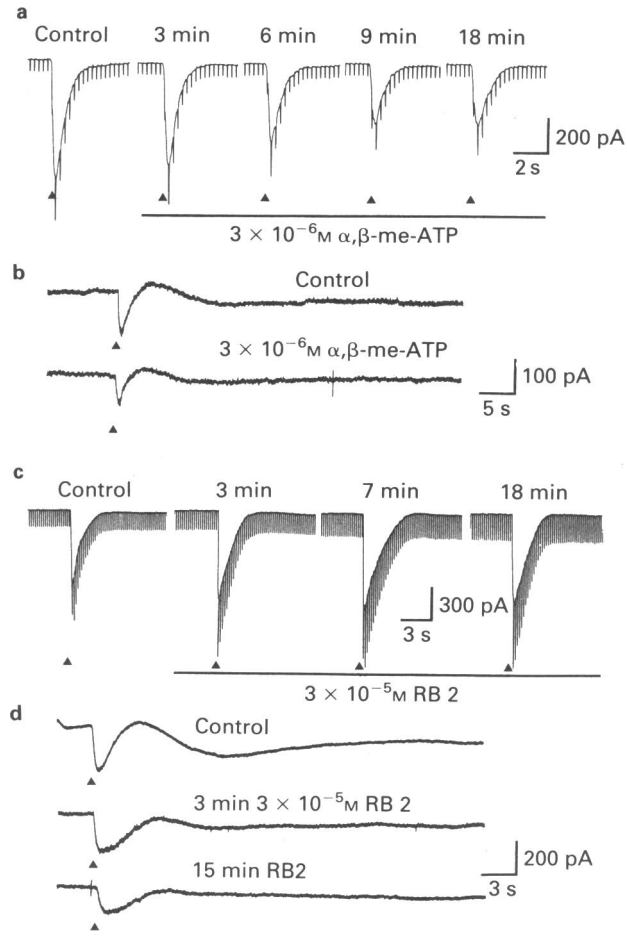

Figure 6 The actions of α,β-methylene ATP (α,β-me-ATP) and reactive blue 2 (RB2) on AH and M type intracardiac neurones. (a) Prolonged superfusion with α,β-me-ATP (3×10^{-6} M) reduced the amplitude of the fast transient inward current evoked by focal application of ATP (500 μM/70 ms) in an M type cell. The ATP-evoked current was maximally inhibited after 9 min superfusion, at which point the current was reduced by approximately 50%. Holding potential −68 mV. A similar reduction in the ATP-induced currents was also seen in cells (all AH type) displaying multi-component responses to ATP (see (b); holding potential −58 mV). (c and d) Show the effect of prolonged superfusion with RB2 (3×10^{-5} M). In cells displaying only a single transient inward current in response to ATP, RB2 produced an initial increase in the evoked current and a slight increase in resting membrane conductance. During prolonged application input resistance continued to decline and the duration of the response was increased. Continued superfusion (up to 30 min) subsequently led to a slow decline in the amplitude of the current and further reduction in input resistance. Washout of RB2 back to control values was never obtained in any cell studied. Holding potential −62 mV. (d) Superfusion with RB2 on to cells displaying multi-component responses produced a similar prolongation of the initial transient inward current and fall in resting membrane conductance. With prolonged exposure to RB2 all of the different component currents were substantially reduced and input resistance continued to decline. Holding potential −61 mV.

brane conductance resulting in a large inward current. In rat dorsal horn and cat vesical parasympathetic ganglia (Jahr & Jessell, 1983; Akasu *et al.*, 1984) as in the present study, application of ATP frequently evoked action potential discharge. The underlying ionic conductance changes observed were similar, in that a large proportion of the current was carried by sodium ions. In dorsal horn and dorsal root ganglion cells, sodium appears to be the sole charge carrier, whilst in sensory, vesical parasympathetic and intracardiac neurones the response to ATP appeared to be mediated via non-selective cationic channels (Krishtal *et al.*, 1983; Akasu *et al.*, 1984). Interestingly, the inward current in AH and M cells giving single component responses to exogenous application of ATP, differed from the initial inward current displayed by AH cells exhibiting multi-component responses. In the latter type of cell, the latency of the response was approximately 100 ms slower and was never observed to evoke action potential discharge. In addition, the ionic- and voltage-dependence of the underlying current was also different. The null/equilibrium potential was 7–8 mV more negative and the current was only slightly reduced in low extracellular calcium- and sodium-containing solutions, but was greatly enhanced when the chloride concentration was reduced, indicating that

this response may have resulted primarily from an increase in chloride conductance.

The inhibitory response which was frequently observed following the initial inward current in AH type cells which displayed multi-component responses to ATP, resulted from an increase in membrane conductance and had a null potential close to the potassium equilibrium potential (E_K; Allen & Burnstock, 1987), which suggests that it may result from an increase in potassium conductance. Similar biphasic responses to ATP, consisting of an initial depolarization followed by hyperpolarization, have also been reported in cat vesical parasympathetic neurones and in chick skeletal muscle cells (Akasu *et al.*, 1984; Hume & Thomas, 1988). As in intracardiac neurones, the inhibitor response in these cells also appeared to be the result of an increase in potassium conductance. In vesical parasympathetic neurones, this response was mimicked by adenosine, indicating that it was mediated via P_1-purinoceptors. Adenosine-induced hyperpolarization resulting from an increase in potassium conductance has also been reported in rat hippocampal, mouse striatal and guinea-pig myenteric plexus neurones (Segal, 1982; Haas & Greene, 1984; Trussell & Jackson, 1985; Palmer *et al.*, 1987). In the current study of intracardiac neurones, however, adenosine was never observed to mimic the ATP-induced hyperpolarization which indicates that this response was not mediated by P_1-purinoceptors.

Two different types of ATP-induced slow depolarizations were observed in AH type cells but were never observed in M type cells. In the majority of cells, the ATP-evoked depolarization and inward current was not associated with any measurable change in membrane conductance. Furthermore, it was not antagonized by indomethacin or the P_1-purinoceptor antagonist 8-phenyltheophylline, suggesting that it did not arise as a result of increased prostaglandin synthesis or from the breakdown of ATP to adenosine. Unlike the most commonly observed slow depolarization, the second type of ATP-induced slow depolarization was associated with a distinct decrease in membrane conductance and was not preceded by a transient depolarization or hyperpolarization. This current most probably resulted from inhibition of a tonically active potassium conductance since it displayed a null potential close to E_K (Allen & Burnstock, 1987). ATP and adenosine have similar actions on a variety of different neurones (Akasu *et al.*, 1983; Morita *et al.*, 1984; Katayama & Morita, 1989).

Both P_1- and P_2-purinoceptor subtypes are present in guinea-pig intracardiac neurones. All of the observed actions of ATP appeared to result from the direct action of ATP on P_2-purinoceptors rather than as a consequence of its breakdown to ADP, AMP or adenosine. Exogenous application of adenosine was never observed to mimic any of the actions of ATP. However, (although the actions of adenosine were not studied in detail in the current report), adenosine, acting on P_1-purinoceptors, was observed to inhibit the outward post-spike calcium-sensitive potassium current in a significant population of AH type cells.

In a number of tissues, α,β-methylene ATP, a slowly degraded analogue of ATP has been shown to act potently (>ATP) and selectively on P_{2X}-purinoceptors. Whilst 2-methylthio ATP acts most potently ($\geqslant$ATP) upon P_{2Y}-purinoceptors (for review see Burnstock & Kennedy, 1985). In the current study, α,β-methylene ATP only weakly mimicked the initial transient inward currents evoked by ATP and was never seen to mimic the slow outward or inward currents. On the other hand, 2-methylthio ATP mimicked all the observed actions of ATP, but was only slightly more potent than ATP. Thus the order of potency for these purines on intracardiac neurones was 2-methylthio ATP $\geqslant$ ATP > α,β-methylene ATP. According to the original classification proposed by Burnstock & Kennedy (1985), α,β-methylene ATP also selectively desensitizes P_{2X} receptors. Prolonged exposure to α,β-methylene ATP generally reduced, but never abolished the response to ATP, indicating that these responses to ATP were not mediated via P_{2X}-purinoceptors. Reactive blue 2, an anthraquinone sulphoric acid derivative, has been shown to display a degree of selectivity in antagonizing P_{2Y}-purinoceptors in a variety of tissues (Kerr & Krantis, 1979; Manzini *et al.*, 1986; Burnstock & Warland, 1987; Hopwood & Burnstock, 1987; Houston *et al.*, 1987). In the current study, reactive blue 2 initially increased and prolonged the actions of ATP, whilst prolonged exposure generally reduced the amplitude of the ATP-induced responses and also slowly reduced the input resistance of all the cells studied. Furthermore, reactive blue 2 (10 μM) also reduced responses to exogenously applied GABA and acetylcholine, which indicates a non-selective action.

The presence of receptors for ATP and adenosine raises the possibility that in the heart they may act pre- or post-junctionally to modulate the transmitter release from the intramural neurones and thereby regulate the activity of the effector tissue. In addition, the presence of quinacrine-positive intramural neurones and nerve fibres in guinea-pig atria suggests that some intracardiac neurones may be purinergic (Crowe & Burnstock, 1982). ATP and adenosine are known to produce potent vasodilatation of coronary vessels and also to have pronounced effects upon heart muscle, particularly in the atrium and the sino-atrial node (Drury & Szent-Györgyi, 1929; Yatani *et al.*, 1978; Berne, 1980; West & Bellardinelli, 1985). Therefore, it is possible that release of ATP from intracardiac neurones may be responsible, at least in part, for mediating some of these actions. Furthermore, the present finding that a considerable proportion of the intracardiac neurones were responsive to ATP and adenosine, raises the possibility that ATP may be released from one population of intracardiac neurones to modulate the excitability of other intracardiac neurones through local reflex pathways.

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Nimodipine has no effect on the cerebral circulation in conscious pigs, despite an increase in cardiac output

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1 We studied the effects of four doses of nimodipine (0.5, 1, 2 and 4 $\mu g\,kg^{-1}\,min^{-1}$) on systemic haemodynamics and on regional vascular beds, in particular the cerebral circulation, in conscious pigs.

2 Nimodipine caused dose-dependent, probably reflex-mediated, increases in heart rate (42% with the highest dose) and cardiac output (54%), while arterial blood pressure was only minimally affected. Left ventricular end-diastolic pressure and systemic vascular resistance decreased dose-dependently (35–40% at the highest dose) while stroke volume remained unchanged.

3 Total brain blood flow was not affected by the drug. Furthermore, we could not demonstrate any regional cerebral differences, as blood flows to both cerebral hemispheres as well as the diencephalon, cerebellum and brain stem remained unchanged.

4 Blood flow to the kidneys, liver, small intestine and skin also did not change. Nimodipine caused dose-dependent increases in blood flow to the stomach (95%), myocardium (97%) and adrenal glands (102%), while blood flow to skeletal muscles (267%) increased most.

5 It is concluded that in the conscious pig, nimodipine is an arterial vasodilator which shows some selectivity for the skeletal muscle vasculature but does not increase total or regional cerebral blood flow.

Introduction

The calcium channel blockers exhibit considerable heterogeneity with respect to their effects on the heart and different vascular smooth muscle preparations (Cauvin *et al.*, 1983; Fleckenstein, 1983; Nayler, 1983; Katz & Leach, 1987). The 1, 4-dihydropyridine derivative, nimodipine, appears to have a preferential action on cerebral vessels *in vitro* (Towart, 1981; Towart & Perzborn, 1981; Towart *et al.*, 1982; Kazda & Towart, 1982; White *et al.*, 1982). Results of *in vivo* studies, however, are variable. In conscious rabbits the drug was reported to have a greater effect on cerebral blood flow than on perfusion of other organs and tissues (Haws *et al.*, 1983). However, we failed to confirm these observations in anaesthetized pigs as nimodipine like the related compound nisoldipine (Duncker *et al.*, 1986a), did not affect cerebral blood flow but more than doubled skeletal muscle blood flow (Duncker *et al.*, 1986b). The decrease in arterial blood pressure is much more pronounced in anaesthetized than in conscious pigs after acute administration of systemic vasodilators apparently due to a lack of reflex tachycardia and concomitant increase in cardiac output in the anaesthetized animals (Duncker *et al.*, 1988). It is therefore possible that this pronounced decrease in blood pressure elicited by nimodipine in anaesthetized pigs negated the expected increase in cerebral blood flow. In order to eliminate possible complications introduced by the use of anaesthetic drugs, we have investigated the vascular responses in various tissues to nimodipine in conscious instrumented pigs. Particular attention has been devoted to regional cerebral blood flows in view of growing clinical evidence that the drug may exert a beneficial effect on a number of cerebral vascular disorders (Auer *et al.*, 1982; Grotenhuis *et al.*, 1984; Kostron *et al.*, 1984; Gelmers *et al.*, 1988) and in the treatment of migraine headaches (Gelmers, 1983; Meyer & Hardenberg 1983).

Methods

Animal model

Experiments were performed in conscious, Landrace x Yorkshire cross-bred pigs (18–24 kg, $n = 14$), which were instrumented by methods described earlier by Duncker *et al.* (1987a). The investigations were performed according to the *Guide for the Care and Use of Laboratory Animals* (DHEW publication No. (NIH) 80–23, 1980). Briefly, the animals were sedated with ketamine HCl ($30\,mg\,kg^{-1}$) and connected to a ventilator after endotracheal intubation. Anaesthesia was maintained with enflurane (1–4 vol%), added to a mixture of O_2: N_2O (1:2, v/v). Under sterile conditions, cannulae were placed in the superior vena cava (via the right jugular vein) and the thoracic aorta (via the common carotid artery), for the administration of drugs and the measurement of arterial blood pressure, respectively. After a left lateral thoracotomy, an electromagnetic flow probe (13–15 mm, Skalar, Delft, The Netherlands) was positioned around the ascending aorta. A tip-manometer pressure transducer (Konigsberg Instruments Inc., Pasadena, CA, USA) was implanted through the apex into the left ventricle of the heart. The left atrium was cannulated for recording of left atrial pressure which, together with the aortic blood pressure, was used for calibration of the Konigsberg transducer signals. A second left atrial cannula was used for the injection of tracer microspheres (diameter $15 \pm 1\,\mu m$; ^{141}Ce, ^{113}Sn, ^{103}Ru, ^{95}Nb or ^{46}Sc labelled; NEN Chemicals GmbH, Dreieich, F.R.G.) for the measurement of organ blood flows (Saxena *et al.*, 1980; Saxena & Verdouw, 1982). Catheters and wires were externalized subcutaneously via the back or the neck. The thorax was then closed and the animals allowed to recover from surgery. Catheters were flushed daily with heparinized saline and antimicrobial prophylaxis was obtained with amoxycillin (500 mg daily). At least 6–8 sessions were held to adapt the animals to the experimental and laboratory conditions. When haemodynamic parameters remained stable for at least 90 min the animals were regarded as being suitable to enter the experimental protocol. All experiments were carried out while the animals were quietly resting in a constraining jacket.

Experimental protocol

After baseline recordings had been made of arterial pressure, aortic blood flow, left ventricular pressure and its first derivative ($LVdP/dt$), approximately 1 million microspheres labelled with one of the isotopes were injected while an arterial reference blood sample ($8.8\,ml\,min^{-1}$) was withdrawn,

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starting 10s before the injection of the microspheres and lasting up to 60s after completion of the injection. All measurements were repeated at the end of each of four consecutive 10min i.v. infusions of nimodipine (0.5, 1, 2 and $4\,\mu g\,kg^{-1}\,min^{-1}$; $n = 7$) or equivalent volumes of the solvent ($n = 7$). After the last measurements, the animals were killed with an overdose of pentobarbitone sodium and the organs removed for the determination of regional blood flows. The heart was fixed in formaldehyde (10%) for later dissection of the left ventricular wall into three layers of equal thickness (epicardium, mesocardium and endocardium). All other organs were dissected without fixation. The brain was divided into cerebral hemispheres, diencephalon, cerebellum and brain stem. Both iliopsoas muscles were dissected for the analysis of skeletal muscle blood flow. Full details of the procedures and the calculation of flow data with this technique have been described earlier (Saxena *et al.*, 1980; Duncker *et al.*, 1986b). In our laboratory the variability of repeated measurements is less than 6% (Schamhardt, 1980).

Vascular resistances in the particular organs were calculated as the ratio between the mean arterial blood pressure and the corresponding organ blood flow. Left ventricular O_2 demand was estimated by the product of the heart rate and systolic left ventricular blood pressure.

Drugs

Nimodipine (Bay e 9736; Bayer AG, Wuppertal, F.R.G.) was dissolved at $0.2\,mg\,ml^{-1}$ in a mixture of glycerol (60g), H_2O (100g) and polyethylene glycol 400 (to 1129g) and this stock solution was diluted with 0.9% w/v NaCl solution immediately before use and administered while protected from light.

Statistical analysis

Data are expressed as mean ± s.e.mean. The significance of the effects of nimodipine was evaluated by Duncan's new multiple range test, after two-way analysis of variance had revealed that the samples represented different populations. Only values of $P < 0.05$ (two-tailed) were considered statistically significant.

Results

Systemic haemodynamics

Infusion of the solvent did not affect systemic haemodynamic variables (Figure 1). Nimodipine caused a dose-dependent increase in cardiac output (from 2.4 ± 0.2 at the baseline to $3.7 \pm 0.4\,l\,min^{-1}$ at the highest dose; +54%) which was almost entirely due to an increase in heart rate (from 124 ± 6 to 176 ± 2 beats min^{-1}; +46%). Mean arterial blood pressure decreased (from 108 ± 8 to 97 ± 7mmHg; −9%), with the decrease in systolic arterial blood pressure (5mmHg at the highest dose; $P < 0.05$) being considerably smaller than that of diastolic arterial blood pressure (14mmHg; $P < 0.05$). Since the blood pressure response was accompanied by an increase in cardiac output, calculated systemic vascular resistance decreased substantially (by 39%) at the highest dose. Although both left ventricular filling pressure and diastolic arterial pressure decreased, there was a marked increase in $LVdP/dt_{max}$ (60%) at the highest dose.

Left ventricular blood flow

In the solvent-treated animals, myocardial O_2 demand, estimated by the product of heart rate and left ventricular systolic blood pressure, did not change ($15 \pm 1\,mmHg\,min^{-1}\,10^3$ at the baseline and $17 \pm 1\,mmHg\,min^{-1}\,10^3$ at the end of the last infusion). This was also reflected by the unchanged transmural left ventricular blood flow (Figure 2). The increase in left ventricular O_2 demand in the nimodipine-treated animals (from $15 \pm 1\,mmHg\,min^{-1}\,10^3$ at the baseline to $21 \pm 1\,mmHg\,min^{-1}10^3$ at $4\,\mu g\,kg^{-1}\,min^{-1}$; +40%) was statistically significant compared to the solvent-treated animals, but the rise in myocardial blood supply (80%; Figure 2), which was uniformly distributed over the different layers, was far in excess of that required to meet the increased demand. Consequently, there was an appreciable decrease (from 86 ± 8 to $64 \pm 6\,mmHg\,ml^{-1}$ 100g) in the ratio between O_2 demand and blood supply.

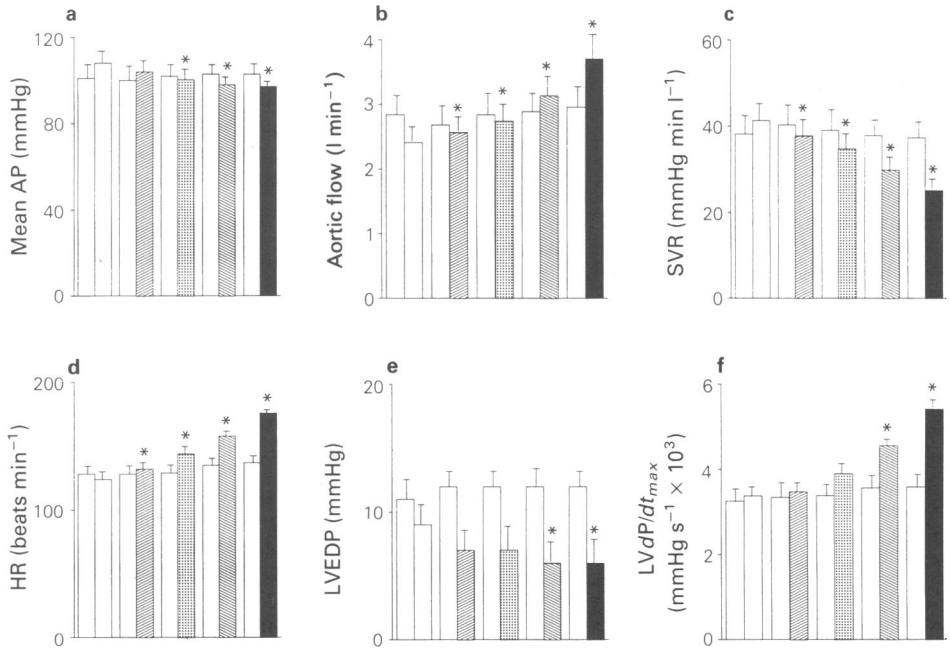

Figure 1 Systemic haemodynamic effects of four consecutive doses of nimodipine (hatched columns; $n = 7$) or its solvent (open columns; $n = 7$) in conscious pigs. The pair of open columns at the left side of each graph represent the pre-drug baseline values for the animals receiving the solvent (left) or nimodipine (right). Nimodipine □ 0, ▨ 0.5, ▩ 1.0, ▧ 2.0, ■ $4.0\,\mu g\,kg^{-1}\,min^{-1}$. (a) Mean AP = mean arterial pressure; (b) aortic flow; (c) SVR = systemic vascular resistance; (d) HR = heart rate; (e) LVEDP = left ventricular end-diastolic pressure; (f) $LVdP/dt_{max}$. *$P < 0.05$, drug-induced changes statistically significant versus solvent.

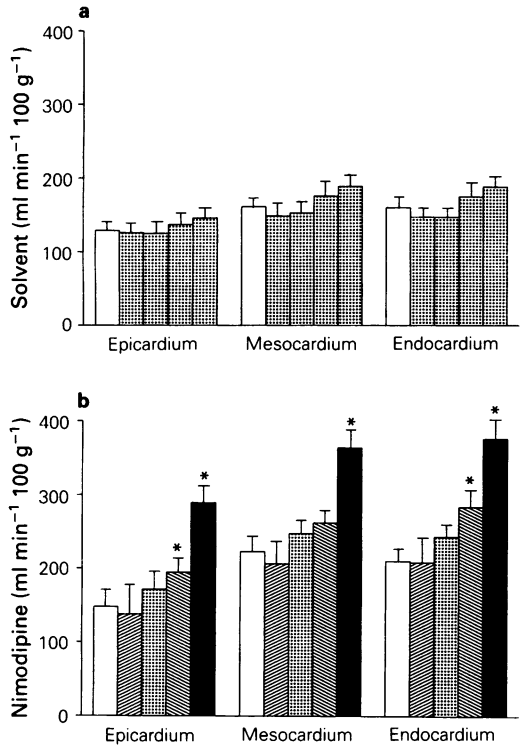

Figure 2 Regional myocardial blood flows at the baseline (open columns) and after four consecutive doses of nimodipine (b; $n = 7$) or its solvent (a; $n = 7$). Nimodipine □ 0, ▨ 0.5, ▩ 1.0, ▧ 2.0, ■ 4.0 $\mu g\,kg^{-1}\,min^{-1}$. In (a) solvent = ▩. *$P < 0.05$, drug-induced changes statistically significant versus solvent.

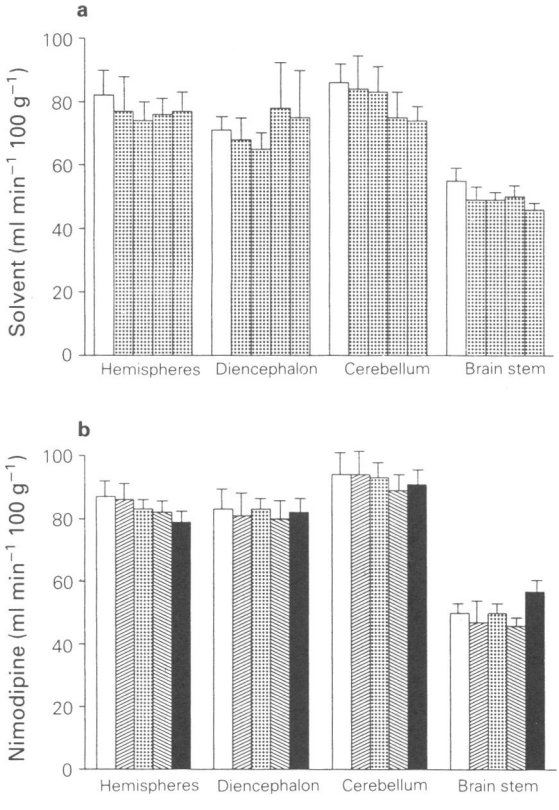

Figure 3 Regional brain blood flows at the baseline (open columns) and after four consecutive doses of nimodipine (b; $n = 7$) or its solvent (a; $n = 7$). Nimodipine □ 0, ▨ 0.5, ▩ 1.0, ▧ 2.0, ■ 4.0 $\mu g\,kg^{-1}\,min^{-1}$. In (a) solvent = ▩.

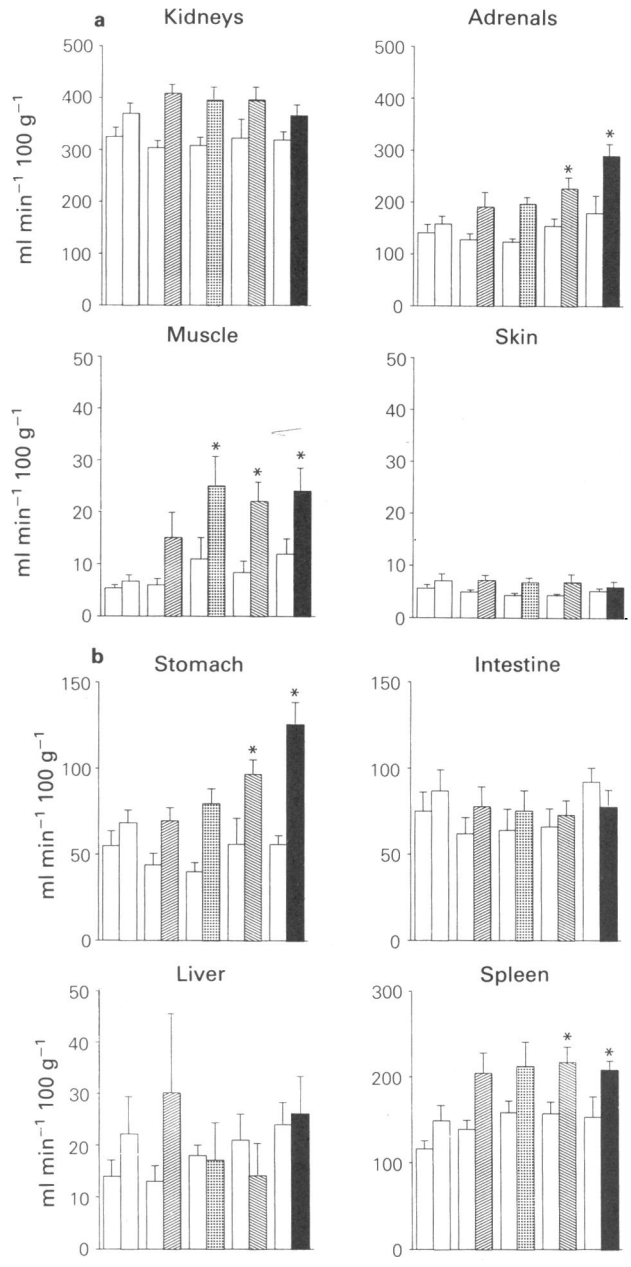

Figure 4 The effects of four consecutive doses of nimodipine (shaded columns; $n = 7$) or its solvent (open columns; $n = 7$) on organ blood flows in conscious pigs: (a) kidneys, adrenals, muscle and skin; (b) stomach, intestine, liver and spleen. The pair of open columns at the left side of each graph represent the pre-drug baseline values of the animals receiving the solvent (left) or nimodipine (right). Nimodipine □ 0, ▨ 0.5, ▩ 1.0, ▨ 2.0, ■ 4.0 $\mu g\,kg^{-1}\,min^{-1}$. *$P < 0.05$, drug-induced changes statistically significant versus solvent.

Cerebral blood flow

Total cerebral blood flow was not affected by nimodipine. Analysis of the flows to the different parts of the brain (hemispheres, diencephalon, cerebellum and brain stem) revealed that no redistribution had occurred in favour of any of these parts (Figure 3). Vascular conductances in the different brain areas were also not affected.

Blood flows to other organs

During administration of the incremental doses of nimodipine, blood flow to the kidneys, liver, small intestine and skin remained unchanged (Figure 4). Perfusion of the spleen increased, but this change was not dose-dependent. Blood flows to the stomach, adrenal glands and skeletal muscle also

increased with the changes being most marked in the skeletal muscles.

Discussion

Systemic haemodynamics

In the present study the effects of four incremental dosages of nimodipine on systemic haemodynamics and the regional distribution of cardiac output in conscious pigs were compared with those of its solvent. Nimodipine caused dose-dependent decreases in left ventricular filling pressure as well as in systemic vascular resistance. Cardiac output increased by more than 50% at the highest rate of infusion, probably because of a baroreflex-mediated increase in heart rate. Accordingly, mean arterial blood pressure decreased only 9% at the highest dose of nimodipine. These effects are comparable with the systemic haemodynamic actions of other dihydropyridines in conscious pigs (Duncker *et al.*, 1988). In anaesthetized pigs, however, nimodipine (in a comparable dose-range) caused marked decreases in mean arterial blood pressure, heart rate and cardiac output (Duncker *et al.*, 1986b), apparently because of suppression of baroreceptor reflex effects by the anaesthetic drugs used. The reduction in cardiac output observed in the latter study was due to a closure of arteriovenous anastomoses, as the nutrient part of cardiac output remained unaffected.

Myocardial blood flow

In the present investigation nimodipine caused dose-dependent increases in myocardial blood flow. As the ratio between O_2 demand and O_2 supply decreased by up to 25%, this higher myocardial blood flow cannot be explained by an autoregulatory process because of a higher O_2 demand. It therefore seems likely that nimodipine elicits 'active' vasodilatation in the coronary vascular bed.

Blood flows to other organs

A small part of the increase in aortic blood flow was directed to the spleen, stomach and adrenals. Vasodilatation was most pronounced in the iliopsoas muscle as blood flow increased more than two fold. This observation was also made in anaesthetized pigs with nimodipine and nisoldipine (Duncker *et al.*, 1986a,b). Interestingly, Haws *et al.* (1983) reported selective increases in cerebral and coronary blood flow after nimodipine administration in conscious rabbits. In fact, at doses of 0.1 and 1.0 $\mu g\,kg^{-1}\,min^{-1}$, the drug induced $70 \pm 25\%$ and $110 \pm 25\%$ increases in cerebral blood flow, respectively, whereas masseter muscle blood flow was enhanced by $50 \pm 50\%$ and $450 \pm 320\%$, respectively. Although the latter responses did not reach statistical significance, it is possible that nimodipine might have elicited a greater response in the skeletal muscle vasculature compared to the cerebral circulation in some rabbits.

Cerebral blood flow

Contrary to the general belief, but in agreement with our experiments in anaesthetized pigs (Duncker *et al.*, 1986b), nimodipine in the doses used in this study did not increase total or regional cerebral blood flows (That is, flows to the hemispheres, midbrain, cerebellum and brain stem). Blood flow measurements with microspheres depend, among other things, on complete trapping of spheres by the tissue under investigation. Since we did not detect any increase in cerebral blood flow, it might be argued that nimodipine dilated small blood vessels to the extent that microspheres escaped to the venous side and, therefore, flow increases remained undetected. For several reasons this possibility is highly unlikely: (i) increases in blood flow to the extent of up to 20 to 30 fold, at least in the skeletal muscles (Hof, 1983; Bolt & Saxena, 1984; Duncker *et al.*, 1986a,b; 1987b; present study) and skin (Saxena & Verdouw, 1982; 1985; Duncker *et al.*, 1987b), can be detected with 15 μm spheres; (ii) if tracer microspheres were escaping to the venous side, lung radioactivity should have increased after calcium antagonists but, as found earlier (Bolt & Saxena, 1984; Duncker *et al.*, 1986a,b; 1987b) and in the present study 'lung' blood flow tended to decrease (from 34 ± 4 to $46 \pm 9\,ml\,min^{-1}\,100\,g^{-1}$ after solvent infusion and from 35 ± 8 to $24 \pm 4\,ml\,min^{-1}\,100\,g^{-1}$ after the highest dose of nimodipine) and (iii) even after the administration of microspheres directly into the carotid artery, thus avoiding their entry into the lungs via the bronchial arteries, local infusions of both nimodipine and nifedipine decreased (not increased) cephalic shunting of microspheres (see Duncker *et al.*, 1987b).

Results in the literature on the effects of nimodipine on cerebral blood flow thus far are equivocal (Table 1). In anaesthetized rats, rabbits, cats, dogs and baboons an increase in cerebral blood flow has been reported (Harper *et al.*, 1981; Kazda *et al.*, 1982; Haws *et al.*, 1983; Mohamed *et al.*, 1984; McCalden *et al.*, 1984). In anaesthetized pigs no such increase in flow could be observed (Duncker *et al.*, 1986b). In conscious rabbits (Haws *et al.*, 1983), and conscious hypertensive rats (Grabowski & Johansson, 1985) nimodipine increased cerebral blood flow, but in conscious normotensive rats, as well as in dogs, no changes were observed (Grabowski & Johansson, 1985; Kanda & Flaim, 1986; Forsman *et al.*, 1986). With respect to the study by Kanda and Flaim, it must be kept in mind that the studies were performed 3 h after recovery from surgery and that the baseline flows were twice as high as in their anaesthetized rats. It could be argued that, in this preparation, further vasodilatation could hardly be expected. The authors, however, also showed that further cerebral vasodilatation was possible when the animals were exercised (treadmill running). Our results in conscious pigs are in agreement with the latter studies. An explanation for the discrepancy in the results between anaesthetized and conscious animals may be the effect of anaesthesia on brain blood flow. Indeed, anaesthetic agents like barbiturates and fentanyl lower total brain flow (Nilsson & Siesjo, 1975; Carlsson *et al.*, 1982), which may result in greater susceptibility of the cerebral circulation to the vasodilator actions of nimodipine. Another variable may be introduced by opening the skull, which was done in several experimental studies (see Table 1). It has been shown that nimodipine infusion increases basal blood flow only in primates with a craniotomy, but not when the skull remains closed (Harris *et al.*, 1982). *In vitro* studies conducted on arteries from several species, including man, showed that nimodipine was a more potent dilator of the large conductance vessels of the cerebral circulation than of other conductance vessels (Towart, 1981; Cauvin *et al.*, 1983; Müller-Schweinitzer & Neumann, 1983). Therefore it is possible that, under physiological conditions, nimodipine dilates the larger cerebral vessels (vertebral and basilar arteries) without dilatation of the smaller cerebral arterioles and thus is without an effect on cerebral blood flow. However, in pathological conditions, where vasoconstrictor mediators act on the cerebral vasculature, or during anaesthesia, the effects of nimodipine may be different. Indeed, in experiments involving ligation of a cerebral artery (Steen *et al.*, 1983; Smith *et al.*, 1983; Mohamed *et al.*, 1985; Newberg Milde *et al.*, 1986) or experimental subarachnoid haemorrhage (McCalden *et al.*, 1986) impairment of cerebral blood flow, due to an increase in vasomotor tone in the post-ischaemic or post-haemorrhagic phase, can be alleviated by nimodipine. However, negative reports have also been published (Barnett *et al.*, 1986; Schuier & Ulrich, 1987; Sahlin *et al.*, 1987; Berger & Hakim, 1988). From the present and other studies the question arises whether the differences in results can be explained by differences in preparations only (conscious versus anaesthetized,

Table 1 Cerebral blood flow and vascular resistance responses to intravenous or intracarotid administration of nimodipine in animals with an intact cerebral circulation

	Animal species	*Rate of administration* ($\mu g\,kg^{-1}\,min^{-1}$)	*Cumulative dose* ($\mu g\,kg^{-1}$)	*Cerebral blood flow* (% increase)	*Mean arterial blood pressure* (% decrease)·	*Cerebral vascular resistance* (% increase)
Anaesthetized						
Harper *et al.*, 1981	Baboon	bolus injection	3–10	NC	NC	NC
	Baboon (os)	2.0	100	25	10	30
Harris *et al.*, 1982	Baboon	0.6 i.c.	27	NC	10	10
	Baboon (os)	0.6 i.c.	27	25	10	30
McCalden *et al.*, 1984	Baboon	0.1–1000	2–22222	NC	0–20	0–25
Haws & Heistad, 1984	Monkey	1.0	5	NC	NC	NC
Kazda *et al.*, 1982	Cat	0.3–3.2	6–118	20–60	10–20	25–50
Haws & Heistad, 1984	Cat (os)	0.5–1.0	2.5–7.5	NC	NC	NC
Mohammed *et al.*, 1984	Rat	1.0–4.0	30–210	0–30[c]	10–25	10–40
Kanda & Flaim, 1986	Rat	0.4–4.0	6–66	10–60	25–40	25–60
Haws *et al.*, 1983	Rabbit	0.1–1.0	0.5–5.5	25–100	20	35–60
Kazda *et al.*, 1982	Dog	bolus injection	1–111	19–54	8–31	23–55
Duncker *et al.*, 1986b	Pig	0.05–6.25	0.5–7.8	0–35	5–60	5–40
Conscious						
Haws *et al.*, 1983	Rabbit	0.1–1.0	0.05–5.5	25–110	5–35	25–70
Grabowski & Johansson, 1985	Rat (WKY)	0.75–7.5[a]	27.5–275	NC	10–38	10–50
	Rat (SHR)	0.75–7.5[a]	27.5–275	0–30	10–46	10–40
Kanda & Flaim, 1986	Rat	0.4–4.0	6–90	NC	5–20	NC
Forsman *et al.*, 1986	Dog[b] (os)	0.1–3.0	1.5–66	NC	10–45	0–40
Present study	Pig	0.5–4.0	5–75	NC	0–10	NC

WKY = Wistar-Kyoto rats; SHR = spontaneous hypertensive rats; [a] bolus injection of 5–50 $\mu g\,kg^{-1}$ followed by an infusion of 0.75–7.5 $\mu g\,kg^{-1}\,min^{-1}$; [b] spinal anaesthesia was employed to circumvent effects of general anaesthesia on the brain; [c] cerebral blood flow increased only in 9 out of 31 brain regions; total cerebral blood flow responses have been estimated from the regional data; NC = no change. Only in the study by Harris *et al.* (1982) was intracarotid (i.c.) administration used. Most studies were performed in animals with a closed skull, for those in which open skull preparations were employed this has been indicated in parentheses (os).

open versus closed skull) or that differences in species may be the dominant factor. Furthermore it should be kept in mind that maintenance of cerebral blood flow when arterial blood pressure decreases does not necessarily mean a drug-induced vasodilatation since Harper *et al.* (1981) have shown that cerebral O_2-consumption does not change in anaesthetized baboons. We feel that further studies are needed to show from which species the results of the studies on the cerebral effects of calcium antagonists can be most reliably extrapolated to that of man.

The question raised above seems to be appropriate as the results of clinical studies point towards a beneficial effect of nimodipine in patients with cerebrovascular disease (Gelmers, 1983; Meyer & Hardenberg 1983; Grotenhuis *et al.*, 1984; Kostron *et al.*, 1984; Gelmers *et al.*, 1988). Though we grant that it remains difficult to extrapolate animal data to clinical situations, our results do question the assertion that nimodipine exerts its beneficial effects by enhancement of cerebral blood flow (Smith *et al.*, 1983; Sahlin *et al.*, 1987; Berger & Hakim, 1988; Lyden *et al.*, 1988).

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Role of adrenoceptors *in vitro* and *in vivo* in the effects of lithium on blood glucose levels and insulin secretion in the rat

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1 Pretreatment of rats with the non-selective α-adrenoceptor antagonist dihydroergotamine counteracts the inhibition of glucose-induced insulin secretion caused by lithium both *in vitro* and *in vivo*. The present study was therefore carried out to specify further which type of adrenoceptor is involved in lithium-induced hyperglycaemia and inhibition of insulin secretion.

2 The lithium-induced effects were reversibly blocked by pretreatment of rats with the α_2-adrenoceptor antagonist yohimbine or a combination of yohimbine and the non-selective β-receptor antagonist propranolol, whereas the α_1-receptor antagonist prazosin and propranolol alone were ineffective in blocking these effects.

3 These findings suggest that the effects of lithium on plasma glucose and insulin levels are mediated mainly by the stimulation of α_2-adrenoceptors.

Introduction

Lithium has been shown to inhibit glucose-stimulated insulin release in the rat after either *in vitro* or *in vivo* administration, leading to impairment of glucose tolerance (Anderson & Blackard, 1978; Shah & Pishdad, 1980). These effects of lithium can be counteracted by pretreatment of rats with dihydroergotamine (DHE), a non-selective α-adrenoceptor antagonist (Fontela *et al.*, 1986; 1987).

α-Adrenoceptors are divided into two groups (Langer, 1974; Starke, 1977; Shattil *et al.*, 1981). The stimulation of α_2-adrenoceptors increased plasma glucose levels (Nakadate *et al.*, 1980) and inhibited immunoreactive insulin (IRI) release from pancreatic islets (Smith & Porte, 1976; Nakadate *et al.*, 1980). However, since DHE has a high affinity for both α_1- and α_2-adrenoceptors (Wood *et al.*, 1979), previous studies have failed to define adequately the α-adrenoceptor subtypes involved in lithium action. Selective α-adrenoceptor antagonists have different effects on the changes in plasma glucose and insulin levels induced by stimulation of α-adrenoceptors (Yamazaki *et al.*, 1982). Yohimbine, a selective α_2-adrenoceptor antagonist, clearly prevented the hyperglycaemia and inhibition of IRI release induced by α-adrenoceptor stimulation, whereas prazosin, a selective α_1-adrenoceptor antagonist, was ineffective (Nakadate *et al.*, 1980; Yamazaki *et al.*, 1982). The present experiments, with a range of adrenoceptor antagonists, were therefore carried out to specify further the types of adrenoceptor involved in lithium-induced hyperglycaemia and inhibition of IRI secretion.

Methods

Animals

Male Wistar rats weighing 250–300 g were used for all experiments. Animals were housed four per plastic cage and received a standard laboratory diet and tap water *ad libitum*. Standard vivarium conditions included a room temperature of 22 ± 2°C and artificial illumination for 12 h day^{-1}. Food was withdrawn the night before each experiment, whereas free access to water was maintained.

Islet experiments

Pancreatic islets were isolated from control and drug-pretreated rats by the collagenase digestion technique (Lacy & Kostianovsky, 1967). The islets were perifused and 50 islets were used per chamber (chamber volume 0.95 ml, Millipore; 5 μm millipore filter) as previously described in detail (Lacy *et al.*, 1972). Four chambers were used in parallel, two chambers containing control islets and two containing islets from the drug-pretreated rats. Two perifusion media were employed: a standard perifusion medium was used for the first 30 min period and throughout the experimental time-period in the control chambers, with a normal ionic composition (i.e. Na^+ 139, K^+ 5, Ca^{2+} 2, Mg^{2+} 2, Cl^- 124 and HCO_3^- 24 mEq l^{-1}: pH 7.4); and a perifusion medium for the last 60 min period in the experimental chambers, in which portions of 0.9% NaCl solution were replaced by equal volumes of 0.65% LiCl (iso-osmotic with 0.9% NaCl) to give the desired concentration of lithium in the medium. Both media were supplemented with 5 mg ml^{-1} bovine serum albumin, fraction V (Sigma Chemical Co., Mo. U.S.A.) and with glucose (E. Merck AG, F.R.G.) at 3.2 mmol l^{-1} in the first 30 min of perifusion and 16.7 mmol l^{-1} in the second 60 min. The media, gassed with 95% O_2 and 5% CO_2, were delivered at a flow rate of 0.9–1.0 ml min^{-1}, and were collected in graduated tubes at 1 or 5 min intervals. The volumes collected were recorded, and aliquots were removed for radioimmunoassay of IRI (Amersham, UK); a rat insulin standard was used. The rate of IRI secretion was expressed as microunits of insulin per islet min^{-1}. At least six experiments were performed for each experimental situation.

In vivo *experiments*

Adrenoceptor antagonists, alone or in combination, were injected i.p. as a bolus. Immediately afterwards the rats were anaesthetized with pentobarbitone 20 mg kg^{-1} i.p., and polyethylene cannulae (PE-10) were inserted into both jugular veins, after which the rats were allowed to stabilize for about 30 min. A blood sample (0.5 ml; baseline) was collected through one of the indwelling catheters with a heparinized syringe. Immediately thereafter, a rapid (30 s) infusion of 0.5 M LiCl or control 0.5 M NaCl (4 mEq kg^{-1}) was given into the jugular vein. Blood samples for the measurement of plasma glucose and IRI were collected 5, 30, 60, 90 and 120 min after LiCl or saline treatment. All blood samples were kept in the refrigerator at 4°C until centrifuged at the end of the experiment. The effect of lithium on glucose-induced IRI release and glucose tolerance was studied as described above, except that glucose (0.5 g kg^{-1}) was administered rapidly 5 min after LiCl or NaCl infusion. Plasma glucose was measured immediately by the glucose-oxidase method and the remaining plasma was frozen at −18°C for determination of IRI by radioimmunoassay.

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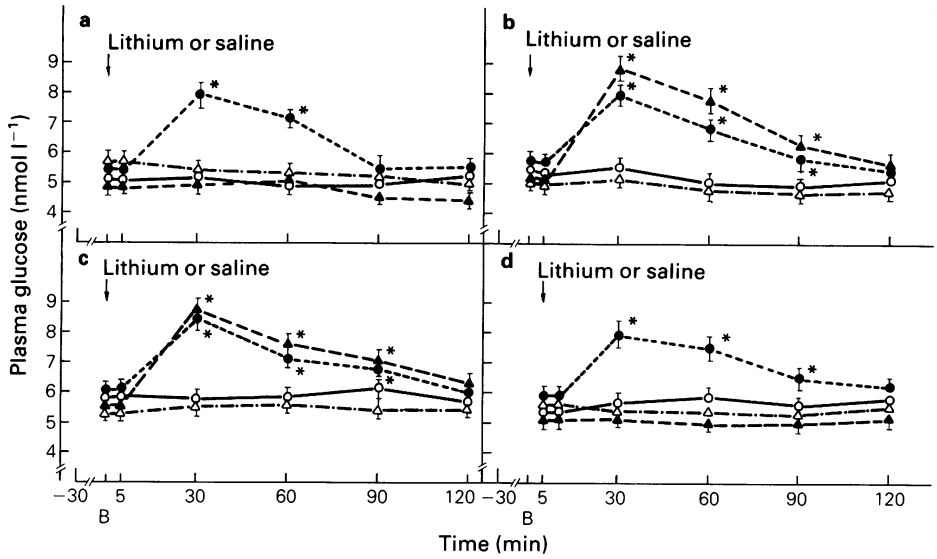

Figure 1 Effects of adrenoceptor antagonists on lithium-induced hyperglycaemia. Drugs were given i.p. 30 min before saline or lithium (4 mEq kg^{-1}) treatment. Effect of (a) yohimbine, (b) prazosin, (c) propranolol and (d) yohimbine + propranolol are shown. (○) Control animals injected with saline, (●) control animals injected with lithium, (△) drug-pretreated animals injected with saline and (▲) drug-pretreated animals injected with lithium. Each result represents the mean of eight rats and vertical lines show s.e.mean. An asterisk indicates that the value is statistically different from the saline control response ($P < 0.05$).

Statistical analysis

The results are expressed as means ± s.e.mean, and differences between means were analysed by Student's *t* test for group or paired differences. The 0.05 level of probability was chosen as the criterion of statistical significance. The rate of plasma glucose reduction, the so-called 'K value', was calculated as percentage fall in plasma glucose during 1 min. The normal 'K value' is around 1.7 and lower values are indicative of glucose intolerance.

Pharmacological agents and dosage

The following drugs were used singly or in combination; yohimbine hydrochloride (Sigma Chemical Co., Mo, U.S.A.) 1 mg kg^{-1}, prazosin hydrochloride (a gift from Pfizer Laboratories, Madrid, Spain) 2 mg kg^{-1} and propranolol hydrochloride (Sigma Chemical Co., Mo., U.S.A.) 5 mg kg^{-1}. They were given i.p. 30 min before LiCl or saline treatment, or killing the animal for the isolation of islets.

Results

In vivo *studies*

Effects of adrenoceptor antagonists on plasma glucose and insulin levels after lithium administration The effects of the adrenoceptor antagonists on hyperglycaemia induced by lithium are shown in Figure 1. Administration of lithium produced a significant elevation of plasma glucose. Yohimbine, a selective α_2-adrenoceptor antagonist, or a combination of yohimbine and propranolol, a non-selective β-adrenoceptor antagonist, significantly inhibited lithium-induced hyperglycaemia (Figure 1a and d). In contrast, prazosin, a selective α_1-adrenoceptor antagonist, and propranolol alone were ineffective in blocking the lithium-induced hyperglycaemia (Figure 1b and c). An analysis of the effect of lithium on plasma IRI values shows that, despite marked hyperglycaemia, plasma insulin levels after LiCl treatment were not significantly different from levels after control treatment (Figure 2). Treatment with yohimbine before lithium, increased the plasma IRI levels significantly

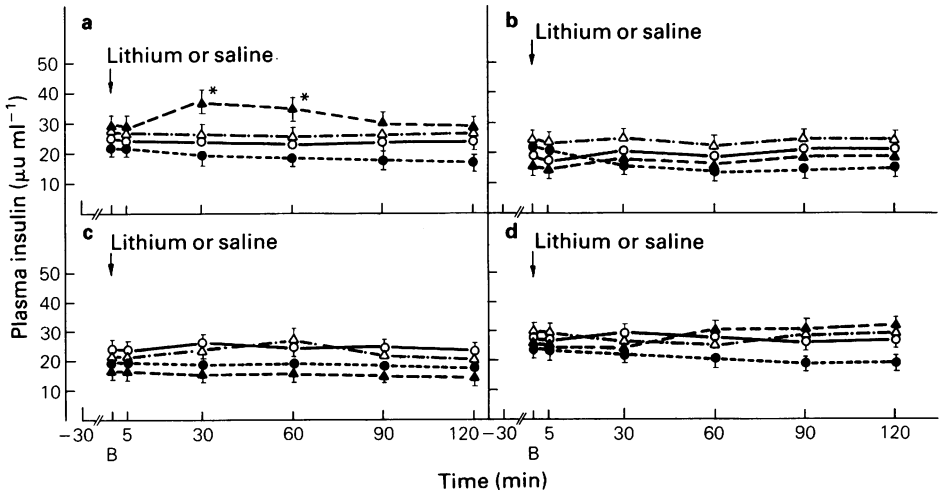

Figure 2 Effects of adrenoceptor antagonists on the effect of lithium on immunoreactive insulin (IRI) levels. Drugs were given i.p. 30 min before saline or lithium (4 mEq kg^{-1}) treatment. Effects of (a) yohimbine, (b) prazosin, (c) propranolol and (d) yohimbine + propranolol are shown. (○) Control animals injected with saline, (●) control animals injected with lithium, (△) drug-pretreated animals injected with saline and (▲) drug-pretreated animals injected with lithium. Each result represents the mean of eight rats and vertical lines show s.e.mean. An asterisk indicates that the value is statistically different from the saline control response ($P < 0.05$).

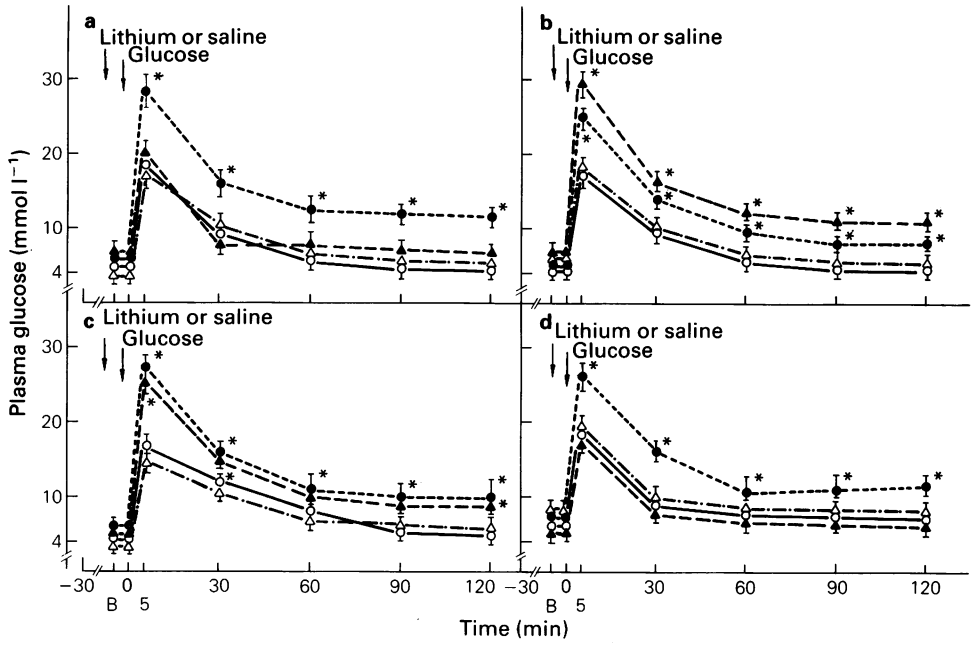

Figure 3 Effects of adrenoceptor antagonists on the effect of lithium on glucose levels after i.v. glucose injection. Drugs were given i.p. 30 min before saline or lithium (4 mEq kg^{-1}) treatment. Glucose (0.5 g kg^{-1}) was administered 5 min after lithium or saline. Effects of (a) yohimbine, (b) prazosin, (c) propranolol and (d) yohimbine + propranolol are shown. (○) Control animals injected with saline, (●) control animals injected with lithium, (△) drug-pretreated animals injected with saline and (▲) drug-pretreated animals injected with lithium. Each result represents the mean of eight rats and vertical lines show s.e.mean. An asterisk indicates that the value is statistically different from the saline control response ($P < 0.05$).

Figure 2a), whereas in prazosin- or propranolol-pretreated animals, injection of lithium did not modify the plasma IRI levels (Figure 2b and c). Pretreatment with a combination of yohimbine and propranolol before lithium caused only a slight increase of the plasma IRI levels (Figure 2d). Adrenoceptor antagonists were without effect on plasma glucose and IRI levels in rats given the control treatment (Figures 1 and 2).

Effects of adrenoceptor antagonists on glucose tolerance and glucose-induced insulin release after lithium administration The effects of adrenoceptor antagonists on glucose tolerance in lithium-treated and control rats is shown in Figure 3. After glucose administration, plasma glucose levels were significantly higher in lithium-treated rats than in control rats. Similarly, the mean 'K value' was 0.7 ± 0.3% after lithium treatment, which was significantly lower than that observed in the controls (1.8 ± 0.2%) (Figure 3). Pretreatment with yohimbine alone or in combination with propranolol inhibited the hyperglycaemia induced by lithium. Lithium-induced glucose intolerance was also blocked by yohimbine with or without

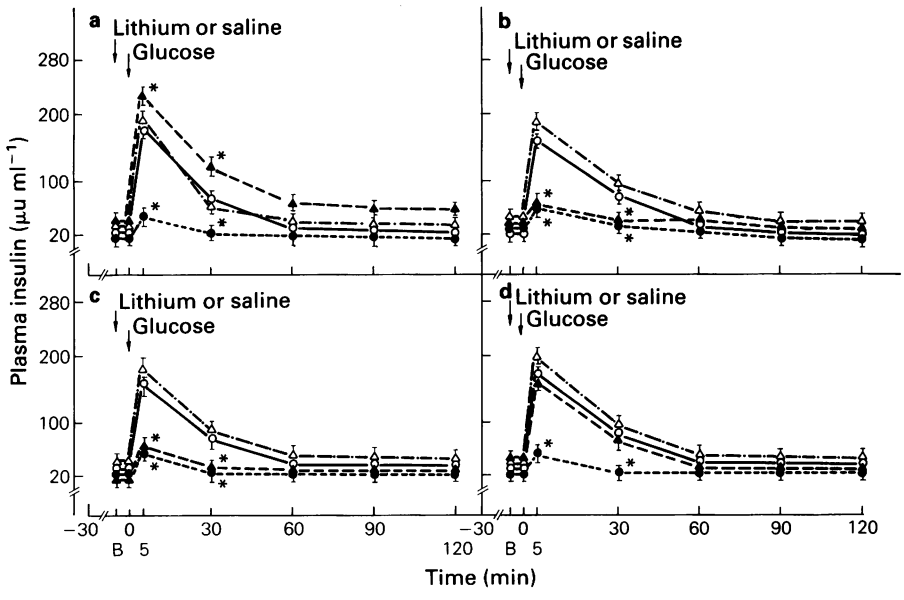

Figure 4. Effects of adrenoceptor antagonists on the effect of lithium on glucose-stimulated IRI release. Drugs were given i.p. 30 min before saline or lithium (4 mEq kg^{-1}) treatment. Glucose (0.5 g kg^{-1}) was administered 5 min after lithium or saline. Effects of (a) yohimbine, (b) prazosin, (c) propranolol and (d) yohimbine + propranolol are shown. (○) Control animals injected with saline, (●) control animals injected with lithium, (△) drug-pretreated animals injected with saline and (▲) drug-pretreated animals injected with lithium. Each result represents the mean of eight rats and vertical lines show s.e.mean. An asterisk indicates that the value is statistically different from the saline control response ($P < 0.05$).

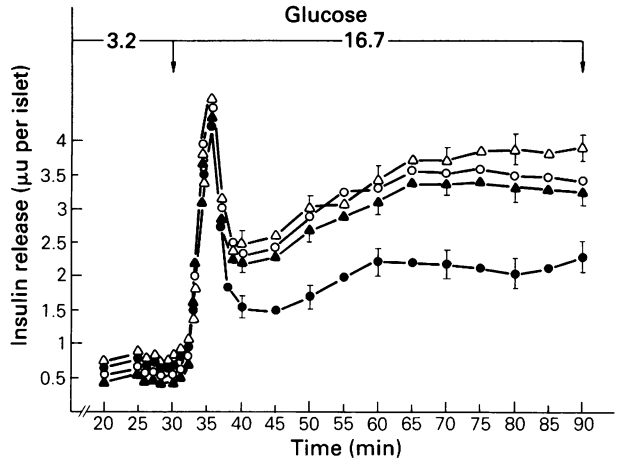

Figure 5 Effect of pretreatment with vehicle or yohimbine (1 mg kg^{-1}) on the inhibitory effect of lithium on glucose-induced IRI release. Following isolation, islets were perifused for 30 min with 3.2 mmol l^{-1} glucose. They were then exposed to 16.7 mmol l^{-1} glucose either in a medium of normal ionic composition (vehicle-pretreated islets (○) and yohimbine-retreated islets (△)) or in a medium containing lithium 5 mmol l^{-1} (vehicle-pretreated (●) and yohimbine-pretreated islets (▲)). Perifusions under different conditions were carried out in parallel chambers. Values are means of results from 6 to 8 separate experiments and vertical lines show s.e.mean.

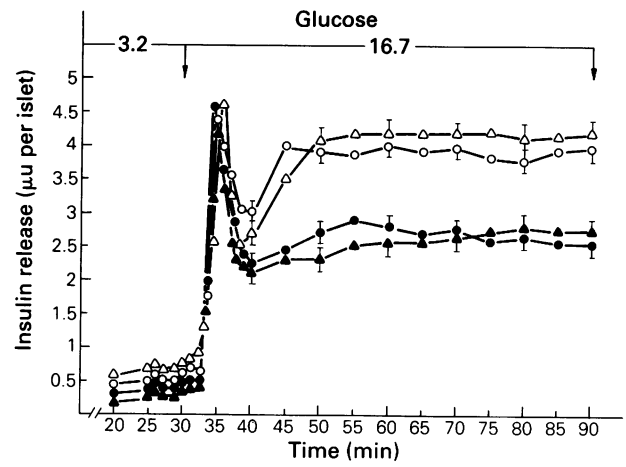

Figure 7 Effect of pretreatment with vehicle or propranolol (5 mg kg^{-1}) on the inhibitory effect of lithium on glucose-induced IRI release. Following isolation, islets were perifused for 30 min with 3.2 mmol l^{-1} glucose. They were then exposed to 16.7 mmol l^{-1} glucose either in a medium of normal ionic composition (vehicle-pretreated islets (○) and propranolol-pretreated islets (△)) or in a medium containing lithium 5 mmol l^{-1} (vehicle-pretreated islets (●) and propranolol-pretreated islets (▲)). Perifusions under different conditions were carried out in parallel chambers. Values are means of results from 6 to 8 separate experiments and vertical lines show s.e.mean.

propranolol; the 'K values' were 1.6 ± 0.2% and 1.9 ± 0.6%, respectively (Figure 3a and d). On the other hand, pretreatment with prazosin enhanced the hyperglycaemia induced by glucose and lithium (Figure 3b). In the propranolol-pretreated rats, plasma glucose levels at each time interval did not differ significantly from the levels in the lithium-treated rats (Figure 3c).

Administration of lithium significantly inhibited glucose-induced IRI release (Figure 4). The effects of different adrenoceptor antagonists on this effect are also shown. Pretreatment with yohimbine alone or in combination with propanolol counteracted lithium-induced inhibition of IRI release (Figure 4a and d). In contrast, prazosin and propanolol alone were ineffective in this respect (Figure 4b and c). Adrenoceptor antagonists were without significant effect on plasma glucose levels and glucose-induced IRI release, unless they were administered together with lithium (Figures 3 and 4).

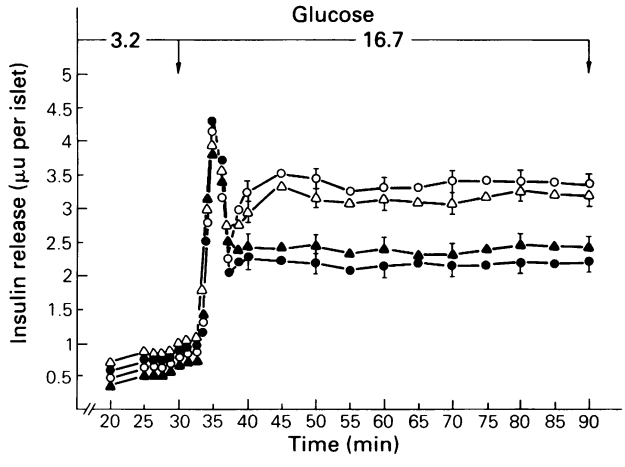

Figure 6 Effect of pretreatment with vehicle or prazosin (2 mg kg^{-1}) on the inhibitory effect of lithium on glucose-induced IRI release. Following isolation, islets were perifused for 30 min with 3.2 mmol l^{-1} glucose. They were then exposed to 16.7 mmol l^{-1} glucose either in a medium of normal ionic composition (vehicle-pretreated islets (○) and prazosin-pretreated islets (△)) or in a medium containing lithium 5 mmol l^{-1} (vehicle-pretreated islets (●) and prazosin-pretreated islets (▲)). Perifusions under different conditions were carried out in parallel chambers. Values are means of results from 6 to 8 separate experiments and vertical lines show s.e.mean.

In vitro *studies*

Effects of adrenoceptor antagonist pretreatment on lithium-induced inhibition of glucose-stimulated IRI release As shown in Figure 5 (control chamber), 16.7 mmol l^{-1} glucose elicited a biphasic release of IRI. IRI release started to rise 5–6 min after the increase in glucose concentration from 3.2 mmol l^{-1} to 16.7 mmol l^{-1}. An initial peak at 7–8 min was followed by a nadir at 10–12 min and a progressively increasing second phase of insulin secretion. Islets in the experimental chamber were perifused during the first 30 min with medium of normal ionic composition. Following this period lithium (5 mmol l^{-1}) was added at the time of glucose stimulation.

The effect of lithium on the biphasic pattern of glucose-induced IRI secretion is illustrated in Figure 5. Lithium produced no significant decrease in the total IRI released in the first phase (first 8 min), whereas the second phase of release was clearly inhibited. Integrated IRI release during the first phase was 16 ± 2 μu per islet for 16.7 mmol l^{-1} glucose alone, and not significantly different from 16.7 mmol l^{-1} glucose + lithium (14 ± 3, $P < 0.10$); second phase release was 154 ± 8 and 93 ± 5 μu per islet, respectively ($P < 0.05$), which corresponds to an inhibition of approximately 40%.

The effect of pretreatment of rats with yohimbine on lithium inhibition of glucose-stimulated IRI release is shown in Figure 5. When islets isolated from yohimbine-pretreated rats were perifused with the lithium medium + 16.7 mmol l^{-1} glucose, the inhibitory effect of lithium on the second phase of glucose-stimulated IRI release was abolished. Integrated IRI release during the second phase was 169 ± 11 μu per islet for the yohimbine-pretreated islets + lithium, and not significantly different from saline-pretreated islets + normal ionic composition medium (154 ± 9, $P < 0.10$). Yohimbine pretreatment had no effect on glucose-stimulated IRI when islets were perifused with medium of normal ionic composition (161 ± 8 μu per islet).

The effect of pretreatment of rats with prazosin on the second phase of glucose-stimulated IRI release is shown in Figure 6. Pretreatment of rats with prazosin did not modify the inhibitory effect of lithium. Integrated IRI release during the second phase was 106 ± 8 uμ per islet for prazosin-pretreated islets + lithium, and not significantly different from saline-pretreated islets + lithium (93 ± 7, $P < 0.10$). Prazosin pretreatment had no effect on glucose-stimulated IRI release when islets were perifused with medium of normal ionic composition (134 ± 10 μu per islet). Finally, as shown in Figure 7, propranolol pretreatment did not significantly modify the inhibition of second phase IRI release induced by lithium (103 ± 6 μu per islet). Also, propranolol pretreatment did not modify the glucose-stimulated IRI release when islets were perifused with medium of normal ionic composition (139 ± 8 μu per islet).

Discussion

In the rat, lithium has been shown to affect sympathoadrenal function at several levels: the evidence ranges from the indirect, for example, a behavioural study showing that both lithium and adrenal catecholamines reduce locomotor activity in rats (Cappeliez & White, 1981), to more direct studies in which various effects of lithium have been reduced or abolished by adrenalectomy or adrenoceptor antagonists.

The effect of lithium relevant to the present study is the induction of marked hyperglycaemia with inappropriately low plasma IRI levels and impaired glucose tolerance (Bhattacharya, 1964; Plenge *et al.*, 1970; Mellerup *et al.*, 1970; Fontela *et al.*, 1986). In addition, lithium impairs IRI secretion *in vitro*, glucose-induced IRI being partially inhibited by lithium (Anderson & Blackard, 1978; Fontela *et al.*, 1987). The participation of the adrenal medulla in the effects of lithium *in vivo* was shown by the finding that adrenalectomy partially counteracted the lithium-induced impairment of glucose tolerance and glucose-induced IRI secretion (Fontela *et al.*, 1986). In this case, however, adrenalectomy would influence other counter-regulatory hormones, which might in turn modify the lithium response. However, it has also been shown that pretreatment of rats with the non-selective α-adrenoceptor antagonist DHE blocked both the *in vivo* effects of lithium on glucose tolerance and IRI secretion and the *in vitro* effect of lithium on the second phase of IRI release from isolated islets (Fontela *et al.*, 1987). Thus the effects of lithium would seem to involve not only circulating catecholamines but also the stimulation of the sympathetic innervation of the islets.

Several different mechanisms that regulate blood glucose are modified by α- and/or β-adrenoceptors (Hornbrook, 1970: Ammon *et al.*, 1973; Ui, 1975). The results of DHE pretreatment suggest that the effects of lithium on glucose regulation are mediated by the stimulation of α-adrenoceptors. Ligand-binding and pharmacological studies on various non-neural cells, such as fat cells and blood platelets, have led to the subdivision of α-adrenoceptors into α_1 and α_2 subtypes (Shattil *et* al., 1981). It is clear from the present data that the inhibitory effects of lithium on glucose-induced IRI release, both *in vivo* and *in vitro*, and on glucose tolerance are blocked by yohimbine, an α_2-adrenoceptor antagonist, whereas the α_1-adrenoceptor antagonist prazosin and the β-adrenoceptor antagonist propranolol are ineffective. This suggests that these effects of lithium are mediated by the stimulation of α_2-adrenoceptors, rather than by α_1-adrenoceptors or β-adrenoceptors.

Morphological studies have shown that in most mammals the islets of Langerhans contain sympathetic and parasympathetic nerve terminals that end blindly near the islet cells (Smith & Porte, 1976). Although there are several reports of effects of lithium on the action of the major neurotransmitters, these have provided conflicting data. The present study confirms that lithium *in vitro* has an inhibitory effect on the second phase of glucose-induced IRI release; this may be due to activation of adrenergic nerve terminals locally within the islets, since the inhibition of IRI release was markedly reduced by α_2-adrenoceptor blockade with yohimbine.

Glucose-induced IRI secretion in the rat is clearly biphasic (Curry *et al.*, 1968; Gold *et al.*, 1982), and it has been suggested that the two phases may be subject to different mechanisms of control (García Hermida & Gómez-Acebo, 1974; Wollheim & Sharp, 1981). We have observed that there is a clear difference in the sensitivity of the two phases of IRI release to lithium. One possible explanation could be that the first phase of glucose-induced IRI release is independent of α-adrenoceptor control, whereas the second phase is subject to inhibition through the stimulation of α_2-adrenoceptors. However, details of the underlying mechanisms remain obscure.

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Evaluation of the bronchodilator properties of Ro 31-6930, a novel potassium channel opener, in the guinea-pig

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1 Ro 31-6930 (0.001–0.3 μM), cromakalim (0.03–3.0 μM), salbutamol (0.001–0.3 μM) and theophylline (0.3–100 μM) evoked dose-related reductions in guinea-pig spontaneous tracheal tone with IC_{50} values of 0.044, 0.20, 0.021 and 21.0 μM respectively. All four agents also relaxed tone supported by betahistine, carbachol, 5-hydroxytryptamine (5-HT), leukotriene D_4 (LTD_4), U46619 and prostaglandin D_2 (PGD_2). The order of potency of tracheal relaxants was always salbutamol > Ro 31-6930 > cromakalim > theophylline.

2 All four agents evoked dose-related reductions in 5-HT- and histamine-induced bronchoconstriction in pithed vagotomised guinea-pigs. The dose of Ro 31-6930 producing 50% inhibition of a 5-HT bronchoconstriction was 11.6 $\mu g\,kg^{-1}$ and the dose producing 50% inhibition of a histamine bronchoconstriction was 4.4 $\mu g\,kg^{-1}$. Salbutamol was approximately 4–5 times more potent than Ro 31-6930 whilst cromakalim was approximately 10 times less potent than Ro 31-6930 as a bronchodilator. Theophylline was markedly less potent than any of the other agents.

3 Ro 31-6930, cromakalim, salbutamol and theophylline each protected conscious guinea-pigs from histamine-induced respiratory distress. Ro 31-6930 and salbutamol were each effective at oral doses of 1.0 and 3.0 $mg\,kg^{-1}$ whilst cromakalim was effective at oral doses of 3.0 and 10.0 $mg\,kg^{-1}$. Theophylline showed activity only at 300 $mg\,kg^{-1}$ p.o.

4 Ro 31-6930 is a novel potassium channel opener which is a potent relaxant of guinea-pig tracheal smooth muscle *in vitro* and a bronchodilator *in vivo*.

Introduction

Ro 31-6930, 2-(6-cyano-2,2-dimethyl-2H-1-benzopyran-4-yl)-pyridine 1-oxide is a novel pyridine N-oxide derivative possessing smooth muscle relaxant properties. The mechanism of action of Ro 31-6930 is believed to be through the opening of potassium channels, thus hyperpolarizing the plasma membrane (Paciorek *et al.*, 1989a). In some smooth muscle membrane hyperpolarization reduces the entry of calcium through dihydropyridine-sensitive voltage-operated channels, e.g. rabbit aorta (Cook *et al.*, 1988), and may also have an action on ion transport systems such as Na^+/Ca^{2+} exchange (Quast & Cook, 1988), resulting in active Ca^{2+} extrusion, or an inhibition of refilling of intracellular Ca^{2+} stores (Bray *et al.*, 1988; Cowlrick *et al.*, 1988). The result of any of these events would be to reduce the availability of Ca^{2+} for contraction of smooth muscle.

It has previously been reported that Ro 31-6930 is a potent relaxant of vascular smooth muscle (Paciorek *et al.*, 1989a). In addition Ro 31-6930 is a potent relaxant of tracheal smooth muscle (Paciorek *et al.*, 1989b) both *in vitro* and *in vivo* thus suggesting it may be of use as a bronchodilator in the treatment of asthma. The aim of this study was to evaluate the bronchodilator properties of Ro 31-6930 in comparison with two standard anti-asthmatic drugs, salbutamol and theophylline, and the potassium channel opener cromakalim, which has been shown to be an effective bronchodilator both in experimental animals (Arch *et al.*, 1988) and man (Williams *et al.*, 1988). A preliminary account of some of these findings has been given previously (Paciorek *et al.*, 1989b).

Methods

In vitro *inhibition of spontaneous and agonist-induced tone*

Female Dunkin-Hartley guinea-pigs (400–500 g) were killed by a blow to the head and exsanguinated. Segments of trachea (≈4 rings) were removed, cut diametrically opposite the trachealis muscle, opened out and placed in organ baths containing Krebs solution of the following composition: (mM) NaCl 118, $NaHCO_3$ 25, D-glucose 10.5, KCl 4.7, $MgSO_4$ 0.49, KH_2PO_4 1.2 and $CaCl_2$ 2.5. The Krebs solution was maintained at 37°C and aerated with 95% O_2/5% CO_2. Tissues were placed under 1 g tension and were connected to Grass FTO3 force displacement transducers for the isometric recording of tone. For the assessment of the inhibition of spontaneous tone, tracheae were left to equilibrate for 90 min before test agents were applied. In the case of agonist-induced tone, tracheae were left for 90 min and were then incubated with indomethacin (3 μM) for 60 min to inhibit prostaglandin-mediated spontaneous tone (Farmer *et al.*, 1974). Indomethacin remained in the bathing solution for the remainder of the experiment. Following the 60 min incubation period tracheae were exposed to ≈EC_{75} concentrations of betahistine (10 μM), carbachol (0.3 μM), 5-hydroxytryptamine (5-HT, 0.6 μM), leukotriene D_4 (LTD_4) (0.02 μM), U46619 (0.1 μM) or prostaglandin D_2 (PGD_2) (2.25 μM). When stable increases in tracheal tone were achieved, inhibitory dose-response curves were constructed to cumulative doses of Ro 31-6930, cromakalim, salbutamol, theophylline or appropriate dilutions of vehicle (< 1.0% ethanol in 0.9% w/v NaCl). Drug-induced relaxation of tone was expressed as a percentage of the maximum relaxation to 1 mM aminophylline given at the end of each experiment.

In vivo *inhibition of agonist-induced bronchoconstriction in anaesthetized guinea-pigs*

Female guinea-pigs (400-500 g) were anaesthetized with urethane (1.75 $g\,kg^{-1}$, i.p.). The right jugular vein was cannulated for the administration of test compounds and the left carotid artery for measurement of blood pressure. The trachea was cannulated and animals were subsequently bilaterally vagotomised and pithed. Guinea-pigs were then artificially ventilated with a Palmer pump set at 10 $ml\,kg^{-1}$ stroke volume and a respiratory rate of 55–60 inflations min^{-1}. Lung resistance was measured by a modified air overflow technique of Konzett & Rössler (1940) where the volume of air not entering the lungs on inspiration was measured by a Gould model 0000 flow transducer connected to a Gould pneumotachograph. Following an initial dose-response curve, ≈ED_{65} doses

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of 5-HT (1.5–8.0 $\mu g kg^{-1}$ i.v., as base) or histamine (1.0–4.0 $\mu g kg^{-1}$ i.v., as base) were administered every 5 min until reproducible bronchospasm was established. Ro 31-6930, cromakalim, salbutamol, theophylline or appropriate dilutions of vehicle (< 1.0% ethanol in 0.9% w/v NaCl), were then administered i.v. in a cumulative fashion 10 min before each agonist challenge. Inhibition of the bronchoconstriction was expressed as a percentage of the pretreatment air overflow.

In vivo *histamine-induced bronchoconstriction in conscious guinea-pigs*

Female guinea-pigs (400–500 g) were placed in a clear perspex container of 2.5 l capacity and challenged with a histamine aerosol generated from a 0.5 $mg ml^{-1}$ solution of histamine diphosphate using a Mistogen E 145 electronic nebulizer. The duration of the period of exposure to the aerosol resulting in respiratory distress, the 'preconvulsive time' (PCT) (Herxheimer, 1952) was recorded. The first sign of respiratory distress was taken as deep abdominal respiration. Guinea-pigs were removed from the perspex container at this time and were fully recovered within 5 min. Animals were then dosed orally with Ro 31-6930, cromakalim, salbutamol, theophylline or vehicle (< 1.0% ethanol in 0.5% carboxymethyl cellulose dissolved in 0.9% w/v NaCl) and the PCT reassessed at 30 min intervals for up to 240 min.

Drugs

Betahistine dihydrochloride (Aldrich), carbachol (Koch-Light), aminophylline, 5-HT hydrochloride, histamine diphosphate, indomethacin, leukotriene D_4, prostaglandin D_2, salbutamol hemisulphate, theophylline, 11,9 epoxymethano-PGH_2 (U46619, Sigma). Ro 31-6930 and cromakalim were synthesized at Roche Products. For *in vitro* studies and for i.v. administration Ro 31-6930 and cromakalim were dissolved in absolute ethanol and diluted to the required concentrations in 0.9% w/v NaCl. Indomethacin was dissolved in absolute ethanol. All other substances were dissolved in 0.9% w/v NaCl. For oral administration Ro 31-6930 and cromakalim were dissolved in absolute ethanol and diluted to the required concentration in 0.5% carboxymethyl cellulose dissolved in 0.9% w/v NaCl. Salbutamol and theophylline were suspended in 0.5% carboxymethyl cellulose dissolved in 0.9% w/v NaCl.

Statistical analysis

Results are expressed as mean ± s.e.mean. Statistical evaluation of changes in PCT were performed by a split plot analysis of variance and significance accepted where $P < 0.05$.

Results

In vitro *inhibition of spontaneous and agonist-induced tone*

Figure 1a shows an original trace of the effects of Ro 31-6930 on spontaneous tracheal tone and also the maximal relaxant effects to 1 mM aminophylline. Ro 31-6930 (0.001–0.3 μM), cromakalim (0.03–3.0 μM), salbutamol (0.001–0.3 μM) and theophylline (0.3–100 μM) evoked dose-related reductions in spontaneous tracheal tone with IC_{50} values of 0.044 ± 0.019, 0.2 ± 0.006, 0.021 ± 0.001 and 21.0 ± 2.0 μM ($n = 4$) respectively (Figure 1). In each case the relaxation obtained was between 85–95% of the maximum relaxation obtained with 1 mM aminophylline. This dose of aminophylline produced a complete relaxation of spontaneous and agonist-induced tracheal tone, irrespective of the agonist employed. Over a similar concentration range all four agents evoked dose-related reductions in betahistine, 5-HT, LTD_4, U46619 and PGD_2-induced tone. The IC_{50} values obtained are presented in Table 1. The degree of relaxation was between 85–100% of the maximum relaxation obtained with 1 mM aminophylline. The concentration range over which agents relaxed carbachol-induced tone was approximately 10 fold higher than that needed for the other agonists (Table 1). On carbachol-induced tone, salbutamol and theophylline evoked a relaxation of between 85–100% of the maximum obtained with aminophylline whilst the potassium channel openers only managed between 60–70% of the aminophylline maximum. In time-matched control tissues administration of vehicle evoked < 20% reduction in tone.

Irrespective of the agonist employed, salbutamol was consistently the most potent bronchodilator, closely followed by Ro 31-6930. Cromakalim was between 5–28 times less potent than Ro 31-6930, whilst theophylline was consistently the least potent agent tested.

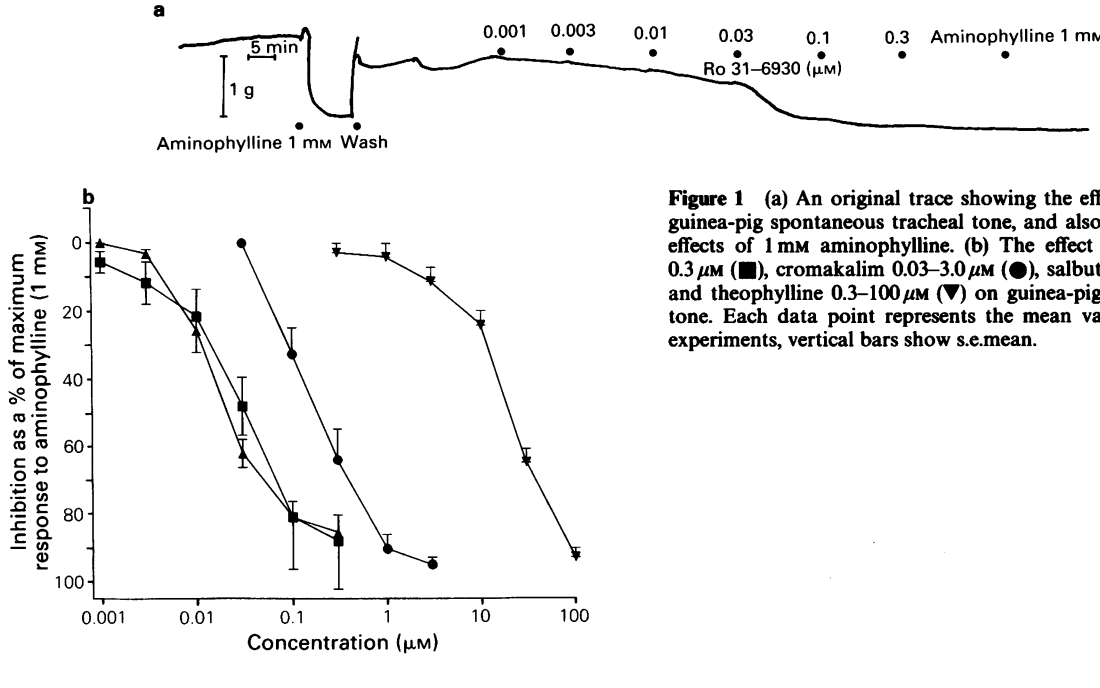

Figure 1 (a) An original trace showing the effects of Ro 31-6930 on guinea-pig spontaneous tracheal tone, and also the maximal relaxant effects of 1 mM aminophylline. (b) The effect of Ro 31-6930 0.001–0.3 μM (■), cromakalim 0.03–3.0 μM (●), salbutamol 0.001–0.3 μM (▲) and theophylline 0.3–100 μM (▼) on guinea-pig spontaneous tracheal tone. Each data point represents the mean value derived from four experiments, vertical bars show s.e.mean.

Table 1 The effect of Ro 31-6930, cromakalim, salbutamol and theophylline on agonist-induced guinea-pig tracheal tone

	Tracheal relaxant (μM)			
Tone	*Ro 31-6930*	*Cromakalim*	*Salbutamol*	*Theophylline*
Betahistine (10 μM)	0.053 ± 0.013	0.35 ± 0.12	0.006 ± 0.001	7.6 ± 2.4
Carbachol (0.3 μM)	0.63 ± 0.32	17.0 ± 3.0	0.12 ± 0.02	53.0 ± 8.0
5-HT (0.6 μM)	0.051 ± 0.009	0.13 ± 0.02	0.011 ± 0.008	9.0 ± 2.0
LTD_4 (0.02 μM)	0.043 ± 0.009	1.20 ± 0.34	0.012 ± 0.004	7.2 ± 3.0
U46619 (0.1 μM)	0.091 ± 0.023	0.61 ± 0.13	0.016 ± 0.001	53.5 ± 7.8
PGD_2 (2.25 μM)	0.11 ± 0.04	1.30 ± 0.47	0.008 ± 0.002	44.0 ± 4.0

IC_{50} values presented are mean ± s.e.mean, $n = 4$.
In the case of carbachol-induced tone, the maximal relaxation obtained with Ro 31-6930 and cromakalim was only 60–70% of the maximum relaxation to 1 mM aminophylline. In all other cases the relaxation obtained was between 85–100% of that obtained with aminophylline.

In vivo *inhibition of agonist-induced bronchoconstriction in anaesthetized guinea-pigs*

Ro 31-6930 (1.0–100.0 $\mu g\,kg^{-1}$, i.v.), cromakalim (10.0–1000 $\mu g\,kg^{-1}$, i.v.), salbutamol (1.0–10.0 $\mu g\,kg^{-1}$, i.v.) and theophylline (1000–30000 $\mu g\,kg^{-1}$, i.v.) evoked dose-related reductions in the bronchoconstriction evoked by 5-HT (Figure 2) with ID_{50} values of 11.6 ± 2.6, 123 ± 28.0, 4.3 ± 1.1 and 15600 ± 2300 $\mu g\,kg^{-1}$ ($n = 4$) respectively. Salbutamol was the most potent bronchodilator with Ro 31-6930 being some 4 times less potent. Cromakalim was approximately 10 times less potent than Ro 31-6930 whilst theophylline was more than 1000 times less potent than Ro 31-6930. Ro 31-6930 (1.0–30.0 $\mu g\,kg^{-1}$, i.v.), cromakalim (10.0–300 $\mu g\,kg^{-1}$, i.v.), salbutamol (0.1–3.0 $\mu g\,kg^{-1}$, i.v.) and theophylline (300–30000 $\mu g\,kg^{-1}$, i.v.) evoked dose-related reductions in the bronchoconstriction evoked by histamine (Figure 3) with ID_{50} values of 4.4 ± 0.98, 56.1 ± 15.7, 0.80 ± 0.38 and 8750 ± 1160 $\mu g\,kg^{-1}$ ($n = 4$) respectively. The rank order of potency was similar to that observed for 5-HT-induced bronchoconstriction. Vehicle-treated animals exhibited <20% reduction in air overflow, throughout the time course of the experiment (data not shown).

The mean pre-dose diastolic blood pressure of pithed, vagotomised guinea-pigs was 13.8 ± 0.8 mmHg ($n = 5$). Drug treatment had little or no effect on this parameter.

In vivo *histamine-induced bronchoconstriction in conscious guinea-pigs*

The mean pre-dose preconvulsive time (PCT) was 68.2 ± 3.2 s ($n = 54$). Ro 31-6930 1.0 and 3.0 $mg\,kg^{-1}$ p.o. evoked maximal dose-related increases in the PCT of 68.2 ± 14.8 s and 92.0 ± 21.3 s ($n = 6$) respectively (Figure 4). A significant increase in PCT was observed for up to 90 min following the highest dose employed. Cromakalim 3.0 and 10.0 $mg\,kg^{-1}$ p.o. evoked dose-related increases in the PCT with peak increases of 38.5 ± 13.8 s and 74.7 ± 20.7 s ($n = 6$) respectively (Figure 5). Significant increases in the PCT were observed for 120 min following drug administration. Following the highest doses of both potassium channel openers, guinea-pigs appeared subdued. This may have been due to the vasodilator properties of these agents. Salbutamol 1.0 and 3.0 $mg\,kg^{-1}$ p.o. evoked dose-related increases in the PCT with peak increases of 72.7 ± 32.7 s and 155.2 ± 11.4 s ($n = 6$) respec-

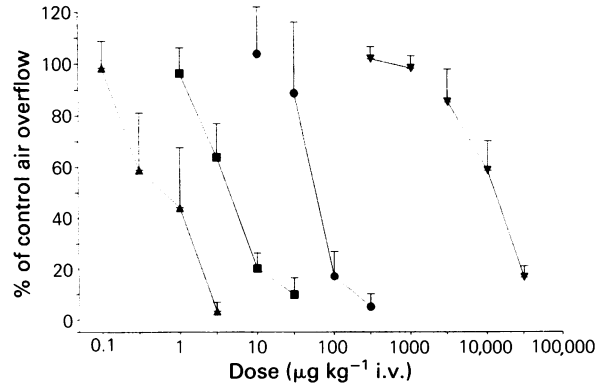

Figure 3 The effect of Ro 31-6930 1.0–30.0 $\mu g\,kg^{-1}$, i.v. (■), cromakalim 10.0–300 $\mu g\,kg^{-1}$, i.v. (●), salbutamol 0.1–3.0 $\mu g\,kg^{-1}$, i.v. (▲) and theophylline 300–30000 $\mu g\,kg^{-1}$, i.v. (▼) on intravenous histamine-induced bronchoconstriction in pithed vagotomised guinea-pigs. Each data point represents the mean value derived from 4 experiments, vertical bars show s.e.mean.

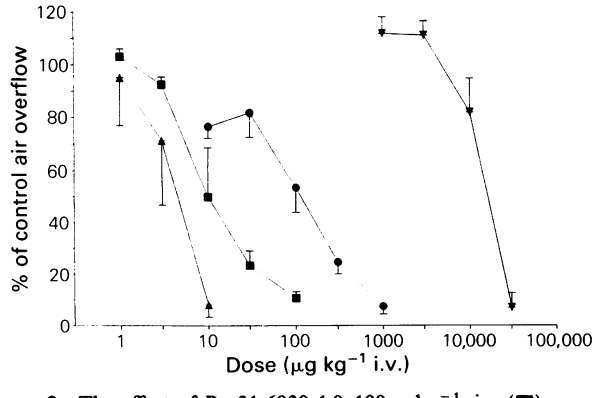

Figure 2 The effect of Ro 31-6930 1.0–100 $\mu g\,kg^{-1}$, i.v. (■), cromakalim 10.0–1000 $\mu g\,kg^{-1}$, i.v. (●), salbutamol 1.0–10.0 $\mu g\,kg^{-1}$, i.v. (▲) and theophylline 1000–30000 $\mu g\,kg^{-1}$, i.v. (▼) on intravenous 5-hydroxytryptamine-induced bronchoconstriction in pithed vagotomised guinea-pigs. Each data point represents the mean value derived from 4 experiments, vertical bars show s.e.mean.

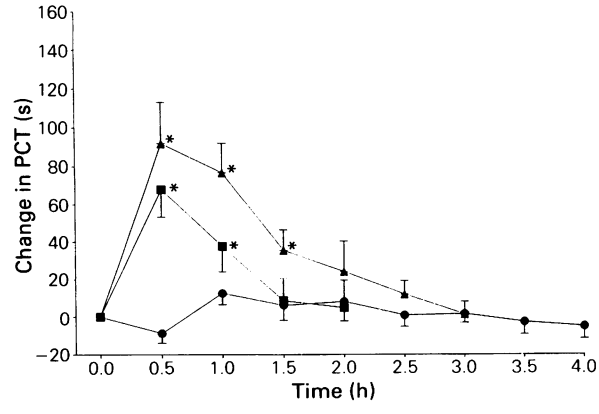

Figure 4 The effect of vehicle 10.0 $ml\,kg^{-1}$, p.o. (●), Ro 31-6930 1.0 $mg\,kg^{-1}$, p.o. (■) and 3.0 $mg\,kg^{-1}$, p.o. (▲) on preconvulsive time (PCT) in the conscious guinea-pig. Each data point represents the mean value derived from 6 experiments, vertical bars represent s.e.mean. *$P < 0.05$ split plot ANOVA.

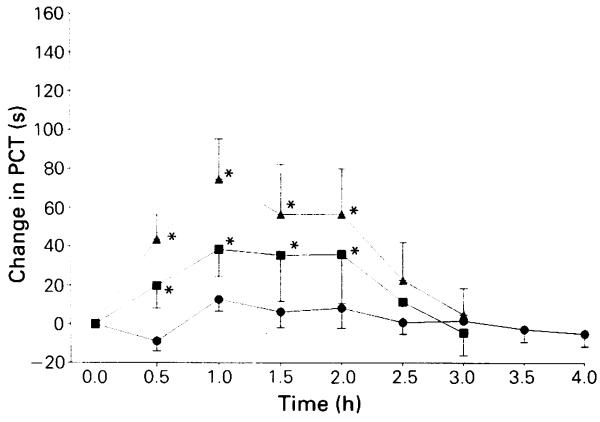

Figure 5 The effect of vehicle 10.0 ml kg^{-1}, p.o. (●), cromakalim 3.0 mg kg^{-1}, p.o. (■) and 10.0 mg kg^{-1}, p.o. (▲) on preconvulsive time (PCT) in the conscious guinea-pig. Each data point represents the mean value derived from 6 experiments, vertical bars show s.e.mean. *$P < 0.05$ split plot ANOVA.

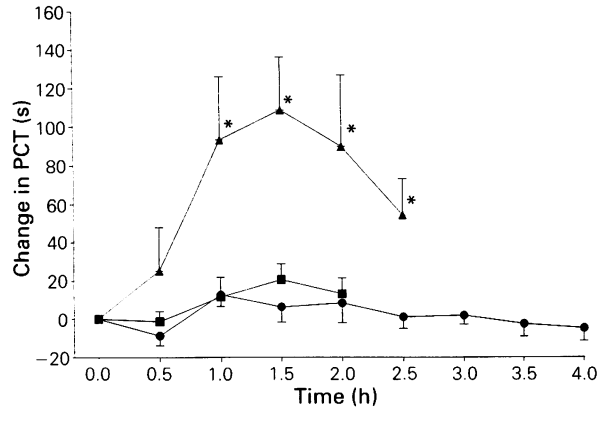

Figure 7 The effect of vehicle 10.0 ml kg^{-1}, p.o. (●), theophylline 100 mg kg^{-1}, p.o. (■) and 300 mg kg^{-1}, p.o. (▲) on preconvulsive time (PCT) in the conscious guinea-pig. Each data point represents the mean value derived from 6 experiments, vertical bars show s.e.mean. *$P < 0.05$ split plot ANOVA.

tively (Figure 6). The duration of action was 150 min following the highest dose administered. Theophylline prolonged the PCT only following a dose of 300 mg kg^{-1} p.o. with a peak increase of 108.7 ± 27.6 s ($n = 6$). Significant protection was observed for approximately 150 min following drug administration (Figure 7). All theophylline experiments were terminated early due to the side effects observed, which were consistent with excessive CNS stimulation. Vehicle-treated animals exhibited no change in the PCT over a 4 h observation period.

Discussion

A number of studies have shown potassium channel openers to be effective in reducing agonist-induced contractions in guinea-pig tracheal preparations (Allen *et al.*, 1986; Arch *et al.*, 1988; Nielsen-Kudsk *et al.*, 1988; Paciorek *et al.*, 1989b). In this study Ro 31-6930 and cromakalim, like the currently employed bronchodilators salbutamol and theophylline, were effective in relaxing tracheal tone supported by a variety of agonists. Since a number of spasmogens are implicated in asthma, these data suggest that potassium channel openers may be of particular value as bronchodilators.

Both Ro 31-6930 and salbutamol were potent relaxants of spontaneous tracheal tone with cromakalim being some 10 times less potent than salbutamol. Theophylline was consistently the least potent agent tested. As spontaneous tracheal tone is believed to be maintained by an interaction of endogenous cyclo-oxygenase products (Coleman & Kennedy, 1980), experiments on the effects of tracheal relaxants on individual agonist-induced tone were performed in the presence of indomethacin.

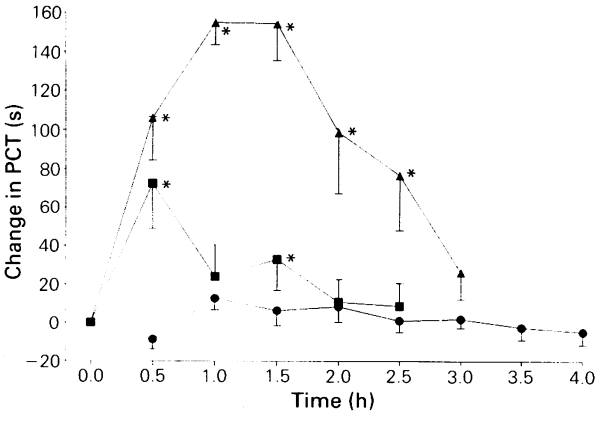

Figure 6 The effect of vehicle 10.0 ml kg^{-1}, p.o. (●), salbutamol 1.0 mg kg^{-1}, p.o. (■) and 3.0 mg kg^{-1}, p.o. (▲) on preconvulsive time (PCT) in the conscious guinea-pig. Each data point represents the mean value derived from 6 experiments, vertical bars show s.e.mean. *$P < 0.05$ split plot ANOVA.

Cholinergic mechanisms are of great importance in mammalian airways, mediating airway tone and bronchial responsiveness (Olsen *et al.*, 1965; Nadel, 1980). It was observed that both Ro 31-6930 and cromakalim were less potent relaxants of carbachol-induced tone than of any other agonists tested. This potency difference could not be accounted for by experimental errors in the estimation of agonist EC_{75} values although these errors may have contributed to the small variations in IC_{50} values seen for the potassium channel openers with the other agonists. It is believed that a major source of Ca^{2+} for muscarinic receptor-mediated tracheal smooth muscle contraction is intracellular stores (Creese & Denborough 1981). As a result, potassium channel openers, which are believed to impede the movement of Ca^{2+} into the cell are likely to have reduced efficacy against carbachol-induced contractions. The lack of effect of cromakalim against muscarinic responses in the guinea-pig has been previously documented (Allen *et al.*, 1986; Taylor *et al.*, 1988). However, this is not the case in man where cromakalim relaxes carbachol-induced tension in human bronchioles (Taylor *et al.*, 1988). Theophylline did not show such marked differences in potency against carbachol-induced tone. This is perhaps not surprising as its mechanism of action is believed to involve directly intracellular secondary messengers.

The potassium channel openers were potent relaxants of betahistine (Brown *et al.*, 1986) -induced tone. Betahistine is a histamine H_1 receptor agonist which evokes a more sustained contraction of the guinea-pig trachea than histamine. Responses to histamine are more dependent on entry of extracellular calcium into smooth muscle than responses to carbachol (Kirkpatrick *et al.*, 1975; Creese & Denborough, 1981), hence the greater potency of potassium channel openers against this spasmogen.

The potassium channel openers possessed the same order of potency as seen with betahistine-induced tone when tracheal tone was maintained by 5-HT, LTD_4, U46619, a thromboxane A_2 (TXA_2)-mimetic (Coleman *et al.*, 1981) and PGD_2. Although 5-HT induces bronchoconstriction in experimental animals, it is not thought to be important in human asthma (Barnes *et al.*, 1988). However, leukotrienes, thromboxanes and prostaglandins are important in asthma (Coleman & Kennedy, 1980). TxA_2 is a potent bronchoconstrictor *in vitro* and may be involved in the potentiation of cholinergic reflexes (Chung *et al.*, 1985), and has been implicated in the bronchoconstriction and airway hyperreactivity induced by PAF (Joseph *et al.*, 1983). LTD_4 is a potent constrictor of human

airways (Weiss *et al.*, 1983) and is a potent stimulant of mucus secretion (Coles *et al.*, 1983). PGD_2 contracts human airway smooth muscle *in vitro* (Gardiner & Collier, 1980) and is a pro-inflammatory agent mediating vasodilatation and histamine release from basophils (Peters *et al.*, 1984).

As observed with histamine, the tracheal contractions produced by the prostanoids are dependent to a large extent upon the influx of extracellular Ca^{2+} (Creese & Denborough, 1981), and hence a greater efficacy against these responses than those obtained with carbachol would be expected. However there is substantial evidence that the mechanism of action of potassium channel openers is not simply a result of the prevention of Ca^{2+} entry via voltage-operated calcium channels, as Arch *et al.* (1988) could show only a partial inhibition of spontaneous tracheal tone with nifedipine whereas cromakalim was highly effective. We have confirmed these findings with nitrendipine (unpublished observations). Additional theories for the mechanism(s) of action of potassium channel openers have been speculated on by Arch *et al.* (1988) and others (see introduction).

In vivo Ro 31-6930 and cromakalim inhibited both 5-HT- and histamine-induced bronchoconstriction in the pithed vagotomised guinea-pig. The results obtained for cromakalim are in broad agreement with those reported by Arch *et al.* (1988), who in addition demonstrated nifedipine to be inactive in this *in vivo* model of bronchoconstriction. The standard anti-asthma drugs, salbutamol and theophylline were also effective in this model. Salbutamol was some 3–5 times more potent than Ro 31-6930 whilst theophylline was markedly less potent.

Ro 31-6930 also protected conscious guinea-pigs from a histamine-induced bronchospasm, further demonstrating its efficacy as a bronchodilator. As before, the data obtained for cromakalim in this experimental model were in good agreement with those of Arch *et al.* (1988) who in addition demonstrated cromakalim to be effective against an antigen-induced dyspnoea in the conscious guinea-pig. These data coupled with *in vitro* data from this study demonstrate potassium channel openers to be effective antagonists of a wide variety of mediators involved in asthma.

Preclinical studies have shown cromakalim to be effective in reversing histamine-induced bronchoconstriction in healthy volunteers (Baird *et al.*, 1988), and in the management of morning dipping in asthmatic patients (Williams *et al.*, 1988). In addition cromakalim has been demonstrated to show little hypotensive activity at bronchodilator doses in normotensive volunteers (Fox *et al.*, 1987). It is thus envisaged that hypotension would not be a problem of potassium channel openers in the treatment of asthma. They may have advantages over β-adrenoceptor agonist bronchodilators where β_2-mediated side effects such as tremor limit the usefulness of oral formulations.

In summary, the novel potassium channel opener Ro 31-6930 is a potent relaxant of spontaneous and agonist-induced tracheal tone *in vitro*. Ro 31-6930 is also effective in two *in vivo* models of bronchoconstriction and compares favourably with cromakalim whôse efficacy as a bronchodilator has been established in preliminary clinical trials. These data would indicate that Ro 31-6930 has potential as a bronchodilator in the treatment of asthma.

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FPL 63012AR: a potent D_1-receptor agonist

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1 FPL 63012AR is a D_1-receptor agonist in the dog kidney, 10 times as potent as dopamine, reducing renal vascular resistance by 20% with an intra-arterial dose of 0.42 nmol kg^{-1}.

2 No prejunctional inhibitory D_2-receptor agonist activity was detected in either the isolated ear artery of the rabbit or in the conscious dog as D_2-receptor-mediated emesis.

3 Unlike dopamine, FPL 63012AR had no significant agonist activity at α_1-, α_2-, β_1- or β_2-adrenoceptors.

4 FPL 63012AR is a potent inhibitor of [^{3}H]-noradrenaline uptake (Uptake$_1$) into brain synaptosomes, with an IC_{50} of 29.5 nM, i.e. 9.2 times more potent than dopamine.

5 The ability to block Uptake$_1$ in the anaesthetised dog was confirmed by inhibition of the tyramine-induced pressor and inotropic responses.

6 Intravenous infusion of FPL 63012AR in anaesthetized and conscious dogs (0.3 to 3 nmol kg^{-1} min^{-1}) reduced vascular resistance and increased blood flow to the kidney which was accompanied by hypotension and tachycardia.

7 It is concluded that FPL 63012AR is an example of a novel class of potent agonists at the D_1-receptor. Such compounds may have the potential for use clinically in improving renal perfusion and reducing afterload.

Introduction

Peripheral dopamine receptors have been subclassified into prejunctional D_2- and postjunctional D_1-receptors (previously DA_2 and DA_1 respectively). Stimulation of the latter leads to clinically useful actions, namely increased renal blood flow, natriuresis and diuresis, and elevation of blood flow to other vascular beds containing D_1-receptors (Goldberg & Kohli, 1983; Lokhandwala, 1987). However, dopamine lacks selectivity, stimulating both D-receptor subtypes as well as α- and β-adrenoceptors and this limits the clinical usefulness of dopamine (Goldberg *et al.*, 1977; Makabali *et al.*, 1982). During our investigations aimed at improving the receptor profile of dopamine we have discovered and developed a D_1-receptor agonist (dopexamine hydrochloride) which lacks agonist activity at both α- and β_1-adrenoceptors but is a more potent β_2-adrenoceptor agonist than dopamine (Brown *et al.*, 1985a). Subsequently a group of compounds was discovered in a series of chemical analogues related to dopexamine in which potent D_1-receptor agonism resided but with no agonist activity at the D_2-receptor or at the above receptors. In this study the activities of one of these compounds, FPL 63012AR (Figure 1) is described.

Methods

The biological properties of FPL 63012AR were examined in several preparations to characterize its pharmacological and cardiovascular properties. Since most of these methods have been fully described before, only brief descriptions are given here.

D_1-receptor

D_1-receptor stimulation was studied as renal vasodilatation in pentobarbitone-anaesthetized beagle dogs (10.0–15.2 kg) of either sex (Brown *et al.*, 1985a). Left renal blood flow (RBF, electromagnetic flow probe) and arterial blood pressure (BP), were used to derive electronically renal vascular resistance (RVR). A 23 G needle inserted into the renal artery allowed injection of drugs into the renal vascular bed and selective antagonists were given (i.v.) to block the α-(phenoxybenzamine, 10 mg kg^{-1}) and β-(propranolol, 0.5 mg kg^{-1} and 0.25 mg kg^{-1} h^{-1}) adrenoceptors, and D_2-receptors (domperidone, 0.1 mg kg^{-1}). Dopamine, the drug of comparison, and FPL 63012AR were administered by bolus administration into the renal artery before and after SCH 23390 (10 μg kg^{-1} with 5 μg kg^{-1} h^{-1}, i.v.) to block D_1-receptors.

D_2-receptor

Stimulation of prejunctional D_2-receptors reduces neurogenic vasoconstriction in the rabbit isolated ear artery, caused by field-stimulation (3 Hz, 10 s duration, every 2.5 min), by inhibiting noradrenaline release. FPL 63012AR was compared with the selective D_2-receptor agonist 6,7-ADTN (Brown *et al.*, 1985a). In some experiments, the stimulator was switched-off in the presence of the highest inhibitory concentrations of FPL 63012AR and 6,7-ADTN studied, to examine the effect of either drug on the vasoconstriction produced by a sub-maximal concentration of exogenous noradrenaline (60 or 100 nM). The inhibitory effect of both drugs on the neurogenic vasoconstriction was re-examined 20 min after addition of the selective D_2-receptor antagonist metoclopramide (2.5 μM).

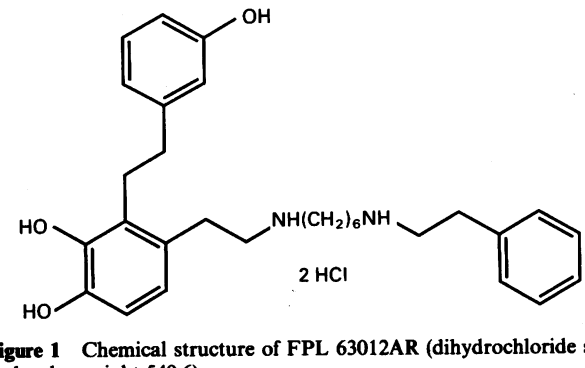

Figure 1 Chemical structure of FPL 63012AR (dihydrochloride salt, molecular weight 549.6).

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α-Adrenoceptors

Agonist activity at postjunctional α_1- and α_2-adrenoceptors was studied as contracture of the canine isolated saphenous vein, prepared as previously described (Brown *et al.*, 1985a) with the exception that they were cut into 3 to 5 mm long tubes. Cumulative doses of FPL 63012AR were examined after recovery from noradrenaline.

β_1-Adrenoceptor

Spontaneously beating guinea-pig atria Increases in the rate of contraction of guinea-pig isolated right atria evoked by FPL 63012AR were expressed as a percentage of the response produced by a maximally effective concentration (0.3 μM) of isoprenaline (Brown *et al.*, 1985a). Blockade of the response by propranolol (1 μM) was used to confirm activity at the β_1-adrenoceptor.

Paced guinea-pig papillary muscle The ability of FPL 63012AR to increase the isometric developed tension was examined in paced isolated left papillary muscles of guinea-pig (1 Hz, 0.5 ms, supramaximal voltage) bathed in Krebs-Henseleit at 37°C (95% O_2 : 5%CO_2) under 1 g resting tension.

β_2-Adrenoceptor

Activity at this receptor was examined as relaxation of spontaneously contracted guinea-pig isolated tracheal chains (Brown *et al.*, 1985a). Cumulatively added FPL 63012AR was examined following characterization with salbutamol.

$Uptake_1$ block

Rabbit brain synaptosomes The ability to block the Na^+-dependent uptake of [^{3}H]-noradrenaline was examined *in vitro* in rabbit cerebral cortex synaptosomes prepared either as crude or as purified fractions (Mitchell *et al.*, 1987).

Tyramine inhibition in the dog Confirmation of $Uptake_1$ blockade in 4 anaesthetized dogs was determined as inhibition of the pressor and positive inotropic actions of bolus tyramine injection (60 $\mu g\,kg^{-1}$, i.v.) during i.v. infusion of FPL 63012AR (10^{-9} mol $kg^{-1}\,min^{-1}$) as previously described (Smith & Naya, 1987).

Cardiovascular actions

Anaesthetized dog Four pentobarbitone-anaesthetized male Beagle dogs (10–15 kg) were surgically prepared as described by Brown *et al* (1985b). Left ventricular contractility ($dP/dt.P^{-1}$) was calculated by use of a pressure-sensitive catheter (Gaeltec 5F) placed in the left ventricle. BP, HR, RBF and RVR were measured as described above. FPL 63012AR was administered as discontinuous 30 min i.v. infusions of 3×10^{-10}, 10^{-9} and 3×10^{-9} mol $kg^{-1}\,min^{-1}$ (0.17, 0.55 and 1.65 $\mu g\,kg^{-1}\,min^{-1}$) with 30 min between infusions to allow recovery. A 10 min infusion of the highest rate was repeated 15 min after administration of the selective D_1-receptor antagonist SCH 23390 (10 $\mu g\,kg^{-1}$ and 30 $\mu g\,kg^{-1}\,h^{-1}$). Lead II ECG was also monitored.

Conscious dog Under aseptic conditions, 5 male beagle dogs (11.6–13.7 kg) were catheterized with Tygon tubing placed in a femoral artery (BP) and in a femoral vein (drug administration). The catheters were exteriorized between the scapulae by means of titanium skin buttons with teflon valves (Omnifit) as previously described (O'Connor *et al.*, 1982). In 3 of these dogs, left renal artery Doppler flow probes (Crystal Biotech, USA) were implanted through a flank retroperitoneal incision to measure RBF. The dogs were allowed a minimum of 2 weeks recovery prior to dosing studies. FPL 63012AR was given as 10 min stepwise infusions of 3×10^{-10} to 10^{-7} mol $kg^{-1}\,min^{-1}$.

Drugs

FPL 63012AR prepared as the dihydrocholoride salt and 6,7-ADTN (2-amino-6,7-dihydroxy 1,2,3,4-tetrahydro- naphthalene hydrobromide) were made in the Department of Medicinal Chemistry of Fisons plc and dissolved in ascorbic isotonic saline (0.02% w/v). SCH 23390 ((R)-(+)-8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-benazazepine-7-ol) (Schering Plough), domperidone (Janssen), cocaine hydrocholoride (May & Baker), (−)-propranolol hydrochloride (ICI) and phenoxybenzamine (SK & F) were gifts. Other drugs obtained from commercial sources were:- sodium pentobarbitone (May & Baker), metoclopramide hydrochloride (Beecham), dopamine hydrochloride (Sigma), noradrenaline hydrogen tartrate (Winthrop), (−)-isoprenaline hydrochloride (Pharmax), phentolamine mesylate (Ciba-Geigy) and (−)-salbutamol sulphate (Allen & Hanbury).

Statistical analysis

Results are expressed either in terms of mean ± s.e. mean or of geometric mean with 95% confidence limits (95% CL). Student's paired *t* test was used to assess statistical significance ($P < 0.05$).

Results

D_1-receptor

The basal RVR and RBF values in the 13 dogs were 1.57 ± 0.12 mmHg min ml^{-1} and 71 ± 7 ml min^{-1} respectively. Both FPL 63012AR and dopamine, given as arterial bolus injections, produced dose-related renal vasodilatation resulting in increased RBF as shown in Figure 2. The geometric mean dose reducing RVR by 20%, the EC_{20} (95% CL), was 0.42 nmol kg^{-1} (0.31–0.55, $n = 13$) for FPL 63012AR and 3.63 nmol kg^{-1} (2.51–5.25, $n = 13$) for dopamine. FPL 63012AR was therefore 10 (9–12) times more potent than dopamine. The renal vasodilatation produced by both drugs was antagonized to the same degree by SCH 23390 (10 $\mu g\,kg^{-1}$ with 5 $\mu g\,kg^{-1}\,min^{-1}$, i.v.) producing rightward

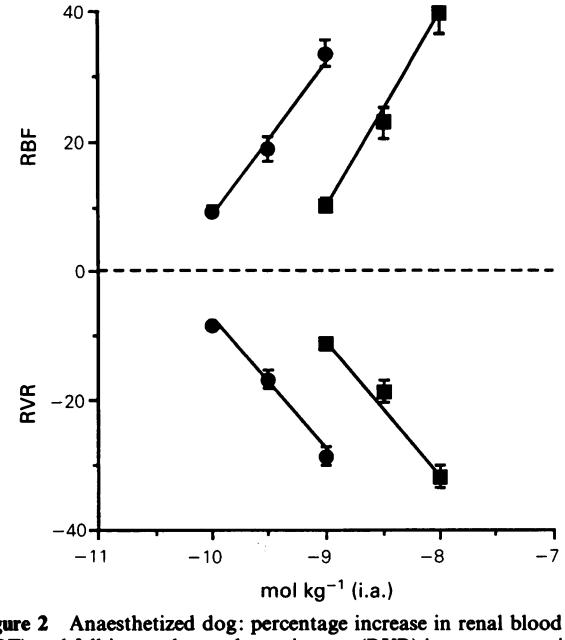

Figure 2 Anaesthetized dog: percentage increase in renal blood flow (RBF) and fall in renal vascular resistance (RVR) in response to intra-arterial injection of FPL 63012AR (●) and dopamine (■). Values are the mean (13 dogs) with s.e.mean shown by vertical bars.

shifts of 19.4 ± 2.4 and 22.2 ± 3.6 fold respectively. These results confirmed that FPL 63012AR, like dopamine, owes its renal action to D_1-receptor stimulation.

D_2-receptor

FPL 63012AR and 6,7-ADTN both produced concentration-dependent inhibition of the neurogenic vasoconstriction of the field-stimulated rabbit ear artery as shown in Table 1. FPL 63012AR was 140 times weaker than 6,7-ADTN in inhibiting the vasoconstriction but its response was not prevented by metoclopramide, indicating that its action does not involve stimulation of D_2-receptors. By contrast, the inhibitory response of 6, 7-ADTN was shifted 38 fold to the right (Table 1). In 6 experiments, in which the effect of the maximum inhibitory concentration of either drug was examined against a submaximal concentration of noradrenaline (60 or 100 nM), FPL 63012AR produced 83.3 ± 7.1% inhibition of the exogenous noradrenaline contracture at a concentration of 3 and 10 μM which inhibited the neurogenic contraction to a similar degree (73.7 ± 5.7%). By contrast, 6,7-ADTN (0.1 μM) did not significantly affect the response to noradrenaline (14.8 ± 6.3%, NS) despite reducing the neurogenic response by 87.0 ± 4.9%.

α-Adrenoceptors

Noradrenaline caused a concentration-dependent contracture (EC_{50} of 80 nM, $n = 7$) of canine isolated saphenous vein tubes. By contrast, FPL 63012AR was inactive at up to 10 μM.

β_1-Adrenoceptor

Spontaneously beating guinea-pig atria A maximally effective concentration of isoprenaline (0.3 μM) increased rate by 110 ± 10 beats min^{-1} from a resting value of 182 ± 5 beats min^{-1}. FPL 63012AR produced an increase no greater than 10% of the maximal increase in rate caused by isoprenaline at concentrations of 0.1 to 1 μM but reduced rate at a concentration above 10 μM (24.6 ± 4.3% at 30 μM, $n = 7$). Propranolol (1 μM) abolished the small rise but did not prevent the fall in rate (14.9 ± 4.3% at 30 μM, $n = 4$) produced by FPL 63012AR.

Paced guinea-pig papillary muscle Isoprenaline (10 nM–0.3 μM) produced a concentration-related increase in tension (EC_{50} 30 nM, $n = 6$) but FPL 63012AR was inactive at up to 100 μM.

β_2-Adrenoceptor

Salbutamol (10 nM–3 μM) caused a concentration-dependent relaxation (EC_{50} of 61 nM, $n = 9$) of tracheal chains under spontaneous tone. FPL 63012AR was virtually inactive in this preparation reaching only 9.8 ± 2.4% of the maximum salbutamol response at 100 μM.

Uptake$_1$ block

Rabbit brain synaptosomes FPL 63012AR inhibited the sequestration of [^{3}H]-noradrenaline into rabbit brain synaptosomes, with a geometric mean IC_{50} of 29.5 nM (20.4–42.6, $n = 12$). The results were similar in the crude and the purified fractions.

Table 1 Rabbit ear artery: inhibition of sympathetically mediated vasoconstriction and antagonism by metoclopramide 2.5 μM

Drug	*IC_{50}* (μM)	*Metoclopramide dose-ratio*
FPL 63012AR	1.65 (1.12–2.42)	1.3 (0.8–2.0)
6,7-ADTN	0.0117 (0.0083–0.0167)	38 (28–52)

Values are the geometric mean (95% CL) of 16 experiments.

Table 2 Anaesthetized dog: prevention of the tyramine-induced responses by FPL 63012AR infusion

Treatment		*HR*	*BP*	*dP/dt.P^{-1}*
Control	a	150 ± 7	135 ± 12	48 ± 3
	b	−12 ± 4*	+12 ± 2*	+48 ± 5*
FPL	a	149 ± 7	127 ± 12	51 ± 2
	b	0 ± 3	+5 ± 2	+9 ± 9

Response to i.v. bolus tyramine (60 μg kg^{-1}) as mean ± s.e.mean in 4 anaesthetized dogs before (control) and during infusion of 10^{-9} mol kg^{-1} min^{-1} FPL 63012AR (FPL). Basal absolute (a) values are shown for HR (beats min^{-1}), BP (mmHg) and dP/dt.P^{-1} (s^{-1}) as well as percentage changes over baseline (b) produced by tyramine. * indicates significant changes (Student's two tailed t test, $P < 0.05$).

Tyramine inhibition in the dog Intravenous infusion of FPL 63012AR (10^{-9} mol kg^{-1} min^{-1}) abolished the tyramine-induced pressor, positive inotropic effects and reflex bradycardia (Table 2).

Cardiovascular actions

Anaesthetized dog Basal cardiovascular parameters at the start of the experiment are shown in Table 3. Intravenous infusion of FPL 63012AR at 3×10^{-10} to 3×10^{-9} mol kg^{-1} min^{-1} resulted in rapid cardiovascular changes which were quick to recover (within 10 min) following termination of the infusion (Figure 3). RBF was elevated, despite a fall in BP, due to renal vasodilatation, as shown by the fall in calculated RVR. The fall in diastolic BP was slightly greater than the systolic fall e.g. 16 ± 3 mmHg compared with 10 ± 2 mmHg at the highest infusion rate and was accompanied by small, though dose-related, increases in HR (maximum 18 ± 2 beats min^{-1}, 12 ± 1%) and contractility (maximum 8.3 ± 2.0 s^{-1}, 16 ± 4%). No change was seen in the ECG. The effect of the highest infusion of FPL 63012AR was completely blocked during infusion of the selective D_1-receptor antagonist SCH 23390 (10 μg kg^{-1} with 30 μg kg^{-1} h^{-1}, i.v.) as shown in Table 4.

Conscious dog Prior to infusing FPL 63012AR into the conscious dogs, the basal parameters were mean BP 96 ± 7 mmHg, HR 73 ± 9 beats min^{-1}, RBF 3.07 ± 0.60 kHz and RVR 31.5 ± 5.4 mmHg kHz^{-1}. Following infusion of isotonic saline, which was ineffective in changing these parameters, stepwise infusion of FPL 63012AR produced a dose-related fall in RVR and a rise in RBF with maximal effects at approximately 10^{-8} mol kg^{-1} min^{-1} of 46 ± 9% and 76 ± 17% respectively in the 3 dogs examined (Figure 4). At and below this infusion rate, the HR response was variable, one dog showing a small bradycardia and there was no significant change in BP. At the higher infusion rates, tachycardia occurred in each dog with a small fall in BP. The compound was well tolerated and no behavioural changes or emesis were seen.

Table 3 Anaesthetized dog: basal parameters at start of experiment

RBF (ml min^{-1})	*RVR* (mmHg min ml^{-1})	*BP* (mmHg)	*HR* (bpm)	*dP/dt.P^{-1}* (s^{-1})
106 ± 15	1.18 ± 0.33	126 ± 7	144 ± 4	49 ± 3

Values are the mean ± s.e.mean of 4 dogs prior to FPL 63012AR infusion.

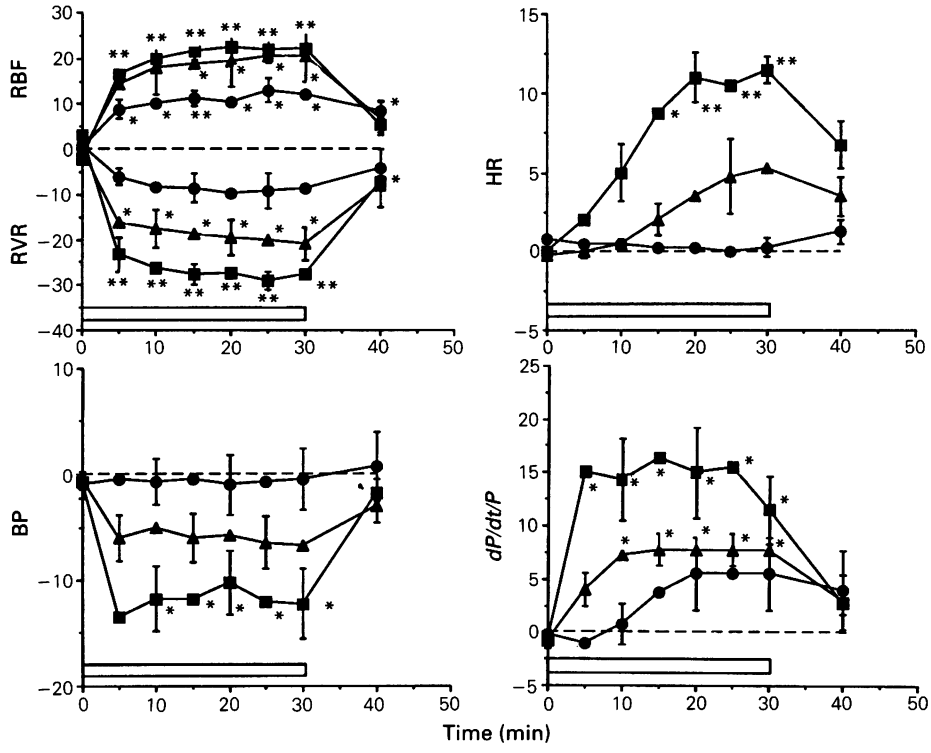

Figure 3 Anaesthetized dog: effect of 30 min intravenous infusions of FPL 63012AR at 3×10^{-10} (●), 10^{-9} (▲) and 3×10^{-9} (■) mol kg^{-1} min^{-1} (0.17–1.65 μg kg^{-1} min^{-1}), as indicated by the horizontal bar, on renal blood flow (RBF) and vascular resistance (RVR), heart rate (HR), contractility ($dP/dt.P^{-1}$) and mean blood pressure (BP). Values are percentage changes, (mean with s.e.mean shown by vertical bars) in 4 dogs. * and ** indicate $P < 0.05$ and 0.01 respectively (Student's paired t test).

Table 4 Anaesthetized dog: antagonism of FPL 63012AR by SCH 23390

Treatment		*mBP*	*HR*	*dP/dt.P*$^{-1}$	*RBF*	*RVR*
Control	a	125 ± 8	156 ± 7	53 ± 3	110 ± 17	1.09 ± 0.23
	b	−12 ± 3*	+5 ± 2	+14 ± 4*	+20 ± 2*	−26 ± 3*
SCH	a	110 ± 10	146 ± 7	48 ± 5	94 ± 14	1.12 ± 0.24
	b	−1 ± 2	−1 ± 1	+3 ± 3	−4 ± 1*	+2 ± 3

Values are mean ± s.e.mean ($n = 4$) of basal absolute values (a) for parameters as described in Table 2 as well as for RBF (ml min^{-1}) and RVR (mmHg min ml^{-1}) and percentage changes (b) induced by FPL 63012AR infusion (3×10^{-9} mol kg^{-1} min^{-1}, after 10 min) both before (control) and during infusion of SCH 23390 (SCH). *indicates significant changes (Student's two-tailed t test, $P < 0.05$).

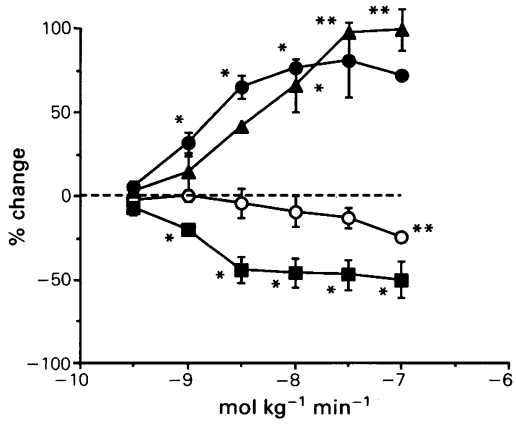

Figure 4 Conscious dog: effect of stepwise 10 min infusions of FPL 63012AR of 3×10^{-10} to 10^{-7} mol kg^{-1} min^{-1} (0.17–55 μg kg^{-1} min^{-1}) on blood pressure (BP) (○) and heart rate (HR) (▲) in 5 dogs and on renal blood flow (RBF) (●) and renal vascular resistance (RVR) (■) in 3 of these dogs. Values are percentage changes, mean with s.e.mean shown by vertical bars. * and ** indicate $P < 0.05$ and 0.01 respectively (Student's paired t test).

Discussion

The action of FPL 63012AR in the dog kidney is clearly due to the stimulation of vascular D_1-receptors, since it is abolished by the selective antagonist SCH 23390 (Goldberg *et al.*, 1984). Moreover it is 10 times more potent than dopamine. FPL 63012AR has been shown to be an agonist at the central D_1 dopamine receptor, approximately 11.4 times as potent as dopamine in the D384 human astrocyte cell line (E. Wells, personal communication).

No agonist activity could be detected at the prejunctional D_2-receptor of the field-stimulated perfused ear artery since metoclopramide was ineffective against the inhibitory action of FPL 63012AR. The lack of stimulant activity at the D_2-receptor (or more correctly the central D_2-receptor, which is regarded as similar in character) was confirmed in the conscious dog by the absence of emesis at infusion rates 30 to 300 times greater than that required to produce D_1-receptor mediated renal vasodilatation.

FPL 63012AR was also inactive in stimulating postjunctional α_1-and α_2-adrenoceptors of the dog saphenous vein and therefore should be devoid of vasoconstrictor activity. Although a small degree of agonist activity was seen at the

atrial β_1-adrenoceptor, this could not be confirmed in ventricular muscle and the reason for this disparity is unclear. Above 10 μM, FPL 63012AR depressed the sino-atrial node by an unknown mechanism. In addition only a small degree of β_2-adrenoceptor agonist activity could be detected, with an intrinsic activity of less than 0.1. In the anaesthetized dog, only weak cardiac stimulation was observed and since this was abolished by SCH 23390, this implies that the cardiac stimulation was a consequence of the baroreflex secondary to D_1-receptor stimulation and not due to the direct stimulation of either cardiac β_1- or β_2-adrenoceptors.

In addition to showing selective agonism for the D_1-receptor, FPL 63012AR is also a potent blocker of noradrenaline uptake (Uptake$_1$) into rabbit brain synaptosomes. Using previously published data obtained from this preparation (Mitchell *et al.*, 1987), FPL 63012AR is calculated to be 9.2 times as potent as dopamine (IC_{50} 270 nM) and 3.7 times more potent than cocaine (IC_{50} 108 nM). Blockade of Uptake$_1$ by FPL 63012AR was confirmed in the anaesthetized dog by the abolition of the response to tyramine. The actions of tyramine, an indirect sympathomimetic, rely on its ability to enter the sympathetic neurone via Uptake$_1$ to displace noradrenaline. Dopexamine hydrochloride, which is of similar potency to FPL 63012AR as an Uptake$_1$ blocker (Mitchell *et al.*, 1987), also prevented the action of tyramine in the anaesthetized dog and in addition potentiated the cardiac effects of exogenous noradrenaline and sympathetic nerve stimulation in the dog (Smith & Naya, 1987; Bass *et al.*, 1987).

The weak inhibitory action of FPL 63012AR against neurogenic vasoconstriction in the rabbit ear artery was due to prevention of the postjunctional action of noradrenaline since the effect of exogenously applied noradrenaline was reduced to the same degree as the neurogenic vasoconstriction. This property was not apparent in the anaesthetized dog at the infusion rates examined, since the cardiovascular actions of FPL 63012AR were completely prevented by SCH 23390 and therefore solely attributable to D_1-receptor stimulation. Inhibition of the postjunctional actions of noradrenaline may well, however, occur at higher infusion rates than are necessary to stimulate D_1-receptors.

We have previously confirmed the moderately potent actions of dopamine *in vitro* at vascular postjunctional α- and cardiac β_1-adrenoceptors (Brown *et al.*, 1985a), properties which limit the clinical usefulness of dopamine by producing intense vasoconstriction, arrhythmias and tachycardia. Moreover the D_2-receptor-stimulant action of dopamine can lead to nausea and emesis (Goldberg *et al.*, 1977; Makabali *et al.*, 1982). The lack of these properties in a more potent D_1-receptor agonist as displayed by FPL 63012AR potentially represents a clinical advantage over dopamine.

Compared with dopexamine hydrochloride (Brown *et al.*, 1985a) the introduction of the 2-[3-hydroxyphenyl]ethyl group in FPL 63012AR leads to a dramatic alteration in the pharmacological profile by way of suppression of agonist activity at both the β_2-adrenoceptor and D_2-receptor with a 30 fold increase in the D_1-receptor potency.

Fenoldopam, is the most potent and selective D_1-receptor agonist of a series of benzazepines which are chemically dissimilar to FPL 63012AR. It is reported to be approximately 10 times more potent than dopamine (Hahn *et al.*, 1982) and therefore of similar potency to FPL 63012AR. It possesses a similar pharmacological profile to FPL 63012AR in that it also prevents the postjunctional effect of noradrenaline, by antagonizing α_2-adrenoceptors (Ohlstein *et al.*, 1985) but this is only seen at intravenous doses exceeding those necessary for D_1-receptor stimulation (Goldberg *et al.*, 1986).

There is now good evidence that endogenous renal dopamine plays a physiological role in the handling of sodium by the kidney. For example in saline-loaded rats, SCH 23390 prevents the subsequent natriuresis and diuresis (Hegde *et al.*, 1989) and in conscious dogs, intra-renal arterial infusion of SCH 23390 reduces sodium and urine output without affecting renal haemodynamics (Siragy *et al.*, 1988). Moreover these effects of endogenous dopamine can be reproduced by infusion of either dopamine (Frederickson *et al.*, 1984) or fenoldopam (Hilditch *et al.*, 1988) into the dog renal artery and are prevented by SCH 23390. Evidence in the rabbit suggests that dopamine acts on the proximal tubule to reduce sodium and water reabsorption (Bello-Reuss *et al.*, 1982). These findings strengthen the case for the use of selective D_1-receptor agonists in the treatment of acute renal failure (Goldberg, 1972; Hahn, 1984). A recent report demonstrated beneficial effects of fenoldopam in patients with advanced chronic renal failure (Garcia Robles *et al.*, 1989).

Selective D_1-receptor agonists have also been advocated for the acute treatment of heart failure, by reducing afterload and improving renal perfusion. Fenoldopam produces beneficial haemodynamic changes in rabbits with experimental congestive heart failure (Jover & McGrath, 1988) and favourable findings have also been reported with i.v. fenoldopam in heart failure patients (Young *et al.*, 1988).

In conclusion, FPL 63012AR is an example of a novel class of potent D_1-receptor agonists which may have potential in the treatment of renal and cardiovascular diseases.

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On the blockade of acetylcholine release at mouse motor nerve terminals by β-bungarotoxin and crotoxin

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1 β-Bungarotoxin and crotoxin are phospholipose A_2 neurotoxins, which block irreversibly the evoked release of acetylcholine from motor nerve terminals of mouse triangularis sterni preparations.

2 Extracellular recording of nerve terminal action potentials reveal that inhibition of transmitter release is not associated with failure of the action potential to invade nerve terminals.

3 When evoked transmitter release (measured as intracellularly recorded endplate potentials) was blocked by β-bungarotoxin, spontaneous acetylcholine release was stimulated as in control experiments by K^+-induced depolarization and by the Ca^{2+}-ionophore A23187.

4 The site of action of the toxins remains to be elucidated but would appear to be associated with the coupling of action potential induced-depolarization to the release mechanism, rather than with the release mechanism itself.

Introduction

Neurotoxins from snake venoms with phospholipase A_2 activity (e.g. β-bungarotoxin, crotoxin, taipoxin) cause muscle paralysis by blocking acetylcholine release (see Chang, 1985; Harris, 1985 for reviews). The mechanisms responsible are unknown, but several have been proposed. These include depletion of energy stores, inhibition of choline uptake, excessive accumulation of Ca^{2+}, a decrease in Ca^{2+} influx and a decrease in the efficacy of Ca^{2+} in promoting release (see Chang, 1985). Some of these suggestions could be tested if the ionic currents controlling acetycholine release could be monitored. The small size of nerve terminals of mammalian neuromuscular junctions makes intracellular recording impossible but the local electrical activity of nerve terminals can be recorded with extracellular electrodes. Contributions related to Na^+, K^+ and Ca^{2+} currents can be distinguished (Mallart, 1985; Penner & Dreyer, 1986). Using this technique, we found that the initial phase of facilitation of acetylcholine release, which is characteristic of the actions of β-bungarotoxin and crotoxin at mouse neuromuscular junctions, is associated with a reduction in terminal K^+ currents (Rowan & Harvey, 1988). We have now tested these two toxins under conditions in which transmitter release is blocked to determine if the toxins block the invasion of the action potential into the nerve terminal or the Ca^{2+} current of the terminal. We have also examined whether the release mechanisms may be affected by the toxins by testing for effects of β-bungarotoxin on the stimulation of release by K^+-induced depolarization and by the Ca^{2+} ionophore A23187.

Methods

Nerve-muscle preparation

Experiments were performed on the mouse triangularis sterni nerve-muscle preparation, as described previously (Rowan & Harvey, 1988). Preparations were perfused at a rate of 5–10 ml min^{-1} with physiological solution (composition, mM: NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11.1 and $NaHCO_3$ 25 to buffer at pH 7.3).

Studies on evoked release and on nerve terminal action potentials

The intercostal nerves were stimulated every 2 s with pulses of 50 μs duration and supramaximal voltage. The potential difference between a silver/silver chloride reference electrode in the bath and the recording microelectrode (containing 3 M KCl, resistance 5-10 MΩ) was recorded as previously (Rowan & Harvey, 1988). In preparations paralysed by tubocurarine (1–2 μM), endplate potentials (e.p.ps) were recorded continuously from one endplate before and throughout application of toxin. Solution (10–20 ml) containing toxin at the desired concentration was perfused through the tissue bath for 30 min, with recycling of the solution after aeration; then toxin-free solution was used to perfuse the tissue. The preparations were maintained at 30°C. One preparation was used for each condition.

Presynaptic waveforms were recorded by glass microelectrodes (containing 2 M NaCl, resistance 5–15 MΩ) placed inside the perineural sheath (near endplate areas) of a branch of an intercostal nerve. The waveforms were displayed and recorded as for intracellular recording. Two preparations were used for each experimental condition, and values quoted are means ± s.e.mean.

Studies on spontaneous release during exposure to KCl and A23187

Triangularis sterni preparations were used as described above, except that the temperature of the perfusing solution was 34°C. Miniature endplate potentials (m.e.p.ps) were recorded before and after addition of 10 mM KCl or 20 μM A23187 for 2 min. The preparations were then stimulated at 0.5 Hz and 140 nM β-bungarotoxin was added. After the muscle stopped twitching, a fibre was impaled with a microelectrode and recordings of e.p.ps and m.e.p.ps were made. Recordings of m.e.p.ps were then made after addition of 10 mM KCl or 20 μM A23187. As the effects of A23187 were difficult to reverse, separate preparations were used for control and β-bungarotoxin-treated samples. Three preparations were used for each treatment.

Materials

Crotoxin and β-bungarotoxin were gifts from Dr C. Bon, Institut Pasteur, Paris, and Dr E. Karlsson, University of

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Uppsala, respectively. Some β-bungarotoxin was also obtained from Calbiochem. A23187 (Calbiochem) was prepared as a stock solution of 2 mg ml^{-1} in 95% ethanol. Other chemicals were obtained from Sigma Chemical Co., Poole, Dorset.

Results

Effects on evoked release and nerve terminal action potentials

In mammalian nerve-muscle preparations, the early facilitatory phase produced by β-bungarotoxin and crotoxin is apparently independent of the toxins' phospholipase activity. Hence, our previous experiments (Rowan & Harvey, 1988) were performed at room temperature to reduce enzymatic activity. To examine the blocking effects of the toxins, experiments were performed at 30°C and in the presence of normal concentrations of Ca^{2+}.

β-Bungarotoxin (150 nM) and crotoxin (130 nM) reduced the amplitude of e.p.ps in a time-dependent manner (Figure 1A and B). There was little change in the membrane potentials of the cells: for example, membrane potential before addition of β-bungarotoxin was −80 mV; after 60 min, it was −84 mV. At 30°C, there was no evidence for a facilitation of e.p.ps, probably because high concentrations of toxins were used and the blocking activity overwhelmed the facilitatory effect.

The perineural waveform consists of a small positive component followed by two larger negative components, which correspond to Na^+ and K^+ currents (see Rowan & Harvey, 1988 and Anderson *et al.*, 1988). In two control experiments where the temperature was raised to 30°C, the perineural waveform was stable for over 60 min. The waveform was markedly faster than at room temperature, but it showed the same sensitivity to ion channel blocking compounds as at room temperature. Thus, 200 μM 3,4-diaminopyridine abolished the second negative waveform and revealed a delayed positive component. Tetraethylammonium (1 mM) increased both amplitude and time course of the positive component, which was reduced by the Ca^{2+} channel blocker Cd^{2+} (200 μM).

When β-bungarotoxin (150 nM) or crotoxin (130 nM) was added to preparations maintained at 30°C, there was a selective reduction in the second negative deflection (Figure 1c and d). This occurred 5–10 min after exposure to the toxin, and the reduction, which was 31 ± 6% with β-bungarotoxin and 53 ± 2% with crotoxin, was stable for at least 60 min. The first negative component of the waveform did not change significantly from control. After exposure to toxin, 3,4-diaminopyridine still abolished the remainder of the second negative waveform and revealed a positive component that was enhanced by subsequent addition of tetraethylammonium. This positive component was similar to that seen in control preparations.

Effects of KCl and A23187

M.e.p.p. frequency in control preparations was approximately doubled during 2 min exposure to 10 mM KCl or 20 μM A23187 (Figure 2). Preparations were then exposed to 140 nM β-bungarotoxin. Visible twitch responses to motor nerve stimulation could not be observed after 75–90 min. The m.e.p.p. frequency recorded after abolition of twitching was apparently normal (i.e. 1–4 Hz). M.e.p.ps could be recorded for at least 2.5 h after addition of the toxin; during this late phase, m.e.p.ps often occurred in high frequency bursts, as described by Abe *et al.* (1976) and large amplitude m.e.p.ps occurred more frequently.

After the muscle fibres stopped twitching, e.p.ps could be recorded for a short period before they were abolished. Quantal contents during this period were around 4–9. Shortly

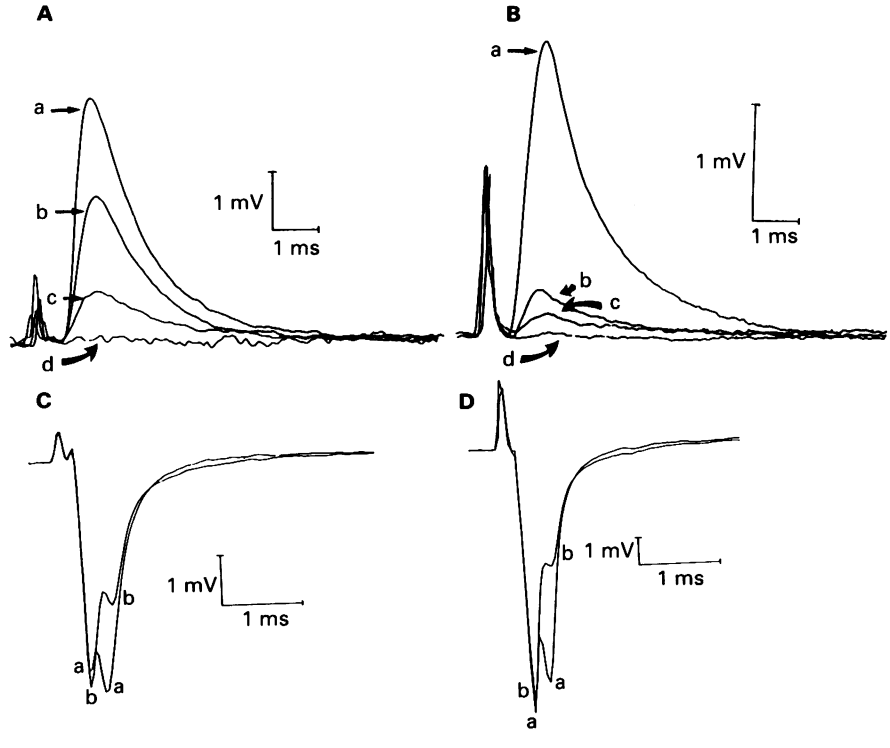

Figure 1 Effect of β-bungarotoxin and crotoxin on nerve-evoked end plate potentials (e.p.ps) and perineural waveforms recorded from mouse triangularis sterni preparations at 30°C in the presence of 2.5 mM Ca^{2+}. (A) (a) Averaged control e.p.p.; (b–d) averaged e.p.ps from the same neuromuscular junction 5, 10, and 60 min after addition of β-bungarotoxin (150 nM). (B) Averaged control e.p.p.; (b–d) averaged e.p.ps from the same neuromuscular junction 5, 20, and 30 min after addition of crotoxin (130 nM). (C) (a) Averaged control waveform; (b) averaged waveform after 60 min exposure of β-bungarotoxin (150 nM). (D) (a) Averaged control. waveform; (b) averaged waveform after 60 min exposure of crotoxin (130 nM). Note the large decrease in the second component of the waveforms in the presence of the toxins. Calibration bars: 1 ms and 1 mV.

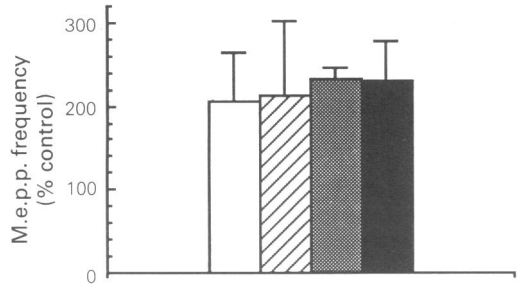

Figure 2 Increase of miniature endplate potential (m.e.p.p.) frequency by KCl and A23187. Left-hand columns, responses to 10 mM KCl: open column, control; hatched column, after twitch blockade by 130 nM β-bungarotoxin. Right-hand columns, responses to 20 μM A23187: stippled column, control; solid column, after twitch blockade by 140 nM β-bungarotoxin. Frequencies in the absence of stimulation were taken as 100%. Each column represents the mean of three separate experiments; bars indicate s.e.means.

after twitch responses were blocked, preparations were exposed to 10 mM KCl or 20 μM A23187. Both treatments increased the frequency of m.e.p.ps to about the same extent as in non-toxin treated preparations (Figure 2). Once the frequency of m.e.p.ps had dropped to zero (about 150 min after addition of β-bungarotoxin), neither KCl nor A23187 restored m.e.p.ps.

Discussion

β-Bungarotoxin and crotoxin abolished e.p.ps with little alteration in the perineural recordings made from terminal regions. The changes that were seen in the nerve terminal action potential were similar to those seen at room temperature, which are associated with a block of some presynaptic K^+ channels (Rowan & Harvey, 1988). Thus, alterations in nerve terminal action potentials cannot account for the block of transmitter release. Nerve terminal action potentials and Ca^{2+} currents (as revealed by application of 3,4-diaminopyridine and tetraethylammonium) were still active even after acetylcholine release had been abolished. Previously, β-bungarotoxin (Chang *et al.*, 1973) and crotoxin (Breithaupt, 1976) were reported not to block conduction of action potentials in rat or rabbit phrenic nerves, respectively. Additionally, extracellularly recorded nerve terminal spikes in frog sartorius nerve-muscle preparations were unaffected by paralysis by β-bungarotoxin (Chang *et al.*, 1973). Chang *et al.* (1973) and Hawgood & Smith (1977) provided further evidence that action potentials could still invade nerve terminals of rat and mouse diaphragm preparations paralysed by β-bungarotoxin or crotoxin, respectively, because tetanic stimulation continued to increase the frequency of m.e.p.ps after failure of e.p.ps. This implies that the blockade of transmitter release must occur at some site of critical importance for the release mechanism but after the action potential.

We attempted to test the functioning of Ca^{2+}-dependent processes by directly depolarizing the nerve terminal with elevated extracellular K^+, and by introducing Ca^{2+} ions via an ionophore in order to bypass the physiological Ca^{2+} channels. After β-bungarotoxin had induced muscle paralysis, evoked acetylcholine release was severely impaired: quantal contents were usually 4–5, while in normal mouse neuromuscular junctions values of at least 30 are expected (Hong & Chang, 1989).

However, both K^+-induced depolarization and the Ca^{2+} ionophore could still enhance the frequency of m.e.p.ps, as in control preparations. A brief report of apparently similar findings with the notexin homologue notechis II-5 was published by Kamenskaya & Satybaldina (1979). After prolonged exposures to β-bungarotoxin, KCl and A23187 had no effect, as previously reported for K^+ during β-bungarotoxin- (Oberg & Kelly, 1976) and crotoxin-induced paralysis (Chang & Lee, 1977).

Our results imply that when β-bungarotoxin has prevented evoked acetylcholine release, the nerve terminal still has functional release sites with apparently normal sensitivity to intracellular Ca^{2+}. The terminals are presumably not continuously depolarized because the perineural recordings are normal, m.e.p.p. frequency is not elevated, and the terminals respond normally to 10 mM KCl. The question remains: what is the site of action of β-bungarotoxin? One possibility is that the apparently normal functioning of the terminal Ca^{2+} channels is misleading. Prolonged depolarization, as induced by KCl or in the presence of 3,4-diaminopyridine and tetraethylammonium, may open Ca^{2+} channels additional to those involved in the physiological regulation of acetylcholine release. For example, acetylcholine release or the perineural waveform is not usually affected by Ca^{2+}-channel blockers such as verapamil, but verapamil-sensitive Ca^{2+} currents are revealed during prolonged depolarization (Penner & Dreyer, 1986; Anderson & Harvey, 1987). Also, Ca^{2+} channels differing in their sensitivity to depolarization have been characterized in other neurones (Nowycky *et al.*, 1985). Hence, the physiologically important Ca^{2+} current may be obscured during a K^+-induced depolarization, and it could be blocked specifically by β-bungarotoxin and similar toxins.

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Effects of platelet activating factor on contractile force and ^{45}Ca fluxes in guinea-pig isolated atria

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1 The effects of platelet activating factor (PAF) were studied on the electromechanical properties and $^{45}Ca^{2+}$ fluxes of guinea-pig isolated atria.

2 Both in spontaneously beating and electrically driven atria, PAF (10^{-12}–10^{-7} M) increased atrial rate but produced a biphasic effect on contractile force. At low concentrations (up to 10^{-10} M) it produced a positive inotropic effect, while at higher concentrations PAF exerted a negative inotropic effect. A similar biphasic effect was observed in the slow contractions elicited by isoprenaline in K^+-depolarized atrial fibres.

3 The positive inotropic effect of PAF was prevented by verapamil, whereas pretreatment of atria with propranolol, phentolamine, indomethacin or atropine did not modify its positive and negative inotropic actions. BN 52021, a specific PAF antagonist, abolished both the positive and negative inotropic effects.

4 PAF had no effect on the characteristics of the action potentials recorded in either normally polarized or K^+-depolarized (slow action potential) atrial fibres.

5 At concentrations at which it increased contractile force, PAF potentiated the contractile responses to Ca^{2+} (0.9–9 mM), whereas at negative inotropic concentrations it inhibited them. The negative inotropic effect of PAF was partially reversed in 70% Na^+ medium.

6 At 10^{-11} M, PAF increased $^{45}Ca^{2+}$ uptake and reduced the rate coefficient (k_{cm}) for the $^{45}Ca^{2+}$ efflux. This increase in $^{45}Ca^{2+}$ uptake was abolished in atria pretreated with verapamil or BN 52021. However, 10^{-7} M PAF modified neither $^{45}Ca^{2+}$ uptake nor efflux in atrial muscle.

7 These results suggest that in guinea-pig atria the biphasic inotropic effects of PAF cannot be explained through modifications in the slow inward Ca^{2+} current or in Na^+–Ca^{2+} exchange, but may be related to changes in trans-sarcolemmal Ca^{2+} entry mediated by specific PAF receptors.

Introduction

Platelet-activating factor [PAF, 1-0-hexadecyl-2-0-acetyl-sn-glyceryl-3-phosphorylcholine] is a polar phospholipid which exhibits a wide range of biological activities (Braquet *et al.*, 1987). In anaesthetized animals, PAF produced hypotension, transient apnoea and increased vascular permeability (Halonen *et al.*, 1980; Sánchez-Crespo *et al.*, 1982; Bessin *et al.*, 1983). In guinea-pig isolated perfused hearts, PAF reduced coronary blood flow and contractile force and produced conduction arrhythmias (Benveniste *et al.*, 1983; Levi *et al.*, 1984; González-Morales & Tamargo, 1988) and thus, it was proposed that PAF may play a major role in cardiac abnormalities occurring during cardiac anaphylaxis (Levi *et al.*, 1984). However, discordant results on contractile force have been reported. Thus, while some authors have described a concentration-dependent negative inotropic effect on guinea-pig isolated atria and papillary muscles (Camussi *et al.*, 1984; Levi *et al.*, 1984; Alloatti *et al.*, 1987; Robertson *et al.*, 1988) and human papillary muscles (Alloatti *et al.*, 1986), others have described a biphasic effect of PAF, a positive inotropic effect being observed at low concentrations, whereas high concentrations produced a negative one (Tamargo *et al.*, 1985; Nayaka & Tohse, 1986). The mechanism(s) of the inotropic effects of PAF are unknown at present, but it is unlikely that they are secondary to formed blood elements or changes in coronary blood flow, since they have also been observed in noncoronary-perfused isolated atria and papillary muscles. Previous experimental evidence attributed the negative inotropic effect to a decrease in the slow inward Ca^{2+} current (Camussi *et al.*, 1984; González-Morales & Tamargo, 1988) and/or to a decrease in intracellular Na^+ activity mediated by the Na^+–Ca^{2+} exchanger (Robertson *et al.*, 1988), whereas the positive inotropic effect was attributed to an increase in Ca^{2+} influx via the slow inward Ca^{2+} current (Tamargo *et al.*, 1985). In fact, PAF has been found to increase $^{45}Ca^{2+}$ influx in mouse macrophages (Braquet *et al.*, 1987), human (Valone & Johnson, 1985) and rabbit platelets (Lee *et al.*, 1981) and $^{45}Ca^{2+}$ uptake in guinea-pig papillary muscles (Tamargo *et al.*, 1988).

Therefore, the present study was undertaken to characterize further the mechanisms responsible for the inotropic effects of PAF, particularly as they may be mediated through changes (increase/decrease) of transmembrane Ca^{2+} movements. Thus, experiments were designed to (1) characterize the effects of PAF on contractile force and transmembrane action potentials in normal and depolarized media, and (2) determine its effect on $^{45}Ca^{2+}$ movement in guinea-pig atria, a cardiac preparation independent of coronary supply.

Methods

General procedure

Guinea-pigs of either sex weighing 350–450 g were stunned by a sharp blow on the head and their hearts were rapidly removed. Right and left atria were dissected and mounted vertically in 10 ml organ baths containing Tyrode solution of the following composition (mM): NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaHCO_3$ 11.9, NaH_2PO_4 0.42 and glucose 5.5. The solution was bubbled with 95% O_2 : 5% CO_2 and maintained at 34°C. In some experiments 30% of the Na^+ concentration of the Tyrode solution was replaced by isosmotic sucrose. In others, Ca^{2+} concentration was varied from 0.9 to 9.0 mM. Left atria were electrically driven at a basal rate of 1 Hz through bipolar platinum electrodes with rectangular pulses (1 ms duration, twice threshold strength) delivered from a multipurpose programmable stimulator (Cibertec CS 220). Right atria were allowed to beat spontaneously. Rate and amplitude of contractions were measured isometrically by a force-displacement transducer and recorded on a Letica 2000 polygraph. Resting tension was adjusted to 1 g and a 30 min

equilibration period was allowed to elapse before control measurements were taken. The different parameters of isometric contractions and the sinus node recovery time (SNRT) were determined as previously described (Tamargo, 1980; Díez & Tamargo, 1987). After control values for each parameter were obtained, incremental concentrations of PAF were added to the bath to obtain a complete dose-response curve. The interval between doses of PAF was 10 min, since preliminary time-response studies indicated that its effect had stabilized in less than 10 min. The values for the different parameters obtained in the absence of PAF were used as a control and compared with those obtained after each increment in drug concentration. To study the interaction of different antagonists with the inotropic effects of PAF, dose-response curves to PAF were performed as above. The atria were washed and stabilized for 30 min. The antagonists were then added to the bath and after 15 min a new dose-response curve to PAF was obtained. Only one antagonist was used in each experiment.

For experiments on the interaction between PAF and the positive inotropic responses to $CaCl_2$, the following procedure was used (Díez *et al.*, 1985). After control recordings were taken, increasing concentrations of $CaCl_2$ (0.9–9.0 mM) were added to the bath and a dose-response curve to Ca^{2+} was obtained. Once stable curves were obtained, the same procedure was repeated 10 min after the addition of PAF to the bathing media. Dose-response curves were expressed as percentages of the contractile response induced by 0.9 mM Ca^{2+} under control conditions.

Intracellular microelectrode recordings

Right and left atria were pinned to a Sylgard resin (Dow Corning) base of a chamber and perfused at a rate of 7 ml min^{-1} with Tyrode solution (34°C) bubbled with 95% O_2 : 5% CO_2. Preparations were stimulated as described earlier. Action potentials were recorded with glass microelectrodes (10–20 MΩ) filled with 3 M KCl as previously described (Díez *et al.*, 1985; Delpón *et al.*, 1989) and displayed on a Tektronix 5104 storage oscilloscope for photography. To study the effects of PAF on slow action potentials and contractions, atria were equilibrated in Tyrode solution and then rendered inexcitable by depolarizing with high K^+ (27 mM) Tyrode solution (Delpón *et al.*, 1989). Under these conditions atrial fibres became inexcitable despite intense electrical stimulation. Excitability was restored (i.e. slow action potentials and contractions) in atria driven at a basal rate of 0.4 Hz by adding isoprenaline (10^{-6} M) to the bathing media.

$^{45}Ca^{2+}$ uptake

To determine $^{45}Ca^{2+}$ uptake, left atria driven at a basal rate of 1 Hz were incubated in Tyrode solution for 30 min. In each experiment half of the atrium served as control and the other half as the experimental preparation. Following the equilibration period the experimental half was treated with PAF for 10 min and then both halves were exposed for various time intervals (2, 5 and 10 min) to $^{45}Ca^{2+}$-labelled Tyrode solution (sp. act. 37 kBa ml^{-1}; Amersham International, Buckinghamshire). At the desired time intervals, atria were removed, blotted on filter paper, dipped into Tyrode solution, reblotted and weighed. The atria were then placed in scintillation vials and 0.5 ml of Protosol 0.5 M (New England Nuclear) added and digested overnight at 50°C. Radioactivity was assayed on a liquid scintillation counter (LKB-Wallac 1211) as previously described (Barrigón *et al.*, 1982; Díez & Tamargo, 1987).

$^{45}Ca^{2+}$ efflux

To determine $^{45}Ca^{2+}$ efflux following the equilibration period, left atria were incubated in $^{45}Ca^{2+}$-labelled Tyrode solution (sp. act. 74 kBq ml^{-1}) for 2 h. Thereafter, the preparations were placed every 5 min for 60 min and every 10 min for another period of 60 min in successive tubes containing 5 ml of Tyrode solution to complete a 120 min washout period. In each experiment one atrium served as control and another atrium was exposed to the action of the desired concentration of PAF (10^{-11} M and 10^{-7} M) from min 20 to the end of the washout period. After the efflux period of 120 min, radioactivity lost into the tubes and present at the end of the experiment in the atria was determined as described for the uptake experiments. From the final activity in muscles and from the amount of radioactivity lost from the atria at 5–10 min intervals the percentage of $^{45}Ca^{2+}$ remaining in the atria at each collecting time was obtained. Washout data were expressed as a percentage of radioactivity remaining in the atria at each time interval and were plotted semilogarithmically as desaturation curves. The rate coefficient for the $^{45}Ca^{2+}$ efflux, k_{cm}, was calculated as described by Holland *et al.* (1978).

Drugs

The following drugs were used: PAF (Bachem, Bubendorf, Switzerland), lyso-PAF (Bachem), BN 52021 (Institut Henri Beaufour, France), L 652,731 (Merck Sharp & Dohme), (−)-isoprenaline hydrochloride (Sigma), verapamil hydrochloride (Knoll), (−)-propranolol (ICI-Farma), atropine sulphate (Sigma), phentolamine hydrochloride (Ciba), indomethacin (Merck Sharp & Dohme) and bovine serum albumin (Sigma). Powdered PAF was dissolved in Tyrode solution containing bovine serum albumin (0.5%) to obtain a concentrated solution. Further dilutions were performed in Tyrode solution to obtain the desired final concentration between 10^{-12} M and 10^{-7} M. Other drugs were dissolved in distilled water. Ascorbic acid (10^{-4} M) was added to each solution of isoprenaline to prevent its oxidation. All concentrations refer to the salt.

Statistical analysis

Throughout the paper results are expressed as mean ± s.e.mean. Statistical analysis was performed by use of Student's *t* test based on paired or unpaired observations when appropriate, and differences were considered significant when $P < 0.05$. Regression analysis was used to study the relationship between k_{cm} and percentage of $^{45}Ca^{2+}$ remaining in the atria.

Results

Effects of PAF on right and left atria

The effects of PAF in a range of concentrations between 10^{-12} M and 10^{-7} M on rate and amplitude of spontaneous contractions were studied in 8 right atria. Control values for both parameters averaged 157.9 ± 13.0 beats min^{-1} and 440.1 ± 67.3 mg, respectively. When added to the bathing medium, PAF produced a concentration-dependent increase in atrial rate which reached significant values ($P < 0.05$) at concentrations higher than 10^{-12} M (Figure 1a). Thus, at 10^{-8} M, PAF increased atrial rate by 11.3 ± 4.0% ($P < 0.01$). On contractile force PAF induced a biphasic effect, a positive followed by a negative inotropic effect (Figure 1a). At low concentrations (10^{-12} M–10^{-10} M) PAF increased peak contractile force (+14.3 ± 5.1% at 10^{-10} M, $P < 0.05$), while at higher concentrations it produced a concentration-dependent negative inotropic effect which reached significant values at 10^{-7} M (−15.7 ± 5.0%, $P < 0.05$). The onset of inotropic effects occurred within 2 min of its addition to the bath and reached steady-state values within 5–7 min. The effect of PAF on the SNRT was analyzed in another 8 spontaneously beating right atria. Control values for this parameter averaged 439.7 ± 29.5 ms. Figure 1a shows that PAF produced a significant shortening of the SNRT at all concentrations tested and thus, 10^{-7} M PAF shortened the SNRT by 17.1 ± 3.9% ($P < 0.01$) below control values. At the same range of concen-

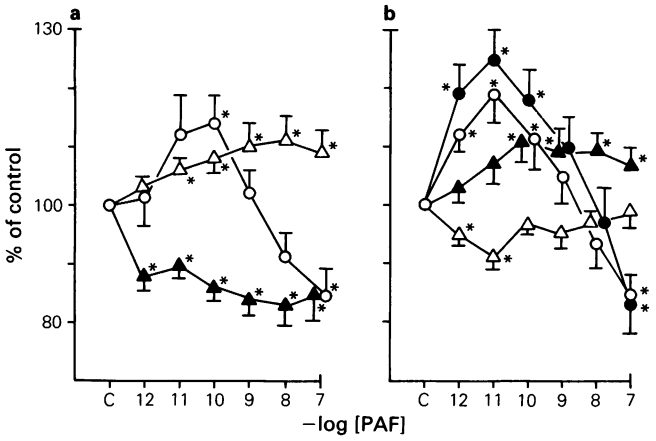

Figure 1 (a) Effect of PAF on peak contractile force (○) and rate (△) of spontaneous contractions and of the sinus node recovery time (▲) in isolated right atria. (b) Effect of PAF on peak contractile force (○), df/dt_{max} (●), time to peak tension (△) and time for total contraction (▲) in electrically drive left atria. Each point represents the mean of 8 experiments; vertical bars show the s.e.mean. $^*P < 0.05$. C = control.

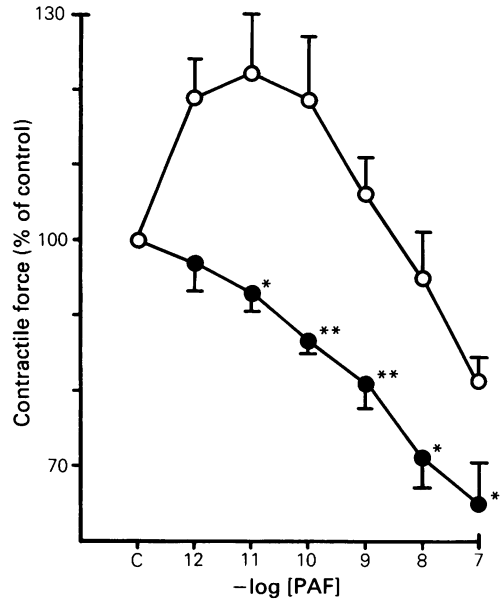

Figure 2 Effect of PAF on peak contractile force in electrically driven left atria in control conditions (○) and after pretreatment of the preparations with verapamil, 10^{-6} M (●). Each point represents the mean of 8 experiments, vertical bars show the s.e.mean. $^*P < 0.05$; $^{**}P < 0.01$.

trations lyso-PAF did not modify atrial rate or contractile force.

As the inotropic effects of PAF were associated with an increase in atrial rate, the effects of cumulative concentrations of PAF on different parameters of isometric contractions were also studied in 13 left atria driven at a constant rate of 1 Hz. Control values of isometric contractions were: peak contractile force 592.6 ± 73.6 mg, df/dt_{max} 20.4 ± 2.8 mg ms^{-1}, time to peak tension 57.3 ± 3.0 ms and time for total contractions 200.3 ± 15.9 ms. Figure 1b shows that PAF also caused a biphasic effect on peak contractile force similar to that observed in right atria. In fact, at low concentrations (10^{-12} M–10^{-10} M), PAF significantly increased peak contractile force as well as the df/dt_{max}, whereas at higher concentrations it produced a concentration-dependent negative inotropic action. The maximum positive inotropic effect was observed at 10^{-11} M PAF, which augmented peak contractile force by 18.8 ± 4.9% ($P < 0.01$) and df/dt_{max} by 25.4 ± 4.7% ($P < 0.001$). Both parameters were reduced by 15.5 ± 4.4% ($P < 0.01$) and 16.7 ± 5.3% ($P < 0.01$), respectively, at the highest concentration tested. At concentrations up to 10^{-10} M the positive inotropic effect was accompanied by a significant shortening of the time to peak tension (9.0 ± 2.4% at 10^{-11} M, $P < 0.05$), but this effect was partly reversed at higher concentrations. PAF also prolonged the time for total contraction which reached significant values at 10^{-10} M (11.4 ± 3.8%, $P < 0.05$) and remained constant at higher concentrations.

In both right and left atria the positive and negative inotropic effects of PAF were completely reversed by washing the preparations with drug-free Tyrode solution and the negative effect by increasing the extracellular Ca^{2+} concentration to 3.6 mM or by adding isoprenaline (10^{-6} M) to the bathing medium.

Effect of different pretreatments on inotropic actions of PAF

In the presence of 10^{-6} M propranolol, PAF exerted similar positive (+12.3 ± 2.9% as compared to +18.6 ± 8.6% at 10^{-11} M PAF, $n = 6$) and negative inotropic effects (−12.7 ± 2.9% as compared to −16.3 ± 10.5% at 10^{-7} M PAF, $P > 0.05$). Similar results were obtained in atria pretreated with atropine (10^{-6} M), indomethacin (10^{-6} M) or phentolamine (10^{-6} M).

The effects of verapamil were studied on the inotropic responses to PAF (Figure 2). Verapamil, 10^{-6} M, by itself, decreased peak contractile force by 33.7 ± 4.6% ($n = 8$, $P < 0.001$), completely abolished the positive inotropic effect produced by low concentrations of PAF, and potentiated the negative inotropic action induced by higher concentrations of PAF ($P < 0.05$). Thus, the dose-response curve to PAF was displaced downwards and to the left. Thus, the maximum negative inotropic effect of PAF in the absence and presence of verapamil was −18.7 ± 2.7% and −35.3 ± 5.6% ($P < 0.05$), respectively.

The PAF antagonist BN 52021 (10^{-7} M) (Braquet *et al.*, 1987) did not itself modify atrial contractile force, but suppressed both the positive inotropic effect of 10^{-11} M PAF (112.6 ± 2.0% of control in the absence of BN 52021 vs. 98.6 ± 1.9, $n = 7$, $P < 0.05$) and the negative inotropic effect of 10^{-7} M PAF (83.0 ± 2.2% of control in the absence of drug vs. 97.6 ± 2.2% of control, $n = 7$, $P < 0.01$).

Effect of extracellular concentrations of calcium and sodium on the inotropic actions of PAF

It is known that as the Ca^{2+} concentration of the bathing medium is increased, or Na^+ is decreased, Ca^{2+} influx and amplitude of contractions are augmented (Niedergerke, 1963). The influence of PAF on inotropic responses induced by increasing extracellular Ca^{2+} concentrations, $[Ca^{2+}]_o$, was evaluated on a paired basis in 8 left atria. A stepwise increase in $[Ca^{2+}]_o$ from 0.9 to 9.0 mM dose-dependently increased atrial contractile force. At 10^{-11} M PAF significantly increased the positive inotropic effect of each $[Ca^{2+}]_o$ and thus shifted the concentration-response curve of Ca^{2+} upwards (Figure 3). At 10^{-7} M PAF significantly decreased the positive inotropic effect of each $[Ca^{2+}]_o$ and shifted the curve downwards and to the right. Thus at concentrations which produced a positive inotropic effect, PAF was able to potentiate the inotropic responses to Ca^{2+}, whereas negative inotropic concentrations decreased these responses.

In another group of experiments the inotropic actions of PAF were studied in 6 left atria equilibrated for 30 min in Tyrode solution with Na^+ concentration reduced to 70%. In 70% Na^+ solution, peak contractile force was significantly increased over values obtained in normal Tyrode solution (759.4 ± 79.9 mg vs 561.4 ± 68.8 mg, $n = 6$, $P < 0.05$). Under these conditions PAF again exerted a biphasic effect on contractile force. However, when the effects observed in normal and 70% Na^+ solution were compared (Figure 4) it was observed that the maximum positive inotropic effect of

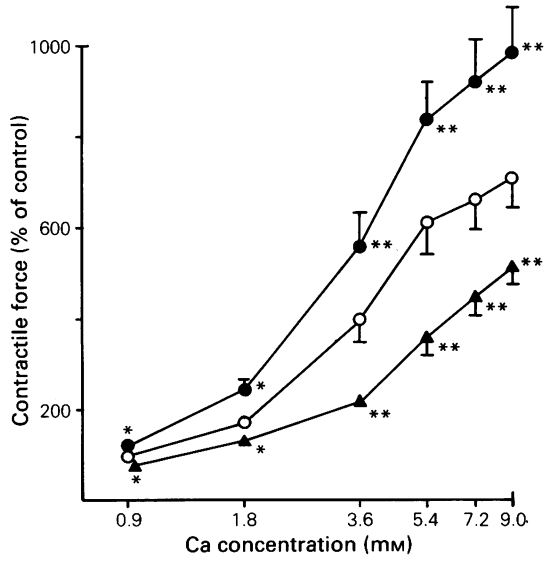

Figure 3 Effect of PAF on the calcium-induced positive inotropic effect in electrically driven left atria. Each point represents the mean of 8 experiments; vertical bars show the s.e.mean. (○) Control; (●) PAF, 10^{-11} M; (▲) PAF, 10^{-7} M. *$P < 0.05$; **$P < 0.01$.

10^{-11} M PAF was similar in both media ($+17.3 \pm 5.2\%$ change in contractile force as compared to $+12.8 \pm 6.1\%$), whereas at 10^{-7} M the negative inotropic effect observed in 70% Na^+ solution was significantly reduced when compared with that observed in normal Tyrode solution ($-5.7 \pm 25\%$ in contractile force as compared to $-14.8 \pm 4.1\%$, $P < 0.01$).

Effect of PAF on transmembrane action potentials

The electrophysiological effects of PAF in a range of concentrations between 10^{-11} M and 10^{-7} M, were studied on atrial transmembrane action potentials in 9 atria driven at a basal rate of 1 Hz. In contrast to ventricular muscle fibres, where PAF shortened the action potential duration (Camussi *et al.*, 1984; Tamargo *et al.*, 1985), PAF had no effect on resting membrane potential, amplitude, maximum rate of depolarization (V_{max}) or action potential duration at any of the concentrations tested.

Effect of PAF on slow responses

In order to ascertain the effects of PAF on the slow inward current, the fast inward Na^+ current was voltage-inactivated by a partial depolarization by addition of 27 mM K^+ to the Tyrode solution and atria become inexcitable despite intense electrical stimulation. Addition of isoprenaline (10^{-6} M) to the bath restored electromechanical activity, i.e., slow action potentials and contractions, of the atria when they were stimulated at a basal rate of 0.2 Hz. In 8 right atria, peak contractile force of slow contractions induced by isoprenaline averaged 480.2 ± 48.9 mg. Addition of PAF produced an initial, non-significant, increase in peak contractile force ($6.1 \pm 3.9\%$ at 10^{-11} M), followed by a concentration-dependent decrease which reached significant values at concentrations higher than 10^{-9} M (Figure 5). This effect of PAF was rapidly reversed by increasing the Ca^{2+} concentration in the bathing medium. In contrast, under similar experimental conditions PAF (10^{-11} M, 10^{-10} M and 10^{-7} M) had no effect on amplitude, V_{max} and duration of the slow action potentials or on the resting membrane potential of atrial fibres depolarized to -46.3 ± 1.2 mV ($n = 6$).

Effect of PAF on $^{45}Ca^{2+}$ uptake and $^{45}Ca^{2+}$ efflux

The effects of PAF (10^{-11} M to 10^{-7} M) on $^{45}Ca^{2+}$ uptake were studied in paired atria driven at a basal rate of 1 Hz and maintained in labelled solution for different time periods. As is shown in Figure 6, an increase in the incubation period in radioactive solution from 2 to 5 and 10 min augmented $^{45}Ca^{2+}$ uptake progressively from 0.526 ± 0.065 to 0.896 ± 0.137 and to 1.130 ± 0.105 mmol kg^{-1}. PAF, 10^{-11} M and 10^{-9} M, significantly increased $^{45}Ca^{2+}$ uptake by $51.8 \pm 5.4\%$ and $48.0 \pm 3.9\%$, respectively ($P < 0.01$) after incubation for 10 min but no significant modifications were observed with incubations of only 2 and 5 min. However, at 10^{-7} M, PAF did not modify $^{45}Ca^{2+}$ uptake at any time interval. Table 1 shows that the increase in $^{45}Ca^{2+}$ uptake after

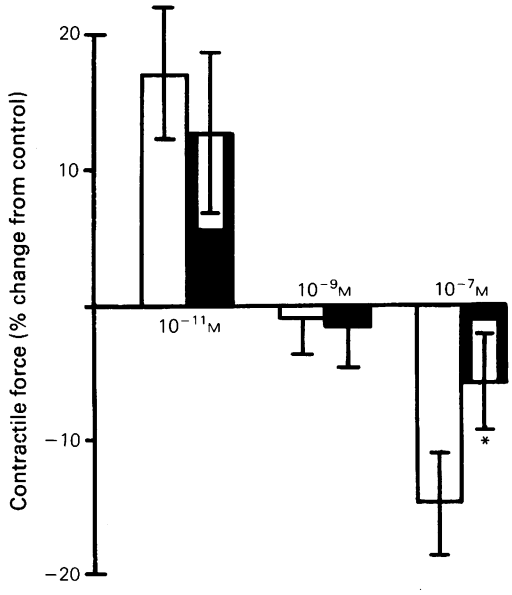

Figure 4 Comparison of inotropic effects induced by PAF (10^{-11} M, 10^{-9} M and 10^{-7} M) in electrically driven left atria incubated in normal Tyrode solution (open columns) or in 70% Na^+ Tyrode solution (solid columns) expressed as percentage change from control contractile force. Values are the mean of 6 experiments; vertical bars show s.e.mean. *$P < 0.01$.

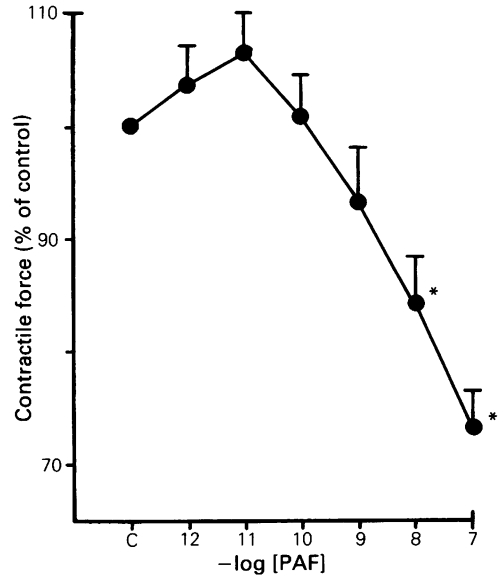

Figure 5 Effect of PAF on slow contractions induced by isoprenaline (10^{-6} M) on K^+ (27 mM)-depolarized atria driven at 0.2 Hz. Each point represents the mean of 8 experiments; vertical bars show the s.e.mean. *$P < 0.05$.

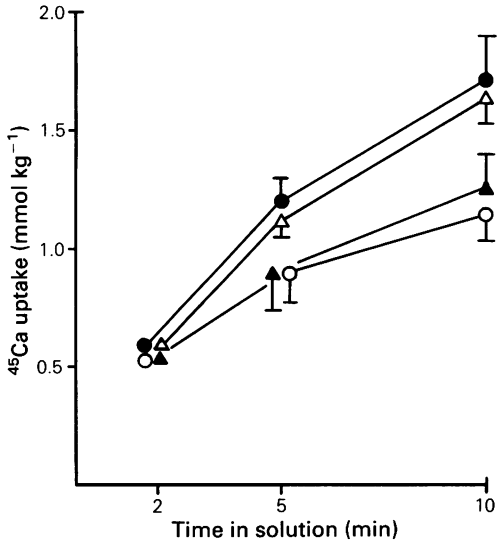

Figure 6 Effect of PAF on $^{45}Ca^{2+}$ uptake in electrically driven left atria. Each point represents the mean of 6 to 18 experiments; vertical bars show the s.e.mean. (○) Control; (●) PAF, 10^{-11} M; (△) PAF, 10^{-9} M; (▲) PAF 10^{-7} M. *$P < 0.01$.

10 min incubation produced by 10^{-11} M PAF was inhibited in atria pretreated with verapamil (10^{-6} M) or BN 52021 (10^{-7} M). The effect of PAF (10^{-11} M and 10^{-7} M) on $^{45}Ca^{2+}$ efflux was evaluated in left atria driven at a basal rate of 1 Hz and incubated in $^{45}Ca^{2+}$-labelled Tyrode solution for 2 h. In 4 atria PAF, 10^{-7} M, modified neither the percentage of $^{45}Ca^{2+}$ remaining in the atria nor the k_{cm} for $^{45}Ca^{2+}$ efflux from the tissue (data not shown). Figure 7 shows the effect of 10^{-11} M PAF on $^{45}Ca^{2+}$ remaining and k_{cm} when added from min 20 to the end of the washout period. In 6 atria the percentage of $^{45}Ca^{2+}$ remaining in the control atria after 120 min of washout was 3.59 ± 0.58%, whereas in the experimental preparations it was 4.96 ± 1.05. Moreover, PAF induced an upward shift of the washout curve, i.e., originated a decrease in $^{45}Ca^{2+}$ efflux from atrial cells. This effect was accompanied by a decrease of k_{cm} which reached significant values during the first 20 min following drug application (see inset in Figure 7). Therefore, the increase in uptake previously described can be interpreted as due to an increase of $^{45}Ca^{2+}$ influx.

Table 1 $^{45}Ca^{2+}$ uptake after 10 min incubation in radioactive solution in control left atria and in atria treated with PAF (10^{-11} M) and verapamil (10^{-6} M) or BN 52021 (10^{-7} M) plus PAF

Drug	n	$^{45}Ca^{2+}$ uptake
Control	6	1.233 ± 0.167
PAF	8	1.811 ± 0.152*
Verapamil + PAF	8	1.274 ± 0.123**
BN 52021 + PAF	6	0.892 ± 0.221**

*$P < 0.05$ vs. control; **$P < 0.01$ vs. PAF.
n = number of experiments.

The semilogarithmic plot of percentage of $^{45}Ca^{2+}$ remaining against time did not become linear even after 120 min of washout. Therefore, and to study further the effect of PAF on $^{45}Ca^{2+}$ efflux, the empirical approach of Reuter & Seitz (1968) was used. They found that the rate efflux of $^{45}Ca^{2+}$ from guinea-pig atria in Ca^{2+}-free washout medium was a function of the square of the amount of Ca^{2+} remaining in the tissue. Such a relationship would produce a straight line when the k_{cm} of the $^{45}Ca^{2+}$ efflux is plotted against the percentage of $^{45}Ca^{2+}$ remaining in atria, which expresses the rate of efflux as a function of the amount of isotope remaining in the tissue. As shown in Figure 8, this relationship fitted well to a straight line both in the absence ($r = 0.9634$, $P < 0.001$) and in the presence of PAF, 10^{-11} M ($r = 0.8696$, $P < 0.001$) but PAF produced a parallel shift to the right of the straight line. Because this shift is independent of the percentage of $^{45}Ca^{2+}$ remaining in the tissue, it may be concluded that the rate coefficient for $^{45}Ca^{2+}$ efflux in the presence of PAF is a constant. The shift was determined at each of the 14 plotted points on the control curve with a linear interpolation to obtain corresponding values in the presence of PAF. The value obtained for the PAF-induced shifts was 0.0101 ± 0.0013 min^{-1} ($P < 0.001$), which means that the drug decreased the rate coefficient to this extent independently of the percentage of $^{45}Ca^{2+}$ remaining in the atria.

Discussion

The results of the present paper demonstrate that both in spontaneously beating and in electrically driven guinea-pig

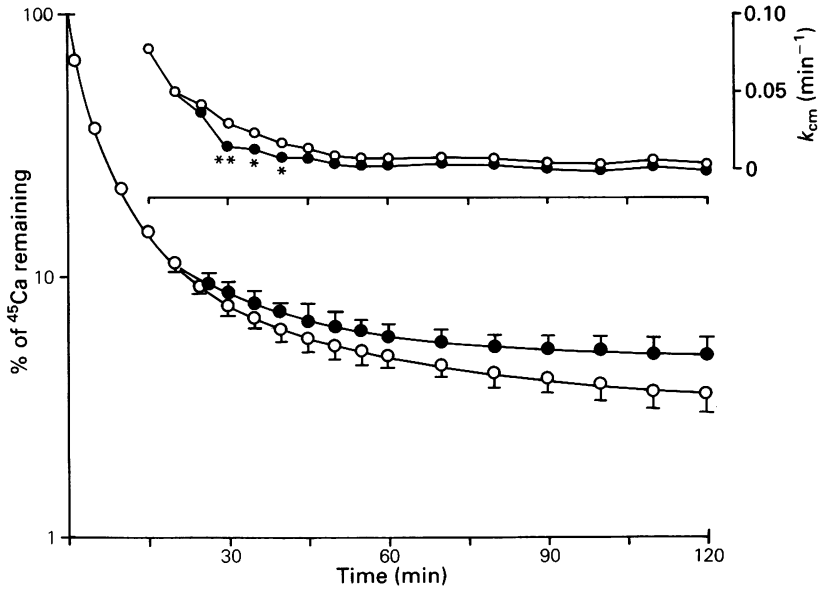

Figure 7 Effect of PAF (10^{-11} M) on $^{45}Ca^{2+}$ efflux in left atria after 2 h incubation in radioactive Tyrode solution. The inset shows the effect of PAF on the rate coefficient for $^{45}Ca^{2+}$ efflux (k_{cm}, min^{-1}). Each point represents the mean of 6 experiments; vertical lines shown s.e.mean. (○) Control; ● PAF, 10^{-11} M. *$P < 0.01$; **$P < 0.001$.

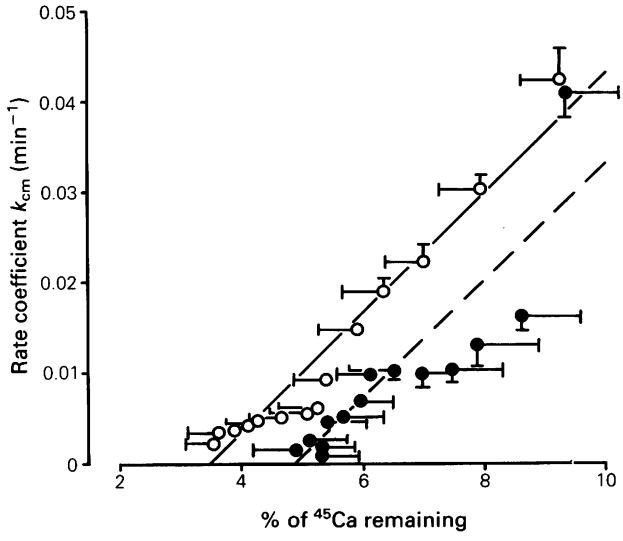

Figure 8 Relationship between the rate coefficient of $^{45}Ca^{2+}$ efflux (k_{cm}, min^{-1}, ordinate scale) and the percentage of $^{45}Ca^{2+}$ remaining in the guinea-pig atria in the absence of (○, $r = 0.9634$, $P < 0.001$) and in the presence of 10^{-11} M PAF (●, $r = 0.8696$, $P < 0.001$). Data were taken from experiments of Figure 7.

left atria, PAF exhibited a biphasic inotropic effect. Thus, low concentrations of PAF (10^{-10} M) produced a positive inotropic effect whereas at higher concentrations it produced a negative inotropic effect. Changes in contractility were accompanied by a moderate increase in atrial rate and a shortening of the SNRT, which suggests that PAF may exert a stimulatory effect on cardiac pacemakers (Tamargo *et al.*, 1985; Alloatti *et al.*, 1988). These inotropic responses are independent of coronary supply, the presence of formed blood elements and frequency variations since they were almost similar both in noncoronary-perfused right and left atria. Pretreatment with phentolamine, propranolol, atropine or indomethacin did not modify these responses, thus indicating that they were not mediated through cardiac α- or β_1-adrenoceptors, by muscarinic mechanisms or through prostaglandin synthesis. However, they were suppressed by the specific PAF antagonist BN 52021 (Braquet *et al.*, 1987), which suggests that PAF exerts direct and specific effects on atrial muscle fibres.

Even though the mechanism(s) responsible for the cardiac effects of PAF are unknown, they can be partly explained through modifications of trans-sarcolemmal Ca^{2+} fluxes. Thus, pretreatment with verapamil suppressed the positive inotropic effect of PAF. Previous evidence has demonstrated that verapamil prevented different effects of PAF both in isolated cardiac preparations and in anaesthetized animals (Tamargo *et al.*, 1985; Alloatti *et al.*, 1987). Furthermore, as previously described for platelets (Lee *et al.*, 1981; Valone & Johnson, 1985), macrophages (Braquet *et al.*, 1987) and ventricular papillary muscles (Tamargo *et al.*, 1988), low concentrations of PAF also increased $^{45}Ca^{2+}$ uptake in atrial fibres, this effect being suppressed in atria pretreated with verapamil or BN 52021. Since verapamil inhibits Ca^{2+} entry through voltage-dependent Ca^{2+} channels (Reuter, 1983) but had no effect on the Na^+-Ca^{2+} exchange mechanism (Coraboeuf *et al.*, 1981), our data would suggest that the increase in $^{45}Ca^{2+}$ uptake induced by PAF may be related to an enhanced Ca^{2+} influx via slow channels. Nevertheless because verapamil also exhibits PAF receptor antagonist properties (Tuffin & Wade, 1985) it is difficult to ascertain whether its inhibitory effects on contractility and $^{45}Ca^{2+}$ uptake are due to a specific Ca^{2+} antagonism or to a competition at the cardiac receptor for PAF. In this regard the finding that in contrast to PAF antagonists, verapamil abolished the positive but potentiated the negative inotropic effect of PAF, may suggest that it acts more as a Ca^{2+} antagonist than as an antagonist of atrial PAF receptors. However, despite all this evidence, since PAF had no effect on the V_{max} of the slow action potentials elicited in K^+-depolarized atria, a direct index of cardiac Ca^{2+} current (Malecot & Trautwein, 1987), it seems unlikely that an increase in Ca^{2+} entry via the slow channels can be responsible for its positive inotropic effect. At higher concentrations ($>10^{-9}$ M) PAF decreased atrial contractility as previously reported (Levi *et al.*, 1984; Alloatti *et al.*, 1987; Robertson *et al.*, 1987). In ventricular muscle fibres where PAF shortened the action potential duration (Camussi *et al.*, 1984; Tamargo *et al.*, 1985; Robertson *et al.*, 1988) this negative inotropic effect has been attributed to the reduction in the time available for Ca^{2+} entry through the slow channels (Camussi *et al.*, 1984; Tamargo *et al.*, 1985) or to a reduced Na^+ influx (Robertson *et al.*, 1988). However, both explanations seem unlikely in atrial fibres where PAF had no effect on duration and V_{max}, an indirect index of the fast inward Na^+ current of the atrial action potential. Moreover, some evidence suggests that the negative inotropic effect of PAF may be due to a decrease in Ca^{2+} entry through the slow channels. Thus, verapamil potentiated this negative inotropic effect and at the same range of concentrations at which it exerted its negative inotropic effect, PAF also decreased the amplitude of the slow contractions. Furthermore, the negative inotropic effect was reversed by adding isoprenaline, which increased the slow inward Ca^{2+} current in atrial fibres (Reuter, 1983). However, because at this range of concentrations PAF did not decrease the V_{max} of the slow action potentials it appears unlikely that inhibition of atrial contractility can be related to a decrease in Ca^{2+} entry through the slow channels.

If changes in Ca^{2+} entry through slow channels are not responsible for the inotropic effects of PAF, entry of Ca^{2+} by passive perfusion down its concentration gradient or in exchange for Na^+ are two alternative and viable possibilities. The contractile force of guinea-pig isolated atria is a function of $[Ca^{2+}]_o$ (Scholz, 1969). Low concentrations of PAF shifted to the left the curve relating tension to $[Ca^{2+}]_o$, so that less Ca^{2+} is required to produce the same amount of tension. Thus, it is possible that PAF could enhance atrial contractility acting on the cardiac sarcolemma increasing its conductance for Ca^{2+}. The inwardly directed transmembrane electromechanical gradient for Ca^{2+} ions would result in an increased net influx of Ca^{2+} into the cell which could then potentiate the contractile effect of Ca^{2+} entering during the cardiac action potential. Such an effect may explain an increased net extra influx of Ca^{2+} independent of the slow channels as well as the PAF-induced increase of $^{45}Ca^{2+}$ uptake reported in platelets (Valone & Johnson, 1985) where there is no evidence of voltage-dependent Ca^{2+} channels (Doyle & Ruegg, 1985). This increase in Ca^{2+} entry together with the decrease in $^{45}Ca^{2+}$ efflux would lead to an increase in $[Ca^{2+}]_i$ and contractile force. The opposite results were found at concentrations of PAF producing a negative inotropic effect, thus suggesting that it may reduce Ca^{2+} entry into atrial fibres. The negative inotropic effect was reduced when the Ca^{2+} concentration was increased or Na^+ concentration in the bathing media was decreased, i.e. when Ca^{2+} entry into atrial fibres was increased (Niedergerke, 1963; Langer, 1973). These results suggest not only that Ca^{2+} antagonized the negative inotropic effect of PAF but also that this effect can be related to a decrease in Ca^{2+} entry into atria.

In conclusion, this study suggests that in guinea-pig atria the inotropic effects of PAF may be related to changes in trans-sarcolemmal Ca^{2+} entry. Since both the inotropic effects and the increase of $^{45}Ca^{2+}$ uptake induced by PAF were abolished by BN 52021, a specific antagonist of PAF receptors, these results suggest that in atrial fibres, PAF exerts direct effects which seem to be mediated by specific receptors possibly similar to those described in platelets (Hwang & Lam, 1986).

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Cerebrovascular responses to capsaicin *in vitro* and *in situ*

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1 The cerebrovascular effects of capsaicin have been examined *in vitro*, in feline isolated cerebral arteries (circular segments, 2–3 mm long, 300–400 μm extended diameter) and, *in situ*, in pial arterioles (diameter 40–200 μm) on the cortical surface of chloralose-anaesthetized cats.

2 In isolated middle cerebral arteries, low concentrations of capsaicin (10^{-14}–10^{-10} M) effected a concentration-dependent relaxation of vessels precontracted with prostaglandin $F_{2\alpha}$. This relaxant response was markedly attenuated by repeated administration of capsaicin but was minimally affected by the presence of atropine, propranolol, cimetidine or spantide in the tissue bath.

3 In isolated middle cerebral arteries, higher concentrations of capsaicin effected a marked concentration-dependent contraction. This contraction was not modified by 10^{-6} M phentolamine or 10^{-6} M ketanserin. A markedly reduced contraction by capsaicin was found upon the removal of calcium ions from the buffer solution. Also the calcium entry blocker nimodipine reversed the capsaicin-induced contraction.

4 Subarachnoid perivascular microapplication of capsaicin around individual pial arterioles *in situ* elicited a biphasic response (an immediate vasoconstriction followed by a sustained vasodilatation). The maximum vasoconstriction was a 60 ± 6% reduction in diameter from base line and the maximum vasodilatation a 38 ± 7% increase in diameter. Vasodilatation occurred at lower concentrations of capsaicin (EC_{50}, approximately 5×10^{-8} M) than those required for vasoconstriction (EC_{50} 3×10^{-7} M).

5 Trigeminal ganglionectomy 10–16 days before the microapplication abolished the *in situ* vasodilator effects of capsaicin (10^{-6} M) applied perivascularly, but was without effect on the vasoconstrictor actions of this agent.

6 Repeated administration of capsaicin (10^{-6} M) around the same arteriole resulted in a progressive attenuation of the vasodilator phase of the response, with no modification of the vasoconstrictor phase.

7 The present study suggests that capsaicin-induced cerebral vasodilatation is due to the release of vasoactive agents from cerebrovascular trigeminal nerve fibres, whereas the vasoconstrictor effect of capsaicin is due to a direct effect on the cerebral vasculature which is mediated via the transmembrane passage of extracellular calcium.

Introduction

Cerebral blood vessels are innervated by a population of small diameter unmyelinated nerve fibres which originate in the trigeminal ganglion (Mayberg *et al.*, 1984). The trigemino-cerebrovascular nerve fibres and perikarya in the trigeminal ganglion contain, often co-localized in the same cellular elements, substance P, calcitonin gene-related peptide (CGRP), cholecystokinin, dynorphin B, neurokinins A and B, as well as putative precursors such as preprotachykinin A (Edvinsson, 1985; Uddman *et al.*, 1985; Hanko *et al.*, 1985; Liu-Chen *et al.*, 1985; Saito & Goto, 1986; Moskowitz *et al.*, 1986; McCulloch *et al.*, 1986; Edvinsson *et al.*, 1987a,b,c). The trigemino-cerebrovascular system has long been considered the primary sensory afferent system involved in the transmission to the CNS of nociceptive information of a vascular origin. However, many of the peptides which are present in trigemino-cerebrovascular fibres are vasoactive in the cerebral circulation (Edvinsson *et al.*, 1987a,b; Edvinsson & Jansen, 1987) and recent evidence suggests that this system is of major vasomotor significance in restoring normal cerebrovascular calibre after excessive vasoconstriction (McCulloch *et al.*, 1986).

Capsaicin, 8-methyl-N-vanillyl-6-nonenamide is a pungent constituent of some red peppers and its uses, both chronically, as a neurotoxin, and acutely to release peptides, in the elucidation of the function of primary sensory afferent neurones, is well established (Nagy, 1982). As in peripheral blood vessels (Nagy, 1982; Furness *et al.*, 1982; Wharton *et al.*, 1986), chronic administration of capsaicin results in a specific depletion of perivascular substance P- and CGRP-immunoreactivity from cerebral vessels (Duckles & Buck, 1982; Duckles & Levitt, 1984; Saito & Goto, 1986; Saito *et al.*, 1988). In peripheral blood vessels, the acute administration of capsaicin produces a sustained vasodilatation and increased vascular permeability, which are putatively mediated via the release of neuropeptides from sensory nerve endings (Lembeck, 1983). Acute capsaicin administration is now being extended to cerebrovascular research to explore the influence of the trigeminal system upon blood-brain barrier permeability (Reid & McCulloch, 1987). Capsaicin has, however, a diverse range of effects on smooth muscle. In some arterial preparations *in vitro*, capsaicin provokes contraction whereas in other vessels, capsaicin elicits relaxation (Toda *et al.*, 1972; Duckles, 1986). In guinea-pig ureter, the effects are dose-dependent, with low concentrations causing an inhibition of ureter motility and high concentrations stimulating motility (Hua *et al.*, 1985; 1986). In the present study, we have characterized in detail the cerebrovascular effects of capsaicin by use of sensitive *in vitro* and *in situ* methods and the insight gained adds to our understanding of the functional significance of the trigemino-cerebrovascular system.

Methods

Responses of middle cerebral artery segments in vitro

Ten adult cats (weighing 2–4 kg) were exsanguinated under pentobarbitone anaesthesia (Nembutal 30 mg kg^{-1} i.p.). The brains were removed, the middle cerebral arteries dissected free and placed in cold Krebs-Ringer solution aerated with

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95% O_2 plus 5% CO_2. The composition of the buffer solution was (mM): NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 15, NaH_2PO_4 1.2 and glucose 11, pH 7.4. Circular vessel segments, 2–3 mm long, were mounted between two L-shaped metal prongs in 2.5 ml mantled tissue baths at 37.5°C for recording of vasomotor activity (Högestätt *et al.*, 1983). Isometric tension was measured with Grass FT03C force displacement transducers and recorded on a Grass Polygraph. The segments were given a passive load of 4 mN, allowed to attain a steady level of tension and to stabilize for 90 min before testing. In an initial set of tests each vessel segment was first exposed to a buffer solution containing 60 mM potassium (achieved by an equimolar substitution of NaCl for KCl in the above buffer solution). This resulted in strong contractions, which in the present series of experiments amounted to 14 ± 3 mN ($n = 19$). In experiments performed in Ca^{2+}-free buffer the calcium was replaced with 0.1 mM EGTA (ethylene glycol-bis (β-aminoethyl ether)-N,N,N,N,-tetraacetic acid). After an incubation time of 0.5–1 h, during which the Ca^{2+}-free buffer in the tissue bath had been exchanged every 5 min, capsaicin was given in cumulative (10^{-9}–10^{-6} M) concentrations until maximum contraction was achieved. In subsequent tests we were only able to observe a contractile effect of capsaicin (see Saito *et al.*, 1988). These contractile responses to capsaicin were reproducible. When relaxation of the vessels was being examined, by cumulative application of capsaicin in the concentration range 10^{-14}–10^{-10} M, the vascular segments were first constricted by the addition of 3×10^{-6} M prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)(EC_{80}), which resulted in a contraction that was stable for at least 30 min. However, in vessels precontracted by potassium chloride (60 mM) no vasodilator response was observed.

In some experiments the effect of capsaicin was examined in the presence of different specific receptor antagonists, each given approximately 20 min before and being present during the tests.

Exposure to capsaicin was performed only once. Experiments with antagonists were performed in parallel tests, six vessel segments were run in separate tissue baths in order to avoid tachyphylaxis or depletion of stored neurotransmitter, with one or two segments serving as control.

The integrity of the endothelium in the present set-up was assessed in separate experiments where vasodilator responses to acetylcholine (10^{-9}–10^{-4} M) in $PGF_{2\alpha}$-precontracted vessel segments were found to be unaltered (60 ± 7% of the $PGF_{2\alpha}$-induced contraction).

Vasomotor responses of pial arterioles in situ

Eight cats (weighing 3–4 kg) were anaesthetized with a mixture of alphaxolone (6.75 mg kg^{-1}) and alphadolone acetate (2.25 mg kg^{-1}) i.v., intubated and connected to an intermittent positive-pressure ventilation system delivering room air in an open circuit. The right femoral artery and vein were cannulated. Anaesthesia was maintained during the subsequent course of the experiments with α-chloralose (60 mg kg^{-1} i.v.). The animals were maintained normocapnic (arterial carbon dioxide tension, P_aCO_2, close to 32 mmHg throughout the course of the experiments). The end-tidal concentration of carbon dioxide was monitored continuously by means of an infra-red analyzer, and samples of arterial blood were taken frequently during the experiments for the estimation of arterial oxygen tension, P_aCO_2 and pH. In each cat, the mean arterial blood pressure was always greater than 80 mmHg. Rectal temperature was maintained at 38°C with a heating blanket. The animals were placed in a stereotaxic frame, and a craniotomy, measuring 2.5 cm × 1.5 cm, was made over the left parietal cortex. The dura was removed carefully and any bleeding from the cut dural edges was sealed by bipolar diathermy. The exposed cortex was bathed by prewarmed liquid paraffin maintained at 38°C. Vascular calibre was measured by an image-splitting technique (Baez, 1966). Individual pial vessels on the convexity of the brain were viewed in focus through a microscope, and the image was passed through a Vickers image-splitting eye-piece to a closed circuit television camera and displayed on a television monitor. Vascular diameter was measured from the degree of shear applied to the image splitter that had been calibrated against wire and thread of known diameter. Capsaicin was dissolved in artificial cerebrospinal fluid immediately before use. The composition of the artificial cerebrospinal fluid was (mM): NaCl 133, KCl 3, $CaCl_2$ 2.5, $NaHCO_3$ 12 and glucose 3.3. It was adjusted to pH 7.2 by aeration with 5% CO_2 and 95% O_2. Glass micropipettes were filled with artificial cerebrospinal fluid under mineral oil. By use of a micromanipulator, the micropipettes were inserted through the arachnoid into the perivascular space close to a cerebral arteriole. For further details see Harper & MacKenzie (1977). Approximately 5 μl of artificial cerebrospinal fluid was injected into the perivascular space over 15 s, and any resulting alterations in vascular calibre were monitored until the vessel diameter was restored, often for periods of up to 5 min following the injection. The maximum changes in calibre in response to the perivascular microinjection of the drug (expressed as % changes from the diameter of the vessel before drug administration) were compared with those following the administration of artificial cerebrospinal fluid alone. Microinjection was usually made only on one occasion at each site.

Surgical division of the trigeminal nerve

In five cats anaesthetized with pentobarbitone, the trigeminal nerve was unilaterally divided under sterile operating conditions by a neurosurgeon (T.A.K.) using a previously described technique (Liu-Chen *et al.*, 1983). The temporal muscle was resected to expose the calvarium and a subtemporal craniectomy was made with a dental drill. Each division of the left trigeminal nerve was surgically sectioned immediately distal to the trigeminal ganglion. Care was taken not to enter the subdural space and to prevent bleeding from the bone and cavernous sinus. In five sham-operated animals the nerve was exposed, but not divided. Post-operative recovery was generally excellent, with all animals eating normally within 48 h of the procedure. The vasomotor responses of pial arterioles *in situ* were investigated 10–16 days after surgery (see above).

Statistical analysis

Data are presented as mean values ± s.e.mean. Differences between the mean values were assessed by Student's t test and the Bonferroni correction factor was employed to maintain an α-level of 0.05 or smaller.

Drugs

Capsaicin (Sigma, U.S.A.), prostaglandin $F_{2\alpha}$ (Amoglandin, Astra, Sweden or Sigma, U.S.A.), atropine (Atropin, ACO, Sweden), propranolol (Inderal, ICI, U.K.), cimetidine (Sigma, U.S.A.), spantide (Ferring AB, Sweden), phentolamine (Regitin, Ciba-Geigy, Switzerland), ketanserin (Janssen, Belgium) and nimodipine (Bayer AG, F.R.G.) were used.

For the investigations *in vitro*, capsaicin was dissolved in ethanol (70%) and then further diluted in saline. Spantide was dissolved and diluted in saline containing 0.1 mM ascorbic acid. All other drugs were dissolved and/or diluted in saline. The concentrations given below are the final concentrations (M) in the tissue bath.

Results

Vasomotor responses of middle cerebral artery segments in vitro

Under the condition of resting tone, the administration of capsaicin markedly increased the tension developed by the

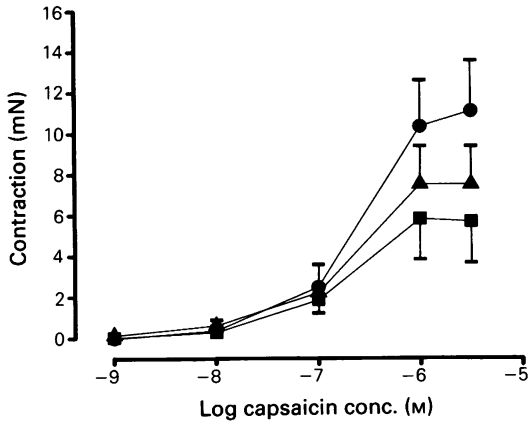

Figure 1 Contractions induced by capsaicin alone (●) or in presence of the α-adrenoceptor antagonist phentolamine (10^{-6} M) (▲), or the 5-hydroxytryptamine-antagonist ketanserin (10^{-6} M) (■). Mean values are shown with vertical lines indicating s.e.mean; number of vessels, 6–8.

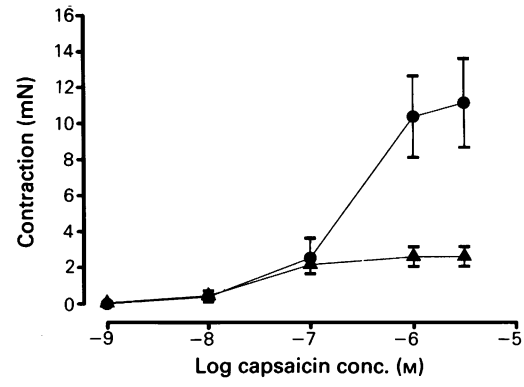

Figure 2 Contraction induced by capsaicin in Ca^{2+}-free media (▲) compared to control (●). Mean values are shown with vertical lines indicating s.e.mean; number of vessels, 5.

pial arteries. The threshold concentration of capsaicin for the induction of contraction was between 10^{-9} and 3×10^{-8} M (Figure 1) and the maximum contraction, at a concentration of 3×10^{-6} M was 11.1 ± 2.5 mN or 63.4 ± 12.1% of the contraction produced by potassium (60 mM). The contractile response to capsaicin was reproducible upon a second exposure (not shown). However, as the experimental design of this study was to avoid a second capsaicin exposure to a particular vessel segment, the antagonist studies were performed in studies in which six parallel tissue baths with one or two ring segments as control were utilized.

The contractions induced by capsaicin were minimally attenuated by the presence of phentolamine (10^{-6} M) or ketanserin (10^{-6} M) in the tissue bath (Student's t test; $P > 0.05$) (Figure 1). The contractions induced by capsaicin were significantly ($P < 0.05$) reduced in calcium-free medium (Figure 2). Nimodipine produced a concentration-related reversal of the contraction to capsaicin (at concentrations between 10^{-11}–10^{-6} M) with the maximum relaxation being 99.1 ± 0.6% and the IC_{50} value $7.6 \pm 3.6 \times 10^{-10}$ M.

The administration of prostaglandin $F_{2\alpha}$ (3×10^{-6} M) elicited a strong and stable contractile response (6.1 ± 1.1 mN, $n = 13$). The cumulative administration of capsaicin to precontracted cerebral arteries elicited a concentration-dependent relaxation in the majority of animals examined (7 of the 10 cats) (Figure 3). In arterial segments from the three remaining cats, capsaicin failed to relax the precontracted arteries despite the testing of multiple segments from each animal. The concentration of capsaicin producing half-maximum relaxation in the seven responding cats was $3.7 \pm 1.3 \times 10^{-14}$ M and the maximum relaxation was 24.5 ± 7.1% of the $PGF_{2\alpha}$-induced contraction. The cerebrovascular relaxation induced by low concentrations of capsaicin was unaffected by the presence of propranolol (10^{-7} M), cimetidine (10^{-6} M), atropine (10^{-4} M) or spantide (3×10^{-6} M) (Figure 4). Repeated exposure of cerebral arterial segments to capsaicin successively reduced the relaxant response to the agent (Figure 3). Furthermore, a slight contraction was seen with 10^{-10} M capsaicin.

Vasomotor responses of pial arterioles in situ

The perivascular microapplication of capsaicin around individual pial arterioles elicited time-dependent and concentration-dependent constrictions and dilatations. Capsaicin, particularly at higher concentrations, effected an immediate transient (duration less than one minute) reduction in arteriolar calibre, followed, two to three minutes after the microinjection, by a sustained increase in arteriolar calibre which was sustained for more than 15 min after the microinjection. A statistically significant dilatation of pial arterioles was observed with capsaicin (10^{-7}–10^{-5} M) whereas significant constriction was noted only with capsaicin (10^{-6} and 10^{-5} M) (Table 1). The concentration of capsaicin eliciting the half-maximal relaxation of pial arterioles was approximately 5×10^{-8} M whereas that for constriction was approximately 3×10^{-7} M (Figure 5, Table 1). The administration of the appropriate vehicle (CSF containing 0.8% Tween 80 and 0.2% ethanol) was without significant effect upon arteriolar calibre (mean alteration in calibre, -4.1 ± 1.5%, $n = 8$).

Repeated microapplication of capsaicin (10^{-6} M), every 15 min at the same site indicated that the initial vasoconstrictor response was unaltered by prior exposure to capsaicin, but that the delayed vasodilator component was markedly

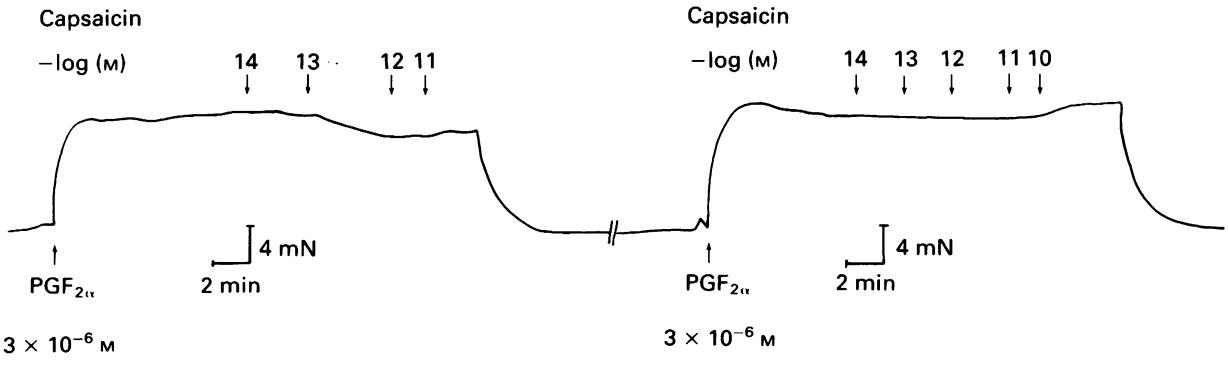

Figure 3 Typical response obtained when capsaicin was given to arteries precontracted by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Repeating the experiment reduced the relaxant response to capsaicin. Calibration bars are inserted. The concentrations of capsaicin, −log (M), are given above the traces.

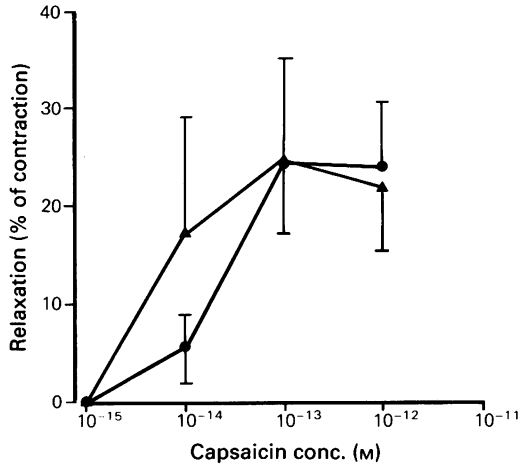

Figure 4 Capsaicin-induced relaxation of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-contracted vessels in the presence of the substance P antagonist [D-Pro^2, D-Trp^{7,9}]-SP (3×10^{-6} M) (▲) compared to control (●). Mean values are shown and vertical lines indicate s.e.mean; number of vessels, 4–6.

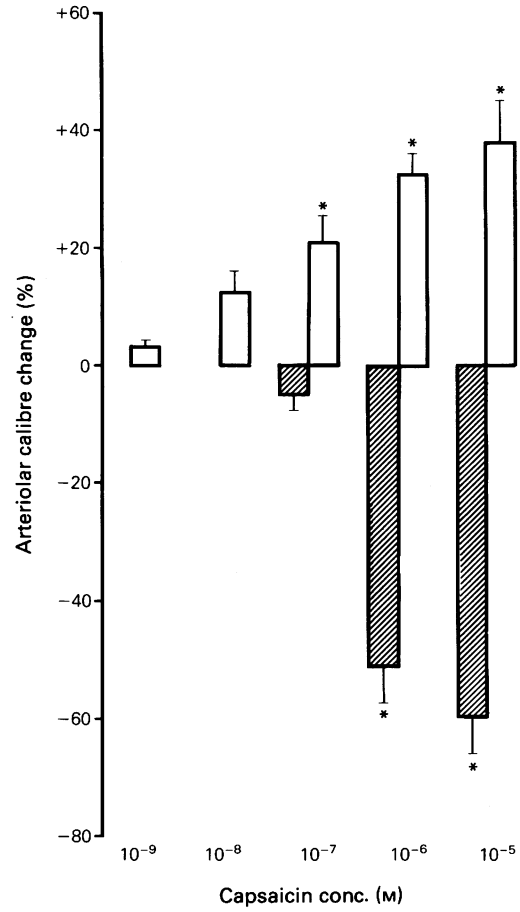

Figure 5 Vasomotor responses of feline pial arterioles *in situ* to perivascular microapplication of capsaicin eliciting concentration-dependent reduction followed by a sustained increase in arteriolar calibre. The maximal arteriolar calibre changes induced by the different capsaicin concentrations are shown; vertical bars indicate s.e.mean. For details on number of tests and statistics, see Table 1; *, $P < 0.05$. Shaded columns represent initial shortlasting constriction upon capsaicin administration; open columns, the more sustained increase in vessel calibre.

reduced after a single prior microapplication of capsaicin at the same site (Figure 6).

A separate series of experiments was performed in cats in which the ipsilateral trigeminal ganglion had been lesioned surgically 10–16 days before the microapplication study, and in cats subjected to a sham-operation. The perivascular microapplication of capsaicin (10^{-6} M) elicited an initial vasoconstriction of a similar magnitude in sham-operated and trigeminal-lesioned cats (Figure 7). However, the duration of the initial vasoconstriction in the sham-operated animals (time for half restoration of preinjection calibre 27 ± 4 s, $n = 12$) was significantly less ($P < 0.01$) than in cats with trigeminal lesions (time for half restoration of preinjection calibre 78 ± 13 s, $n = 11$). Moreover, no delayed vasodilatation was observed after perivascular microapplication of capsaicin (10^{-6} M) in cats in which the trigeminal nerve had been surgically lesioned before the micro-application study (Figure 7).

Discussion

In this detailed characterization of the cerebrovascular effects of capsaisin, the dual vasomotor effects of capsaicin upon cerebral arteries *in vitro* and arterioles *in situ* have been defined. A vasodilator effect was observed at lower concentrations of capsaicin and required the integrity of the trigeminal system. A vasoconstrictor effect of capsaicin was found at higher concentrations and this was not dependent upon the trigeminal system.

The acute administration of capsaicin is known to cause the release of neuropeptides such as substance P, neurokinin A and CGRP from perivascular nerve fibres (Duckles & Buck, 1982; Saria *et al.*, 1983; Franco-Cereceda & Lundberg, 1985; Hua *et al.*, 1986). Although the release from cerebral vessels *in vitro* of substance P and CGRP has been demonstrated with capsaicin administration (Moskowitz *et al.*, 1983; Saito & Goto, 1986), it seems likely in view of the evidence from other tissues that the release of a range of other neuropeptides (neurokinin A, neurokinin B, dynorphin, *inter alia*) may contribute to the cerebral vasomotor effects of capsaicin. Although capsaicin administration has been demonstrated to relax the thoracic aorta and carotid artery of the guinea-pig, only contractions of the cerebral vasculature have been shown with capsaicin (Toda *et al.*, 1972; Duckles, 1986; Saito *et al.*, 1988). In the present study, vasodilatation of cerebral blood vessels could be demonstrated both *in vitro* and *in situ* after

Table 1 Cerebral vasomotor effects to the microapplication of capsaicin *in situ*

Capsaicin conc. (M)	*Initial vasoconstriction*	*No. of arterioles responding/ No. of arterioles tested*	*Delayed vasodilatation*	*No of arterioles responding/ No. of arterioles tested*
10^{-9}	None	0/5	103.0 ± 0.9	0/5
10^{-8}	None	0/9	112.7 ± 3.6	4/9
10^{-7}	95.1 ± 2.5	1/6	121.3 ± 4.3*	6/6
10^{-6}	49.1 ± 6.1*	9/9	132.8 ± 4.1*	9/9
10^{-5}	39.9 ± 5.9*	9/9	138.0 ± 7.2*	9/9

Data on vessel calibre are expressed as a percentage of calibre before microinjection of an agent (i.e. 100%). Values given represent means ± s.e.mean. Statistical comparison between microapplication of cerebrospinal fluid and different concentrations of capsaicin were analysed; *$P < 0.05$ (derived from t statistic with use of Bonferroni's inequality).

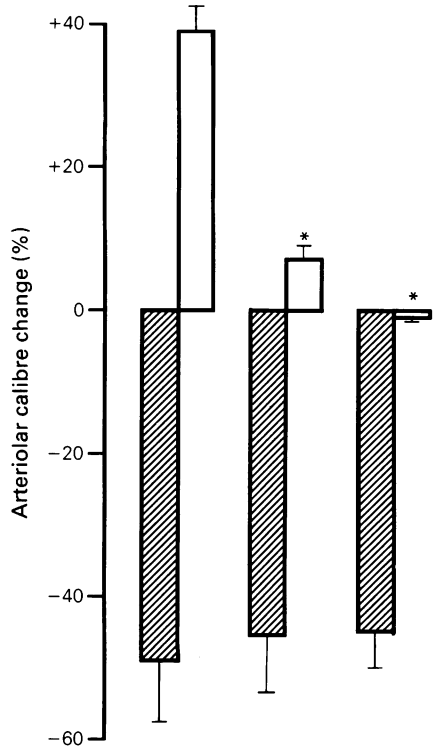

Figure 6 Vasomotor responses of feline pial arterioles *in situ* to repeated perivascular microapplication of capsaicin (10^{-6} M) every 15 min. The initial vasoconstrictor response (shaded columns) was unaltered while the delayed vasodilator response (open columns) was markedly reduced (Student's *t* test with Bonferroni correction; $*P < 0.05$). Arteriolar calibre changes are given and vertical bars show s.e.mean. Number of experiments, 8–9.

capsaicin administration. Notably the vasodilator response was seen at concentrations lower than those required to produce vasoconstriction. The loss of the vasodilator response to capsaicin after trigeminal lesions *in situ* or after repeated

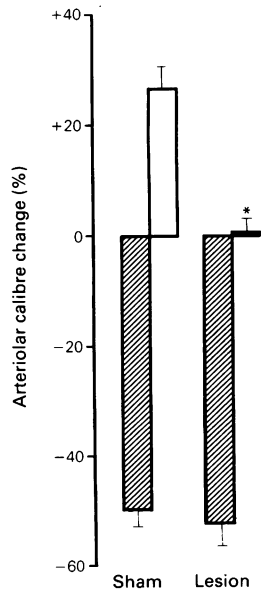

Figure 7 Vasomotor responses of feline pial arterioles *in situ*. The magnitude of the vasoconstrictor response (shaded columns) capsaicin (10^{-6} M) was unchanged while the delayed vasodilatation (open column) was abolished in cats in which the trigeminal nerve had been surgically lesioned before the microapplication study. Arteriolar calibre changes are given and vertical bars show s.e.mean. Number of experiments, 11–12. Student's *t* test; $*P < 0.01$.

exposure to the agent *in vitro* or *in situ* is consistent with the view, well established in peripheral tissue, that relaxation induced by capsaicin is produced indirectly via the release of vasoactive agents from the trigeminal nerve endings. The failure of previous studies *in vitro* to observe relaxation of cerebral vessels after capsaicin (Toda *et al.*, 1972; Duckles, 1986; Saito *et al.*, 1988) may be a consequence of the widespread use of depolarization with potassium, at the outset of the study, to assess the maximal contractile capacity of the vessel. This was avoided in the present study because high concentrations of potassium may effect the release of stored perivascular neurotransmitters such as substance P (Edvinsson *et al.*, 1983; Moskowitz *et al.*, 1983). In some cases we were unable to demonstrate the relaxation of middle cerebral arteries induced by capsaicin after exposure of the vessel to potassium (see Methods). Moreover, in vessels from a minority of cats (30%), no relaxation was observed upon capsaicin administration. This may reflect either the release of neuropeptides during the killing of the animal or interanimal variability in endogenous levels of neuropeptides. There is support for both views (see Uddman *et al.*, 1985; McCulloch *et al.*, 1986).

The trigemino-cerebrovascular system putatively contains substance P, neurokinin A, calcitonin gene-related peptide, gastrin-releasing peptide, dynorphin B and cholecystokinin (Edvinsson *et al.*, 1981; 1987a,c; Uddman *et al.*, 1983; 1985; Hanko *et al.*, 1985; Liu-Chen *et al.*, 1985; Moskowitz *et al.*, 1986). Substance P, neurokinin A and particularly CGRP are cerebral vasodilators, both *in vitro* and *in situ* (Edvinsson *et al.*, 1981; 1987a,c; McCulloch *et al.*, 1986), whereas gastrin-releasing peptides, dynorphin B and cholecystokinin have no direct effects on the cerebral vasculature (Uddman *et al.*, 1983; McCulloch & Kelly, 1984; Moskowitz *et al.*, 1986). The cerebrovascular effects of substance P and neurokinin A are markedly attenuated by the substance P analogue, spantide, which does not influence the effects of CGRP (Edvinsson & Jansen, 1987). The capsaicin-induced relaxation of cerebral vessels was not modified by atropine, cimetidine, propranolol or spantide. The weak relaxing effects of substance P on cat middle cerebral artery (Edvinsson *et al.*, 1981) compared to the stronger CGRP relaxation (Edvinsson *et al.*, 1985) suggest that it is the release of CGRP or an equally potent agent rather than substance P or neurokinin A which is responsible for the relaxation of the cerebral vessels elicited by capsaicin in the cat. Our recent observations (Jansen, Alafaci, Brodin, Edvinsson & Uddman, unpublished observations) that the relaxant response to capsaicin is not dependent on an intact endothelium, is further indicative of the involvement of a strong vasodilator such as CGRP in the response. However, one cannot exclude the possibility that, it is the release of some presently unknown material from the trigeminal nerve fibres that is crucial for the effect of capsaicin.

Vasoconstrictor responses to capsaicin have been seen in many smooth muscle preparations, although there is considerable variability between different tissues in their sensitivity to capsaicin (Toda *et al.*, 1972; Nagy, 1982; Wahlestedt *et al.*, 1984; Duckles, 1986). Capsaicin produces contractions of a similar magnitude to noradrenaline in canine mesenteric and renal arteries whereas in rabbit mesenteric or canine pulmonary arteries, capsaicin elicits only small contractions (less than 5% of that to noradrenaline). In contrast, the present study of cerebral vessels revealed that capsaicin is considerably more potent as a vasoconstrictor than noradrenaline (Wahl *et al.*, 1972; Edvinsson & Owman, 1974; Edvinsson *et al.*, 1982). In most tissues, the contractile effects of capsaicin are reproducible and, as in the present study, not dependent upon an intact sensory afferent innervation (persists after trigeminalectomy), although in some muscle preparations (e.g. pupillary sphincter), the contractile effect of capsaicin requires an intact trigeminal system (Wahlestedt *et al.*, 1984). Previous studies have shown that substance P and neurokinin A act as vasoconstrictors in this preparation while CGRP was ineffective but potentiated the substance P-induced contractions

(Wahlestedt *et al.*, 1986). The contractile effect of capsaicin in all tissues including the cerebral vasculature is dependent upon the mobilization of extracellular calcium, as shown here by reduced capsaicin contraction in calcium-free medium with EGTA and reversal of contraction by the calcium entry blocker nimodipine. Furthermore, phentolamine and ketanserin were without blocking effect. Our study complements that of Saito *et al.* (1988), who noted that capsaicin directly constricted cat cerebral arteries *in vitro* via a mechanism that was independent of endothelium and nerve components.

The present data have provided a detailed description of the vasomotor effects of capsaicin in the feline cerebral circulation. They describe not only the sensitivity and selectivity of capsaicin in this vascular bed but also the difficulties which are encountered (biphasic responses) when capsaicin is used *in vivo*.

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δ-Opioid receptor binding sites in rodent spinal cord

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1 The δ-opioid receptor agonist [D-Pen^2,D-Pen^5]enkephalin showed an antinociceptive effect in the mouse tail-flick test, following intrathecal administration. This action was reversed by naloxone and by the selective δ-opioid receptor antagonist ICI 174864.

2 High affinity, saturable binding of [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin has been demonstrated in spinal cord homogenates from guinea-pig, hamster, rat and both adult and young (18–20 g) mice. The binding was shown by autoradiography to be concentrated in the superficial laminae of the dorsal horn.

3 Competition studies confirmed that the binding of [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin was to the δ-opioid site. However, anomalies were seen with displacement assays using μ-ligands, which may suggest some common high affinity site for δ- and μ-opioid receptor agonists in the spinal cord.

4 The results add further evidence for a role of the δ-opioid receptor in spinally-mediated antinociception.

Introduction

Opioids have analgesic properties following intrathecal injection in man, by an action mediated at the spinal level (Yaksh & Noueihed, 1985). However, the spinal cord of several species including rat (Traynor & Wood, 1987), guinea-pig (Zarr *et al.*, 1986), rabbit (Meunier *et al.*, 1983) and man (Czlonkowski *et al.*, 1983) contains all three types, μ-, δ- and κ-, of major opioid-binding sites. Consequently the role of these various receptor types in spinally mediated antinociception has been of interest. Previous studies point to a role for μ-receptor activation in blocking nociceptive responses in various test models (Martin *et al.*, 1976; Yaksh, 1983) whilst κ-receptors may be mainly involved in modulating chemically-induced, but to a lesser extent thermally-induced responses (Schmauss & Yaksh, 1984; Porreca *et al.*, 1987b; Leighton *et al.*, 1988). A role for the δ-receptor is more controversial.

The stable enkephalin analogue [D-Ala^2,D-Leu^5]enkephalin (DADLE) is active in antinociceptive tests following intrathecal (i.t.) administration (Tung & Yaksh, 1982). However, this compound has much cross-reactivity with the μ-receptor and the results are thus difficult to interpret. More recent studies have employed the selective δ-opioid receptor agonist [D-Pen^2,D-Pen^5]enkephalin (DPDPE) (Mosberg *et al.*, 1983; Cotton *et al.*, 1985), although even with this ligand results are not conclusive. For example Rodriguez and colleagues (1986) have demonstrated an antinociceptive effect of intrathecal DPDPE in the rat, by an action blocked by the selective δ-opioid receptor antagonist ICI 174864 (Cotton *et al.*, 1984). However, a reported antinociceptive action in mice shows cross-tolerance with morphine, perhaps suggesting that the δ- and μ-agonists are acting at the same receptor (Porreca *et al.*, 1987a). This idea is supported by studies with the antagonist naloxone which blocks the antinociceptive effects of i.t. selective μ- and δ-opioid receptor agonists with similar affinity *in vivo* (Heyman *et al.*, 1986). Recently two contradictory findings have been obtained concerning the action of ICI 174864 in blocking the antinociceptive effect of i.t. DPDPE in the mouse. Heyman *et al.* (1987) demonstrated that this compound administered i.t. blocked the antinociceptive effects of i.t. DPDPE, but not the i.t. effects of the μ-selective ligand [D-Ala^2,MePhe^4, Glyol^5]enkephalin (DAMGO). However, Birch *et al.* (1987) were unable to antagonise i.t. DPDPE with i.t. ICI 174864, but the effects were blocked by low doses of i.t. naloxone suggesting an action at μ-receptors.

The reported differences in the specificity of action of DPDPE at the spinal level could be due to species or strain differences resulting in different receptor involvement, or methodological differences. In order to clarify the situation we have studied the antinociceptive action and binding properties of DPDPE in mouse spinal cord and compared this with binding in cords from other rodent species. A preliminary account of part of this work has been presented (Traynor *et al.*, 1988).

Methods

Behavioural tests

Male CFLP mice (18–20 g) (Interfauna plc) were used. Animals were housed in groups of twelve in a room with the temperature controlled at 21°C on a 12 h light-dark cycle and with free access to food and water.

[D-Pen^2,D-Pen^5]enkephalin (DPDPE) (2 μg) in saline (5 μl), or vehicle, was injected intrathecally (i.t.), by the direct lumbar puncture method of Hylden & Wilcox (1980), 10 min after the subcutaneous injection of naloxone (1 mg kg^{-1}), ICI 174864 (1 mg kg^{-1}) or saline vehicle (0.2 ml). Antinociception was determined 5 min after the intrathecal injections as follows:

Tail-flick test A beam of light from a 300 W quartz projection bulb was focused onto the tail and the time to withdrawal was recorded. The cut-off time for non-responding animals was 10 s.

Hot-plate test The latency to 'front-paw-lick' was measured for mice placed on a hot-plate maintained at 55 ± 0.2°C. The cut-off time for non-responding animals was 15 s.

Rotarod test Motor performance of the mice was assessed with an accelerating rotarod apparatus. The animals were placed on the stationary treadmill, following which the rotation speed was increased. The end-point was taken as the time at which the mice failed to stay on the rotating treadmill. Before the test sessions the mice had been trained to achieve a minimum level of proficiency of 150 s. This was taken as the cut-off point.

In the above experiments the order of testing the animals was tail-flick, hot-plate then rotarod. Tests were performed by

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an operator who was unaware of which treatment the animals were receiving.

Ligand binding assays

Spinal cord tissue from the following male animals was used: Dunkin-Hartley guinea-pigs (300–350 g), CFLP mice (18–20 g and 30–35 g), Syrian hamsters (200–250 g) and Wistar rats (200–250 g). All animals were supplied by Interfauna plc.

Homogenates of the above tissues were prepared and the binding experiments with [^{3}H]-DPDPE or [^{3}H]-DAMGO performed at 25°C for 40 min. The methods previously described (Cotton *et al.*, 1985) were employed except that HEPES (25 mM) buffer (pH 7.4) containing 10 mM $MgCl_2$ rather than Tris-HCl (50 mM) buffer was used. Specific binding was defined with naloxone (10 μM). Total bound ligand did not exceed 10% of added tritiated ligand.

Equilibrium dissociation constants (K_D) and binding capacities (B_{max}) were determined from Scatchard (1949) transformation of the specific binding data by use of the programme 'EBDA' (McPherson, 1985). Competition data were analysed by use of the programme 'ALLFIT' (DeLean *et al.*, 1978) with IC_{50} values converted to K_i values with the Cheng & Prusoff (1973) equation.

Autoradiography

This was performed essentially as described previously (Clark *et al.*, 1988). Briefly, fresh spinal cords were rapidly frozen, sectioned (10 μm) and thaw mounted onto gelatinised microscope slides. After being dryed, the slide-mounted sections were incubated with [^{3}H]-DPDPE (3–4 nM), in the presence or absence of naloxone (10 μM), for 60 min at 20°C in HEPES buffer containing 10 mM $MgCl_2$, then washed at 4°C in buffer (3 × 30 s) followed by a dip (2 s) in distilled water. After drying in a stream of cool air the slides were apposed to tritium-sensitive LKB Ultrafilm for 3 months before being developed.

Materials

[^{3}H]-DPDPE (34 Ci mmol^{-1}) and [^{3}H]-DAMGO (60 Ci mmol^{-1}) were obtained from Amersham International. The following unlabelled drugs and peptides were used: DPDPE (Bachem); DAMGO and ICI 174864 (N,N-diallyl-Tyr-Aib-Aib-Phe-Leu, Aib = α-aminobutyric acid, Cambridge Research Biochemicals); naloxone (Endo-Dupont); U-69563 ((5α,7α,8β)-(−)-N-methyl-N-(7-(1-pyrrolidinyl)-1-oxaspiro[4, 5]dec-8-yl) benzeneacetamide, Upjohn).

Results

Antinociceptive effects of intrathecal DPDPE

DPDPE (2 μg) administered i.t. in the mouse increased the latency time to response against heat nociceptive stimuli as measured in the radiant heat tail-flick test. The i.t. injection of saline vehicle (5 μl) caused no change in the latency time (Figure 1). With the hot-plate test the difference between saline and DPDPE groups was not significant (Wilcoxon matched pairs signed rank test) and observed responses in the DPDPE group varied from no effect to an effect which reached the cut-off time of 15 s.

The observed antinociceptive action of i.t. DPDPE in the radiant heat test was reduced by s.c. administration of the antagonists naloxone and ICI 174864 at 1 mg kg^{-1} (Figure 1).

ICI 174864 (1 mg kg^{-1} s.c.) alone had no effect on the latency periods in either test for up to at least 60 min after administration. No motor dysfunction was observed with any treatment.

Binding characteristics of [^{3}H]-DPDPE in spinal cord homogenates

Saturation studies of [^{3}H]-DPDPE binding to membrane homogenates, prepared from whole spinal cords taken from young mice (18–20 g) similar to those used in the i.t. dosing studies, revealed a homogeneous population of saturable, high affinity [^{3}H]-DPDPE binding sites (Figure 2). The equilibrium dissociation constant (K_D) and maximum capacity of binding sites (B_{max}) obtained following Scatchard transformation of the specific binding data were 4.3 nM and 106 fmol mg^{-1} protein respectively (Table 1). The Hill (1910) slopes were not significantly different from unity.

The characteristics of [^{3}H]-DPDPE binding obtained by such analysis varied between species (Table 1). Highest binding capacity (B_{max}) was observed in mouse cord homogenates, with young mice containing approximately twice the number of binding sites as hamster, rat and guinea-pig. A wide variation in the K_D values obtained for [^{3}H]-DPDPE at

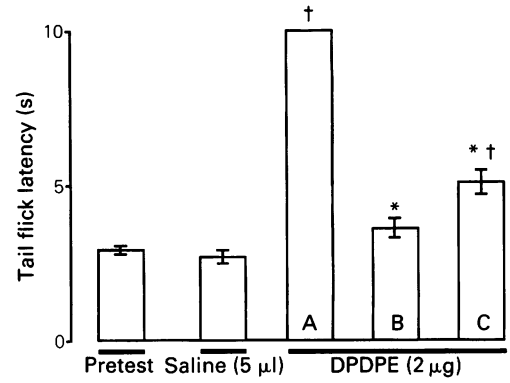

Figure 1 The effects of i.t. [D-Pen^2,D-Pen^5]enkephalin (DPDPE, 2 μg) on tail-flick latency 15 min after pretreatment (s.c.) with saline (column A), naloxone (1 mg kg^{-1} s.c.) (B) or ICI 174864 (1 mg kg^{-1} s.c.) (C). Data are expressed as mean values with s.e.mean shown by vertical lines; $n = 5$. * $P < 0.05$ compared to saline control group and † $P < 0.05$ compared to pretest values (Wilcoxon matched pairs signed rank test). The lack of an effect of saline vehicle (5 μl, i.t.) ($n = 4$) is also shown.

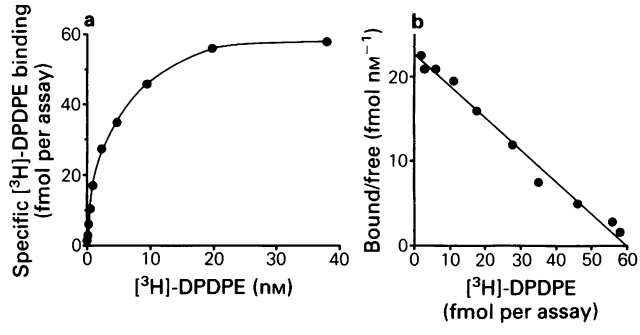

Figure 2 Saturation binding curve (a) and Scatchard plot (b) of the specific binding of [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin ([^{3}H]-DPDPE) to homogenates of spinal cords from 18–21 g mice. Representative experiment which was repeated twice more with similar results.

Table 1 Characteristics of specific [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin binding to homogenates prepared from whole spinal cords of various rodents

Species	B_{max} (pmol g^{-1} tissue)	B_{max} (fmol mg^{-1} protein)	K_d (nM)
Guinea-pig	2.92 ± 0.15	61.8 ± 5.4	1.83 ± 0.11
Hamster	1.93 ± 0.11	42.1 ± 2.6	2.39 ± 0.19
Mouse (adult)	3.60 ± 0.61	78.1 ± 4.9	2.62 ± 0.52
Mouse (18–20 g)	5.42 ± 0.46	106.4 ± 5.1	4.33 ± 0.30
Rat	1.96 ± 0.15	55.1 ± 1.0	3.76 ± 0.21

Each value represents the mean ± s.e.mean of three separate experiments. Data were derived from Scatchard plots analysed with the programma EBDA.

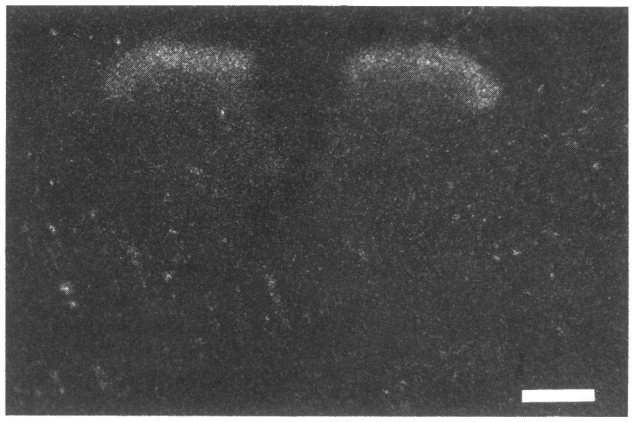

Figure 3 Autoradiographic localisation of [^{3}H]-[D-Pen^2,D-Pen]enkephalin ([^{3}H]-DPDPE, 4 nM) in hamster lumbosacral spinal cord. The calibration bar represents 0.5 mm. An adjacent section in the presence of 10 μM naloxone showed no evidence of [^{3}H]-DPDPE binding.

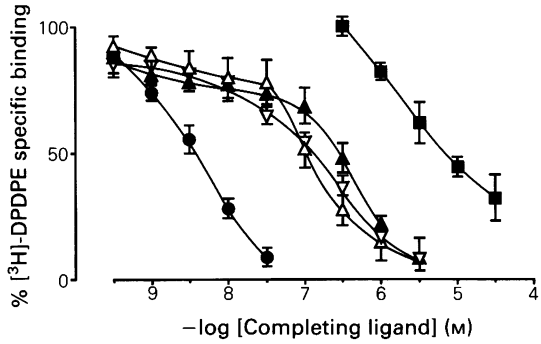

Figure 4 Competitive inhibition of [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin ([^{3}H]-DPDPE, 3.6 nM) specific binding to homogenates of Wistar rat spinal cord by DPDPE (●), [D-Ala^2,MePhe^4, Glyol]enkephalin (△), fentanyl (▲), morphine (▽) and U-69593 (■). Values represent mean of 3 or 4 determinations; vertical lines show s.e.mean.

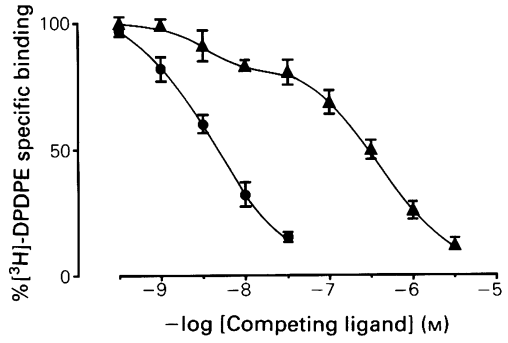

Figure 5 Competitive inhibition of [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin ([^{3}H]-DPDPE, 3.6 nM) specific binding to homogenates of spinal cord from 18–20 g mice by DPDPE (●) and [D-Ala^2,MePhe^4, Glyol]enkephalin (▲). Points represent means of three determinations; vertical lines show s.e.mean.

Table 2 Characteristics of unlabelled [D-Pen^2,D-Pen^5]- enkephalin (DPDPE) and [D-Ala^2,MePhe^4, Glyol^5]enkephalin (DAMGO) inhibition of [^{3}H]-DPDPE (3.6 nM) specific binding to homogenates of mouse (18–20 g) spinal cords

	K_i (nM)	*Hill coefficient*
DPDPE	2.32 ± 0.20	0.99 ± 0.02
DAMGO (high affinity)	1.97 ± 0.69	1.08 ± 0.07
DAMGO (low affinity)	207 ± 40	1.05 ± 0.05

Values are means ± s.e.mean from three separate experiments.

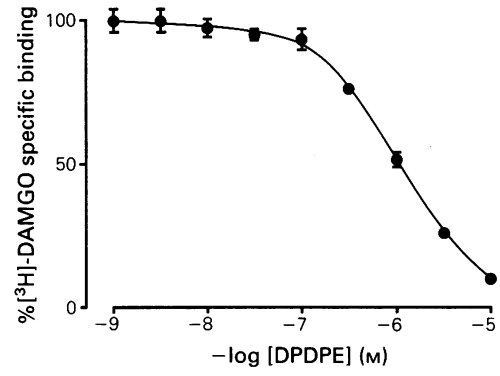

Figure 6 Competitive inhibition of [^{3}H]-[D-Ala^2,MePhe^4, Glyol]-enkephalin ([^{3}H]-DAMGO, 2 nM) specific binding to homogenates of Wistar rat cord by [D-Pen^2,D-Pen^5]enkephalin (DPDPE). Values represent means of 3 determinations; vertical lines show s.e.mean.

its binding sites in the various species was also observed. Affinities decreased in the order guinea-pig > hamster > adult mouse > rat > young mouse, such that the affinity in spinal cords from young mice represented approximately 40% of that in the guinea-pig.

Qualitative study of the distribution of [^{3}H]-DPDPE binding sites in the rodent spinal cord is exemplified by the hamster and demonstrates that the sites are highly concentrated in the outer laminae of the dorsal horn (Figure 3).

Competitive inhibition of [^{3}H]-DPDPE binding in spinal cord homogenates

The binding of [^{3}H]-DPDPE to rat spinal cord homogenates was readily displaced by unlabelled DPDPE with a K_i of 2.6 ± 0.4 nM (Figure 4). In contrast, exceptionally high concentrations (μM) of the κ-ligand U-69593 were needed to displace [^{3}H]-DPDPE. The competitive inhibition of specific [^{3}H]-DPDPE binding by the μ-ligands DAMGO, morphine and fentanyl was complex. Much of the bound ligand (approximately 80%) was displaced only poorly by these agonists in line with their profile as μ-receptor agonists. However, 10–20% of the specifically bound [^{3}H]-DPDPE was displaced with higher affinity, thus affording biphasic curves (Figure 4). A similar displacement profile with DAMGO was observed in the spinal cord homogenates of young mice (Figure 5). The competition curves could be separated into high and low affinity components (Table 2).

The two components of the binding of [^{3}H]-DPDPE could not be distinguished in the reverse experiment, i.e. the displacement of [^{3}H]-DAMGO by unlabelled DPDPE (Figure 6).

Discussion

The results confirm that DPDPE has an antinociceptive action at the spinal level in thermally-induced behavioural tests. The dose of i.t. DPDPE found to be effective in this study agrees with previously obtained results in the warm-water tail-withdrawal test (Porreca *et al.*, 1987a) and the hot-plate test (Porreca *et al.*, 1984). In the present study the data from the tail-flick test suggest that the effect of DPDPE is mediated by δ-receptor activation. This can be concluded from the selectivity of the agonist, but more importantly from the ability of the selective antagonist ICI 174864 to block the antinociceptive action following s.c. administration. Indeed, the difference in potency between ICI 174864 and the non-selective antagonist naloxone agrees with their relative affinities for the δ-receptor as measured by *in vitro* bioassay

(Cotton et al., 1984). Although ICI 174864 is a peptide and usually administered directly into central nervous tissue (e.g. Porreca et al., 1987a), it has previously been used successfully as a selective δ-opioid receptor antagonist following s.c. administration. For example, 1 mg kg^{-1} ICI 174864 s.c. reverses the anti-nociceptive action of i.t. DPDPE, but not of i.t. morphine in the rat (Rodriguez et al., 1986). Also, rearing behaviour in the rat which is induced by the i.c.v. administration of DPDPE, but not other agonists selective for μ-, κ- and δ-receptors, is blocked by ICI 174864 administered s.c. (Cowan et al., 1986). In addition the related, but weaker, antagonist ICI 154129 reverses δ-opioid agonist-mediated head turning in the rat (Gormley et al., 1982) following s.c. administration.

The presence of δ-binding sites in the cords of all species studied, together with the localisation of these sites in the superficial laminae of the dorsal horn, support the proposal of a role for the δ-site in spinal analgesic mechanisms. The lower level of binding in the rat cord, compared with the mouse cord, may explain the higher levels of i.t. DPDPE needed to modulate nociceptive responses in this species (Rodriguez et al., 1986). The binding characteristics of [^{3}H]-DPDPE in spinal cord homogenates of guinea-pig and mouse are similar to those found in the brain tissue of these species (Robson et al., 1985). However, the δ-binding capacity in rat cord homogenates is much less than in brain tissue (Robson et al., 1985). Indeed, the level of specific [^{3}H]-DPDPE binding in brain homogenates of Wistar rats used in the present study (12.4 ± 2.0 pmol g^{-1}) is much higher than seen in the cord, although the affinity constants are similar.

On the other hand the δ-binding capacity in homogenates of spinal cords of the Wistar rats used is five-times higher than observed in cord homogenates from rats of the Alderley Park Wistar strain (Traynor & Rance, 1984), suggesting strain differences are important. Indeed, marked differences in the binding of [^{3}H]-DPDPE to homogenates of brains from various rat strains has been demonstrated (Cotton et al., 1985). Such strain variations may explain the apparent discrepancies between earlier findings regarding the presence of δ-binding sites in rodent cords (Gouarderes et al., 1983; Traynor & Rance, 1984). Importantly, this means that great care is required in the collation and interpretation of *in vivo* data from different laboratories using different rodents, or even different strains of rodent.

The binding of [^{3}H]-DPDPE in spinal cord homogenates appears homogeneous as determined by the analysis of binding isotherms and displacements by the δ- and κ-ligands. The results with μ-ligands in the rat cord suggests the presence of more than one binding site, a finding confirmed by further analysis of the competition by DAMGO for [^{3}H]-DPDPE binding sites in mouse cord, which can be resolved into high and low affinity components. A similar displacement profile has been observed in guinea-pig brain homogenates (Cotton et al., 1985). In both cases the high and low affinity components were not identified by analysis of saturation binding isotherms, suggesting the high affinity site represented only a minor component of [^{3}H]-DPDPE binding. This latter site is unlikely to be residual μ-receptor binding of [^{3}H]-DPDPE, since this ligand has a relative affinity preference for the δ-site of 260 times (Leslie, 1987), a finding we have confirmed which also holds in the cord homogenates used in the present study (data not shown). This means that of the [^{3}H]-DPDPE used in the assays less than 2% can be attributed to binding to μ-sites, and could thus not account for the displacements obtained.

The component of [^{3}H]-DPDPE binding recognised with high affinity by DAMGO could represent a common-high affinity site for μ- and δ-receptor agonists. The presence of a common analgesic site for μ- and δ-receptor agonists at the spinal level is suggested by the existence of cross-tolerance between i.t. morphine and i.t. DPDPE (Porreca et al., 1987a). The fact that intracerebroventricular DAMGO is more potent than i.t. DAMGO, whilst the reverse is true for DPDPE, has led to the suggestion that this common site may have the properties of a δ-receptor (Porreca et al., 1984). On the other hand, the reversal of δ-receptor-mediated spinal modulation of nociceptive responses by ICI 174864, but not of μ(DAMGO)-mediated antinociception (Heyman et al., 1987) would argue against this. However, taken together the data do not rule out some form of allosteric interaction between the two classes of receptor (Rothman et al., 1985) which may be responsible for the observations in binding assays. Obviously further definition of this site is required, and in particular whether it plays a role in spinally-mediated analgesia.

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Development of tolerance to the effects of vigabatrin (γ-vinyl-GABA) on GABA release from rat cerebral cortex, spinal cord and retina

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1 The effects of acute and chronic vigabatrin (γ-vinyl-GABA) (GVG) administration on γ-aminobutyric acid (GABA) levels and release in rat cortical slices, spinal cord slices and retinas were studied.

2 GVG (250 mg kg^{-1} i.p.) administered to rats 18 h before death (acute administration) produced an almost 3 fold increase in GABA levels of the cortex and spinal cord and a 6 fold increase in retinal GABA. The levels of glutamate, aspartate, glycine and taurine were unaffected.

3 When GVG (250 mg kg^{-1} i.p.) was administered daily for 17 days (chronic administration) a similar (almost 3 fold) increase in cortical GABA occurred but the increases in spinal and retinal GABA were reduced by approximately 40%.

4 Acute administration of GVG strikingly increased the potassium-evoked release (KCl 50 mM) of GABA from all three tissues. This enhanced evoked release was reduced by about 50% in tissues taken from rats that had been chronically treated with GVG.

5 Acute administration of GVG reduced GABA-transaminase (GABA-T) activity by approximately 80% in cortex and cord and by 98% in the retina. Following the chronic administration of GVG, there was a trend for GABA-T activities to recover (significant only in cortex). Acute administration of GVG had no effect on glutamic acid decarboxylase (GAD) activity in cortex or spinal cord. However, chronic treatment resulted in significant decreases in GAD activity in both the cortex and cord (35% and 50% reduction respectively).

6 The K-evoked release of glutamate, aspartate, glycine and taurine from cortical slices and the K-evoked release of glycine from spinal slices and retinas were not affected by either acute or chronic GVG treatment.

7 These experiments indicate that GVG treatment increases specifically the K-evoked release of GABA and that tolerance can develop to this enhancing effect of GVG on central GABA release. This tolerance may result from increased feedback inhibition of GAD with a consequent reduction of presynaptic GABA stores.

Introduction

γ-Vinyl-GABA (GVG) is an enzyme-activated irreversible inhibitor of γ-aminobutyric acid (GABA)-transaminase (GABA-T) (Lippert *et al.*, 1977) that has anticonvulsant activity in both animals (Meldrum & Horton, 1978); Schechter & Trainer, 1978) and epileptic patients (Gram *et al.*, 1983; Rimmer & Richens, 1984). The drug increases GABA levels in the brain and it is generally believed that it exerts its anticonvulsant effects by acting as an indirectly acting GABA-mimetic and increasing GABA release. This is supported by release experiments in animals where GVG has been shown to increase GABA release from the cerebral cortex (Abdul-Ghani *et al.*, 1980; 1981) and from mouse embryonic cultured neurones (Gram *et al.*, 1988). The evidence in human epileptics is less direct, but GVG increases the GABA concentration in the CSF and this probably reflects an increase in the central release of GABA (Grove *et al.*, 1981; Schechter *et al.*, 1984).

A problem with some anti-epileptics, notably the benzodiazepines, is that tolerance develops and recently tolerance to the antiseizure action of GVG in epileptic gerbils was described (Löscher & Frey, 1987).

The present study was undertaken to examine the mechanism underlying the development of tolerance to the antiseizure action of GVG. It was found that the increase in the K-evoked release of GABA produced by GVG was reduced when the drug was administered chronically. This decrease in evoked GABA release, which occurred in three areas of the CNS, might explain the development of tolerance to the antiseizure activity of GVG.

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Methods

Preparation of tissues

Male Wistar rats (150–200 g) were killed by cervical dislocation. The eyes were enucleated and the retinas dissected in Krebs bicarbonate medium at room temperature. The brains were removed and slices of cerebral cortex (0.2 mm thick) were prepared with a McIlwain tissue chopper as described previously (Cunningham & Neal, 1981). The spinal cord was removed following an extensive laminectomy and transverse (0.3 mm thick) slices were again prepared with a McIlwain chopper (Hopkin & Neal, 1971).

Incubation of tissues

In order to measure the release of GABA from the tissues, slices of cortex (5–6 slices, weight 25 mg), spinal cord (5–6 slices, weight 25 mg), or two entire retinas (approx. 20 mg) were placed in a small perspex chamber (volume 0.5 ml) containing Krebs bicarbonate medium (250 μl) at room temperature and were gently agitated by a stream of 95% O_2 plus 5% CO_2.

At 10 min intervals, the medium was drained off and replaced with fresh medium. The GABA and other amino acids in each of the resulting 10 min samples were measured

by high performance liquid chromatography (h.p.l.c.) (Yazulla *et al.*, 1985).

Tissue amino acids

Tissue amino acids were measured as described previously (Yazulla *et al.*, 1985). Briefly, slices of cerebral cortex, spinal cord or entire retinas were homogenized in 1 M formic acid 15% and acetone 85% with a glass homogenizer (40 mg ml^{-1}). The homogenates were centrifuged at 3000 r.p.m. for 5 min at room temperature and the amino acids in the supernatant were measured by h.p.l.c.

High performance liquid chromatography

The samples were analysed on a Varian 5000 HPLC by pre-column derivatisation with *o*-phthalaldehyde (Turnell & Cooper, 1981).

Glutamic acid decarboxylase activity and GABA-transaminase activity

These were measured by the methods of Lowe *et al.* (1958) and Salvador & Alders (1959), respectively.

Acute GVG administration

Animals were given a single intraperitoneal injection of GVG (250 mg kg^{-1}) 18 h before death. Control rats were given an injection of saline 18 h before death.

Chronic GVG administration

Rats were given an injection of either saline (controls) or GVG (250 mg kg^{-1}) daily for 17 days. The last injections were given 18 h before death. In all cases injections were given between 16 h 00 min and 18 h 00 min.

Analysis of results

Differences in amino acid contents, and in the K-evoked release of GABA were tested for statistical significance by Student's unpaired *t* test.

Materials

Incubation medium Krebs-Ringer bicarbonate of the following composition was used (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.4, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 9.5. The medium was continuously gassed with 95% O_2 plus 5% CO_2. γ-Vinyl-GABA (vigabatrin) was a gift from Merrell-Dow Pharmaceuticals Ltd.

Results

Effect of GVG administration on tissue GABA levels

In the cerebral cortex both acute and chronic administration of GVG more than doubled the GABA levels (Figure 1). There was no evidence of tolerance developing to the effects of GVG on cortical GABA levels. Indeed, the GABA level in cortical tissue from the chronically treated rats was slightly higher than that in the tissue from rats receiving only one injection (2.7 and 2.5 fold increases respectively).

In contrast to the cerebral cortex, the increases in GABA levels caused by GVG in the spinal cord and retina were significantly smaller in the chronically treated animals (Figure 1). Thus, in the spinal cord, a single injection of GVG caused a 2.8 fold increase in GABA levels 18 h later, but with chronic administration this increase was reduced by 65%.

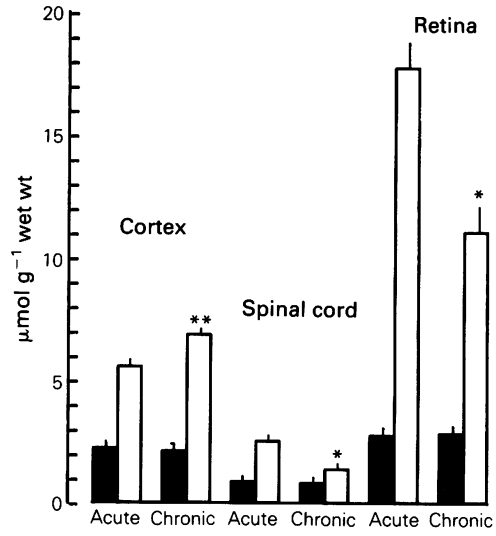

Figure 1 Effect of acute (single injection 18 h before death) or chronic (17 daily injections) treatment with saline (solid columns) or γ-vinyl-GABA (open columns) (250 mg kg^{-1}) on GABA content of cortex, spinal cord and retina. Each column is the mean and the bar s.e.mean of 5–10 experiments. There was a significant difference in GABA content between acutely and chronically treated animals, ** $P < 0.05$, * $P < 0.001$.

In retina GVG produced very large increases in GABA levels. Thus, acute treatment increased retinal GABA levels more than 6 fold, an increase that was reduced by approximately 40% by chronic treatment with GVG (Figure 1).

Effect of GVG administration on tissue glutamate, aspartate, glycine and taurine levels

Neither acute nor chronic administration of GVG affected the tissue levels of these amino acids (Table 1).

Effect of GVG administration on GABA-transaminase and glutamate decarboxylase activities

GABA-transaminase Acute treatment of animals with GVG produced large decreases in the activity of GABA-T when this was measured 18 h after drug administration (Table 2). In the cerebral cortex and spinal cord, GABA-T activity was reduced by approximately 80% but the most striking inhibition was seen in the retina where GABA-T activity was virtually abolished. When GVG was administered chronically, there was a trend in all three areas for the GABA-T activity to recover slightly but this was significant only in the cerebral cortex (Table 2).

Glutamate decarboxylase Acute treatment of animals with GVG had no effect on the activity of glutamate decarboxylase (GAD) in either the cerebral cortex or spinal cord. In contrast, after chronic treatment with GVG, the GAD activity in the cortex and spinal cord was reduced by approximately 35% and 50% respectively (Table 2).

Effect of GVG treatment on the release of GABA

Saline-injected controls When slices of cerebral cortex from control (saline-injected) animals were exposed to high-K (50 mM KCl) the release of GABA was increased approximately 4 fold (acute and chronic saline controls were similar). In contrast, when slices of spinal cord or entire retinas from control rats (acute or chronic) were exposed to high-K, there

Table 1 Effects of acute and chronic γ-vinyl-GABA (GVG) administration on amino acid levels

	Amino acid level (μmol g^{-1} wet wt)		
	Cerebral cortex	*Spinal cord*	*Retina*
Glutamate			
Acute saline	11.49	3.28	4.23
Acute GVG	12.31	3.60	4.40
Chronic saline	11.70	3.84	4.69
Chronic GVG	12.15	3.82	4.11
Aspartate			
Acute saline	2.52	1.65	1.88
Acute GVG	2.46	1.71	1.85
Chronic saline	2.49	1.66	2.06
Chronic GVG	2.35	1.58	1.78
Glycine			
Acute saline	1.58	5.83	1.80
Acute GVG	1.58	5.86	1.85
Chronic saline	1.75	5.31	2.13
Chronic GVG	1.36	5.98	1.85
Taurine			
Acute saline	7.11	2.41	35.3
Acute GVG	6.32	2.65	35.3
Chronic saline	8.11	2.41	38.2
Chronic GVG	7.49	2.44	35.7

Rats were injected (i.p.) with either saline or GVG (250 mg kg^{-1}) once (acute treatment) or daily for 17 days (chronic treatment). Results are the mean of 8–10 measurements, s.e.means are omitted for clarity but were approximately 10%. Drug treatment did not affect the level of any of the amino acids examined.

was no significant increase in GABA release (not illustrated). These results confirm previous observations (Neal & Shah, 1989).

Acute GVG treatment Acute treatment of rats with GVG increased the spontaneous resting release of GABA (not illustrated) and strikingly increased the K-evoked release of GABA from all three tissues (Figure 2, open columns). Thus, in the spinal cord and retina the K-evoked releases were increased from undetectable to 4.5 fold and 2.8 fold the resting release respectively. In cortical slices from rats given one injection of GVG, the K-evoked GABA release was strikingly greater than that seen in tissue from saline-injected rats (Figure 2). These results are very similar to the effects of acute GVG administration on GABA release from cortex, spinal cord and retina that have recently been described in detail (Neal & Shah, 1989).

Table 2 Effects of acute and chronic γ-vinyl GABA (GVG) administration on GABA-transaminase (GABA-T) and glutamate decarboxylase (GAD) activities

	Enzyme activity (% of control)			
	GABA-T		*GAD*	
	Acute	*Chronic*	*Acute*	*Chronic*
Cerebral cortex	23 ± 4.1	33.3 ± 4.5*	102 ± 8.5	64.5 ± 4.4†
Spinal cord	21.3 ± 3.6	29.2 ± 4.1	99.1 ± 7.2	48.3 ± 3.6†
Retina	2.3 ± 0.9	8.0 ± 3.8		

Rats were injected (i.p.) with either saline or GVG (250 mg kg^{-1}) once (acute) or daily for 17 days (chronic). The results are expressed as the % of the appropriate controls (±s.e.mean, $n = 6$). Values obtained in saline-injected acute and chronic controls were not significantly different. Acute and chronic GVG significantly inhibited GABA-T activity ($P < 0.02$). Chronic significantly different from acute, * $P < 0.05$, † $P < 0.01$. In saline-injected controls, the specific activities in cortex, cord and retina of GAD (nmol h^{-1} mg^{-1} protein) were 85.9 ± 10.6, 36.7 ± 10.9 and 94 ± 8, respectively and GABA-T (μmol h^{-1} mg^{-1} protein) 1.52 ± 0.13, 1.19 ± 0.12 and 2.27 ± 0.76, respectively.

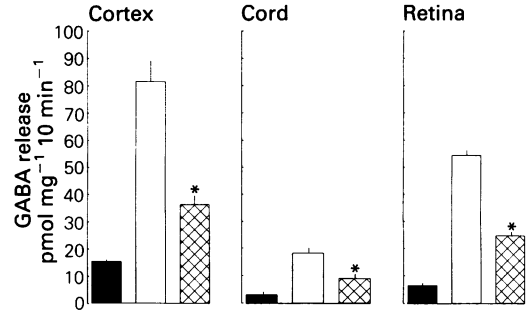

Figure 2 Release of GABA from cortex, spinal cord and retina depolarized with 50 mM KCl. Solid columns, saline-injected controls (acute and chronic were not different). Open columns, single injection of γ-vinyl GABA (GVG). Hatched columns, chronic administration of GVG. Each column is the mean and bar s.e.mean of 6–10 experiments. * Significant difference between evoked releases from acutely and chronically treated animals $P < 0.001$.

Chronic GVG treatment The chronic administration of GVG to rats had little effect on the resting release of GABA from any of the tissues when compared with the resting release from tissues taken from acutely treated rats (not illustrated). However, in all three tissues the K-evoked release of GABA was significantly less in tissues taken from chronically treated rats compared with that found in tissues taken from acutely treated rats (Figure 2). Thus, in all tissues the absolute amount of GABA released in the presence of high-K was reduced by more than 50% in tissues taken from rats treated chronically with GVG.

Effects of GVG treatment on the release of glutamate, aspartate, glycine and taurine from cortical slices

The K-evoked releases of glutamate, aspartate, glycine and taurine from cortical slices taken from rats treated acutely or chronically with GVG were not significantly different (not illustrated).

Effects of GVG treatment on the release of glycine from retina and spinal cord

The K-evoked release of glycine from retinas and spinal slices from rats treated acutely or chronically with GVG were not significantly different (not illustrated).

Discussion

The mechanism of action of anti-epileptic drugs remains unclear. However, there is reasonable evidence that the benzodiazepines and GABA-T inhibitors exert their antiseizure activity by mechanisms involving GABAergic synapses.

The benzodiazepines are potent anticonvulsants but their use is limited by the development of tolerance. There have not, to our knowledge, been any reports of tolerance developing to the anticonvulsant actions of GVG in epileptic patients. On the contrary, it is claimed that tolerance is not a problem in man (Remy & Beaumont, 1989). However, Löscher & Frey (1987) found that tolerance developed to the anticonvulsant action of GVG in gerbils which exhibited seizures in response to an air blast. In these experiments it was found that the daily administration of GVG at a dose that initially gave 60% seizure protection resulted in marked tolerance so that after 17 days, seizure protection was reduced to 13%. Tolerance to the anticonvulsant effects of the GABA-T inhibitors, γ-acetylenic-GABA and aminooxyacetic acid (AOAA), after prolonged treatment has also been demonstrated in both gerbils (Löscher, 1986) and mice (Löscher,

1982). GVG is believed to be an anticonvulsant because it increases central GABA release. In the present experiments a single injection of GVG caused large increases in GABA release from three areas of the CNS (cerebral cortex, spinal cord and retina). However, this enhanced release was strikingly reduced when GVG was given chronically and this could provide an explanation for the development of tolerance to the anticonvulsant action of the drug.

The mechanism that underlies this reduction in enhanced GABA release following chronic GVG administration is not altogether clear but it cannot be simply related to tissue GABA levels. Thus, the GABA levels in spinal cord and retinas taken from rats subjected to prolonged GVG treatment were significantly lower than in those given a single injection (although they were still above saline-injected controls). However, the GABA levels in cortical slices taken from chronically treated rats were actually higher than those in cortex taken from rats given in a single injection of drug.

It seems unlikely that changes in GABA-T inhibition with chronic GVG treatment are important. Although there was a trend for the enzyme inhibition to decrease slightly with chronic administration, this was only significant in the cerebral cortex. Furthermore, in the retina, the GABA-T activity was still only 8% of control values, even after chronic treatment.

A possible explanation for the present results is that the increase in presynaptic nerve terminal GABA stores produced by GVG is not completely maintained when the drug is given for a long period. This implies a reduction in GABA synthesis. In the present experiments, chronic GVG administration resulted in a substantial reduction in GAD activity in both the cortex and spinal cord. GAD is the rate limiting enzyme for GABA synthesis and is highly localized in presynaptic nerve endings (Neal & Iversen, 1969). GAD activity is reduced by high levels of GABA and chronic treatment of rats, mice or gerbils with GABA-T inhibitors has been shown previously to reduce the activity of the enzyme (Fletcher & Fowler, 1980; Löscher, 1982). This feedback inhibition of GABA synthesis in nerve endings offers an explanation for both the decrease in GABA levels and release from cord and retina following prolonged treatment with GVG.

In the cerebral cortex the situation is more complicated because the total GABA level in the tissue was increased by prolonged GVG treatment, although tolerance still developed to the K-evoked release. These seemingly paradoxical results may result from a change in the cellular distribution of cortical GABA pools. Normally, GABA released into the synaptic cleft is inactivated by uptake systems in the nerve-endings and glial cells. The GABA taken up by the glia is quickly degraded and does not accumulate there. However, when the GABA-T is inhibited, the glia can accumulate substantial amounts of GABA. This redistribution of cellular GABA pools produced by GVG has been shown indirectly in biochemical experiments (Iadarola & Gale, 1981) and clearly demonstrated in the rat retina by use of immunocytochemical techniques (Neal *et al.*, 1989a). A possible explanation for the present results is that in cerebral cortex, prolonged GVG treatment results in a glial accumulation of GABA which outweighs the decrease in nerve-ending GABA. This causes an increase in tissue GABA together with a decrease in evoked release (high-K causes little GABA release from glial cells, Neal *et al.*, 1989b). In cord and retina the decrease in nerve-ending GABA produced by chronic GVG treatment may outweigh the increase in glial GABA and so there is a decrease in total GABA, together with a decrease in evoked release. The failure of increased GABA levels in various brain areas produced by GABA-T inhibitors to correlate with anticonvulsant actions has been shown previously (Löscher & Frey, 1987).

In summary, we have found in the present experiments that GVG administration increases the K-evoked release of GABA from the CNS, but this enhanced release is smaller when the drug is given chronically. This may be because the initially very high concentration of GABA in the nerve terminals results in feedback inhibition of GAD and a reduction in GABA synthesis.

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Membrane hyperpolarization, cyclic nucleotide levels and relaxation in the guinea-pig internal anal sphincter

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1 Changes in membrane potential (measured with an intracellular microelectrode) and in cyclic nucleotide (adenosine 3′:5′-cyclic monophosphate, cyclic AMP and guanosine 3′:5′-cyclic monophosphate, cyclic GMP) levels (measured by radioimmunoassay) in response to inhibitory non-adrenergic non-cholinergic (NANC) field stimulation and drugs were investigated in the guinea-pig internal anal sphincter (gpIAS) in the presence of phentolamine and atropine (each 10^{-6} M).

2 Inhibitory NANC nerve stimulation (single pulse, 5 pulses at 5, 10 and 20 Hz, 0.5 ms supramaximal voltage) and adenosine triphosphate (ATP, 10^{-7}–10^{-3} M) inhibited spike discharge, hyperpolarized the membrane and relaxed the sphincter. The effects of inhibitory nerve stimulation were blocked by tetrodotoxin (TTX, 10^{-6} M) and, with those of ATP, were blocked by apamin (5×10^{-6} M).

3 Isoprenaline (10^{-9}–10^{-4} M), cromakalim (10^{-9}–10^{-5} M), sodium nitroprusside (NaNP 10^{-5} M), M&B 22948 (10^{-4} M) and 8-bromocyclic GMP (8-Br-cyclic GMP, 10^{-4} M) also inhibited spike discharge, hyperpolarized the membrane and relaxed the sphincter. The effects of isoprenaline were blocked by propranolol (10^{-6} M). However, forskolin (10^{-9}–10^{-7} M), M&B 22948 (10^{-9}–10^{-5} M) and lower concentrations of NaNP (10^{-9}–10^{-6} M) relaxed the sphincter without affecting the membrane potential.

4 The characteristics of the membrane potential changes in response to different inhibitory stimuli in the gpIAS differed. Hyperpolarization produced by inhibitory NANC nerve stimulation and ATP were rapid in onset, of brief duration and of comparable amplitude. Isoprenaline and direct electrical stimulation also hyperpolarized the membrane and relaxed the muscle although the extent of the relaxation in these two cases was much less than that with nerve stimulation and ATP. In each case, the membrane potential change preceded relaxation and probably accounted for it.

5 Both inhibitory NANC nerve stimulation (80 pulses 8 Hz supramaximal voltage 0.5 ms) and ATP (10^{-4} M) raised levels of cyclic GMP significantly and to a comparable degree and relaxed the sphincter. The effect of nerve stimulation was prevented by TTX (10^{-6} M) but not by apamin (5×10^{-6} M). Isoprenaline (10^{-4} M), cromakalim (10^{-5} M) and forskolin (10^{-5} M) were ineffective.

6 Inhibitory NANC nerve stimulation (80 pulses 8 Hz 0.5 ms supramaximal voltage) and ATP (10^{-4} M) raised levels of cyclic AMP significantly to a comparable degree and relaxed the sphincter. The increase produced by nerve stimulation was abolished by TTX (10^{-6} M) and apamin (5×10^{-6} M). Isoprenaline (10^{-4} M), cromakalim (10^{-5} M) and forskolin (10^{-5} M) raised levels of this nucleotide significantly.

Introduction

Smooth muscle relaxation in response to nerve stimulation or drugs is often accompanied by changes in membrane potential and/or second messenger systems. The extent to which these changes occur depends on the species of animal, the type of muscle and the nature of the stimulus employed. In the lower oesophageal sphincter (LOS) electrical field stimulation of inhibitory nerves produces relaxation, membrane hyperpolarization (Gonella *et al.*, 1977) and raised guanosine 3′:5′-cyclic monophosphate (cyclic GMP) levels (Torphy *et al.*, 1986). On the other hand, nerve-mediated relaxation of the bovine retractor penis muscle (BRP) is accompanied by little change in membrane potential (Byrne & Muir, 1984) but by an elevation of the cyclic GMP content (Bowman & Drummond, 1984). Cromakalim, a novel K^+ channel activator, relaxes the BRP but changes neither the cyclic GMP nor the adenosine 3′:5′-cyclic monophosphate (cyclic AMP) content (Gillespie & Sheng, 1988). Sodium nitroprusside, in contrast, relaxes the aorta and raises cyclic GMP levels but does not alter membrane potential (Lincoln, 1983).

The above results were obtained from a variety of tissues by different investigators. It is not clear whether or how second messenger systems relate to the widely differing degrees of membrane potential change which accompany relaxation to different stimuli in the various tissues or indeed whether the membrane potential change is independent of the second messenger system involved. The present investigation was carried out, therefore, to permit a more direct comparison of the electrical and biochemical responses that accompany different inhibitory stimuli in the same tissue. The circular muscle of the guinea-pig internal anal sphincter (gpIAS), a muscle with a powerful non-adrenergic, non-cholinergic (NANC) inhibitory innervation (Costa & Furness, 1973) was chosen for examination. Changes in membrane potential and in cyclic nucleotide content (cyclic GMP and cyclic AMP) of the gpIAS were correlated with mechanical responses to both neuronal and non-neuronal stimuli. Preliminary accounts of these results have been presented by Muir & Stirrat (1988) and Baird & Muir (1989).

Methods

Adult guinea-pigs (250–350 g) were killed by cervical dislocation, and bled. The gpIAS was dissected by the method of Lim & Muir (1985).

Electrical and mechanical recordings

Intracellular electrical and mechanical recordings were made simultaneously from the gpIAS. One end of each tissue was attached by thread to a force displacement transducer (Grass FT03C) and the other passed through Ag/AgCl ring electrodes for field stimulation (supramaximal voltage; 0.5 ms) and pinned to the Sylgard base of a horizontal organ bath (2 ml). The effect of displacing membrane potential on the mechanical effects, by passing electrotonic current through the tissue, was measured with the partition method of Abe &

Tomita (1968). In all experiments, tissues were perfused with Krebs solution of the following composition (mM): NaCl 118.4, $NaHCO_3$ 25.0, NaH_2PO_4 1.13, KCl 4.7, $CaCl_2$ 2.7, $MgCl_2$ 1.3, glucose 11.0. This was bubbled with 95% O_2 and 5% CO_2 (pH = 7.4) and contained atropine and phentolamine (each 10^{-6} M). This eliminated responses from cholinergic and from adrenergic nerves in residual longitudinal and circular muscle layers.

Intracellular electrical recordings were made with capillary glass micro-electrodes (resistance 20–40 MΩ) filled with 3 M KCl. Signals were passed to a unity gain impedance pre-amplifier (W.P. Instruments), displayed on a storage oscilloscope and u.v. recorder and stored on an instrumentation tape recorder (Racal Store 4DS).

Drugs were applied (a) in the Krebs solution perfusing the preparation, (b) by microsyringe directly into the bath when perfusion was impracticable or if the drugs were unstable, or (c) locally by hydrostatic pressure ejection from a broken off micropipette (2–10 μm in diameter) by Picospritzer (General Valve Corporation, N.J., U.S.A.). Pressure ejection ensured direct application of small amounts of drug to the preparation. However, because of these small amounts only a few muscle bundles were activated and the overall tension of the muscle was not altered. To minimize the occurrence of desensitization, a dose interval of at least 15 min was allowed. When drugs were perfused, a 15 min equilibration period elapsed before cell penetration began. In the case of 8-bromo-cyclic GMP, 30 min, at least, were allowed for permeation of the cell; recovery of membrane potential took about 1.5 h. Accordingly it was impracticable to employ sufficient doses of this drug in the same tissue to assess dose-response effects. Hence, only one dose of drug which is known to permeate other smooth muscle cells (Napoli *et al.*, 1980) was investigated on several tissues.

Cyclic nucleotide measurement

Cyclic nucleotide (cyclic AMP and cyclic GMP) levels were measured at the point of maximum relaxation to a variety of inhibitory stimuli. To monitor the mechanical response, each tissue was placed in a vertical jacketed organ bath (10 ml), maintained at 37°C by circulating, thermostatically controlled water and perfused with Krebs solution. One end of each tissue was attached by thread to a force displacement transducer (Grass FT03C) and the other passed through Ag/AgCl ring electrodes for field stimulation (supramaximal voltage; 0.5 ms) and attached to a metal hook.

After maximal relaxation, to either field stimulation or drugs, each tissue was removed from the organ bath and rapidly (within 10 s) frozen in liquid nitrogen-cooled isopentane. The frozen tissue was then thawed in trichloroacetic acid (1 ml 10% w/v) and homogenized with a ground glass homogenizer. Precipitated proteins were removed by centrifugation (3000 *g*; 15 min; 4°C). The acid-soluble fraction was removed and extracted with water-saturated diethyl-ether (4 volumes, 4 times). Residual diethyl-ether was driven off by evaporation in a water bath (2 min at 70°C).

The cyclic GMP and cyclic AMP contents (membrane bound and soluble nucleotides) of the smooth muscle cells were analysed by radioimmunoassay by the acetylation method of Harper & Brooker (1975) with commercially-available assay kits (Dupont).

Drugs

The following were used and except where stated they were dissolved in saline 0.9% w/v. Apamin (Sigma), atropine sulphate (Sigma), adenosine 5′-triphosphate disodium salt (ATP, Sigma), bombesin (Sigma), bradykinin (Sigma), 8-bromo-cyclic GMP (Sigma), cromakalim (Beecham), forskolin (as 10^{-2} M Sigma) dissolved in 95% ethanol then diluted with saline 0.9% w/v or Krebs, isoprenaline sulphate (Aldrich), Leu-enkephalin (Sigma), Met-enkephalin (Sigma), neuropeptide Y (Sigma), phentolamine mesylate (Ciba), (±)-propranolol hydrochloride (Sigma), 2-0-propoxyphenyl-8-azapurin-6-one (M&B 22948, May & Baker), sodium nitroprusside (Sigma), somatostatin (Sigma), tetrodotoxin (TTX, Sigma), vasoactive intestinal polypeptide (Sigma). Concentrations in the text refer to the salts except for TTX, apamin and vasoactive intestinal polypeptide which are expressed as base.

Analysis of results

Since each experiment on the gpIAS required one animal, the number of experiments was limited but in no case did the number fall below three. Results were expressed as means ± s.d. of n experiments for electrical and mechanical investigations and of tissues in cyclic nucleotide measurements. Student's t test was used to determine significance ($^*P < 0.05$; $^{**}P = 0.01$; $^{***}P < 0.001$) between mean values.

Results

Electrical and mechanical properties of gpIAS

When set up, the gpIAS had no tone; it was stretched gently (by 1 g) and during the next 15–20 min, a further 2–3 g tone developed which was maintained throughout the experiment (cf. Lim & Muir, 1985). The development of tone depended on the maintenance of a spontaneous spike discharge. Spike potentials reached 50 mV in amplitude and were discharged at a rate of 1–2 Hz. In the absence of tone, spikes were not observed and therefore resting tone and membrane electrical activity were closely related. The mean resting membrane potential, between spikes, was −44 ± 3 mV (n = 229).

Effects of inhibitory stimuli on electrical and mechanical responses in the gpIAS

Inhibitory NANC nerve stimulation Inhibition of spike discharge and frequency-dependent inhibitory junction potentials (i.j.ps) accompanied field stimulation (single pulse; 5 at 5, 10 and 20 Hz; 0.5 ms; supramaximal voltage) of intramural NANC nerves. The i.j.ps, at 10 Hz, had a mean amplitude of −16 ± 9 mV, a rapid rate of onset (41 ± 20 mV s^{-1}) a short duration (2.7 ± 0.8 s, n = 69) and were accompanied by relaxation (cf. Lim & Muir, 1985; 1986) (Figure 1). The electrical and mechanical responses to field stimulation were closely related. Antagonism of hyperpolarization, for example, by blocking K^+ channels, inhibited relaxation. Hyperpolarization by direct application of electrotonic current pulses in the Abe & Tomita bath also caused relaxation. Electrical responses to nerve stimulation were inhibited by apamin (5×10^{-6} M) which blocks certain Ca^{2+}-mediated K^+ channels, (Figure 2a and c) and by TEA (8×10^{-2} M) which blocks K^+ channels.

Effects of putative transmitters ATP (10^{-7}–10^{-3} M applied by Picospritzer, Figure 1b, or by microsyringe) produced rapid, brief, dose-dependent hyperpolarizations. When applied by microsyringe, ATP also relaxed the sphincter by some 80%. The mean amplitude of the membrane potential change (ATP 10^{-4} M by Picospritzer) was −14 ± 6 mV (n = 12), the rate of onset 18 ± 24 V s^{-1} (n = 12) and the duration 14 ± 9 s (n = 12). The relaxation induced by ATP was always associated with a membrane hyperpolarization. As with field stimulation, the hyperpolarizations to ATP were sensitive to apamin (5×10^{-6} M) and TEA (8×10^{-12} M).

In contrast, vasoactive intestinal polypeptide, neuropeptide Y, substance P, somatostatin, Leu-enkephalin, Met-enkephalin, bombesin and bradykinin (each 10^{-7}–10^{-4} M by Picospritzer) each produced insignificant effects on membrane potential and on the amplitude and rate of spike potential discharge.

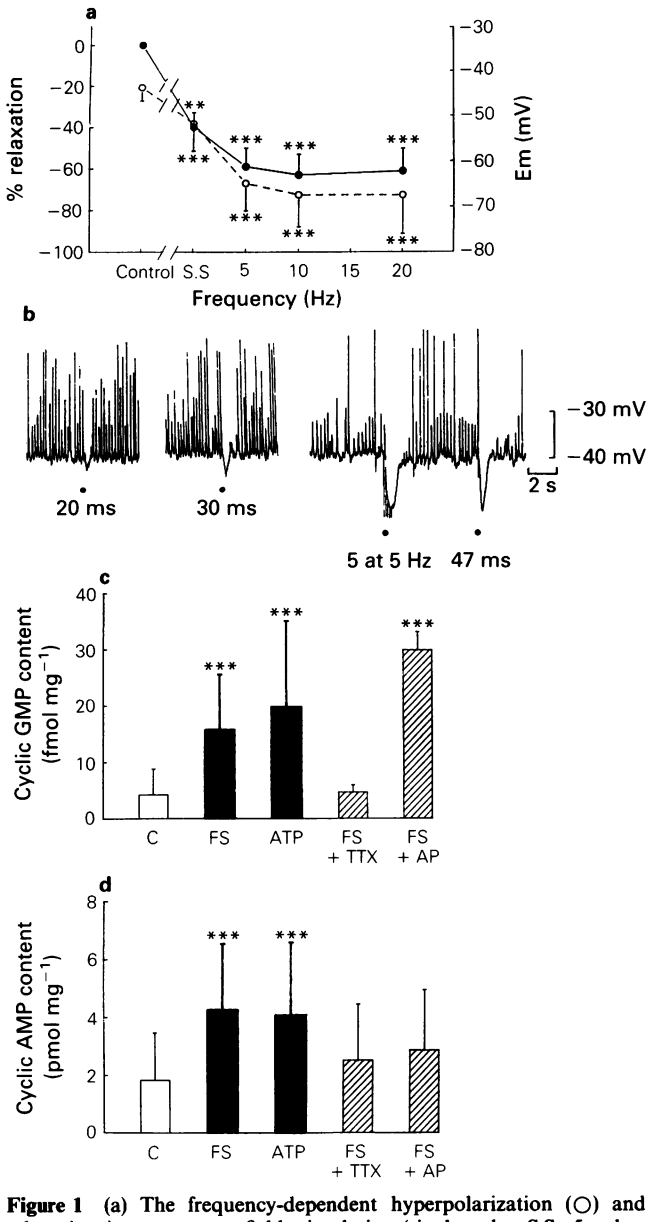

Figure 1 (a) The frequency-dependent hyperpolarization (○) and relaxation in response to field stimulation (single pulse, S.S., 5 pulses at 5, 10 and 20 Hz; 0.5 ms; supramaximal voltage) in the guinea-pig internal anal sphincter (gpIAS). Each point represents the mean of at least 10 observations; s.d. shown by vertical bars. (b) A comparison of the electrical responses of the gpIAS to ATP (● 5.8×10^{-4} M, applied by pressure ejection) and to field stimulation (5 pulses at 5 Hz; 0.5 ms; supramaximal voltage) in the same cell. (c and d) The effects (± s.d. of at least 10 observations) of ATP (10^{-4} M), field stimulation (FS, 80 pulses at 8 Hz; 0.5 ms; supramaximal voltage) alone and in the presence of tetrodotoxin (TTX, 10^{-6} M, FS + TTX) or apamin (5×10^{-6} M, FS + AP) on the cyclic GMP (fmol mg^{-1}, c) and cyclic AMP (pmol mg^{-1}, d) contents of the gpIAS. Atropine and phentolamine (each 10^{-6} M) were present throughout these experiments.

β-Adrenoceptor stimulation Isoprenaline (10^{-9}–10^{-4} M) inhibited spike discharge, hyperpolarized the membrane and relaxed the gpIAS in a dose-dependent manner (Figure 3). The membrane hyperpolarization (-7 ± 3 mV, $n = 6$) produced by isoprenaline (10^{-4} M) was smaller ($P < 0.025$) and the maximum relaxation only some 20% of that obtained by field stimulation (5 pulses, 10 Hz, 0.5 ms). The duration of the electrical response also differed. The hyperpolarization produced by isoprenaline (10^{-4} M) had a slower rate of onset (0.6 ± 0.5 mV s^{-1}) than that following nerve stimulation ($P < 0.001$) or ATP ($P < 0.001$). Notwithstanding these differences it is clear (Figure 4a) that, as with ATP and nerve stimulation, the electrical and mechanical effects produced by isoprenaline appeared to be related. Hyperpolarization did not occur without relaxation. The effects of isoprenaline involved β-adrenoceptors as they were abolished by propranolol (10^{-4} M) and presumably K^+ channels because they were also abolished by TEA (8×10^{-2} M). These latter responses unlike those to inhibitory nerve stimulation were insensitive to apamin (5×10^{-6} M).

K^+-channel activation Membrane hyperpolarization, inhibition of spike discharge and relaxation were produced by the K^+ channel activator cromakalim (10^{-9}–10^{-5} M) in a dose-dependent manner (Figure 2b). Although the amplitude of the membrane change (-19 ± 6 mV, $n = 12$) and the extent of the relaxation (80% at 10^{-5} M) were comparable to those produced by inhibitory NANC nerve stimulation, there were important differences (Figure 4b). The electrical change was much slower (0.6 ± 0.2 mV s^{-1}, $n = 12$) in reaching the peak response and was more prolonged (596 ± 238 s) lasting up to 15 min. The membrane potential change was antagonised by TEA (8×10^{-2} M) but unaffected by apamin (5×10^{-6} M) (Figure 2d) like that produced by isoprenaline but unlike that to nerve stimulation. Apamin inhibited tone and therefore the effects of apamin on mechanical responses to the K^+ channel activator could not be measured.

Activation of cyclic GMP

The effects of three drugs were investigated: sodium nitroprusside (NaNP) which activates guanylate cyclase directly, M&B 22948, which is a cyclic GMP specific phosphodiesterase inhibitor, and 8-bromo-cyclic GMP, a stable, permeable, cyclic GMP analogue. Each induced small membrane hyperpolarizations and relaxed the muscle (Figure 4c). However, electrical and mechanical events were clearly separated by the dose of drug chosen. With NaNP (10^{-9}–10^{-6} M) or M&B 22948 (10^{-9}–10^{-5} M), relaxation occurred without any significant membrane potential change though at higher concentrations (10^{-5} M NaNP and 10^{-4} M M&B 22948) a small (10 mV or less) hyperpolarization with spike inhibition accompanied the virtual abolition of tone (e.g. Figure 4c).

8-Bromo-cyclic GMP (10^{-4} M) hyperpolarized the membrane potential (by some 10 mV), inhibited spike discharge and relaxed the sphincter (Figure 5). It is interesting that the i.j.p. amplitude (5 pulses at 5, 10 and 20 Hz, 0.5 ms supramaximal voltage) was not diminished when stimuli were applied during the membrane hyperpolarization produced by 8-bromo cyclic GMP (10^{-4} M), NaNP (10^{-5} M) or M&B 22948 (10^{-4} M) at a time when tone was abolished. This suggests that the mechanism underlying the membrane potential change produced by these drugs and by nerve stimulation was different.

Activation of cyclic AMP

The membrane effects produced by raising cyclic AMP levels in the gpIAS were investigated by using forskolin which activates adenylate cyclase. Forskolin (10^{-10}–10^{-7} M) relaxed the gpIAS in a dose-dependent manner without significantly affecting the membrane potential (Figure 4d). Small (< 10 mV) membrane hyperpolarizations were produced by higher concentrations of the drug.

Effects of inhibitory stimuli on cyclic nucleotide levels in the gpIAS

Cyclic GMP content The cyclic GMP content of the gpIAS was increased significantly ($P < 0.05$) from control levels of 4.2 ± 4.6 ($n = 11$) to 15.7 ± 9.8 ($n = 10$) fmol mg^{-1}, by field stimulation (80 pulses at 8 Hz; supramaximal voltage; 0.5 ms). This increase was prevented by TTX (10^{-6} M), but not by apamin (5×10^{-6} M) (Figure 1c).

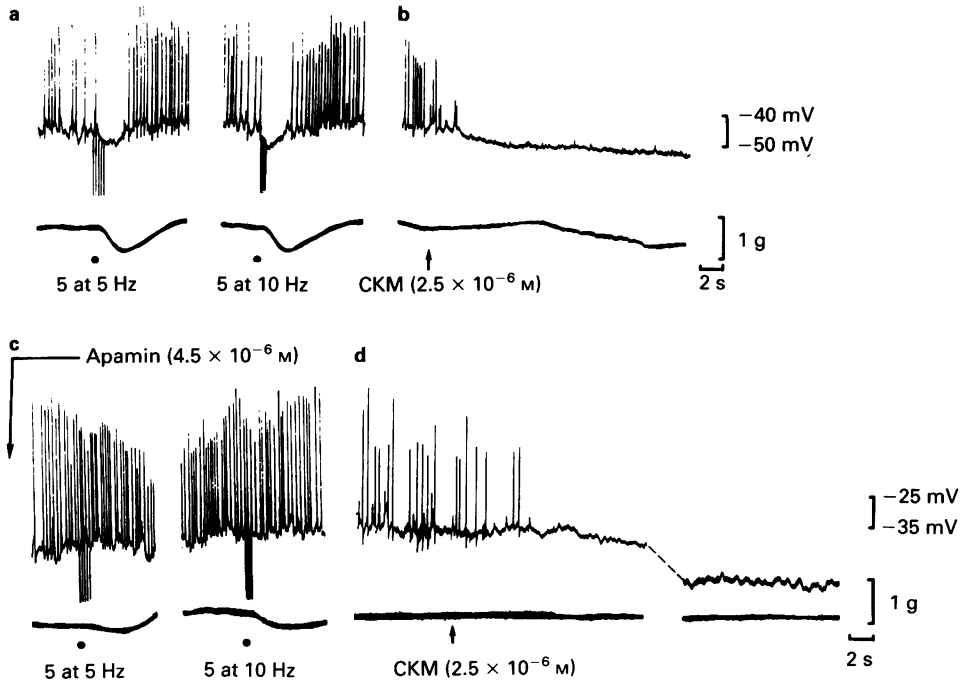

Figure 2 The effect of field stimulation (●, 5 pulses each at 5 and 10 Hz; 0.5 ms; supramaximal voltage) and of cromakalim (CKM, 2.5×10^{-6} M) each alone (a and b, respectively) and in the presence of apamin (4.5×10^{-6} M) (c and d, respectively) on the simultaneously-recorded electrical (upper trace) and mechanical responses of the guinea-pig internal anal sphincter (gpIAS). Field stimulation produced i.j.ps and relaxation which were abolished by apamin (leaving some spontaneous mechanical activity), however, the hyperpolarization produced by cromakalim was unaffected. In the presence of apamin, tone declined and with it the ability to demonstrate mechanical inhibition (c and d). (a) and (b) were from the same cell, (c) and (d) were from the same cell; time between panels joined by dotted line was 1 min. Atropine and phentolamine (each 10^{-6} M) were present throughout.

ATP (10^{-4} M), the putative inhibitory neurotransmitter, induced a quantitatively similar increase in the cyclic GMP content of the cells to that produced by field stimulation (80 pulses 8 Hz 0.5 ms; 19.8 ± 15.4 fmol mg^{-1}, $n = 11$) (Figure 1c), whilst NaNP (10^{-5} M) exhibited a 6 fold increase (26.1 ± 21.9 fmol mg^{-1}, $n = 19$) (Figure 3d). In contrast, isoprenaline (10^{-4} M), cromakalim (10^{-5} M) and forskolin (10^{-5} M) were each ineffective (Figure 3d).

Cyclic AMP content The cyclic AMP content of the gpIAS was increased from 1.84 ± 1.63 pmol mg^{-1} ($n = 11$) to 4.28 ± 2.27 pmol mg^{-1} ($n = 10$; $P < 0.05$) during relaxation in response to field stimulation (80 pulses at 8 Hz; 0.5 ms pulse width; supramaximal voltage). This increase was abolished by TTX (10^{-6} M) and also by apamin (5×10^{-6} M) (Figure 1d). ATP (10^{-4} M) caused a quantitatively similar increase to that produced by field stimulation (4.1 ± 2.5 pmol mg^{-1}, $n = 11$, $P < 0.05$, Figure 1d) as did isoprenaline (10^{-4} M, 4.2 ± 3.3 pmol mg^{-1}, $n = 9$, $P < 0.05$) and cromakalim, (10^{-5} M, 4.51 ± 2.76 pmol mg^{-1}, $n = 10$, $P < 0.05$ Figure 3e). The adenylate cyclase activator, forskolin (10^{-5} M) increased the cyclic AMP level by 6 fold (Figure 3e).

Discussion

In the gpIAS, membrane hyperpolarization accompanies the relaxation produced by directly applied current, inhibitory nerve stimulation and drugs. The characteristics of the membrane potential change varied with the stimulus employed. Inhibitory nerve stimulation and ATP, the proposed neurotransmitter (Lim & Muir, 1986), produced rapid, brief changes in membrane potential. The amplitudes of the electrical and mechanical events were clearly correlated because relaxation was never observed without hyperpolarization. In these cases, hyperpolarization was the likely cause of the relaxation and, (Lim & Muir, 1985; 1986) is likely to be mediated by an increase in gK^+, as shown by its dependence on $[K^+]_o$ and its sensitivity to K^+ channel antagonists (TEA and apamin). The effectiveness of these drugs suggests the involvement of a Ca^{2+}-dependent K^+ channel (Adams *et al.*, 1982). Whether or not the increase in $[Ca^{2+}]_i$ arose from an increased membrane permeability to Ca^{2+} or a release of bound cation from a membrane bound store is unknown (Den Hertog, 1982; Bolton & Lim, 1989).

Relaxation to isoprenaline and cromakalim was accompanied by dose-dependent hyperpolarization. With isoprenaline, the membrane change was significantly less in amplitude ($P = 0.025$), rate of onset ($P < 0.001$) and more prolonged ($P < 0.001$) than with nerve stimulation. With cromakalim, the amplitude of the membrane potential change was comparable to that produced by nerve stimulation. Unlike that to nerve stimulation and ATP, the membrane hyperpolarization produced by isoprenaline and cromakalim was not susceptible to apamin, suggesting the involvement of different K^+ channels (see also Weir & Weston, 1986). Notwithstanding these differences, electrical and mechanical events were clearly linked and could not be dissociated. In contrast, the membrane hyperpolarization and relaxation produced by inhibitory nerve stimulation in quiescent smooth muscles e.g. BRP (Byrne & Muir, 1984), rat anococcygeus (Creed *et al.*, 1975) and rabbit rectococcygeus (Blakeley *et al.*, 1979) for example, may not be closely associated. The electrical change in response to inhibitory nerve stimulation is insignificant and can be inhibited in the BRP without significantly affecting the relaxation (Byrne *et al.*, 1984). In the BRP, both electrical and mechanical responses are insensitive to apamin but inhibited by oxyhaemoglobin and may not involve K^+ channels (Byrne & Muir, 1984). Together these results suggest the presence of an additional non-electrical component in the control of the mechanical event. Whether the absence of a significant membrane change in response to nerve stimulation is due to an alteration in channel properties produced by the raising of tone so making them more sensitive to Ca^{2+}, or to changes in

membrane potential produced by the stimulus, remains unknown. Alternatively, as suggested by Kotecha & Nield (1988), the increase in intracellular Ca^{2+} produced by the stimulus may have been insufficient to open K^+ channels in these muscles.

The cyclic GMP content of the gpIAS was much smaller than that reported in the BRP (Bowman & Drummond, 1984; Gillespie & Sheng, 1988) or the LOS (Torphy *et al.*, 1986) but the cyclic AMP content of these tissues was comparable. Relaxation in the gpIAS was accompanied by increases in cyclic AMP, cyclic GMP or both depending on the stimulus. The rise in cyclic GMP content to nerve stimulation exceeded that in the BRP or LOS (by some 100%). Rises in nucleotide levels following application of drugs in these tissues were more uniform than those produced by nerve stimulation. Cyclic GMP levels were elevated 6 fold by NaNP in the gpIAS, and BRP while cyclic AMP was elevated 6 fold by forskolin in the gpIAS and BRP. In the gpIAS relaxation accompanied a rise in both cyclic nucleotide levels even though a significant membrane hyperpolarization was not observed. Thus, NaNP, which stimulates guanylate cyclase, M&B 22948 which inhibits cyclic GMP phosphodiesterase and the permeable cyclic GMP analogue 8-bromo-cyclic GMP relaxed the sphincter without affecting the membrane potential significantly. Inhibitory NANC nerve stimulation and ATP the proposed inhibitory transmitter raised the level of both cyclic AMP and cyclic GMP. Isoprenaline, acting via β-adrenoceptors, and cromakalim which activates certain potassium channels raised only levels of cyclic AMP. Selective stimulation of either nucleotide also accompanies relaxation in other smooth muscles. In the LOS, inhibitory nerve stimulation raised cyclic GMP but not cyclic AMP levels (Torphy *et al.*, 1986); similar results were reported in the BRP (Bowman & Drummond, 1984) but

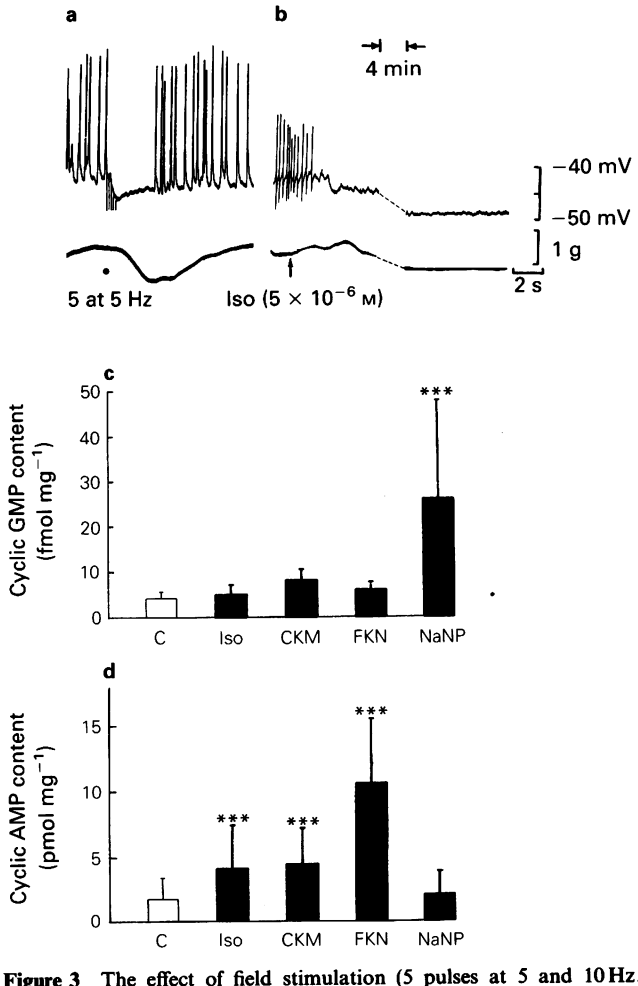

Figure 3 The effect of field stimulation (5 pulses at 5 and 10 Hz; 0.5 ms; supramaximal voltage) and (b) isoprenaline (Iso, 5×10^{-6} M) on the simultaneously-recorded electrical (upper trace) and mechanical responses of the guinea-pig internal anal sphincter (gpIAS). The time between panels (a) and (b) was 30 min. (c and d) The effect (± s.d.) of at least 9 applications of isoprenaline (Iso, 10^{-4} M), cromakalim (CKM, 10^{-5} M), forskolin (FKN, 10^{-5} M) and sodium nitroprusside (NaNP, 10^{-5} M) on the cyclic GMP (fmol mg^{-1}, c) and cyclic AMP (pmol mg^{-1}) contents of the gpIAS. NaNP alone failed to raise the cyclic AMP content and only NaNP raised the level of cyclic GMP significantly compared with controls (C). Atropine and phentolamine (each 10^{-6} M) were present throughout these experiments.

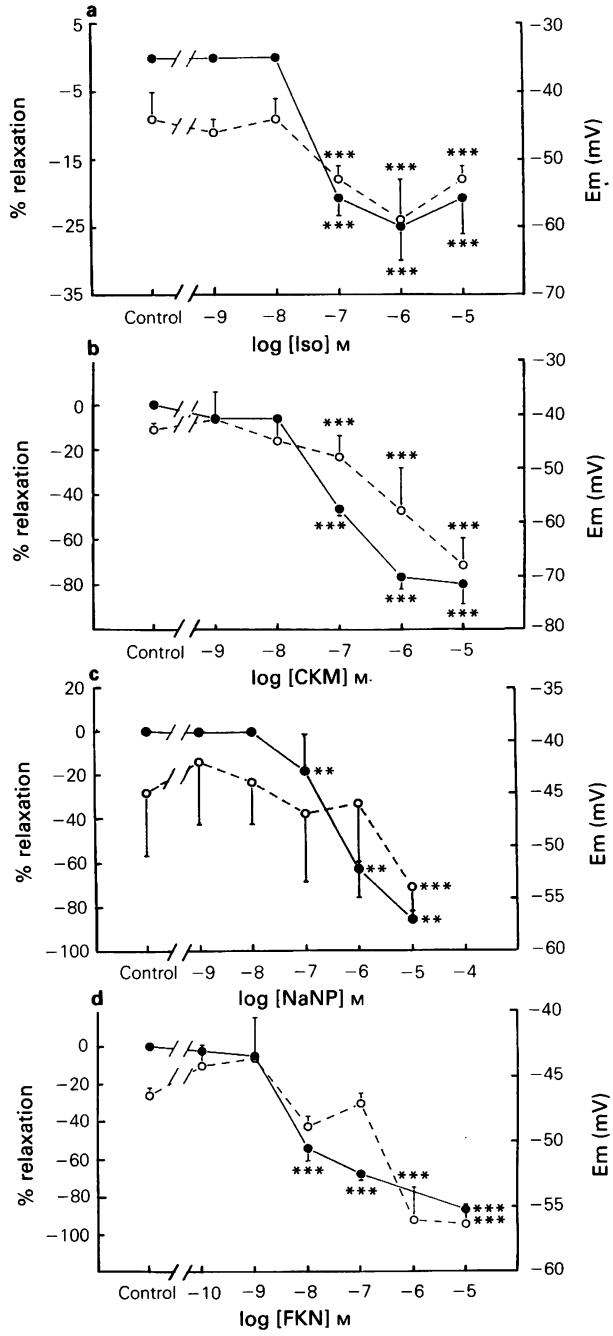

Figure 4 The effects of increasing the concentration of isoprenaline (Iso, a), cromakalim (CKM, b), sodium nitroprusside (NaNP, c) and forskolin (FKN, d) on the electrical (Em, RHS, ○) and mechanical (% relaxation, LHS, ●) responses of the guinea-pig internal anal sphincter. Each point represents the mean of a minimum of 9 observations; s.d. shown by vertical bars. Each drug produced a concentration-dependent membrane hyperpolarization and reduction in tone. NaNP and FKN relaxed the tone initially without significantly affecting membrane potential although higher concentrations hyperpolarized the membrane. Atropine and phentolamine (each 10^{-6} M) were present throughout these experiments.

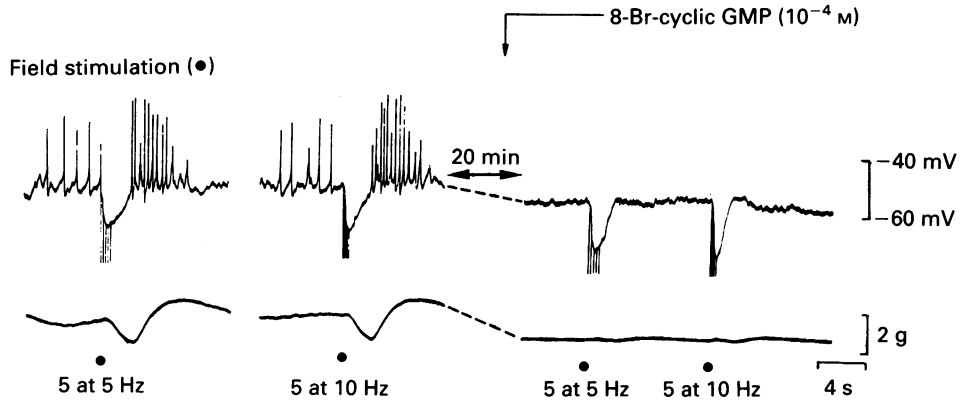

Figure 5 The effect of 8-bromo-cyclic GMP (8-Br-cyclic GMP 10^{-4} M, added at the arrow for the duration of the experiment) on the simultaneously-recorded electrical (upper trace) and mechanical responses of the guinea-pig internal anal sphincter to field stimulation (●, 5 pulses at 5 and 10 Hz; 0.5 ms; supramaximal voltage). 8-Br-cyclic GMP hyperpolarized the membrane, abolished spikes and muscle tone so that further relaxation could not be observed. However, i.j.ps to field stimulation were not diminished during the hyperpolarization produced by the cyclic nucleotide. Time between recordings from two different cells in the same preparation (– – –) was 20 min and between panels showing spontaneous activity in the same cell was 10 s. Atropine and phentolamine (each 10^{-6} M) were present throughout these experiments.

unlike in the gpIAS, cromakalim failed to raise cyclic AMP levels in this tissue (Gillespie & Sheng, 1988).

Although only indirect evidence exists to link a rise in cyclic nucleotide levels with relaxation in the gpIAs, in every case at present investigated, relaxation was accompanied by a rise in either or both cyclic nucleotides. If a causal relationship exists between the two, the question remains as to the mechanism by which the electrical and biochemical events are functionally related to control the Ca^{2+} economy of the cell. The rise in the level of both (rather than one) nucleotide would enable additional relaxation mechanisms to be activated. The main effect of a rise in cyclic GMP (Itoh *et al.*, 1985) is to increase the amount of Ca^{2+} extruded at the sarcolemmal membrane by activating a Ca^{2+} pump; cyclic AMP serves principally to increase the amount of Ca^{2+} stored in the reticulum. Both nucleotides, in addition, inhibit the phosphorylation of myosin light chain kinase and reduce the Ca^{2+} sensitivity of the contractile elements to Ca^{2+} but cyclic AMP is more effective in this respect. On this basis, purinoceptor interaction arising from inhibitory nerve stimulation or ATP, which produced rapid changes in membrane potential and tone may have stimulated both the efflux and the accumulation of Ca^{2+}. Isoprenaline and cromakalim which raised only cyclic AMP levels, may have relaxed the muscle largely by enhancing Ca^{2+} uptake into stores rather than by enhancing extrusion of the ion.

An opportunity to re-assess the similarities between the electrical and mechanical responses of the gpIAS to NANC inhibitory nerve stimulation and ATP the proposed inhibitory transmitter (Lim & Muir, 1986) was taken during the present study. Unlike previous studies from this laboratory (Lim & Muir, 1986), the purine was applied by hydrostatic pressure ejection close to the recording site and at suitable time intervals to avoid desensitization. Intracellular recordings revealed that both ATP and NANC inhibitory nerve stimulation produced membrane hyperpolarizations which were similar in the rates of rise and decline, amplitude and duration. These responses were frequency- (nerve stimulation) or dose-dependent (ATP) and inhibited by TEA and apamin and each accompanied a rise in cyclic GMP and cyclic AMP (see above). These results, together with previous observations (Lim & Muir, 1986), are in keeping with an inhibitory transmitter role for ATP in the tissue.

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Renal vascular responsiveness to arachidonic acid in experimental diabetes

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1 Isolated perfused kidneys from diabetic rats (duration 4–6 and 20–24 weeks) were more sensitive to the vasoconstrictor effects of arachidonic acid than kidneys from age-matched control rats. Sensitivity diminised with age in both control and diabetic groups.

2 The enhanced vasoconstrictor effect of arachidonic acid in diabetic rat kidneys was associated with increased conversion to prostaglandins.

3 The renal vasoconstrictor response to arachidonic acid in both groups was reduced by thromboxane A_2/prostaglandin H_2 receptor antagonism but not by inhibition of thromboxane synthase.

4 Diabetic rat kidneys were also more sensitive to the vasoconstrictor effects of the endoperoxide analogue, U46619, while vasoconstrictor responses to phenylephrine were not markedly different from those of control rat kidneys.

5 In conclusion, prostaglandin endoperoxides appear to mediate arachidonic acid-induced vasoconstriction in diabetic and control rat kidneys. The enhanced renal vasoconstrictor response to arachidonic acid in diabetic rats results from increased sensitivity to endoperoxides and increased formation of endoperoxides from arachidonic acid.

Introduction

Diabetes mellitus results in abnormalities of vascular and renal eicosanoid production. Thus, Halushka *et al.* (1981) found increased platelet thromboxane A_2 (TxA_2) formation in diabetic patients, while several investigators have shown reduced vascular prostacyclin production in human and experimental diabetes (Harrison *et al.*, 1978; Johnson *et al.*, 1979; Valentovic & Lubawy, 1983). Moreover, we demonstrated alterations in the profile of urinary prostanoids in rats made diabetic with streptozotocin (Quilley & McGiff, 1985), while others have shown abnormalities in the generation of glomerular prostanoids (Schambelan *et al.*, 1985). Consequently, we predicted the renal vascular responses to exogenous arachidonic acid would be altered in diabetes depending upon the activity of cyclo-oxygenase, the profile of eicosanoids produced and the sensitivity of the vasculature to these eicosanoids. Indeed, in a preliminary study we have shown enhanced conversion of arachidonic acid by the kidneys of diabetic rats associated with increased vasoconstrictor responses (Sarubbi *et al.*, 1989). However, this study was complicated by the development of tachyphylaxis to repeated administration of arachidonic acid. In a recent study we provided evidence that endoperoxides, intermediates in the metabolism of arachidonic acid by cyclo-oxygenase, are responsible for the vasoconstrictor effects of arachidonic acid in the isolated perfused kidney of the rat (Quilley *et al.*, 1989). If the same pertains for the diabetic rat kidney, then the response to arachidonic acid should depend upon cyclo-oxygenase activity and the responsiveness of the vasculature to endoperoxides. The present study was designed, therefore, to determine renal vascular responsiveness to arachidonic acid in kidneys of rats with short-term (4–6 weeks) and long-term (20–24 weeks) diabetes. The role of endoperoxides in the response was examined by use of a thromboxane synthase inhibitor and a TxA_2/prostaglandin H_2 (PGH_2) receptor antagonist, while sensitivity of the vasculature to endoperoxides was assessed by testing the vascular responses to an endoperoxide analogue. Cyclo-oxygenase activity was assessed by measuring the release of PGE_2 and 6-keto-$PGF_{1\alpha}$ following arachidonic acid administration.

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Methods

Diabetes was induced in male Wistar rats (175–200 g) with streptozotocin, 70 mg kg^{-1}, i.v. Control rats were given an equivalent volume of citrate buffer, pH 4.2. Rats were used either 4–6 weeks or 20–24 weeks later. At the time of experimentation blood glucose levels were measured by a glucometer (Ames). Rats were considered diabetic if blood glucose levels were greater than 300 mg dl^{-1}. Mean blood glucose levels in citrate-treated rats were less than 100 mg dl^{-1}.

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 to provide an exit for the perfusate. The right ureter was also cut and the animal killed by an intracardiac injection of 10 mg pentobarbitone. In some preparations the vena cava was cannulated for the collection of renal venous effluent for the subsequent measurement of immunoreactive 6-keto-$PGF_{1\alpha}$ and PGE_2.

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 flow adjusted to obtain a basal perfusion pressure of 70–80 mmHg. The perfusion pressure was measured with a Harvard pressure transducer and recorded on a Soltec 1246 recorder.

Renal vascular responses to arachidonic acid

Arachidonic acid was administered as random bolus doses (0.3–10 μg) given at 10 min intervals into the perfusate line proximal to the kidney. Each kidney was used for 1 or 2 doses of arachidonic acid only, depending on whether a high or low dose was given first because of the appearance of tachyphylaxis to repeated administrations. Responses in kidneys from rats with diabetes of 4–6 weeks duration (n = 10) were

compared to those in age-matched controls ($n = 8$). Similarly, kidneys from 20–24 week diabetic rats ($n = 9$) were compared to age-matched controls ($n = 10$).

Renal vascular responses to U46619, an endoperoxide analogue

In kidneys from rats with diabetes of 4–6 weeks duration ($n = 5$) and age-matched controls ($n = 5$), U46619 was administered as random bolus doses (10–1000 ng), given at 5–7 min intervals when pressure had returned to baseline levels following the preceding dose.

In kidneys from rats with diabetes of 20–24 weeks duration ($n = 3$) and their corresponding controls ($n = 4$), only one dose of U46619 was compared, 100 ng. These same preparations were also used to test responses to 3 μg arachidonic acid and acted as the vehicle-treated group in those experiments where responses to U46619 and arachidonic acid were tested following inhibition of thromboxane synthase (see below).

Renal vascular responses to phenylephrine

The kidneys of rats with diabetes of 4–6 weeks duration ($n = 5$) and their age-matched controls ($n = 5$) were used to determine perfusion pressure responses to phenylephrine (100–3000 ng), given as random bolus doses at intervals of 5–7 min.

Influence of thromboxane synthase inhibition on the renal vascular responses to U46619 and arachidonic acid

Perfusion pressure increases in response to arachidonic acid (3 μg) and U46619 (100 ng) in vehicle-treated kidneys from 20–24 week diabetic rats ($n = 3$) and age-matched control rats ($n = 4$) were compared to those obtained in diabetic rat kidneys ($n = 3$) and control rat kidneys ($n = 4$) perfused with Krebs-Henseleit buffer containing 5 μM CGS-13080, a thromboxane synthase inhibitor. In addition, the rats were given CGS-13080, 2 mg kg^{-1} i.p., 2 h before the experiment. We have previously shown that this concentration of CGS-13080 is effective in reducing the renal efflux of immunoreactive TxB_2 following arachidonic acid administration by more than 70% (Quilley *et al.*, 1989). The vehicle-treated groups in these experiments correspond to those that were used to compare diabetic and control kidney responses to 100 ng U46619 (see above).

Influence of TxA_2/PGH_2 receptor antagonism on the renal vascular responses to arachidonic acid and U46619

In 6 diabetic (4–6 weeks) and 5 age-matched control rat kidney preparations, perfusion pressure responses to 1 μg and 3 μg arachidonic acid, respectively, were determined after the addition of 1 μM SQ 29548, a TxA_2/PGH_2 receptor antagonist, to the perfusate. These responses were compared to those obtained in the construction of the dose-response curves to arachidonic acid. In some of the preparations used to determine the effects of TxA_2/PGH_2 receptor blockade on responses to arachidonic acid, we verified that SQ 29548 was effective in reducing the responses to U46619 (100 ng), which was administered before and 10 min after addition of the antagonist to the perfusate. Thus, 6 control and 3 diabetic rat kidneys were tested in this way. The responses obtained to U46619 before the addition of SQ 29548 to the perfusate did not differ from those used in the construction of dose-response cuves to U46619 and the data were, therefore, pooled.

The experiments with SQ29548 were only conducted in rats with diabetes of 4–6 weeks duration, while those with CGS-13080 utilized rats with diabetes of 20–24 weeks duration. This design was adopted because of the similar qualitative responses to arachidonic acid and U46619 in both diabetic and control rats irrespective of the duration of diabetes (see Results).

Renal venous efflux of 6-keto-prostaglandin $F_{1\alpha}$ and prostaglandin E_2 in response to arachidonic acid

In additional experiments, kidneys from 3 rats with diabetes of 4–6 weeks duration and 4 age-matched control rats were used to compare the venous release of immunoreactive 6-keto-$PGF_{1\alpha}$ and PGE_2 following the administration of 3 μg arachidonic acid. Thus, the renal venous effluent was collected for 1 min before and for 2 min after the administration of arachidonic acid, beginning at the onset of a change in perfusion pressure. Perfusates were stored at −20°C prior to radioimmunoassay as previously described (Quilley & McGiff, 1985). Stimulated release of 6-keto-$PGF_{1\alpha}$ and PGE_2 was obtained by subtracting basal release from that measured following arachidonic acid administration.

Statistical analyses

Dose-response curves were compared by ANOVA and individual points on the curve by Student's t test for unpaired data. Similarly, responses to individual doses of arachidonic acid and U46619 between diabetic and control and following treatment with a thromboxane synthase inhibitor or a PGH_2/TxA_2 receptor antagonist were compared by an unpaired 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. 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 used. U46619 (11,9 epoxy methano prostaglandin H_2), a gift from the UpJohn Co., Kalamazoo, MI, U.S.A 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. The hydrochloride salt of phenylephrine was obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A and was dissolved in distilled water, 100 μg ml^{-1}, and stored in aliquots at −70°C. Streptozotocin was also obtained from the Sigma Chemical Co. and dissolved in citrate buffer, pH 4.2, immediately before use.

Results

Renal vascular responses to arachidonic acid

In rats with diabetes of 4–6 weeks duration and age-matched control rats the renal perfusate flows were 14.6 ± 1.1 ($n = 10$) and 12.3 ± 1.1 ml min^{-1} ($n = 8$), respectively, which yielded basal perfusion pressures of 76 ± 4 mmHg and 75 ± 3 mmHg, respectively. The dose-response curve for arachidonic acid in the diabetic group was shifted to the left (Figure 1). Thus, at comparable doses, arachidonic acid elicited greater increases in renal perfusion pressure in the diabetic rat. At 1.0 μg arachidonic acid the increases in renal perfusion pressure were 137 ± 34 mmHg and 11 ± 1 mmHg for diabetic and control groups, respectively ($P < 0.01$). Similarly, 3 μg arachidonic acid increased perfusion pressure by 189 ± 3 mmHg in the

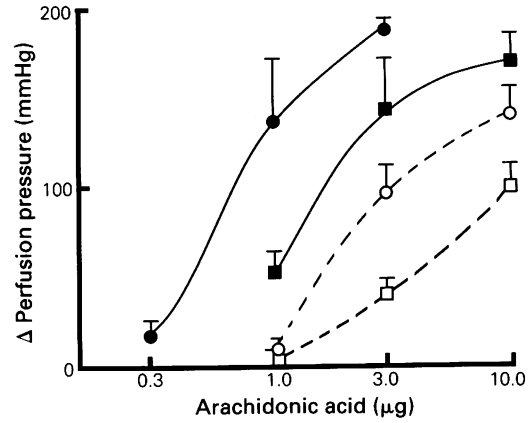

Figure 1 Increases in perfusion pressure in response to arachidonic acid in kidneys from rats with diabetes of duration 4–6 weeks ($n = 10$, ●——●) and 20–24 weeks ($n = 9$, ■——■) and their respective age-matched controls ($n = 8$, ○---○ and $n = 10$, □---□). Data are presented as the mean with s.e.mean shown by vertical bars.

diabetic group compared to 99 ± 13 mmHg in the control group ($P < 0.01$).

In kidneys from rats with diabetes of 20–24 weeks duration ($n = 9$) and age-matched control rats ($n = 10$) the perfusate flows were 23.6 ± 2.5 ml min^{-1} and 17.5 ± 1.7 ml min^{-1} ($P < 0.05$), respectively, which resulted in basal perfusion pressures of 77 ± 6 mmHg and 85 ± 4 mmHg, respectively. As before, the dose-response curve to arachidonic acid in the diabetic group was shifted to the left (Figure 1). Thus, at all doses tested (1–10 μg) the diabetic group responded with greater increases in perfusion pressure. However, the older rats, both diabetic and control, were less responsive than younger rats.

Renal vascular responses to U46619

In kidneys from 4–6 week diabetic rats ($n = 5$) and age-matched controls ($n = 5$) perfusate flow rates were 14.0 ± 0.6 ml min^{-1} and 12.9 ± 0.8 ml min^{-1}, respectively, which resulted in basal perfusion pressures of 71 ± 3 mmHg and 68 ± 2 mmHg, respectively. U46619 elicited significantly greater responses in diabetic rat kidneys compared to control (Figure 2a), such that the dose-response curve was shifted to the left.

In the older diabetic ($n = 6$) and control ($n = 8$) groups perfusate flows were 25.7 ± 1.7 ml min^{-1} and 14.2 ± 1.4 ml min^{-1}, respectively ($P < 0.01$) and basal perfusion pressures were 75 ± 5 mmHg and 77 ± 3 mmHg, respectively. The much greater flow rate in the diabetic rat kidneys could be accounted for by the greater renal mass. Thus, left kidney weight in diabetic rats was 3.95 ± 0.30 g compared to 2.35 ± 0.13 g for control ($P < 0.01$). When this weight difference was taken into account renal perfusate flows were not different, i.e., 6.0 ml min^{-1} g^{-1} for control versus 6.5 ml min^{-1} g^{-1} for diabetic rats. These groups of rats were those used to test the effects of thromboxane synthase inhibition on the vasoconstrictor responses to U46619 (100 ng) and arachidonic acid (3 μg). As thromboxane synthase inhibition did not affect the responses to U46619 the data were pooled. Thus, in the diabetic rat kidneys treated with CGS 13080, 100 ng U46619 increased perfusion pressure by 131 ± 22 mmHg compared to an increase of 47 ± 10 mmHg in control rat kidneys ($P < 0.01$). These perfusion pressure responses are comparable to those seen in the 4–6 week diabetic and control rats when 100 ng U46619 increased perfusion pressure by 146 ± 7 mmHg and 75 ± 17 mmHg, respectively.

Renal vascular responses to phenylephrine

In 4–6 week diabetic ($n = 5$) and age-matched control ($n = 5$) rats, renal perfusate flow rates were 17.2 ± 0.7 and 10.6 ± 0.7 ml min^{-1}, respectively, which resulted in similar basal perfusion pressures of 73 ± 2 and 74 ± 1 mmHg, respectively. In the diabetic rat kidneys, phenylephrine (100–3000 ng) resulted in dose-dependent increases in perfusion pressure which were slightly greater than those obtained in control rat kidneys, the difference being significant at the lower doses (Figure 2b). Thus, the diabetic rat kidney has a lower threshold for the vasoconstrictor response to phenylephrine, although the maximal response was not different from control.

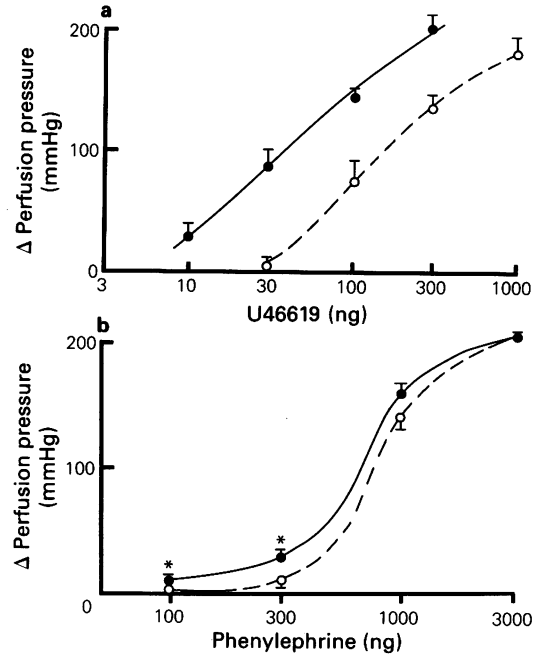

Figure 2 Increases in perfusion pressure in response to (a) the thromboxane A_2/prostaglandin H_2 agonist, U46619, and (b) phenylephrine in kidneys from rats with diabetes of 4–6 weeks duration ($n = 5$ for each agonist) and from age-matched control rats ($n = 5$ for each agonist). (●——●) Diabetic; (○---○) control. Data are presented as the mean with s.e.mean shown by vertical bars.

Effects of inhibition of thromboxane synthase on renal vascular responses to U46619 and arachidonic acid

In both control rats and those with diabetes of 20–24 weeks duration, inhibition of thromboxane formation by CGS-13080 did not alter responses to U46619 (100 ng) or arachidonic acid (3 μg). Thus, in vehicle-treated diabetic rat kidneys perfusion pressure increases to U46619 and arachidonic acid were 140 ± 13 mmHg and 182 ± 13 mmHg, respectively, compared to increases of 121 ± 47 mmHg and 186 ± 5 mmHg, respectively, in kidneys treated with CGS-13080 (Figure 3). Similarly, in control rat kidneys the perfusion pressure responses to U46619 and arachidonic acid were 54 ± 18 mmHg and 78 ± 15 mmHg, respectively, in the absence of CGS-13080 compared to 40 ± 11 mmHg and 66 ± 19 mmHg, respectively, in the presence of CGS-13080.

Effects of TxA_2/PGH_2 receptor antagonism on renal vascular responses to U46619 and arachidonic acid

In rats with diabetes of 4–6 weeks duration and age-matched control rats, the renal perfusate flow rates were 17.7 ± 1.5 ml min^{-1} and 11.8 ± 1.6 ml min^{-1}, respectively, resulting in basal perfusion pressures of 76 ± 3 mmHg and 77 ± 2 mmHg, respectively. In diabetic rat kidneys, the administration of 1 μg arachidonic acid, following SQ 29548 treatment, increased perfusion pressure by 60 ± 22 mmHg compared to 137 ± 34 mmHg in untreated preparations, a reduction of almost 60% (Figure 4). Similarly, in control preparations after SQ 29548, 3 μg arachidonic acid increased pressure by 12 ± 10 mmHg compared to 99 ± 13 mmHg in

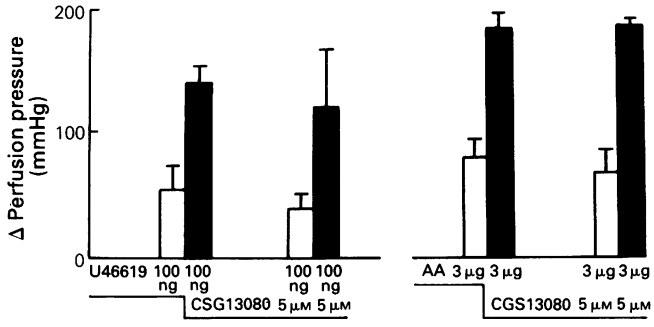

Figure 3 Effects of thromboxane synthesis inhibition with CGS 13080 (5 μM) on perfusion pressure responses to the endoperoxide analogue, U46619, and arachidonic acid (AA) in kidneys from rats with diabetes of 20–24 weeks duration ($n = 3$; solid columns) and from age-matched controls ($n = 4$; open columns). Data are presented as the means with s.e.mean shown by vertical bars.

untreated kidneys ($P < 0.01$), a reduction of 88%. We verified that this concentration of SQ 29548 was effective in blocking the responses to U46619. Thus, in control rat kidneys the perfusion pressure response to 100 ng U46619 was reduced from 85 ± 12 mmHg to zero after SQ 29548 treatment (Figure 4). Similarly, in diabetic rat preparations the response to U46619 was markedly reduced from 181 ± 11 mmHg to 8 ± 7 mmHg after SQ 29548 ($P < 0.01$).

Release of 6-keto-prostaglandin $F_{1\alpha}$ and prostaglandin E_2

In diabetic (4–6 weeks) and control rat kidneys the basal release of 6-keto-$PGF_{1\alpha}$, an index of prostacyclin, into the venous effluent was 0.5 ± 0.1 ng min^{-1} and 0.3 ± 0.1 ng min^{-1}, respectively (NS). In contrast, basal release of PGE_2 was lower in diabetic rats (0.7 ± 0.1 ng min^{-1}) than control (2.3 ± 0.7 ng min^{-1}) rats ($P < 0.05$). However, administration of 3 μg arachidonic acid resulted in greater increases in the release of 6-keto-$PGF_{1\alpha}$ and PGE_2 from diabetic rat kidneys than control rat kidneys. Thus, 6-keto-$PGF_{1\alpha}$ and PGE_2 release increased by 6.0 ± 0.6 ng min^{-1} and 7.1 ± 1.2 ng min^{-1}, respectively, from diabetic rat kidneys and 2.7 ± 0.4 ng min^{-1} ($P < 0.05$) and 3.9 ± 0.5 ng min^{-1} ($P < 0.05$), respectively, from control rat kidneys. The increased release of prostanoids from diabetic rat kidneys was associated with greater perfusion pressure increases to arachidonic acid, i.e., 165 ± 22 mmHg vs 110 ± 22 mmHg in control rat kidneys ($P < 0.05$).

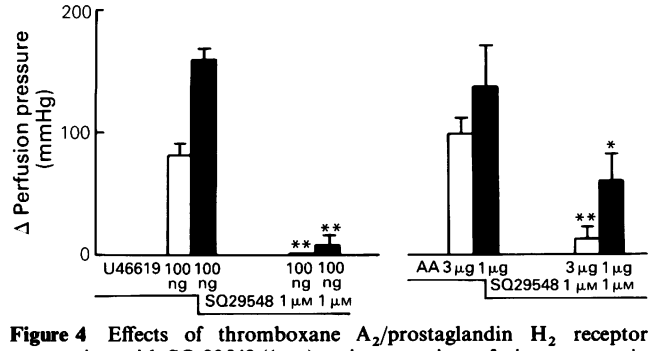

Figure 4 Effects of thromboxane A_2/prostaglandin H_2 receptor antagonism with SQ 29548 (1 μM) on increases in perfusion pressure in kidneys from rats with diabetes of 4–6 weeks duration (solid columns) and from age-matched control rats (open columns) in response to the endoperoxide analogue, U46619 ($n = 6$ for control and 3 for diabetic), and arachidonic acid ($n = 5$ for control and 6 for diabetic). The pretreatment values for U46619 also include data from the dose-response curves to U46619 (Figure 2) so that $n = 11$ for control and 8 for diabetic. The values for the arachidonic acid (AA) responses were obtained from Figure 1. Data are presented as the means with s.e.mean shown by vertical bars. *$P < 0.05$, **$P < 0.01$, compared to responses with SQ 29548.

Discussion

This study demonstrates enhanced renal vasoconstrictor responses to arachidonic acid in rats with diabetes induced by streptozotocin and confirms our earlier preliminary study (Sarubbi *et al.*, 1989). However, the previous study was complicated by the development of tachyphylaxis to the vasoconstrictor effects of arachidonic acid. This vascular tachyphylaxis to arachidonic acid was avoided by administering only one or two doses of arachidonic acid to each kidney depending on whether a high or low dose was given first. Our earlier work has now been extended to characterize more fully the renal vascular response in diabetic rats.

In both diabetic and non-diabetic rat kidneys the vasoconstrictor effect of arachidonic acid is cyclo-oxygenase-dependent, as the response is reduced by indomethacin or meclofenamate (Sarubbi *et al.*, 1989; Quilley *et al.*, 1989). In non-diabetic rat kidneys, we have previously shown that the vasoconstrictor response to arachidonic acid is mediated by the endoperoxides (Quilley *et al.*, 1989). The results of the present study indicate that the same pertains to the diabetic rat kidney, as the vasoconstrictor response to arachidonic acid is not affected by thromboxane synthetase inhibition but is markedly reduced by TxA_2/PGH_2 receptor antagonism. However, we cannot exclude the possibility that prostanoids other than endoperoxides also contribute to the arachidonic acid response in the diabetic rat, although in normal rats we have shown that microgram quantities of PGD_2, PGE_2 and $PGF_{2\alpha}$ are required to elicit vasoconstriction while responses to PGE_2 and $PGF_{2\alpha}$ are unaffected by SQ 29548 (Quilley *et al.*, 1989).

There are several possible mechanisms whereby experimentally-induced diabetes may lead to an increase in renal vascular responsiveness to arachidonic acid. These include a generalized increase in sensitivity to vasoconstrictor agents which would probably reflect an enhancement of signal transducing mechanisms, an increase in conversion of arachidonic acid to endoperoxides or enhanced responsiveness to endoperoxides, possibly resulting from increased TxA_2/PGH_2 receptor number or affinity. A generalized increase in sensitivity to vasoconstrictor agents can be excluded, as previous studies have shown reduced renal vascular responsiveness to angiotensin II and arginine vasopressin in the diabetic rat (Reineck & Kreisberg, 1983; Sarubbi *et al.*, 1989). Moreover, in this study we found that the vasoconstrictor effects of phenylephrine were not markedly different in diabetic versus control rat kidneys. An increase in the conversion of arachidonic acid by cyclo-oxygenase contributes to the enhanced vasoconstrictor effects in diabetes. Thus, the increase in venous efflux of PGE_2 and PGI_2, measured as 6-keto-$PGF_{1\alpha}$, from the diabetic rat kidney after arachidonic acid administration was twice that of control rat kidneys. These results confirm those of a previous study and are in agreement with other studies demonstrating enhanced conversion of exogenous arachidonic acid in various tissues from diabetic rats (Rosen *et al.*, 1983; Roth *et al.*, 1983; Schambelan *et al.*, 1985). Finally, increased sensitivity of the renal vasculature of diabetic rats to endoperoxides also appears to contribute to the enhanced response to arachidonic acid. Thus, the dose-response curve to the endoperoxide analogue, U46619, in the diabetic rat kidney is shifted to the left. This is in contrast to the work of Boura *et al.* (1986) who demonstrated reduced responsiveness to U46619 in the autoperfused hindquarters of the alloxan diabetic rat. However, Boura *et al.* obtained increased vasoconstrictor responses to arachidonic acid, which were attributed to increased TxA_2 formation as blockade of the receptor reduced response. Nonetheless, the observations of Boura *et al.* can be explained by increased platelet formation of either endoperoxides or TxA_2 in diabetic rats. Thus, in human and experimental diabetes there are accounts of increased platelet cyclo-oxygenase and TxA_2 synthesis (Ziboh *et al.*, 1979; Subbiah & Deitemeyer, 1980; Halushka *et*

al., 1981). The increased sensitivity of the diabetic rat kidney to the vasoconstrictor effects of U46619 could be the result of increased receptor number or affinity, although this cannot be determined without radioligand binding studies which we have not done.

Our study indicates that increased renal vascular responsiveness to arachidonic acid in the diabetic rat results from increased cyclo-oxygenase activity, leading to the formation of more endoperoxides as well as enhanced renal responsiveness to the endoperoxides. The age-dependent diminution in vascular responsiveness to arachidonic acid in both diabetic and control rats is most probably due to reduced cyclo-oxygenase activity, as the decline in responsiveness to the endoperoxides is less suggesting that reduced formation of vasoconstrictor products, rather than reduced sensitivity, is responsible. Finally, enhanced renal vascular sensitivity to endoperoxides and increased conversion of arachidonic acid in diabetes may be of pathological significance, particularly under conditions of platelet aggregation whereupon greater vasoconstriction would be anticipated.

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Refractoriness of the gravid rat uterus to tocolytic and biochemical effects of atrial natriuretic peptide

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1 Effects of atrial natriuretic peptide (ANP) on tension development, particulate guanylate cyclase activity and guanosine 3':5'-cyclic monophosphate (cyclic GMP) concentrations of uteri from oestrogen-treated, progesterone-treated, ovariectomized and pregnant rats were determined *in vitro*.

2 ANP inhibited the tension development by myometrial tissues from oestrogen-treated virgin rats and the sterile horn of 10 to 14 day pregnant rats but not of the uterus from pregnant and progesterone-treated rats.

3 Inhibition of cyclo-oxygenase and lipoxygenase activities did not restore the tocolytic activity of ANP on gravid uterus. ANP exerted a tocolytic effect on nongravid uterus submaximally stimulated by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), oxytocin, vasopressin, angiotensin II or 5-hydryoxtryptamine (5-HT).

4 Ovariectomy decreased the tocolytic effects of ANP, which could be restored by oestrogen treatment.

5 The refractoriness to the tocolytic effect of ANP in pregnant rats was not accompanied by a decrease in its relaxant effects on isolated aortic strips.

6 Tocolytic effects of isoprenaline, isobutylmethyl xanthine and hydroxylamine were not influenced by pregnancy or progesterone treatment. Up to a concentration of 3 mM, sodium nitroprusside did not affect myometrial tension development.

7 Pregnancy and progesterone treatment markedly inhibited ANP-induced increases in myometrial particulate guanylate cyclase activity and cyclic GMP concentrations but did not influence the effects of ANP on aortic cyclic GMP concentrations.

8 It is concluded that exposure of the myometrium to circulating and placentally-produced progesterone is responsible for the pregnancy-induced decrease in the effects of ANP on myometrial particulate guanylate cyclase activity and cyclic GMP concentrations and in turn on myometrial tension development.

Introduction

In addition to causing natriuresis (de Bold *et al.*, 1981), atrial natriuretic peptide (ANP) produces several other effects including a relaxation of smooth muscles (Cantin & Genest, 1985). ANP relaxes vascular (Currie *et al.*, 1983; St-Louis *et al.*, 1988), bronchial (Potvin & Varma, 1989) and intestinal (Currie *et al.*, 1983) smooth muscles. A tocolytic effect of ANP on the nongravid myometrium has also been recently reported (Bek *et al.*, 1988). However, to our knowledge, effects of ANP on the activity of gravid uterus have not been studied. A tocolytic effect of ANP on gravid uterus may be of relevance because pregnancy and parturition are associated with an increase in the circulating concentrations of ANP (Otsuki *et al.*, 1987; Thomsen *et al.*, 1987; Olsson *et al.*, 1988; Castro *et al.*, 1989). We therefore studied the effects of ANP on isolated uterus and found that ANP inhibited the activity of nongravid but not the gravid uterus. Our data suggest that the pregnancy-induced refractoriness to the tocolytic effects of ANP is due to an inhibition of the effects of ANP on guanylate cyclase activity and guanosine 3':5'-cyclic monophosphate (cyclic GMP) and appears to be mediated by progesterone.

Methods

Animals

Female Sprague-Dawley rats (Charles River, St. Constant, Quebec, Canada) weighing between 200–350 g were maintained on Purina rat diet and tap water *ad libitum*. Pregnant rats (presence of sperm in vaginal washing denoted day 0), rats with one uterine horn sterile and one gravid, virgin untreated rats, virgin rats treated with progesterone or oestrogen and ovariectomized rats with or without pretreatment with oestrogen were used. Rats were decapitated for the removal of uterine and aortic tissues.

In some rats, one uterine horn was ligated under ether anaesthesia at its origin approximately 2 weeks before mating in order to raise pregnant rats with one sterile and one gravid horn. Ovariectomy was performed under ether anaesthesia 2 weeks prior to experiments. Oestrogen and progesterone were dissolved in sesame oil and injected intraperitoneally; 17β-oestradiol (1 mg kg^{-1}) was injected daily for 2 days and progesterone (2 mg kg^{-1}) for 3 days before the day of experiments.

Isolated uterine strips

Uterine horns were dissected out and placed in a physiological salt solution (PSS) of the following composition (mM): NaCl 118, $MgSO_4$ 1.18, KH_2PO_4 1.18, dextrose 5.5, $NaHCO_3$ 25, $CaCl_2$ 2.5, and KCl 4.7. Foetuses and placentas were removed and uterine horns were cleaned of visible blood vessels and connective tissues. Myometrial strips (~10 mm) were set up at approximately 2 g resting tension in 50 ml tissue organ baths containing PSS which was equilibrated with 5% CO_2 plus 95% O_2 and maintained at 37°C. The preparation was washed every 10 to 15 min with fresh buffer until the force and frequency of spontaneous contraction had become uniform. Tension development was recorded isometrically by means of Grass force-displacement transducers (FTO3C) on a Grass polygraph.

Myometrial tension development was quantitated by integration (Granger *et al.*, 1985). Cumulative concentration-response curves to ANP, isoprenaline, isobutylmethyl xanthine (IBMX), hydroxylamine and sodium nitroprusside were determined by adding the next higher concentration after the response to the previous concentration had reached a plateau. The plateau was reached in 2–3 min after ANP, 10–15 s after isoprenaline, 30–60 s after IBMX and 60–90 s

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after hydroxylamine. The response to each concentration of the agent was measured during a minimum 5 min period after the plateau was achieved. The motor activity during a 5 min period prior to the addition of the drug was treated as control and the tocolytic effects of increasing concentrations of various agents were calculated as percentage inhibition of this control activity; complete suppression of tension development was treated as 100% inhibition. Potencies of various agents were calculated from the regression line of the probit of percentage inhibition in uterine tension development against $-\log_{10}$ M drug concentration for each experiment (Granger *et al.*, 1985). The $-\log(EC_{50})$ was the $-\log$ molar concentration of the drug causing 50% inhibition of uterine tension development. In the case of uteri from ovariectomized animals, $-\log EC_{50}$ was calculated by extrapolation since the maximal tocolytic effect of ANP on these tissues was less than 50%. Because ANP did not inhibit the motor activity of the gravid uterus, experiments were done to determine if this refractoriness was induced by progesterone and prostaglandins. For this purpose, virgin rats were treated with progesterone. The effects of ANP on the gravid uterus were determined after inhibition of cyclo-oxygenase activity with 1 μM indomethacin and lipoxygenase activity with 10 μM nordihydroguaiaretic acid; since these treatments abolished spontaneous activity, myometrial activity was induced by 5-hydroxytryptamine (5-HT, 0.1–1 μM).

Isolated aortic strips

Two spirally-cut strips (approximately 2 mm wide and 15 mm long) from thoracic aorta were set up at approximately 1 g tension as described previously (Varma & Yue, 1986). The PSS and experimental conditions were similar to those described above for uterine strips. After an equilibration period of approximately 2 h, preparations were contracted to approximately 75% of maximal by adding 20 to 40 nM noradrenaline. Cumulative relaxant concentration-response curves to ANP were determined and $-\log(EC_{50})$ was calculated as described above for uterine strips.

Cyclic GMP assay

Cyclic GMP was assayed essentially as described by Tremblay *et al.* (1985). Strips of uterus (300 to 500 mg) and aorta (50 to 100 mg) were incubated at 37°C in 20 ml beakers containing Krebs solution equilibrated with 95% O_2 and 5% CO_2. Tissues were preincubated with 100 μM IBMX for 5 min following which ANP was added into the bath. The reaction was stopped by removing the tissues and placing them into 3 ml ice-cold acidic-ethanol (1% 1N HCl in ethanol) followed immediately by homogenization at 0°C with a Polytron. The homogenate was centrifuged at 4°C for 10 min at 14,000 *g*. The supernatant was aspirated and dried under vacuum and then resuspended in 50 mM Tris buffer containing 4 mM EDTA (pH 7.6). An aliquot was removed and its cyclic GMP content was determined by radioimmunoassay with a commercial kit (Amersham, Oakville, Ontario, Canada). The pellet was resuspended in 2 ml NaOH and the protein content determined according to Lowry *et al.* (1951) with bovine serum albumin as the standard.

Particulate guanylate cyclase activity

Particulate guanylate cyclase activity was assayed according to the method of Winquist *et al.* (1984). Myometrial strips (0.3–0.5 g) were minced with scissors and homogenized by a Polytron at a setting of 10 at 4°C in 5 ml of 50 mM Tris HCl (pH 8) containing 1 mM EDTA, 250 mM sucrose and 1 mM dithiothreitol. The homogenate was centrifuged for 10 min at 500 *g*. The supernatant was aspirated and recentrifuged at 100,000 *g* for 1 h at 4°C. The supernatant was discarded and the pellet was resuspended in 0.5–1 ml of 50 mM Tris HCl (pH 7.6) for the assay of guanylate cyclase activity. Guanylate cyclase activity was assayed in 50 μl of the membrane preparation (50–100 μg protein) at 37°C in the presence of 50 mM Tris HCl (pH 7.6), 10 mM theophylline, 2 mM IBMX, ANP and a GTP-regenerating system which consisted of 15 mM creatinine phosphate and 5 units (20 μg of 250 units mg^{-1} protein) of creatinine phosphokinase in a final volume of 250 μl. The reaction was initiated by the addition of the substrate (1 mM GTP, 4 mM $MnCl_2$) and the reaction was terminated after 3 min by addition of 1 ml of acidic ethanol. The period of 3 min was selected on the basis of time-course studies from 1 to 5 min. Ethanol was removed under vacuum and the sample was assayed for cyclic GMP as described above. Guanylate cyclase activity was calculated as cyclic GMP formed per min per mg membrane protein.

Statistics

Two means were compared by unpaired or paired (in the case of changes in the rate of tension development) Student's *t* test. Multiple means were compared by one-way analysis of variance followed by comparisons of each pair in the group (Bonferroni). A probability of less than 0.05 was assumed to denote a significant difference. Throughout this paper, means ± s.e.mean are presented.

Chemicals

Synthetic 28 amino acid rat atrial natriuretic peptide was purchased from Peninsula Laboratories, Belmont, California. The cyclic GMP RIA kit (TRK.500) was purchased from Amersham, Oakville, Ontario, Canada. The following agents were purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A.: angiotensin II, creatinine phosphate, creatinine phosphokinase, 17β-oestradiol, hydroxylamine, 5-hydroxytryptamine creatinine sulphate, indomethacin, 3-isobutyl-1-methyl xanthine, (−)-isoprenaline bitartrate, (−)-noradrenaline bitartrate, nordihydroguaiaretic acid, oxytocin (synthetic), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), progesterone and [Arg]-vasopressin.

$PGF_{2\alpha}$, indomethacin and nordihydroguaiaretic acid were dissolved in ethanol such that no more than 10 μl ethanol was added to the bath and this quantity of ethanol did not affect uterine activity. All other agents were dissolved in distilled water. Because trifluoroacetic acid was found to be a potent tocolytic agent, this was not used to dissolve ANP as is often done to delay degradation of peptides in solution.

Results

Spontaneous myometrical motor activity

Myometrial strips from all groups of rats used in this study exhibited spontaneous motor activity. In general, the spontaneous activity appeared within 1 to 15 min of uteri being set up in tissue baths and became regular after 30 to 60 min of equilibration. The force of spontaneous tension development by uteri from pregnant rats was generally greater than of uterine strips from oestrogen-treated, progesterone-treated and ovariectomized rats.

Responses of nongravid uterus

Atrial natriuretic peptide ANP (2–100 nM) caused a concentration-dependent decrease in both the amplitude and rate of tension development by uterine horns from virgin untreated (not shown) and oestrogen-treated animals (Figure 1a). The tocolytic effect of ANP was observed within 1 min after the addition of ANP to the bath and reached a maximum within 2–3 min. However, myometrial spontaneous activity tended to reappear after 10 to 15 min even if ANP was not washed out. At low concentrations (2–4 nM), ANP caused a decrease in the rate rather than the force of tension develop-

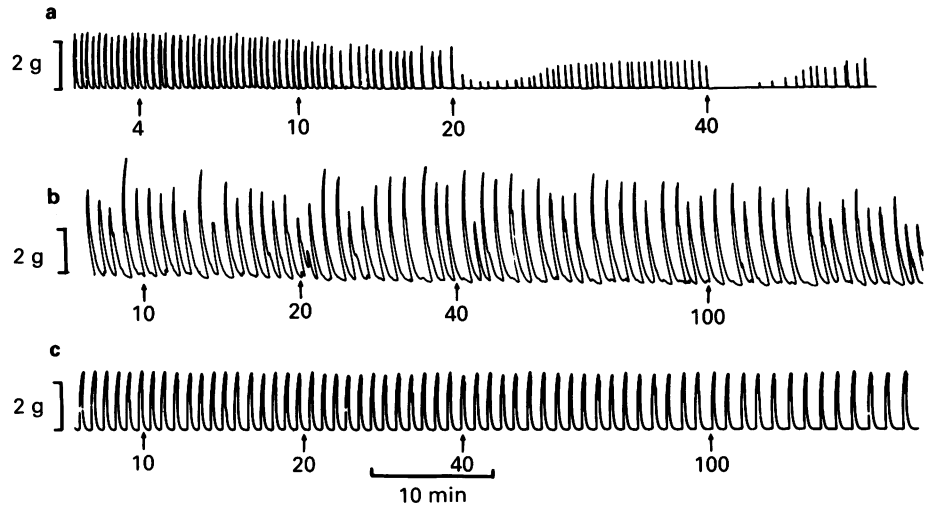

Figure 1 An example of the effect of atrial natriuretic peptide (ANP) on the spontaneous motor activity of (a) oestrogen-treated (17β-oestradiol, 1 mg kg^{-1} day^{-1} × 2), (b) 20-day pregnant and (c) progesterone-treated (2 mg kg^{-1} day^{-1} × 3) rats. Concentrations of ANP (nM) are indicated at the arrows.

ment. The rate (contractions min^{-1}) changed significantly ($P < 0.05$) from a control value of 1.53 ± 0.22 to 1.36 ± 0.21 after 2 nM and to 1.03 ± 0.13 after 4 nM. ANP was able to suppress completely uterine tension development. At concentrations of 10 to 100 nM, ANP exerted a tocolytic effect on oestrogen-treated rat uteri submaximally stimulated with $PGF_{2\alpha}$ (500 nM), oxytocin (0.2 nM), vasopressin (1 nM), and angiotensin II (10 nM) (data not shown). Tocolytic potency (−log EC_{50}) of ANP on spontaneous tension development of nongravid myometrium ($n = 16$) was 7.92 ± 0.07 and did not differ significantly ($P > 0.1$) from its potency (7.81 ± 0.11) on nongravid myometrial strips ($n = 6$) stimulated with 5-HT (0.1–1 μM).

Isoprenaline, isobutylmethyl xanthine, hydroxylamine and sodium nitroprusside Isoprenaline, IBMX and hydroxylamine produced concentration-dependent inhibition of myometrial tension development; the maximum inhibition was 100%. Potencies of ANP, isoprenaline, IBMX and hydroxylamine under different experimental conditions are presented in Table 1. The order of tocolytic potencies on uteri from oestrogen-treated rats was: isoprenaline > ANP > IBMX > hydroxylamine. Up to a concentration of 3 mM, sodium nitroprusside did not exert any tocolytic effect on nongravid or gravid uterus.

Table 1 Tocolytic potencies of atrial natriuretic peptide (ANP), isoprenaline (Iso), isobutylmethyl xanthine (IBMX), hydroxylamine (HA) and sodium nitroprusside (SNP) on myometrial strips from oestrogen- and progesterone-treated virgin rats and 20-day pregnant rats

Agents	n	*Oestrogen treated*	*Progesterone-treated*	*20 day-pregnant*
		Tocolytic −log M (EC_{50})		
ANP	10–16	7.92 ± 0.07[a,b]	<6.0[b]	<6.0[b]
Iso	10	9.52 ± 0.05	9.58 ± 0.07	9.51 ± 0.07
IBMX	4–6	5.43 ± 0.05	5.42 ± 0.03	5.41 ± 0.06
HA	4	3.06 ± 0.04	3.06 ± 0.07	3.04 ± 0.08
SNP	4	<3.0	<3.0	<3.0

Oestrogen (17β-oestradiol, 1 mg kg^{-1}, i.p.) was injected daily for 2 days and progesterone (2 mg kg^{-1} i.p.) for 3 days prior to experiments.
Values represent mean ± s.e.mean.
[a] Denotes significant ($P < 0.05$) difference from the values in the same row; [b] each value significantly ($P < 0.05$) different from all other values in the same column.

Responses of the gravid uterus to various agents

Up to as high as 1 μM concentration, ANP did not inhibit the motor activity of uteri of 20 day pregnant rats (Figures 1b and 2a). Also, ANP did not decrease 5-HT induced tension development by the gravid myometrium after inhibition of cyclooxygenase and lipoxygenase activities by indomethacin and nordihydroguaiaretic acid, respectively.

ANP exerted a tocolytic effect on the sterile uterine horns of 10 to 14 day pregnant rats (Figure 2a); however, its potency

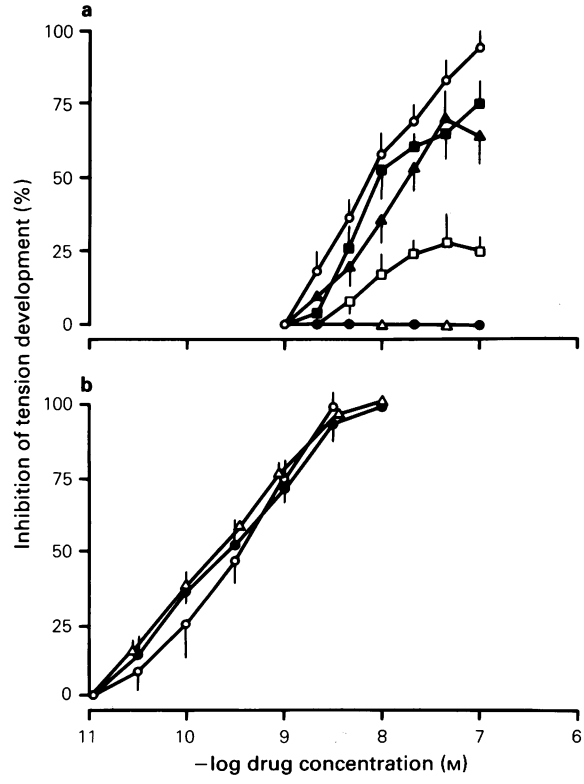

Figure 2 Tocolytic concentration-response curves to (a) atrial natriuretic peptide and (b) isoprenaline on myometrial strips from oestrogen-treated (17β-oestradiol, 1 mg kg^{-1} day^{-1} × 2, ○), progesterone-treated (2 mg kg^{-1} × 3, △), 20-day pregnant (●), sterile horn of 10-day pregnant (▲), ovariectomized (□) and ovariectomized plus oestrogen-treated (■) rats. Data are means of 6–16 separate experiments; vertical lines show s.e.mean.

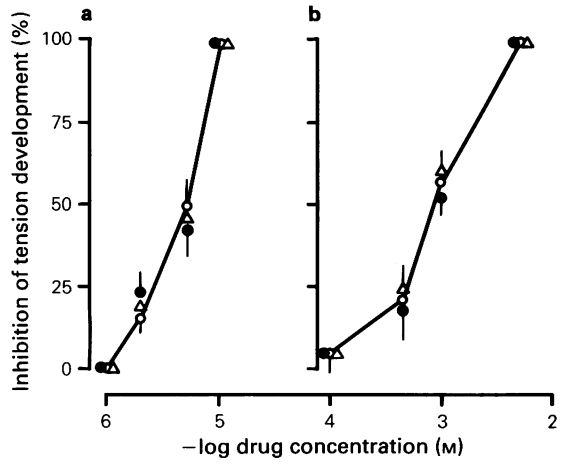

Figure 3 Tocolytic concentration-response curves to (a) isobutylmethyl xanthine and (b) hydroxylamine on myometrial strips from oestrogen-treated (17β-oestradiol $1\,mg\,kg^{-1}\,day^{-1} \times 2$, ○), progesterone-treated ($2\,mg\,kg^{-1}\,day^{-1} \times 3$, △) and 20-day pregnant (●) rats. Data are means of 4–6 experiments; vertical lines show s.e.mean.

($-\log EC_{50} = 7.56 \pm 0.12$) and maximal effect ($70 \pm 11\%$) on the sterile horn ($n = 8$) were significantly ($P < 0.05$) less than its potency ($-\log EC_{50} = 7.92 \pm 0.07$) and maximal effects ($95 \pm 5\%$) on uteri from oestrogen-treated virgin rats ($n = 16$). The sterile horns of 15- to 20-day pregnant rats did not respond to ANP. The gravid horn was completely refractory to the tocolytic effects of ANP after day 10 of gestation (the earliest period studied; data for only day 20 presented in Figure 1, 2a).

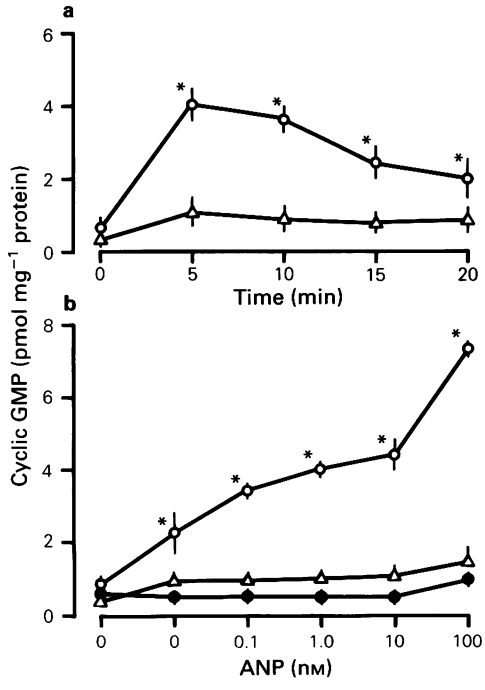

Figure 4 Effect of atrial natriuretic peptide (ANP) on cyclic GMP accumulation in myometrial tissue from oestrogen-treated ($1\,mg\,kg^{-1}$ daily for 2 days, ○), progesterone-treated ($2\,mg\,kg^{-1}$ daily for 3 days, △) and 20-day pregnant (●) rats; (a) time-course of cyclic GMP accumulation to 10 nM ANP in the presence of 100 μM isobutylmethyl xanthine (IBMX); (b) cyclic GMP accumulation 5 min after addition of increasing concentrations of ANP in the presence of 100 μM IBMX. The first set of values on the left in (b) are in the absence of IBMX and all other values are in the presence of 100 μM IBMX. Data points are means of 4 to 6 experiments in duplicate; vertical lines show s.e.mean. *Denotes significant ($P < 0.05$) differences from the corresponding values for uteri from progesterone-treated and pregnant rats.

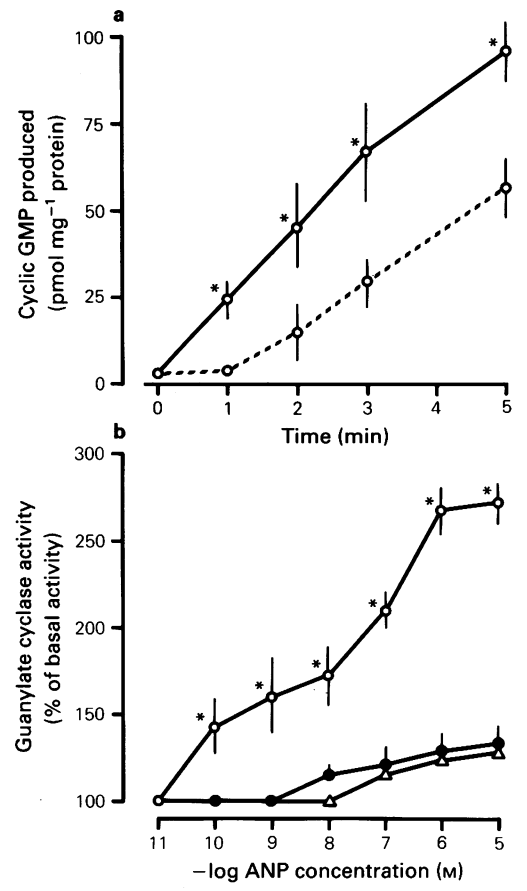

Figure 5 Effect of atrial natriuretic peptide (ANP) on particulate guanylate cyclase activity of myometrial tissues of oestrogen-treated ($1\,mg\,kg^{-1}\,day^{-1} \times 2$, ○), progesterone-treated ($2\,mg\,kg^{-1}\,day^{-1} \times 3$, △) and 20-day pregnant (●) rats; (a) time-course of cyclic GMP accumulation in the absence (○---○) and the presence (○——○) of 1 μM ANP in myometrial tissues from oestrogen-treated rats; (b) guanylate cyclase activity as % of basal activity at 3 min after addition of increasing concentrations of ANP. All measurements were made in the presence of 100 μM isobutylmethyl xanthine and represent means of 4–6 experiments in duplicate; vertical lines show s.e.mean. *Denote significant ($P < 0.01$) difference from the corresponding values in the lower curve in (a) and the two lower curves in (b).

Pregnancy did not change the tocolytic potencies of isoprenaline (Figure 2b), IBMX (Figure 3) and hydroxylamine (Figure 3).

Table 2 Effects of atrial natriuretic peptide (ANP) on the tone and cyclic GMP concentrations of aortic strips from oestrogen- and progesterone-treated virgin rats and 20-day pregnant rats

Animals	*Vasorelaxant* $-\log M\ (EC_{50})$	*ANP* (0) Cyclic GMP (pmol mg^{-1} protein)	*ANP* (10 nM)
Oestrogen-treated	9.10 ± 0.03	0.43 ± 0.16	3.9 ± 0.30
Progesterone-treated	9.06 ± 0.03	0.36 ± 0.11	4.0 ± 0.25
Pregnant	9.07 ± 0.04	0.36 ± 0.08	3.78 ± 0.41

Oestrogen (17β-oestradiol, $1\,mg\,kg^{-1}$) was injected daily for 2 days and progesterone ($2\,mg\,kg^{-1}$) for 3 days prior to experiments. $-\log (EC_{50}s)$ were derived from relaxant concentration-response curves to ANP on aortic strips contracted with 20 to 40 nM noradrenaline. Data are means ± s.e.mean of 4 to 6 experiments; cyclic GMP assays were done in duplicate.

Responses of uteri from progesterone-treated rats

The effects of ANP on myometrial activity was completely abolished by three daily doses of progesterone (Figure 2a). On the other hand, isoprenaline (Figure 2b), IBMX and hydroxylamine (Figure 3) were equipotent on uteri from progesterone- and oestrogen-treated rats.

Effects of atrial natriuretic peptide on uteri from ovariectomized rats

The maximal tocolytic effect and potency ($-\log EC_{50}$) of ANP on uteri from ovariectomized rats ($n = 6$) were $28 \pm 2\%$ and 7.20 ± 0.07 and signficantly ($P < 0.05$) less than the maximal effect ($95 \pm 5\%$) and potency (7.92 ± 0.07) on uteri from oestrogen-treated rats. Pretreatment of ovariectomized rats ($n = 8$) with oestrogen for two days partly restored the maximal tocolytic activity to $75 \pm 7\%$ and potency to 7.68 ± 0.05 (Figure 2a); these values were significantly ($P < 0.05$) different from corresponding values from oestrogen-treated control and untreated ovariectomized rats.

Effects of atrial natriuretic peptide on myometrial cyclic GMP concentrations

Basal concentration of cyclic GMP (pmol mg^{-1} protein) in uteri from oestrogen-treated rats was 0.81 ± 0.15 and significantly ($P < 0.05$) greater than the concentrations in uteri from progesterone-treated (0.32 ± 0.29) and pregnant (0.37 ± 0.09) rats. Also, cyclic GMP concentration (pmol mg^{-1} protein) after inhibition of phosphodiesterase with IBMX in uteri from oestrogen-treated rats (2.22 ± 0.42) was significantly ($P < 0.05$) greater than in uteri from progesterone-treated (0.50 ± 0.09) and pregnant (0.42 ± 0.21) rats. Effects of ANP were studied in the presence of IBMX. ANP caused a concentration- and time-dependent increase in uterine cyclic GMP concentrations (Figure 4) and this increase was significantly ($P < 0.001$) greater in uteri from oestrogen-treated animals than in uteri from progesterone-treated and pregnant animals. The effect of ANP on cyclic GMP concentrations in uteri from pregnant rats was not different from that in tissues from progesterone-treated animals ($P > 0.1$).

Effects of atrial natriuretic peptide on guanylate cyclase activity

ANP produced a time- and concentration-dependent increase in myometrial particulate guanylate cyclase activity (Figure 5). This increase was significantly ($P < 0.01$) greater in myometrial tissues from oestrogen-treated rats than in tissues from pregnant or progesterone-treated animals. Effects of ANP on guanylate cyclase activity of uteri from progesterone-treated and pregnant rats did not differ ($P > 0.2$). Addition of 1 μM progesterone to the assay system did not influence the effect of ANP on guanylate cyclase activity in uteri from oestrogen-treated rats.

Effects of atrial natriuretic peptide on the tone and cyclic GMP concentrations of aortic strips

ANP caused a concentration-dependent relaxation of aortic strips and an increase in aortic cyclic GMP concentrations (Table 2). These effects of ANP were not different in tissues from oestrogen-treated, progesterone-treated and pregnant rats. The effect of ANP on aortic guanylate cyclase activity was not determined.

Discussion

The main finding of this study was that ANP inhibited the motor activity of the nongravid but not of the gravid uterus. Our results on nongravid uterus are in conformity with the recent data of Bek *et al.* (1988). However, to our knowledge a pregnancy-induced refractoriness to the tocolytic effects of ANP has not been previously reported.

ANP is known to relax a variety of smooth muscles (Currie *et al.*, 1983; Cantin & Genest, 1985; Potvin & Varma, 1989). This study merely adds another tissue to this list. However, the present data reveal some important differences between ANP and other smooth muscle relaxants. For example, ANP inhibited the activity of only nongravid myometrium. Isoprenaline, hydroxylamine and IBMX were effective on both gravid and nongravid tissues. Sodium nitroprusside was ineffective on both nongravid and gravid uterus. The differences in the biological activities of these agents seem to be related to their mechanisms of action. ANP increases particulate guanylate cyclase activity (Winquist *et al.*, 1984; Cantin & Genest, 1985; Inagami, 1989). On the other hand, sodium nitroprusside and hydroxylamine increase soluble guanylate cyclase activity (Diamond, 1983; Murad, 1986), which may not contribute to a tocolytic effect (Diamond, 1983). This may be why sodium nitroprusside is not tocolytic although it causes a greater increase in cyclic GMP than does hydroxylamine (Diamond, 1983). Isoprenaline acts by increasing cyclic AMP concentrations (Polacek & Daniel, 1971; Bhalla *et al.*, 1972; Verma & McNeill, 1976). IBMX increases both cyclic AMP and cyclic GMP (Leiber *et al.*, 1978) and thus combines the effects of isoprenaline and hydroxylamine.

The results of this study taken as a whole suggest that the pregnancy-induced refractoriness to the tocolytic effects of ANP is caused by progesterone. Both pregnancy and exogenous administration of progesterone inhibited the effects of ANP on myometrial tension development, guanylate cyclase activity and cyclic GMP accumulation. As in other mammals, pregnancy in rats is associated with an increase in circulating progesterone (Morishige *et al.*, 1973; Mulay *et al.*, 1982); the maximum increase occurs from day 12 to 15 of gestation (Morishige *et al.*, 1973). The observation that the sterile horn became refractory to ANP later than the gravid horn is consistent with the above suggestion. The placentally produced progesterone (Csapo & Wiest, 1969) may be delivered to the myometrium without entering the systemic circulation (Laatikainen *et al.*, 1982). It may be expected that, relative to the sterile horn, the gravid horn is exposed to a higher concentration of progesterone and earlier during pregnancy.

Several known myometrial effects of progesterone provide additional support for our suggestion that pregnancy-induced refractoriness of the uterus to ANP is due to an influence of this hormone. For example, administration of progesterone to rats has been shown to decrease and that of oestrogen to increase numbers of myometrial angiotensin II receptors (Schirar *et al.*, 1980), guanylate cyclase activity (Kuehl *et al.*, 1974; Beatty *et al.*, 1979) adrenoceptor concentrations (Williams & Lefkowitz, 1977) and responses to oxytocic agents (Ruzycky & Crankshaw, 1988). The present studies also show that oestrogen and progesterone exert opposing influences on the tocolytic activity of ANP and on guanylate cyclase activity.

Assuming that ANP exerts its effects on the uterus by increasing particulate guanylate cyclase activity, as is believed to be the case on other tissues (Winquist *et al.*, 1984; Cantin & Genest, 1985), a decrease in the effects of ANP on myometrial activity should accompany a decrease in its effects on cyclic GMP accumulation. This, indeed, was the case. Thus pregnancy and progesterone treatment, both of which abolished the tocolytic activity of ANP also caused a profound decrease in the ability of ANP to increase guanylate cyclase activity and cyclic GMP concentrations. Ovariectomy caused a smaller decrease in the effects of ANP on myometrial activity and cyclic GMP accumulation than did pregnancy or progesterone treatment such that a good correlation ($r = 0.92$, $P < 0.02$) existed between effects of ANP on myometrial activity and cyclic GMP accumulation under different experimental conditions of this study (Figure 6). In contrast, neither

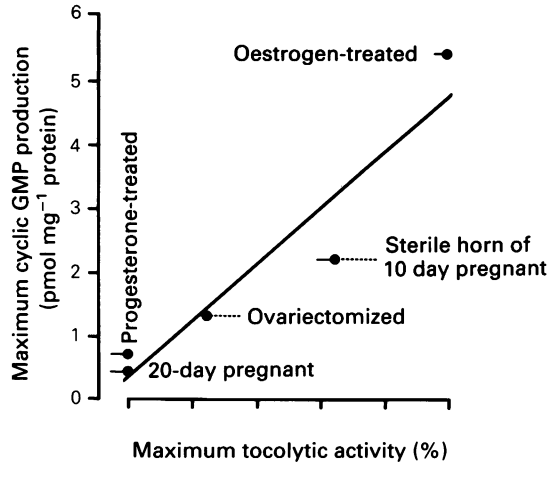

Figure 6 Relationship between maximal effects of atrial natriuretic peptide on motor activity and cyclic GMP concentrations of myometrial tissues from 20-day pregnant, progesterone-treated ($2\,mg\,kg^{-1}\,day^{-1} \times 3$), ovariectomized, the sterile horn of 10-day pregnant and oestrogen-treated (17β-oestradiol, $1\,mg\,kg^{-1}\,day^{-1} \times 2$) rats. Data points (●) used for the regression line ($r = 0.92$, $P < 0.02$) represent means of 4–16 experiments; solid horizontal line on the left of the data points represent + or − s.e.mean for cyclic GMP and dashed line on the right represent + or − s.e.mean for tocolytic activity.

pregnancy nor progesterone treatment altered the effects of ANP on aortic smooth muscle tone as also reported by others (St-Louis *et al.*, 1988) and on cyclic GMP concentrations.

The suggestion that the pregnancy-induced refractoriness to the tocolytic effects of ANP is exerted by progesterone is supported by studies which exclude some of the other possibilities. For example, ANP could inhibit the motor activity of uterus in the presence of $PGF_{2\alpha}$, oxytocin, vasopressin, 5-HT or angiotensin II and inhibition of cyclo-oxygenase and lipoxygenase activity did not restore the tocolytic effects of ANP on the gravid uterus. It can thus be argued that any increase in the synthesis or effects of these agents during pregnancy could not account for the refractoriness of the pregnant uterus to the tocolytic effects of ANP.

In conclusion, this study demonstrates that the tocolytic activity of ANP is abolished by pregnancy, possibly because of high concentrations of progesterone. ANP is considered to offer promise in the treatment of circulatory, volume and salt disturbances (Needleman & Greenwald, 1986). Our previous data that ANP lowers the blood pressure and increases placental blood flow in hypertensive rats (Chemtob *et al.*, 1989) and does not cross the placenta (Mulay & Varma, 1989) point to a therapeutic potential of ANP against pregancy hypertension; in this context the lack of an effect of ANP on gravid myometrium may be an attribute.

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No effect of pertussis toxin on peripheral prejunctional α_2-adrenoceptor-mediated responses and on endothelium-dependent relaxations in the rat

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1 We have investigated the effects of pertussis toxin treatment on a variety of peripheral tissues in the rat.

2 Incubation with pertussis toxin ($1\,\mu g\,ml^{-1}$) *in vitro* failed to alter the negative inotropic actions of acetylcholine in rat left atria.

3 Pretreatment with pertussis toxin ($6\,\mu g\,kg^{-1}$, i.v., 3–4 days) abolished the negative inotropic actions of acetylcholine in rat left atria.

4 Pretreatment with pertussis toxin ($40\,\mu g\,kg^{-1}$, i.v., 3–4 days) failed to alter the prejunctional inhibitory actions of the α_2-adrenoceptor agonist xylazine, either in terms of the isometric contraction to a single stimulus in rat vas deferens or in terms of stimulation-evoked overflow of tritium in atria pre-incubated with [^{3}H]-noradrenaline.

5 Pretreatment with pertussis toxin ($6\,\mu g\,kg^{-1}$, i.v., 3–4 days) failed to affect, and pertussis toxin ($40\,\mu g\,kg^{-1}$, i.v., 3–4 days) potentiated endothelium-dependent relaxations of rat aorta to histamine and acetylcholine.

6 It seems unlikely that peripheral prejunctional actions of α_2-adrenoceptor agonists or endothelium-dependent relaxations of rat aorta involve pertussis toxin-sensitive G proteins.

Introduction

Pertussis toxin catalyses the adenosine 5'-diphosphate (ADP)-ribosylation of the guanine-nucleotide binding regulatory proteins G_0 and G_i which are linked to several responses including inhibition of adenylate cyclase and opening of K^+ channels (Murayama & Ui, 1983; Dolphin, 1987). Various responses have been shown to be attenuated by pertussis toxin, including α_2-adrenoceptor-mediated vasoconstriction in vascular smooth muscle (Boyer *et al.*, 1983), α_2-adrenoceptor-mediated inhibition of noradrenaline release in brain slices (Allgaier *et al.*, 1985) but not in peripheral nerves (Musgrave *et al.*, 1987; Docherty, 1988), endothelium-dependent relaxations of guinea-pig pulmonary artery (Weinheimer & Osswald, 1989) and pig coronary arteries (Flavahan *et al.*, 1989). In the above studies, tissues were either incubated *in vitro* with pertussis toxin, or pretreated with pertussis toxin *in vivo*.

The present study sets out to compare pertussis toxin incubation with pretreatment and to examine the effects of pertussis toxin in 3 systems: negative inotropic actions of acetylcholine (ACh) on rat left atrium, peripheral prejunctional α_2-adrenoceptors of rat vas deferens and right atrium, endothelium-dependent relaxations of rat aorta.

Some of these results have been presented in abstract form (Docherty, 1990).

Methods

Male Wistar rats (200–250 g) were obtained from Biolabs, Ballina, Ireland, and tissues from untreated, vehicle-treated or pertussis toxin-treated animals were employed as outlined below.

Rat left atrium

Left atria were attached to myograph transducers, under a resting isometric tension of 1 g, in organ baths at 37°C in Krebs-Henseleit solution of the following composition (mM): NaCl 119, $NaHCO_3$ 25, D-glucose 11.1, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.0. Tissues were placed between platinum electrodes and stimulated continuously at 1 Hz (0.5 ms pulses, supramaximal voltage) to produce isometric contractions (inotropic response). When consistent isometric contractions had been obtained, ACh was administered cumulatively in 1 log unit increments beginning with 0.1 μM.

Rat vas deferens

Prostatic and epididymal portions of rat vas deferens were placed between platinum electrodes and attached to myograph transducers, under a resting isometric tension of 1 g, in organ baths at 37°C in Krebs-Henseleit solution of the same composition as outlined above. Tissues were stimulated with a single stimulus (0.5 ms pulses, supramaximal voltage) every 5 min, and when consistent isometric contractions had been obtained, xylazine or dopamine was administered cumulatively in 0.5 log unit increments and a response to a single stimulus was obtained in the presence of each agonist concentration. Experiments in epididymal portions were carried out in the presence of the calcium entry blocker nifedipine (10 μM) to eliminate postjunctional actions of agonists (see Docherty, 1984).

Rat aorta

Aortic rings of approximately 3–5 mm in length were attached to myograph transducers under 1 g tension in organ baths at 37°C in Krebs-Henseleit solution of the same composition as outlined above. Tissues were contracted with KCl 40 mM, exposed to ACh 100 μM, and washed. Bathing fluid was then changed every 15 min for the next 60 min. Tissues were again contracted with KCl 40 mM, and when the contraction had reached a maximum, ACh or histamine was administered cumulatively in 1 log unit increments beginning with 0.1 μM (ACh) or 1 μM (histamine). Tissues were then washed, bathing fluid was changed every 15 min for the next 60 min, KCl 40 mM was added and tissues which had received ACh now received histamine, and vice versa. In other experiments, a cumulative concentration contractile response curve to phenylephrine was obtained. In some experiments, the endothelium was deliberately removed by gentle rubbing.

Rat right atrium

Isolated right atria were preincubated for 1 h in 1 ml medium containing [^{3}H]-noradrenaline (0.5 μM, specific activity 39 Ci mmol^{-1}) then placed in organ baths under approximately 1 g tension and superfused with [^{3}H]-noradrenaline-free Krebs-Henseleit solution of the same composition as listed above but containing (mM): ascorbic acid 0.28, tetrasodium EDTA 0.03, corticosterone 0.03, propranolol 0.001. Additionally, following the end of pre-incubation, cocaine (3 μM) was added. Tissues were placed between platinum electrodes in organ baths, and superfused at a rate of 2 ml min^{-1} at 37°C.

In all experiments, tissues were stimulated four times (S_0–S_3) for 3 min at a frequency of 2 Hz at intervals of 27 min, beginning after 2 h of superfusion.

Xylazine or distilled water vehicle was added to the superfusion stream at a rate of 50 μl min^{-1}, in 2 cumulative concentrations beginning 12 min before S_2. Effluent samples were collected in 6 ml aliquots beginning 6 min before S_1, and at the end of the experiment tissues were made soluble in 1 ml of tissue solubiliser. A volume of 1 ml of superfusate or dissolved tissue was added to 9 ml of liquid scintillation solution (Liquiscint) and counted in a liquid scintillation counter. Basal outflow was calculated from the sample obtained immediately before each stimulation period and expressed as a percentage of tissue tritium released per min. The stimulation-evoked overflow of tritium was calculated by subtraction of the basal outflow, and was expressed as a percentage rate (i.e. the evoked overflow during a given stimulation period was expressed as a percentage of the tritium content of the tissue at the onset of that stimulation period).

Pertussis toxin treatments

In some experiments, left atria were incubated for 2 h with pertussis toxin 1 μg ml^{-1} or with vehicle before beginning concentration-response curves to ACh.

In most experiments, rats were anaesthetised with ether and pertussis toxin (1–40 μg kg^{-1}) or vehicle was injected in a volume of 1 ml into the jugular vein. Experiments on isolated tissues were carried out 3–4 days after injection.

Drugs

Acetylcholine chloride (Sigma, Poole, U.K.); cocaine hydrochloride (Sigma); corticosterone (Sigma); dopamine hydrochloride (Sigma), histamine hydrochloride (Sigma); pertussis toxin (Peninsula Labs, St. Helens, U.K.); phenylephrine hydrochloride (Sigma); propranolol hydrochloride (Sigma); xylazine hydrochloride (gift: Bayer, Ireland).

Drugs were dissolved in distilled water with the exception of corticosterone (100% ethanol).

Statistics

Values are geometric mean and 95% confidence limits, or arithmetic mean ± s.e.mean. Differences between groups were compared by Student's *t* test for unpaired data.

Results

Incubation with pertussis toxin

Left atrium In left atria incubated with vehicle, ACh produced a concentration-dependent inhibition of contractions evoked by 1 Hz stimulation (negative inotropic action of ACh). Incubation with pertussis toxin 1 μg ml^{-1} for 2 h failed to alter the negative inotropic actions of ACh (Figure 1a).

Aortic rings Incubation with pertussis toxin 1 μg ml^{-1} for 2 h failed to alter the relaxation to histamine in KCl (40 mM)-contracted tissue as compared with tissues incubated with vehicle (Table 1).

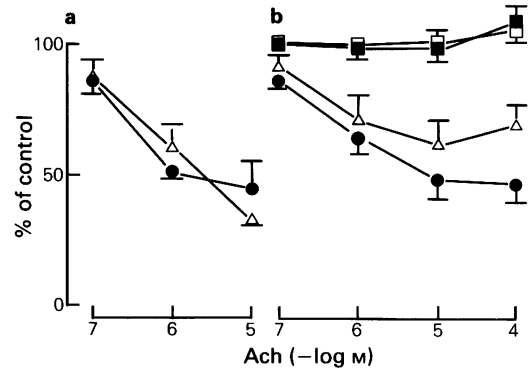

Figure 1 Effects of pertussis toxin on the inhibition by acetylcholine (ACh) of isometric contractions (inotropic response) evoked by 1 Hz continuous electrical stimulation in rat left atria. In (a) tissues were incubated for 1 h with pertussis toxin (1 μg ml^{-1}) (△) or vehicle (●). In (b) animals were pretreated with pertussis toxin 1 (△), 6 (□) or 40 (■) μg kg^{-1}, or with vehicle (●), intravenously 4 days before experimentation. Vertical bars represent s.e.mean from at least 4 experiments.

In all subsequent experiments, tissues were obtained from animals injected with pertussis toxin or vehicle intravenously 3–4 days previously.

Pertussis toxin pretreatment

Left atrium In left atria, pretreatment with pertussis toxin (1, 6 or 40 μg kg^{-1}, i.v.) failed to alter the contraction evoked by 1 Hz stimulation (responses of 0.15 ± 0.09 g, $n = 4$; 0.14 ± 0.03 g, $n = 5$; 0.22 ± 0.08 g, $n = 4$; respectively), as compared with vehicle controls (0.21 ± 0.05 g, $n = 8$). Pretreatment with pertussis toxin (1 μg kg^{-1}, i.v.) caused a small but significant attenuation of the negative inotropic response to ACh (100 μM) (Students' *t* test: $P < 0.05$), and pertussis toxin (6 and 40 μg kg^{-1}, i.v.) abolished the actions of ACh (Figure 1b).

Rat vas deferens

In prostatic portions of rat vas deferens, single pulse stimulation produced an isometric contraction of 2.06 ± 0.32 g ($n = 4$) in tissues from vehicle-treated animals, and 2.12 ± 0.14 g ($n = 4$) in tissues from animals pretreated with

Table 1 Maximum relaxations to histamine and acetylcholine (ACh) in KCl (40 mM)-contracted aortic rings

	Histamine	*ACh*
a *Incubation* (1 h)		
Pertussis toxin (1 μg ml^{-1})	10.1 ± 3.7% ($n = 4$)	/
Vehicle	10.8 ± 6.3% ($n = 4$)	/
b *Pretreatment* (3–4 days)		
Pertussis toxin (6 μg kg^{-1})	14.5 ± 3.7% ($n = 4$)	26.6 ± 1.5% ($n = 4$)
Pertussis toxin (40 μg kg^{-1})	46.5 ± 9.6%* ($n = 4$)	52.8 ± 9.3% ($n = 4$)
Vehicle	18.0 ± 1.3% ($n = 6$)	34.4 ± 4.5% ($n = 6$)

Relaxations are expressed as a percentage of the contraction to KCl (mean ± s.e. from at least 4 experiments). In (a), tissues were incubated for 1 h with pertussis toxin (1 μg ml^{-1}) or with vehicle. In (b), animals were pretreated with pertussis toxin (6 or 40 μg kg^{-1}, i.v.) or vehicle 3–4 days before experimentation. Asterisks denote response significantly different from response in vehicle tissues (Student's *t* test. * $P < 0.05$).

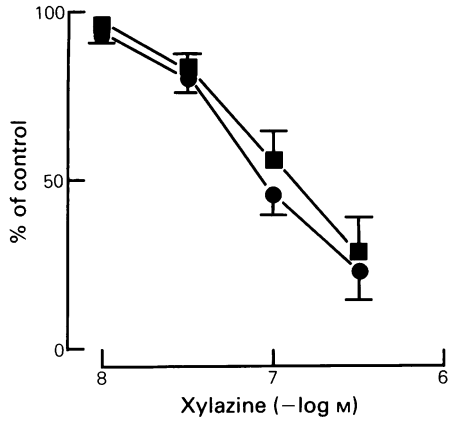

Figure 2 Inhibition by xylazine of the isometric contraction to a single electrical stimulus in prostatic portions of rat vas deferens. (●) Represent responses from vehicle-pretreated animals and (■) represent responses from pertussis toxin ($40\,\mu g\,kg^{-1}$, i.v., 3–4 days) pretreated animals. Vertical bars represent s.e.mean from at least 3 experiments.

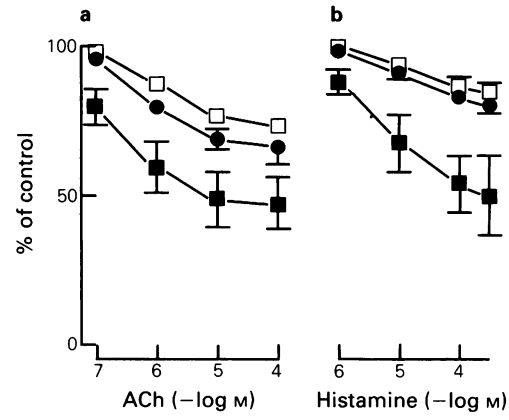

Figure 3 Relaxations to (a) acetylcholine (ACh) and (b) histamine in aortic rings contracted with KCl 40 mM from vehicle pretreated (●), or pertussis toxin 6 (□), or 40 (■) $\mu g\,kg^{-1}$ (i.v., 3–4 days)-pretreated rats. Vertical bars represent s.e.mean from at least 4 experiments.

pertussis toxin ($40\,\mu g\,kg^{-1}$, i.v., 3–4 days) (no significant difference). Pertussis toxin did not significantly alter the inhibition by xylazine of stimulation-evoked contractions (Figure 2 and Table 2).

In epididymal portions of rat vas deferens in the presence of nifedipine (10 μM), single pulse stimulation produced an isometric contraction of 0.92 ± 0.38 g ($n = 3$) in tissues from vehicle-treated animals, and 1.51 ± 0.24 g ($n = 3$) in tissues from animals pretreated with pertussis toxin ($40\,\mu g\,kg^{-1}$ i.v., 3–4 days) (no significant difference). Dopamine (1–300 μM) produced concentration-dependent inhibitions of the stimulation-evoked contraction, but there were no significant differences between tissues from vehicle-treated and pertussis toxin-treated animals in terms of the potency of dopamine (Table 2).

Rat aorta

In aortic rings, KCl (40 mM) produced contractions of 0.82 ± 0.07 g ($n = 6$), 0.69 ± 0.16 g ($n = 4$) and 0.63 ± 0.03 g ($n = 4$) in tissues from vehicle-treated animals and animals pretreated with pertussis toxin $6\,\mu g\,kg^{-1}$ and $40\,\mu g\,kg^{-1}$, respectively (no significant differences). The maximum contraction to phenylephrine was 1.13 ± 0.06 g ($n = 3$) and 0.82 ± 0.05 g ($n = 3$) in tissues from vehicle-treated animals and animals pretreated with pertussis toxin ($40\,\mu g\,kg^{-1}$), respectively ($P < 0.05$). Pertussis toxin ($6\,\mu g\,kg^{-1}$) did not significantly alter the relaxations to histamine (1–300 μM) or ACh (0.1–100 μM) in KCl (40 mM) contracted tissues (Figure 3). However, pertussis toxin ($40\,\mu g\,kg^{-1}$) significantly increased the maximum relaxation to histamine ($P < 0.05$) and significantly increased the potency of ACh ($P < 0.05$) (Figure 3; Tables 1 and 2).

In endothelium-denuded preparations contracted with KCl, ACh did not produce relaxations and histamine (100 μM) produced contractions of 0.07 ± 0.01 g ($n = 3$).

Rat right atrium

Field stimulation of rat isolated right atria at a frequency of 2 Hz for 3 min produced an evoked overflow of tritium: an S_1 of $1.05 \pm 0.18\%$ ($n = 4$) and $1.20 \pm 0.15\%$ ($n = 4$) of tissue tritium in tissues from vehicle-treated and pertussis toxin ($40\,\mu g\,kg^{-1}$)-treated animals, respectively (no significant difference). Pertussis toxin failed to alter the inhibition by xylazine of stimulation-evoked release of tritium (Figure 4).

Table 2 Potency of xylazine or dopamine in rat vas deferens expressed as an IC_{50} (concentration producing 50% inhibition of the isometric contraction to a single stimulus) and potency of histamine and acetylcholine (ACh) in rat aorta expressed as an EC_{50} (concentration producing 50% of maximum relaxation in KCl-contracted tissues)

		Pertussis toxin	
	Vehicle	*6 $\mu g\,kg^{-1}$*	*40 $\mu g\,kg^{-1}$*
Vas deferens			
Xylazine IC_{50} (nM)	98 (44–220)	/	132 (44–398)
Dopamine IC_{50} (μM)	102.3 (40.7–257)	/	66.1 (10.7–407)
Rat aorta			
Histamine EC_{50} (μM)	12.0 (9.3–15.5)	13.2 (8.7–20.0)	4.8 (0.91–25.1)
ACh EC_{50} (μM)	0.59 (0.34–1.02)	1.05 (0.44–2.5)	0.17* (0.035–0.87)

Tissues were pretreated with pertussis toxin (6 or $40\,\mu g\,kg^{-1}$, i.v.) or vehicle 3–4 days before experimentation. Values are mean and 95% confidence limits from at least 3 experiments. Asterisks denote responses significantly different from response in vehicle-treated tissues (Student's t test. * $P < 0.05$).

Discussion

In this study, incubation for 2 h with pertussis toxin ($1\,\mu g\,ml^{-1}$) failed to affect negative inotropic responses of rat

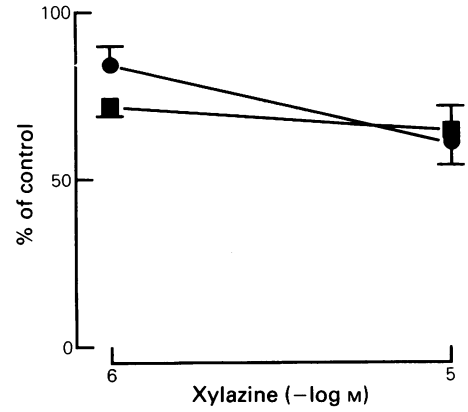

Figure 4 Effects of xylazine on 2 Hz stimulation-evoked overflow of tritium in atria from vehicle (●)- or pertussis toxin $40\,\mu g\,kg^{-1}$ (i.v., 3–4 days) (■)-pretreated rats. Vertical bars represent s.e.mean from 4 experiments.

left atrium to acetylcholine, and in a previous study even 16 h incubation with pertussis toxin ($1\,\mu g\,ml^{-1}$) produced only a limited reduction of the negative inotropic response of mouse atria to carbachol (Musgrave *et al.*, 1987). Although other authors found that incubation with pertussis toxin $200\,ng\,ml^{-1}$ for 1 h (Flavahan *et al.*, 1989) or $1\,\mu g\,ml^{-1}$ for 2 h (Weinheimer & Osswald, 1989) inhibited endothelium-dependent relaxations to 5-hydroxytryptamine or histamine, these authors did not examine the effects of pertussis toxin treatment on negative inotropic responses of atria to muscarinic agonists. In contrast, pretreatment with pertussis toxin ($6\,\mu g\,kg^{-1}$) intravenously 3–4 days before the experimental day abolished the negative inotropic actions of ACh, and even pertussis toxin $1\,\mu g\,kg^{-1}$ produced some attenuation. Similarly, pretreatment with pertussis toxin ($1.5\,\mu g$) intravenously 4 days before the experimental day abolished the negative inotropic action of carbachol in mouse atria (Musgrave *et al.*, 1987), and pretreatment with pertussis toxin ($25\,\mu g$) 40 h before the experimental day abolished the negative inotropic action of carbachol in rat atria. Hence, pretreatment with pertussis toxin *in vivo* is a more effective means of inactivating the G protein, G_i or G_0 (Endoh *et al.*, 1985; Dolphin, 1987), involved in the negative inotropic actions of muscarinic agonists than incubation with the toxin *in vitro*. Although this does not necessarily mean that G_i or G_0 in other tissues is blocked by the pretreatment, it should still imply that G proteins in other tissues are more likely to be blocked by toxin pretreatment than by incubation with toxin.

Pretreatment with pertussis toxin ($40\,\mu g\,kg^{-1}$ i.v. 3–4 days), a dose 6 times higher than necessary to abolish, and 40 times higher than necessary to reduce, negative inotropic actions of ACh in atria, failed to influence the prejunctional inhibitory actions of the α_2-adrenoceptor agonist xylazine, either in terms of the inhibition of isometric contractions in prostatic portions of rat vas deferens or in terms of the inhibition of stimulation-evoked release of tritium in rat right atria. Likewise, inhibitory responses to dopamine (present results) and to 5-hydroxytryptamine (Borton & Docherty, unpublished) are unaffected by pertussis toxin in vas deferens. This is in agreement with our previous findings that pretreatment with pertussis toxin ($50–200\,\mu g\,kg^{-1}$, i.p. 18 h) failed to alter the prejunctional inhibitory actions of xylazine in pithed rat heart and in epididymal portions of rat vas deferens (Docherty, 1988). Similar findings have been obtained for mouse atria (Musgrave *et al.*, 1987). However, other authors have shown that incubation of brain slices with pertussis toxin for 6 h produced a relatively small reduction in the prejunctional actions of α_2-adrenoceptor agonists and antagonists (Allgaier *et al.*, 1985). However, this incubation with pertussis toxin significantly increased the stimulation-evoked release of noradrenaline (Allgaier *et al.*, 1985; see also Allgaier *et al.*, 1987), although such an increase was not found in the present study of rat atria pretreated with pertussis toxin.

Pertussis toxin $6\,\mu g\,kg^{-1}$ (i.v., 3–4 days) failed to affect the endothelium-dependent relaxations to histamine or ACh in rat aorta precontracted with KCl and pertussis toxin $40\,\mu g\,kg^{-1}$ actually potentiated the relaxant actions of these agents. The reason for this is unclear, although the maximum relaxation to a vasodilator in rat aorta can be altered by varying the concentration of contractile agent (see Sawyer & Docherty, 1987), so alterations in the contractile response to KCl might explain such an effect. Admittedly, the contractile response to KCl was not significantly reduced by pertussis toxin ($40\,\mu g\,kg^{-1}$) even though the maximum contraction to phenylephrine was significantly reduced. Other authors have shown that pertussis toxin pretreatment reduced the maximum contraction to α_1-adrenoceptor agonists in rabbit pulmonary artery (Liebau *et al.*, 1989).

Some authors have demonstrated that incubation for 1 h with pertussis toxin ($100\,ng\,ml^{-1}$) virtually abolished endothelium-dependent relaxations produced by 5-hydroxytryptamine in pig coronary artery (Flavahan *et al.*, 1989), and that incubation for 2 h with pertussis toxin ($1\,\mu g\,ml^{-1}$) inhibited endothelium-dependent relaxations to histamine in guinea-pig pulmonary artery (Weinheimer & Osswald, 1989) without affecting endothelium-dependent relaxations to the calcium ionophore A23187 in either tissue. Although these differences from the present results may reflect tissue and species differences in endothelium-dependent relaxations, two points should be noted. In the above studies, the effectiveness of the pertussis toxin incubation was not independently assessed in another test system, such as attenuation of negative inotropic responses in atria. Secondly, in both studies the pertussis toxin incubation attenuated responses to agonists (5-hydroxytryptamine, histamine) which produced maximum relaxations of less than 100% of the response to the contractile agent, whereas responses resistant to pertussis toxin involved agonists (calcium ionophore) which produced maximum relaxations of 100% or more of the response to the contractile agent (Flavahan *et al.*, 1989); Weinheimer & Osswald, 1989). Hence, apparent resistance to pertussis toxin may reflect the maximal nature of the relaxation to some agents, analoguous to the effects of ageing on vascular relaxations in rat aorta, where age-related alterations may be difficult to demonstrate in the response to agents producing relaxations of a magnitude sufficient to abolish the KCl contraction in young animals (Sawyer & Docherty, 1987).

Two questions remain to be considered concerning pertussis toxin treatments. Firstly, is the effect of pretreatment *in vivo* different from the effect of incubation *in vitro*? In our hands, *in vitro* incubation by use of a methodology similar to that of Flavahan *et al.* (1989) or Weinheimer & Osswald (1989) failed to produce any discernible effect, although the source of pertussis toxin differed between laboratories. Secondly, is there a problem of access for pertussis toxin to cells other than atrial cells? This seems unlikely since neuronal responses to opioids (Werling *et al.*, 1989) and dopamine (Bean *et al.*, 1988) in brain slices are attenuated by pertussis toxin pretreatment. Although the possibility remains that peripheral neurones differ from central neurones in the ability of pertussis toxin to penetrate, especially since responses to dopamine and 5-hydroxytryptamine-receptor agonists as well as α_2-adrenoceptor agonists proved to be resistant to pertussis toxin in peripheral nerves. The evidence for a lack of involvement of G_i or G_0 in these responses would have been stronger if a neuronal response had been found which was susceptible to pertussis toxin in vas deferens or atria. However, some but not all effects of adenosine at A_1-receptors in the rat hippocampus are resistant to pertussis toxin (Fredholm *et al.*, 1989), so that even some central inhibitory receptors mediate responses resistant to pertussis toxin. It seems unlikely that peripheral neurones have a large excess of G_i or G_0, since even doses of pertussis toxin 6 times higher than necessary to abolish, and 40 times higher than necessary to attenuate, negative inotropic responses of atria were totally ineffective. However, a combination of reduced access for pertussis toxin and a large excess of G_i or G_0 could explain resistance to the toxin.

In conclusion, although we cannot categorically state that these responses do not involve G_i or G_0, it seems unlikely that endothelium-dependent relaxations of rat aorta to ACh or histamine and prejunctional inhibitory actions of α_2-adrenoceptor agonists in rat vas deferens and atria involve pertussis toxin-sensitive G proteins, G_i or G_0.

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Effects of calmodulin antagonists on calcium-activated potassium channels in pregnant rat myometrium

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1 The effects of W-7, trifluoperazine, and W-5 on Ca^{2+}-activated K^+-channels were investigated with the inside-out patch-clamp method in smooth muscle cells freshly dispersed from pregnant rat myometrium. These drugs are known to have different potencies as calmodulin antagonists.

2 In the presence of 1 μM Ca^{2+} on the cytoplasmic side ($[Ca^{2+}]_i$), the fraction of time the channel was open (open probability, P_o) was about 0.9 and the calmodulin antagonists (1–30 μM) applied to the cytoplasmic face reduced P_o to 0.65–0.55 dose-dependently. In the presence of 0.1–0.16 μM Ca^{2+}, when P_o was very low (0.02), calmodulin antagonists increased P_o. All antagonists used produced almost identical effects at the same concentration.

3 The probability density function of the open time distribution could be described by the sum of two exponentials. W-7 decreased the time constant of the slow component of distribution and at 30 μM the slow component disappeared both at 1 and 0.25 μM $[Ca^{2+}]_i$, reflecting the appearance of flickering channel activity. The probability density function of the closed time distribution could be fitted with three exponentials. The time constants of these components were not significantly altered by W-7.

4 Internally applied calmodulin (1–5 μM) did not produce any significant effect on channel activity.

5 The effects of calmodulin antagonists are considered to be due to a direct action of these compounds on the channel, and suggest that channel activation by Ca^{2+} is not mediated by calmodulin.

Introduction

The plasma membrane of smooth muscles contains potassium (K^+) channels the activity of which is controlled by intracellular calcium (Ca^{2+}-activated K^+-channels) (Inoue *et al.*, 1985; Benham *et al.*, 1986; McCann & Welsh, 1986). Since these channels have a large conductance and are highly selective to K^+, they may play an important role in regulating electrical excitability, for example in alteration of electrical activity during pregnancy (Osa & Fujino, 1978; Kishikawa, 1981; Bengtsson *et al.*, 1984). However, the mechanism of channel activation with Ca^{2+} is still not well understood.

Since calmodulin mediates many intracellular Ca^{2+}-regulated enzymes and processes, it may also be involved in activation of the Ca^{2+}-activated K^+-channel. This has actually been suggested for some tissues (cultured fibroblast: Okada *et al.*, 1986; 1987; erythrocyte: Lackington & Orrego, 1981; Pape & Kristensen, 1984), mainly based on the action of calmodulin antagonists (e.g., phenothiazines). However, in the Ca^{2+}-activated K^+-channel in airway smooth muscle, it has been found that the order of potency as channel antagonists is haloperidol > trifluoperazine > chlorpromazine and that this is different from the order expected from the potency of these drugs as calmodulin antagonists (trifluoperazine > chlorpromazine > haloperidol), suggesting that these antagonists interact directly with the channel, not through a calmodulin-mediated process (McCann & Welsh, 1987). We have re-examined the possibility of calmodulin involvement in the Ca^{2+}-activated K^+-channel in smooth muscle cells freshly dispersed from pregnant rat myometrium, using calmodulin antagonists (a phenothiadine derivative, trifluoperazine, and a naphalenesulphonamide derivative, W-7) and a naphthalenesulphonamide of weak anticalmodulin potency (W-5) (Asano & Stull, 1985; Asano *et al.*, 1985).

Methods

Pregnant (the 14–18th day of pregnancy) Wistar rats (about 250 g) were used. After anaesthetizing the rat with pentobarbitone sodium (50 mg kg^{-1}), the uterine horn was excised and the animals were killed by bleeding. The endometrium was then carefully removed, and the circular muscle layer was separated from the longitudinal muscle layer. Single smooth muscle cells were obtained by enzymatic dissociation from the circular layer, by a technique similar to that described by Benham *et al.* (1985, 1986) and Inoue *et al.* (1985). The solution for cell dispersion contained 0.1% collagenase, 0.1% trypsin inhibitor, 0.5% bovine serum albumin and no Ca^{2+}.

The physiological solution had the following composition (mM): NaCl 127, KCl 6.0, $CaCl_2$ 2.4, $MgCl_2$ 1.2, glucose 12, HEPES buffer (N-2-hydroxyethylpiperazine-N'-2 ethanesulphonic acid, pH adjusted with NaOH to 7.4) 10. Pipettes used for the whole-cell clamp were filled with a solution containing (mM); K-aspartate 106.0, KCl 24.0, Na_2ATP 5.0, $MgCl_2$ 2.4, $CaCl_2$ 0.8, EGTA (ethyleneglycol-*bis*-tetraacetic acid) 5.0, HEPES 10. For the cell-detached patch clamp experiments, pipettes were filled with solution containing (mM): K-aspartate 130, NaCl 18.0, $CaCl_2$ 0.9, EGTA 1.0, HEPES 5.0. The bath solution facing the cytoplasmic surface of the plasma membrane usually contained (mM): KCl 6.0, NaCl 142.0, HEPES 5.0, EGTA 1.0 and the required concentration of Ca^{2+} ($[Ca^{2+}]_i$). $[Ca^{2+}]_i$ was adjusted by adding a correct amount of Ca^{2+} in the presence of 1 mM EGTA, according to the calculation by Fabiato (1981). When the K^+ concentration was increased, NaCl was replaced with KCl isosmotically. All experiments were carried out at room temperature (22–25°C).

The methods of recording whole-cell currents and single channel currents from an inside-out isolated patch were similar to those described by Hamill *et al.* (1981). The currents were recorded with an amplifier (L/M EPC-7, List). Data were recorded on a videotape recorder (GX4, National) at a sampling rate of 28.8 kHz with an analogue-digital converter (RP-880, PCM Data Recording System, NF Electronic Instrument) and a low pass filter (3.3 kHz). Single channel activity was analysed with a computer using the pClamp programme (version 5.03, Axon Instruments, Inc.). Channel opening and closing were determined by setting the threshold at the half-value of current amplitude.

W-7 (N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide) and W-5 (N-(6-aminohexyl)-1-naphthalenesulphonamide) were obtained from Seikagaku Kogyo (Japan). Other

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chemicals used were all obtained from Sigma. The calmodulin used was a product from bovine brain (Sigma).

Results

Outward currents were produced with 400 ms depolarizing steps from a holding potential of −80 mV with the whole-cell clamp method in normal solution. External application of W-7 slowly inhibited the current dose-dependently. The average current was 367 ± 28 pA in normal solution and this was decreased to 232 ± 21, 187 ± 31 and 150 ± 29 pA (mean ± s.d., $n = 5$) with 1, 10 and 30 μM W-7 applied for 5 min. W-7 50 μM produced a similar degree of inhibition ($n = 2$) to 36 μM, but since the recovery became very slow, the effects of concentrations higher than 30 μM were not carefully examined. Figure 1 shows the strongest inhibitory effect on outward currents observed with 30 μM W-7. The currents were produced by constant depolarizing pulses from −80 to +20 mV applied every 12 s. The recovery was very slow, taking 40–60 min after application of 30 μM W-7.

Figure 2 shows the voltage-current relationship under whole-cell clamp conditions. Outward currents were increased with increasing membrane depolarization beyond −40 mV, showing outward-going rectification. W-7 30 μM decreased the current and reduced the rectification. Nicardipine (a Ca^{2+}-channel blocker, 1 μM) produced effects similar to W-7 and in the presence of nicardipine, the W-7 effect was greatly reduced. These results may be explained either by a direct action of W-7 on outward K^+ currents, or by an indirect action on the Ca^{2+}-dependent K^+-channel, as a result of reduced Ca^{2+}-influx produced by W-7.

In order to investigate the mechanisms underlying the inhibition of outward currents with W-7, activities of single K^+-channels were analysed. When $[Ca^{2+}]_i$ was more than 0.1 μM, three or four channels often became open sumultaneously in many membrane patches. For analysis of channel properties, however, we have chosen patches which were considered to contain only a single active channel. The amplitude of single channel currents recorded with patch pipettes containing 130 mM K^+ depended on the internal K^+ concentration ($[K^+]_i$) and the membrane potential. As shown in Figure 3, the voltage-current relationship can be described by the constant field equation (Hodgkin & Katz, 1949) assuming that only K^+ is permeable. When the permeability constant (P_K) was assumed to be 4.2×10^{-13} cm^{-3} s^{-1}, the observed points agreed reasonably well with the theoretical curves at $[K^+]_i$ higher than 65 mM. When $[K^+]_i$ was 6 mM, a P_k value of 3.8×10^{-13} cm^3 s^{-1} gave a better fit. At a symmetrical K^+ concentration (130 mM), the single channel conductance was 204 ± 4 pS ($n = 4$). These values are close to those (4.5×10^{-13} cm^3 s^{-1} for the P_k and 183–198 pS for the conductance at symmetrical 126 mM K^+) reported for the Ca^{2+}-activated K^+-channel in the smooth muscle of guinea-pig jejunum and mesenteric artery (Benham *et al.*, 1986). In the following experiments, the effects of W-7 and trifluoperazine on this type of channel were investigated at 130 mM external and 6 mM internal K^+ concentrations, because single channel currents of reasonable amplitude (about 4.5 pA) could be observed at a holding potential of 0 mV.

Figure 4a and b show the effects of W-7 (1–30 μM) applied to the cytoplasmic side on single channel currents at 1 and 0.16 μM cytoplasmic Ca^{2+} concentrations ($[Ca^{2+}]_i$), respectively. The channel activity strongly depended on $[Ca^{2+}]_i$. The channel was mostly in the open state at 1 μM Ca^{2+}. The fraction of time during that the channel was in an open state (the open probability, P_o) was 0.91 ± 0.03 ($n = 4$). When 1–10 μM W-7 was applied, the channel activity was slightly decreased, as observed in airway smooth muscle cells (McCann & Welsh, 1987), and very fast flickering activity appeared at 10–30 μM (Figure 4a). The amplitude of currents was decreased at concentrations higher than 10 μM, probably due to the high frequency flickering.

The channel activity was low in the presence of a low $[Ca^{2+}]_i$ (0.16 μM, $P_o = 0.02 \pm 0.01$, $n = 3$). In this condition, the channel activity was clearly increased by W-7 dose-dependently (Figure 4b). The channel opening became not only more frequent, but also longer with flickering activity.

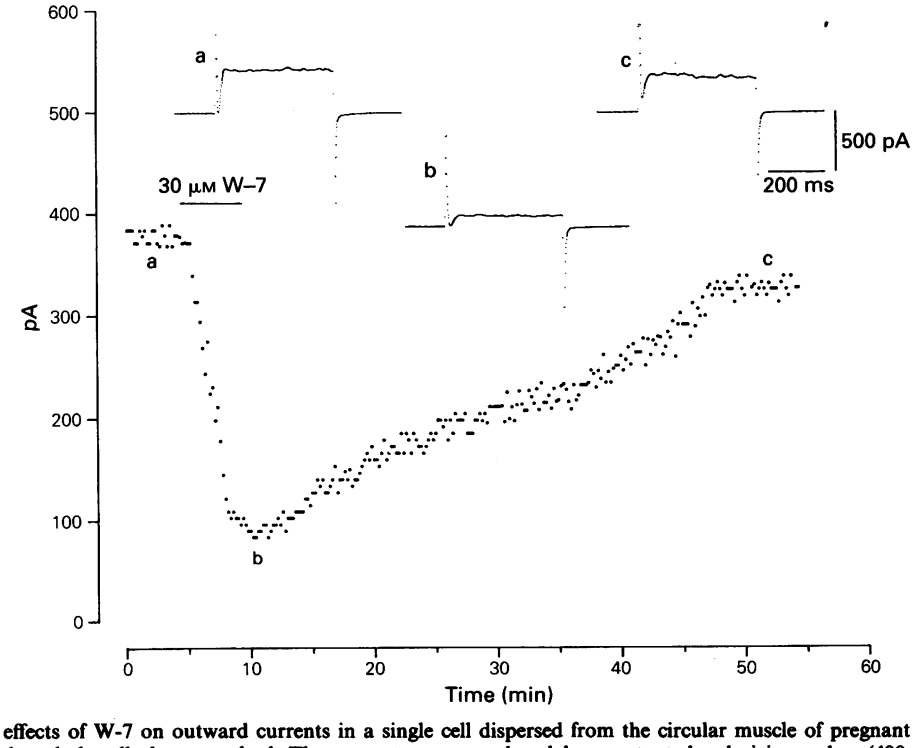

Figure 1 The effects of W-7 on outward currents in a single cell dispersed from the circular muscle of pregnant rat myometrium, recorded with the whole-cell clamp method. The currents were produced by constant depolarizing pulses (400 ms) from −80 to +20 mV every 12 s. The recording chamber (0.2 ml) was perfused with normal solution at a constant rate (2 ml min^{-1}) and 30 μM W-7 was applied to the perfusing solution for 5 min. The current traces shown above were obtained before, 2 min after, and 45 min after W-7 application corresponding to the time indicated by (a), (b) and (c).

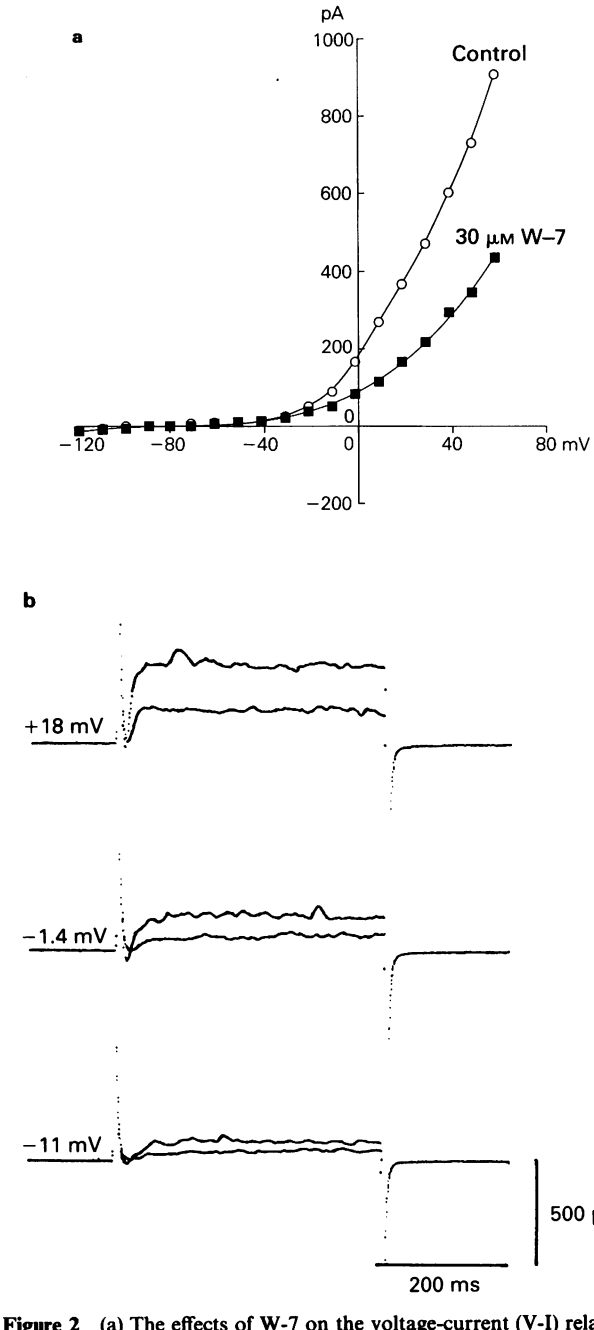

Figure 2 (a) The effects of W-7 on the voltage-current (V-I) relationship obtained with the whole-cell clamp method. After obtaining the V-I relationship in normal solution, 30 μM W-7 was applied and 5 min later the V-I relationship was again obtained in the presence of W-7. (b) Shows superimposed current tracings in the absence and the presence of W-7 at three different voltage steps. The current amplitude was measured at the end of a 400 ms pulse.

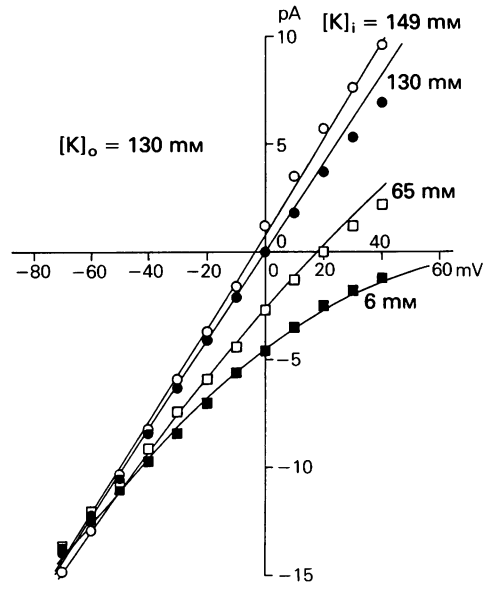

Figure 3 The voltage-current relationship obtained from a single Ca^{2+}-activated K^+ channel on an isolated inside-out patch at different cytoplasmic K^+ concentrations ($[K]_i$). The K^+ concentration in the pipette (external) solution ($[K]_o$) was kept constant at 130 mM. Lines were drawn based on the constant field equation (Hodgkin & Katz, 1949), assuming that only K^+ is permeable. The permeability constant was 4.2×10^{-13} $cm^3 s^{-1}$ for higher than 65 mM K^+ and $3.8 \times 10^{-13} cm^3 s^{-1}$ for 6 mM K^+ (23°C).

These effects were similar to those of local anaesthetics observed at the acetylcholine-receptor channel of frog muscle (Neher & Steinbach, 1978). As W-7 concentrations were increased to 10–30 μM, the current amplitude was reduced, as observed in the presence of 1 μM Ca^{2+}. Compared with the whole-cell clamp condition, recovery from W-7 was much faster in isolated membrane patches, taking about 5 min after removal of 30 μM W-7.

Nearly identical results were obtained at the same concentration (1–30 μM) with trifluoperazine, another calmodulin antagonist (not shown), and also with W-5, a derivative of W-7, which has weak potency as a calmodulin antagonist (Figure 5). In Figure 5, the effects of W-5 were compared with those of W-7 in the presence of 0.16 and 1 μM Ca^{2+} in the same channel. Calmodulin itself was applied internally to three different channels, but it produced no clear effect at a concentration of 1–5 μg ml^{-1} (not shown).

In a solution containing 5 mM EGTA and no added Ca^{2+}, channel activity was absent. Even under these conditions, however, application of 30 μM W-7 started channel activity, although at a very low rate (Figure 6). As seen in the faster current tracings, the channel activity was of a burst type in the presence of W-7. This was confirmed in three channels.

Based on experiments similar to those shown in Figure 4, the effect of W-7 on the relationship between $[Ca^{2+}]_i$ and P_o was studied (Figure 7). In the absence of W-7, the channel remained in a closed state below 0.1 μM Ca^{2+}, and its activity was sharply increased beyond 0.16 μM reaching maximum at 0.4 μM Ca^{2+}. As shown in Figure 4, at low $[Ca^{2+}]_i$ (0.1–0.16 μM), the channel activity was increased by W-7 dose-dependently, whereas at high $[Ca^{2+}]_i$ (0.25–1 μM), it was reduced by W-7. Therefore, the $[Ca^{2+}]_i - P_o$ curve was decreased in amplitude, and flattened by increasing W-7 concentrations from 1 to 30 μM, without a significant shift in the mid-point. The sensitivity to Ca^{2+} varied slightly in different channels, but the effect of W-7 was essentially the same in the three channels studied.

The effects of W-7 on the probability density function of the distributions of open time and closed time were analysed at 1 μM and 0.25 μM $[Ca^{2+}]_i$ in two channels, and the data in the presence of 1 μM are shown in Figure 8. Since the channel activity was very low at 0.16 μM $[Ca^{2+}]_i$, 0.25 μM $[Ca^{2+}]_i$ was chosen for precise analysis. In the absence of W-7, the curve could be fitted with a sum of two exponentials for the open time distribution. W-7 significantly increased the event frequency and shortened the time constant of the slow component of the distribution at both $[Ca^{2+}]_i$ (Table 1). However, the time constants in the presence of 0.25 μM $[Ca^{2+}]_i$ were very similar to those in 1 μM $[Ca^{2+}]_i$ at each W-7 concentration. At 30 μM, the slow component disappeared due to the appearance of a high frequency burst activity. Shortening of the time constant with calmodulin antagonists has previously been reported for dog airway muscle cells (McCann & Welsh, 1987).

The probability density function of closed time distribution could be fitted with three exponentials in the absence and in

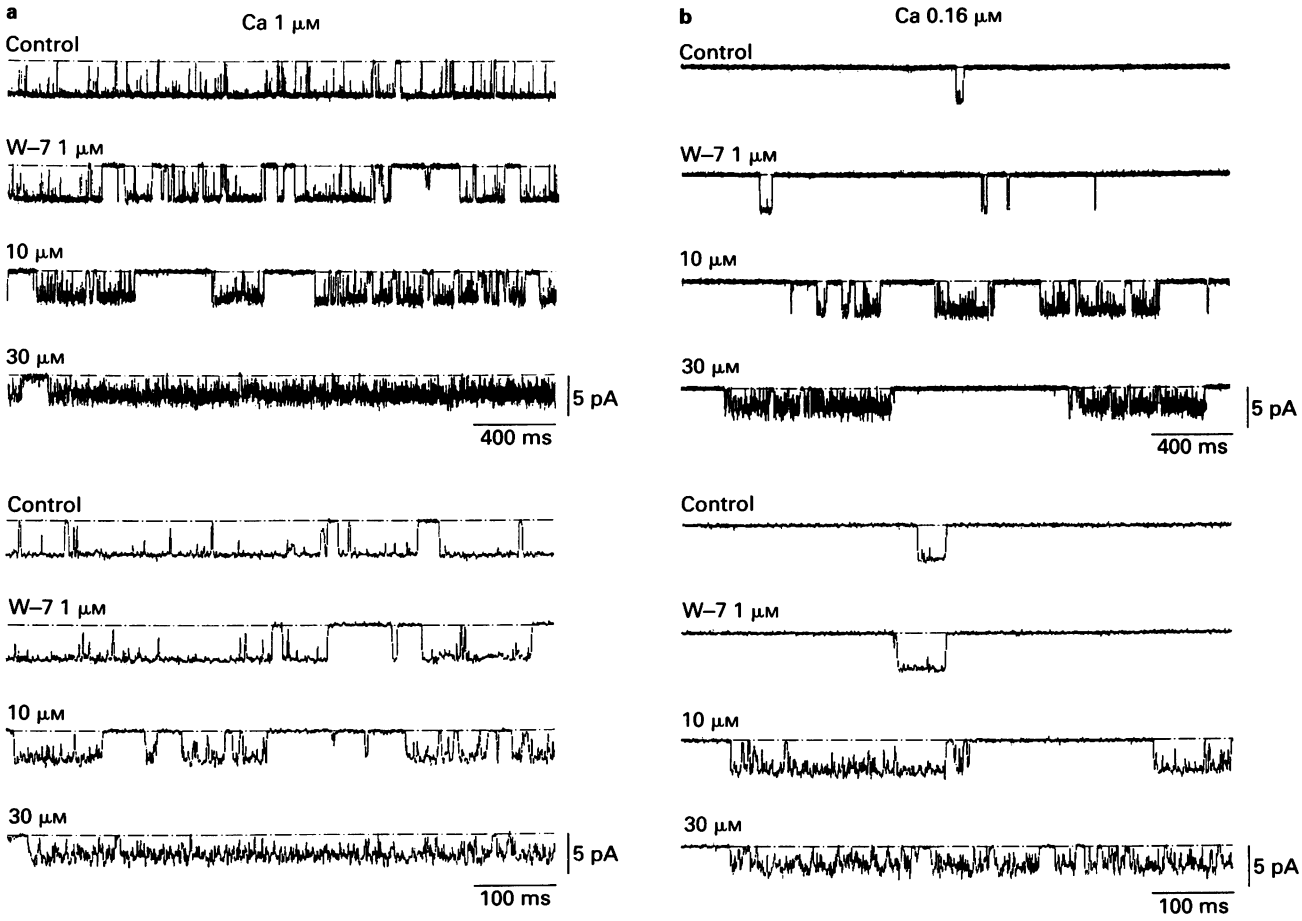

Figure 4 The effects of W-7 on a single Ca^{2+}-activated K^+ channel. $[K^+]_o = 130$ mM, $[K^+]_i = 6$ mM, $[Ca^{2+}]_i = 1\,\mu$M in (a) and $0.16\,\mu$M in (b), and the membrane potential was clamped at 0 mV. W-7 was applied to the cytoplasmic face and its concentration was increased from 1 to $30\,\mu$M stepwise, each for 5 min. The dotted lines correspond to the closed state of the channel. Lower traces are shown at a faster time scale. (a) and (b) are from the same channel. See text for further explanation.

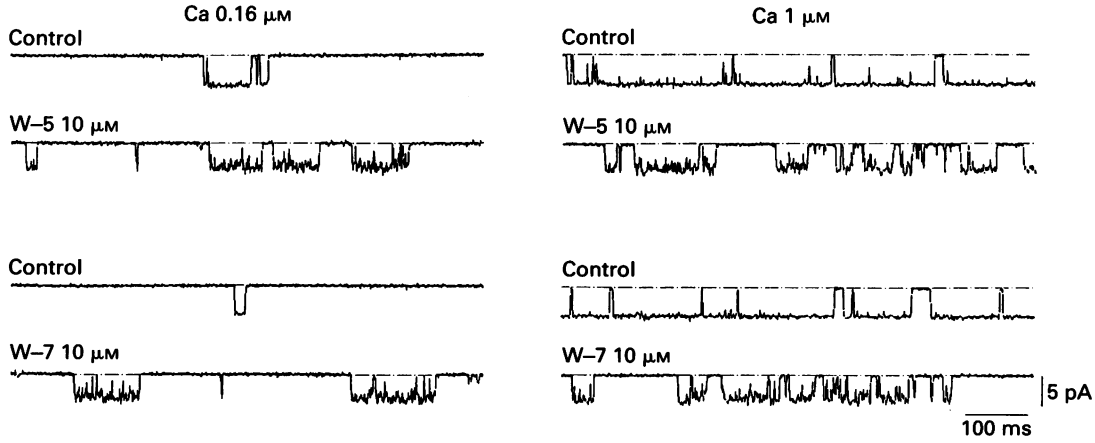

Figure 5 Comparison of the effects of W-5 and W-7 at $10\,\mu$M on channel activity, in the presence of 0.16 and $1\,\mu$M $[Ca^{2+}]_i$. The experimental conditions were the same as in Figure 4, but from a different channel. W-7 was applied 10 min after wash-out of W-5. The Ca^{2+} concentration was increased after observing the effects of $10\,\mu$M W-7 at $0.16\,\mu$M Ca^{2+}.

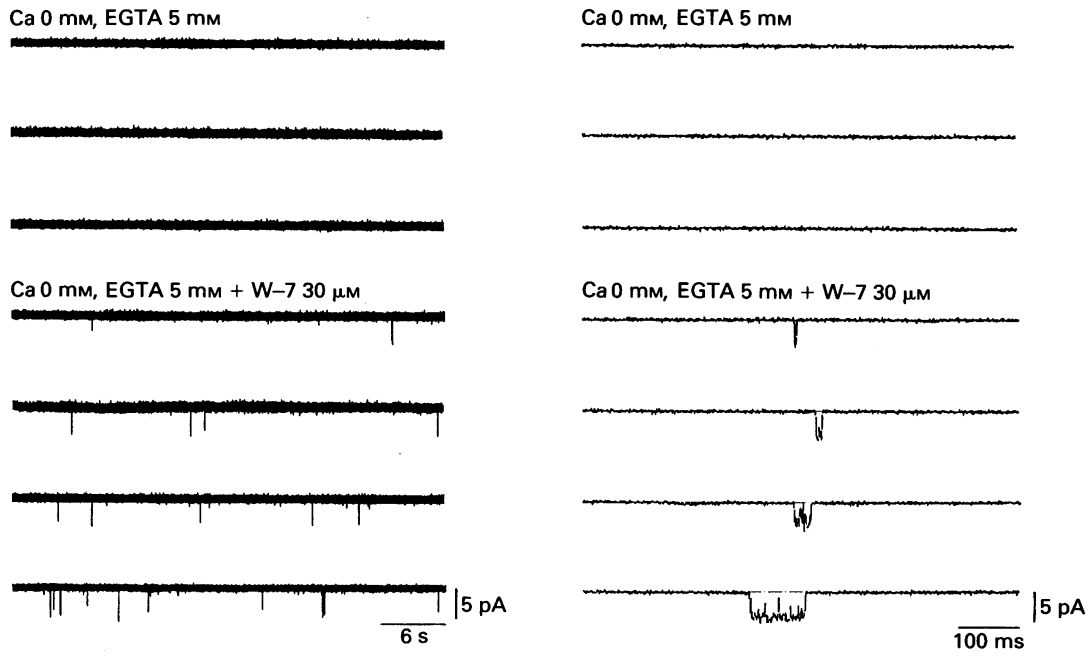

Figure 6 The effects of W-7 30 μM in the absence of Ca^{2+}. The internal solution contained no added Ca^{2+} and 5 mM EGTA. On the left, continuous recordings are shown successively at a slow speed, and W-7 was applied near the end of the third trace. The faster traces (on the right) were selected to show channel activity from the slow records shown on the left.

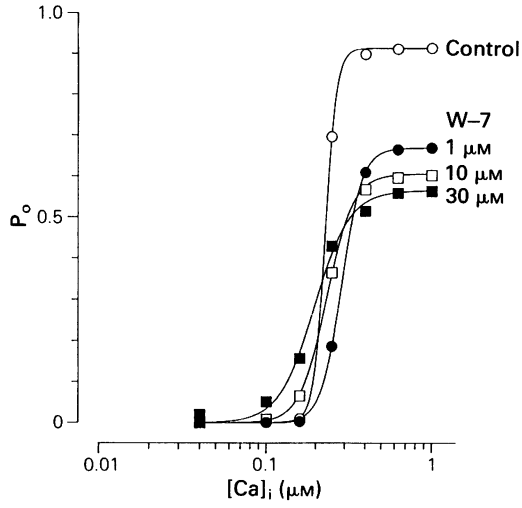

Figure 7 The relationship between open probability (P_o) and Ca^{2+} concentration at the cytoplasmic face ($[Ca]_i$), in the absence and presence of W-7 1–30 μM. All data were obtained from a single channel.

the presence of W-7 (Table 1). When P_o was reduced by decreasing $[Ca^{2+}]_i$ from 1 to 0.25 μM in the absence of W-7, the main change was a lengthening of the time constant of the slowest component. However, the effects of W-7 on the time constants of closed time distribution varied, depending on the concentration of both $[Ca^{2+}]_i$ and W-7, as shown in Table 1.

Discussion

In mouse fibroblasts, a $[Ca^{2+}]_i$-dependent hyperpolarization is reduced by calmodulin antagonists (trifluoperazine, 25 μM, or W-7, 30 μM) and intracellular application of calmodulin induces hyperpolarization of the membrane (Okada *et al.*, 1986; 1987). This result has been interpreted as suggesting that calmodulin is involved in the functioning of Ca^{2+}-activated K^+-channels. In single muscle cells freshly dispersed from pregnant rat myometrium, outward membrane currents recorded with the whole-cell clamp method are also inhibited by W-7. These currents are considered to be mainly carried by K^+ flowing through Ca^{2+}-activated K^+-channels, because the current is reduced by nicardipine. From this result alone,

Table 1 Open probability (P_o), mean open time (MOT), mean closed time (MCT) and time constants (τ) in ms of open and closed time distributions (the mean of two channels)

		Open				*Closed*		
	P_o	*MOT*	τ_1	τ_2	*MCT*	τ_1	τ_2	τ_3
$[Ca^{2+}]_i$ 1 μM								
Control	0.84	36.3	1.5	41.8	6.8	0.7	3.2	16.6
W-7 (μM)								
1	0.61	12.6	2.4	15.9	8.1	0.7	2.9	26.0
10	0.56	4.8	2.2	7.3	4.4	0.6	3.3	23.7
30	0.52	2.0	1.9	—	1.6	0.9	2.7	23.9
$[Ca^{2+}]_i$ 0.25 μM								
Control	0.70	35.8	1.7	34.8	15.2	0.3	2.6	35.3
W-7 (μM)								
1	0.20	9.9	2.4	12.9	15.3	0.6	2.5	44.8
10	0.43	7.2	1.8	8.2	8.1	0.5	2.1	15.4
30	0.47	2.1	1.9	—	2.7	0.9	3.1	19.6

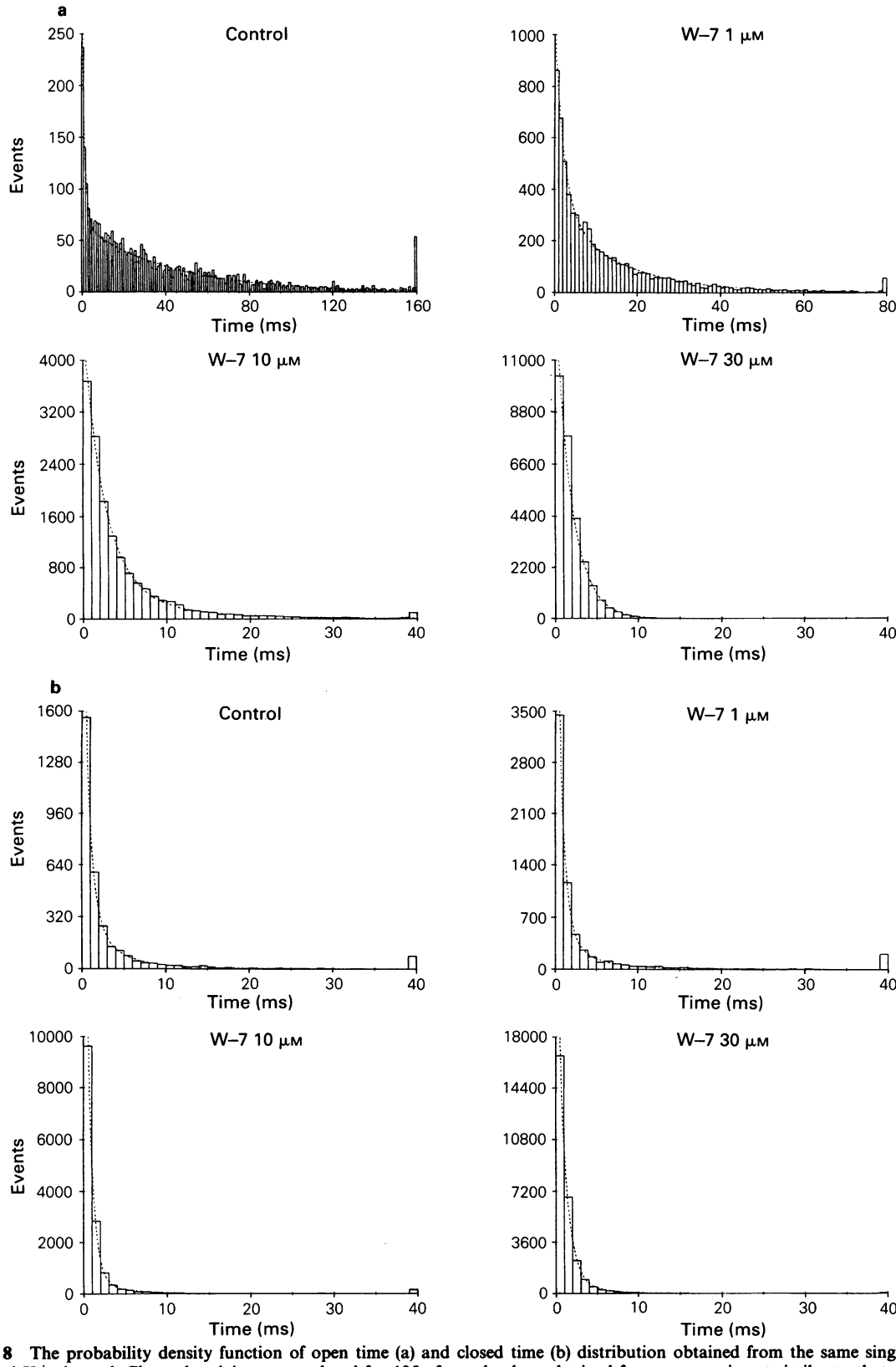

Figure 8 The probability density function of open time (a) and closed time (b) distribution obtained from the same single Ca^{2+}-activated K^+ channel. Channel activity was analysed for 135 s from the data obtained from an experiment similar to that shown in Figure 4. W-7 concentration was increased from 1 to 20 μM, each for 5 min. The effects developed fully in 1–2 min.

however, it is not certain whether W-7 is inhibiting Ca^{2+}-activated K^+-channels directly or is blocking the Ca^{2+} influx responsible for activation of the K^+-channels. It is known that calmodulin antagonists, such as trifluoperazine and W-7, reduce voltage-dependent Ca^{2+} influx (Greenberg *et al.*, 1987; Klöckner & Isenberg, 1987). In smooth muscle cells dispersed from cow portal vein, depolarization-induced outward currents are inhibited by trifluoperazine at concentrations above 1 μM, probably through blocking Ca^{2+} influx, but at a higher concentration (20 μM) the outward currents induced as a result of intracellular Ca^{2+} release by agonists (acetylcholine, prostaglandin, or angiotensin) are also reduced (Klöckner & Isenberg, 1987). Since no recovery from inhibition with trifluoperazine could be observed after dialysis with 100 μM

calmodulin, they concluded that calmodulin is not involved in the activation of K^+ channels by Ca^{2+}.

In the rat myometrium, the single channel activity of Ca^{2+}-activated K^+-channels observed in the presence of 1 μM $[Ca^{2+}]_i$ with the patch-clamp method, is also inhibited by W-7 or trifluoperazine. At a concentration of 30 μM, P_o is reduced by about 40%. However, the effect of calmodulin antagonists is different from that of lowering $[Ca^{2+}]_i$. When P_o is decreased by reducing $[Ca^{2+}]_i$ the main alteration of channel kinetics is a lengthening of the time constant of the slow component of closed time distribution, without a significant change in open kinetics. However, the decrease of P_o with W-7 or trifluoperazine is accompanied by a shortening of the time constant of the slow component of open time distributions, reflecting flickering activity of the channel. Therefore, calmodulin antagonists do not seem to be simply inhibiting the activation process of the channel with Ca^{2+}.

When P_o is low in the presence of 0.1–0.16 μM $[Ca^{2+}]_i$, channel activity is clearly increased by W-7 or trifluoperazine. Furthermore, even when no channel activity is observed in the absence of Ca^{2+}, some channel activity is started by 30 μM W-7 and the $[Ca^{2+}]_i$ for 50% activation is not shifted much by calmodulin antagonists. These results strongly suggest that W-7 and trifluoperazine exert a non-specific action on the channel, not accounted for by calmodulin antagonism.

In Ca^{2+}-activated K^+-channels in dog airway muscle cells, the potency of drugs (haloperidol, trifluoperazine, thioridazine, chlorpromazine) in reducing channel activity was found to be different from their potency as calmodulin antagonists, as mentioned in the Introduction (McCann & Welsh, 1987). The concentration of calmodulin antagonists that inhibit 50% of a calmodulin-dependent phosphodiesterase is reported to be 7–10, 26–67, and 240 μM, for trifluoperazine, W-7 and W-5, respectively (Asano & Stull, 1985; Asano *et al.*, 1985). The low potency of W-5 as a calmodulin antagonist can serve as a reasonable control compound for W-7, which has a similar structure, to specify drug effects. In the present experiments, W-5 and W-7 produced very similar effects on the single channel current of Ca^{2+}-activated K^+-channels, at the same concentration. These results suggest that the effects are not related to their calmodulin antagonistic action. They also suggest that calmodulin is not involved in the activation of the K^+-channel by Ca^{2+}, and that Ca^{2+} probably binds directly to the gating site for channel activation.

W-7, W-5, and trifluoperazine all reduced the $[Ca^{2+}]_i$-dependency of the K^+-channel. In the presence of these compounds, some channel activity continued in the absence of Ca^{2+}. A similar finding has been reported for the Ca^{2+}-activated K^+-channel in smooth muscle cells of the human aorta, following treatment with a fatty acid, 2-decanoic acid (Bregestovski *et al.*, 1989). The effect of 2-decanoic acid is interpreted as due to alteration of membrane-associated protein function. Hydrophobic compounds, such as phenothiadine and naphthalenesulphonamide, may exert a similar effect on the membrane lipid, resulting in modification of the channel activity.

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Effect of diadenosine polyphosphates on catecholamine secretion from isolated chromaffin cells

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1 The action of several diadenosine polyphosphates (AP_3A, AP_4A and AP_5A) on basal, and on nicotine- and high K^+-evoked, catecholamine (CA) release has been investigated. Each of the three diadenosine polyphosphates weakly but significantly increased basal CA secretion. This enhancement represented about 10% of the response evoked by 2 μM nicotine.

2 The evoked secretory response to diadenosine polyphosphates had an absolute requirement for extracellular Ca^{2+}.

3 In contrast, these compounds had an inhibitory action on nicotine-evoked release. This response was concentration-dependent, EC_{50} values being $3.2 \pm 0.4\,\mu$M, $4.0 \pm 1.6\,\mu$M and $19.3 \pm 4.0\,\mu$M for AP_3A, AP_4A, and AP_5A, respectively. The lower the concentration of nicotine used to evoke secretion, the higher the inhibitory power of these compounds.

4 The CA secretion evoked by K^+-rich solutions was further enhanced by AP_3A and AP_5A, whereas AP_4A inhibited it. The possible physiological role of these dual actions is discussed.

Introduction

The use of adrenal medullary chromaffin cells as a model has been largely responsible for the improvement in our understanding of neurosecretory responses, their molecular mechanism and modulation (Winkler & Carmichael, 1982; Burgoyne, 1984; Bader *et al.*, 1986). Many substances, including opioid peptides, substance P, γ-aminobutyric acid and peptides derived from cromogranin A, appear to modulate acetylcholine-mediated catecholamine (CA) release from these cells (Mizobe *et al.*, 1979; Kumakura *et al.*, 1980; Castro *et al.*, 1988; Simon *et al.*, 1988).

Another of these putative neuromodulator substances is adenosine and its analogues. Today, adenosine receptors and their actions are well documented (Williams, 1987), and recently adenosine triphosphate (ATP) itself has been shown to influence many biological processes (Gordon, 1986; Reilly & Burnstock, 1987).

ATP is one of the main components of chromaffin granules and it is released in the exocytotic process. This nucleotide can be degraded extracellularly by the action of ecto-nucleotidases (Richardson *et al.*, 1987; Newby, 1988) to form adenosine. The effect of adenosine and adenosine nucleotides on CA secretion from chromaffin cells has therefore been studied. ATP, adenosine diphosphate (ADP) and adenosine inhibit acetylcholine-evoked CA release, probably by prior conversion to adenosine (Chern *et al.*, 1987). In contrast, adenosine can enhance, in a quite complex manner, forskolin-mediated secretion (Chern *et al.*, 1988). Chromaffin cells present a single class of high affinity adenosine transporters of the neural type (Miras-Portugal *et al.*, 1986; Torres *et al.*, 1986; 1988). These transporters are active enough to control the termination of the effects of adenosine.

ATP is not the only nucleotide component co-stored in secretory granules. In effect, diadenosine polyphosphates (AP_xA) have been demonstrated to exist in platelet (Flodgaard & Klenov, 1982; Lütje & Ogilvie, 1983) and in chromaffin granules (Rodriguez del Castillo *et al.*, 1988). The AP_xA have been demonstrated to be responsible for multiple biological effects inside the cells (Zamecnik, 1983), but their extracellular role, if any, after release, is still not fully known (Lütje & Ogilvie, 1987; 1988). Recently, Louie *et al.* (1988) found an antithrombotic action for AP_4A.

AP_3A, AP_4A and AP_5A are present in chromaffin granules and the purpose of the present experiments was to study the effects of these dinucleotides on CA release from isolated chromaffin cells.

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Methods

Bovine adrenal glands supplied by the local slaughter house were immediately placed in ice-cold physiological saline solution and processed within 1–2 h following the death of the animal.

Isolation of bovine adrenal chromaffin cells

Chromaffin cells were prepared from adrenal medullae according to the method of Miras-Portugal *et al.* (1985). In brief, glands were cannulated and washed by retrograde perfusion with Ca^{2+}-free Locke medium containing 5% bovine serum albumin. Medullary tissue was digested with 0.1% collagenase (Boehringer) perfused continuously for 1 h. Collected cells were washed twice and purified in a percoll gradient (50% isotonic percoll, centrifuged at 15000 *g* for 30 min at 20°C). Collected cells were suspended in Dulbecco's modified Eagle's medium, DMEM (GIBCO), and washed twice. Cell viability was checked by trypan blue exclusion. The purity of the chromaffin cells was assessed by the specific incorporation of neutral red into these cells. Viability and purity were greater than 90%.

Purified cells were dispersed at a density of 10^6 cells ml^{-1} in DMEM containing 10% foetal calf serum (GIBCO), standard antibiotics (100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 40 μg ml^{-1} gentamicin, all from Sigma), 50 μM cytosine arabinoside (Aldrich), 50 μM 5-fluorodeoxyuridine (Aldrich) and 100 μM sodium ascorbate (Sigma). This suspension was kept at 4°C and used during the 2–3 days following cell isolation, as described by Greenberg & Zinder (1982). Under these conditions, third-day cells were able to grow when seeded in plastic Petri dishes (Costar) and maintained at 37°C in 5% CO_2/95% air.

Chromaffin cell secretory response

CA release was measured by direct electrochemical detection of CA eluting from a superfused cell bed, in a monitoring system similar to that described by Green & Perlman (1981) and Kumakura *et al.* (1986). Chromaffin cells (10^6 cells) were introduced into a perfusion chamber, formed by a Millex GS filter (0.22 μm pore size, 25 mm ϕ) and perfused at 2 ml min^{-1} with Locke solution (composition in mM: NaCl 140, KCl 4.4, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 4.0, glucose 5.6 and HEPES 10, pH 7.5). In potassium-rich solution (K^+-rich, 25 mM), KCl concentration was increased at the expense of NaCl.

The cell bed was stimulated by injection of chemical stimuli into the flow stream, in a volume of 50 μl, through a loop injector (Rheodyne 7010, Cotati, California). When taking into account the filter volume and the continuous perfusion, the maximum concentrations of the secretagogue in the cell bed (referred to as final concentrations) were 4.7 times lower than the secretagogue concentration in the injected solution (referred to as initial concentrations). This ratio was measured experimentally, by injecting different adrenaline concentrations under the same experimental conditions and referring peak height to the signal produced by continuous perfusion with those concentrations of adrenaline in the medium. The electrochemical detector was adjusted to +500 mV to avoid K^+ effect on support current. The electrochemical detector provided a continuous signal proportional to the concentration of catecholamines in the perfusate. None of the drugs used in our experiments gave electrochemical signals detected by this system.

Results are presented as the mean ± s.e.mean of at least three experiments, each performed in triplicate. For each experiment, cells from adrenal glands of four animals were pooled. The level of significance was established at $P < 0.05$, obtained by use of Student's t test. EC_{50} values were derived by logit-log regression.

Results

Effects on basal secretion: diadenosine polyphosphate-evoked release

All three diadenosine polyphosphates had a weak secretory action, which was only about 10% of control 2 μM (final concentration) nicotine-evoked release. However, this effect could not be attributed to a reagent artifact since, under the same experimental conditions but without cells present in the perfusion chamber, no signal was observed.

CA release by chromaffin cells was increased when cells were challenged with AP_xA in a concentration-dependent manner. AP_xA-evoked release was low, amounting to 27 ± 5, 19 ± 3 and 23 ± 3% of previous basal release for AP_3A, AP_4A and AP_5A, respectively.

The secretory response had an absolute requirement for extracellular calcium. Challenging cells with each AP_xA, or nicotine as a control, in a Ca^{2+}-free medium failed to elicit a secretory response (Figure 1).

Effect of diadenosine polyphosphates on nicotine-evoked catecholamine release

When CA secretion was stimulated with 10 μM nicotine (initial concentration), the three AP_xA compounds studied exerted an inhibitory effect (Figure 2), in a concentration-dependent fashion. EC_{50} values were 3.2 ± 0.4 μM for AP_3A, 4.0 ± 1.6 μM for AP_4A and 19.3 ± 4.0 μM for AP_5A. The inhibitory effect of

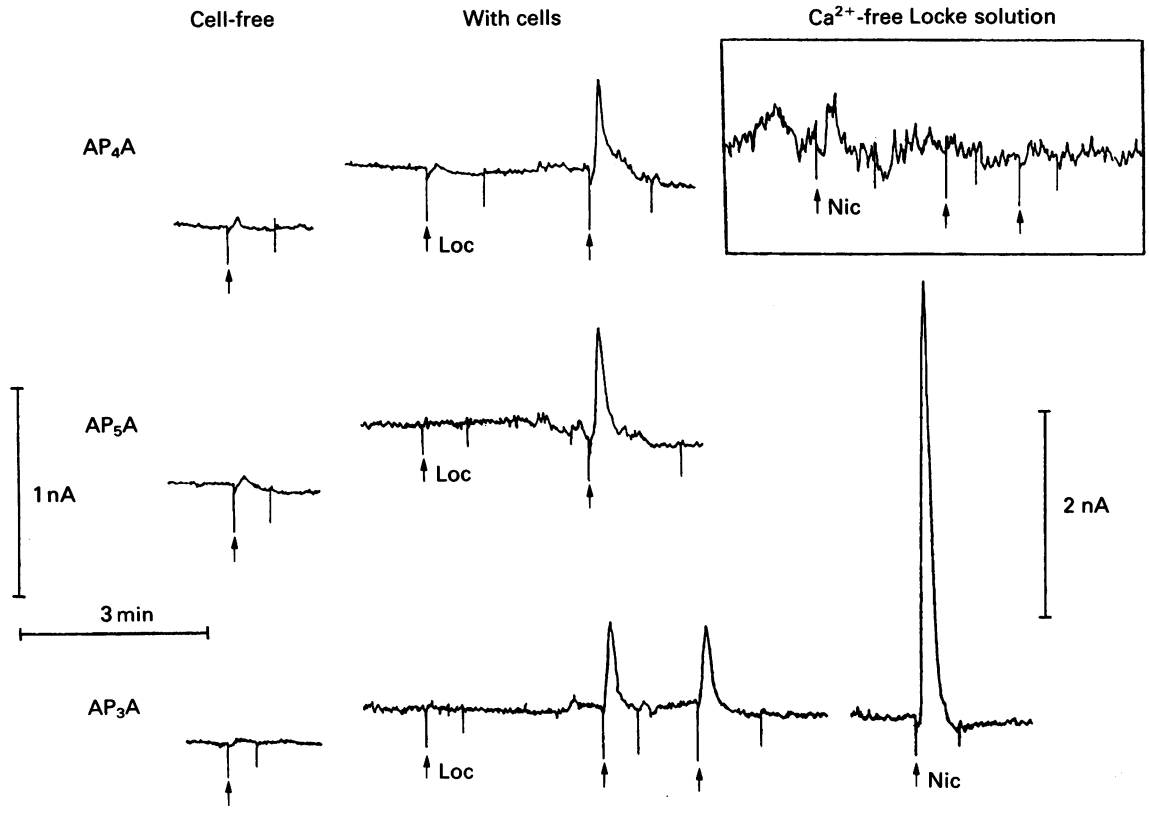

Figure 1 Effect of diadenosine polyphosphates (AP_xA) on basal catecholamine (CA) release. Typical records of electrochemically monitored current-time peaks from a perfusion chamber without cells (cell-free), with cells in normal medium containing Ca^{2+} and in the absence of Ca^{2+}. Drugs (100 μM, initial concentration) were injected at the arrows. Loc, normal Locke solution injected as negative control. The nicotine peak referred to as CA release in normal Ca^{2+}-containing medium evoked by 10 μM (initial concentration) nicotine. Note the different scale.

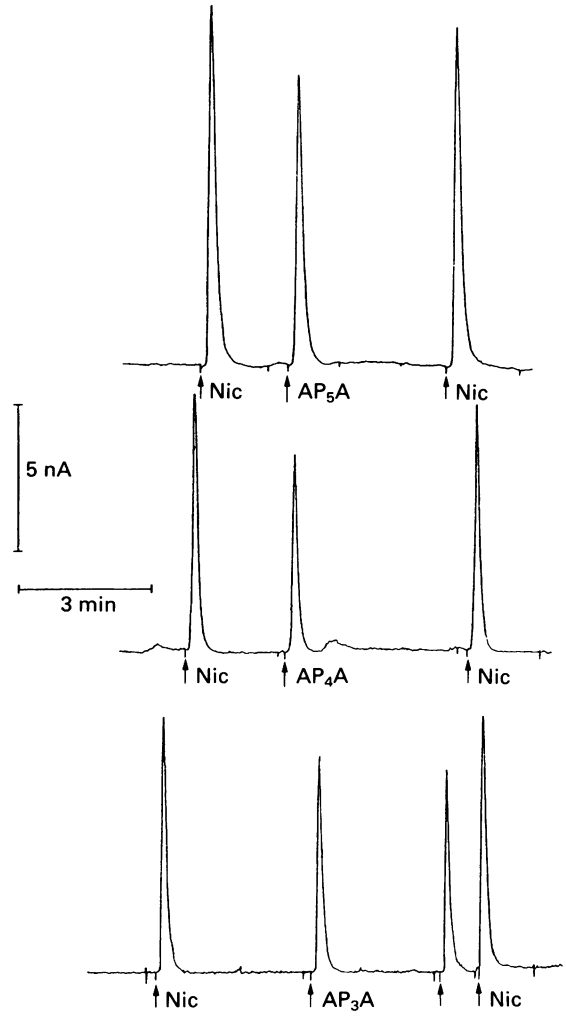

Figure 2 Effect of diadenosine polyphosphates (AP_xA) on catecholamine (CA) release evoked by nicotine. The figure represents typical records of electrochemically monitored current-time peaks from nicotine (Nic) 10 μM (initial concentration) and nicotine (10 μM) plus the respective diadenosine polyphosphate (100 μM, initial concentration).

these polyphosphates was not further increased at concentrations higher than 100 μM (initial concentration). The greatest inhibitory effect was achieved with AP_4A, 40.0 ± 0.5% maximum inhibition, whereas the inhibitions caused by AP_3A and AP_5A, though significant, were only 15 ± 0.5% and 18 ± 3%, respectively (Figure 3).

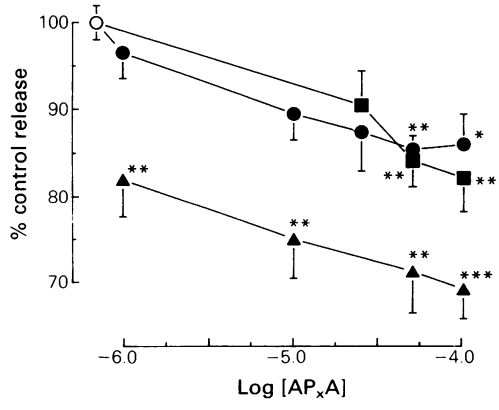

Figure 3 Inhibition of nicotine-evoked catecholamine (CA) release by diadenosine polyphosphates (AP_xA). Control release was evoked by 10 μM (initial concentration) nicotine. Each point was determined as shown in Figure 2 for each concentration of AP_3A (●), AP_4A (▲) and AP_5A (■). $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Vertical lines show s.e.mean.

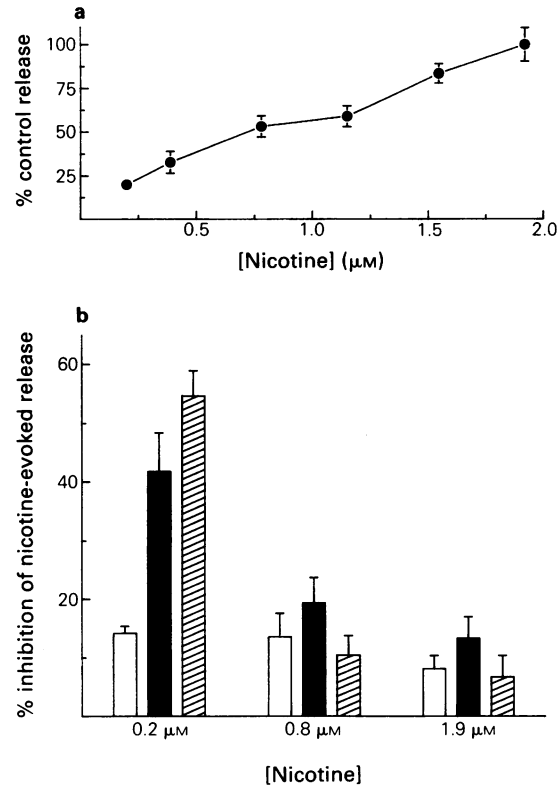

Figure 4 (a) Control values of catecholamine (CA) release evoked by increasing concentrations of nicotine. (b) Effect of 25 μM (initial concentration) diadenosine polyphosphates (AP_xA) on CA release evoked by different nicotine concentrations. Open columns, effect of AP_3A; solid columns, AP_4A; hatched columns, AP_5A.

At a final concentration of 5.3 μM, AP_3A, AP_4A and AP_5A caused a statistically significant reduction in the CA release evoked by nicotine (0.2–1.9 μM, final concentration). This inhibitory effect was greater when cells were stimulated with low concentrations of nicotine, and became reduced with increasing nicotine concentrations (Figure 4).

Effect of diadenosine polyphosphates on catecholamine release evoked by 25 mM K^+

In the presence of K^+-rich solution, CA secretion was elevated. AP_3A and AP_5A further enhanced this evoked release, whereas AP_4A inhibited it (Table 1).

Effect of continuous perfusion with diadenosine polyphosphates

When long-term effects of AP_XA were studied by bathing the cells with the appropriate drug and testing secretory responses to nicotine, the inhibitory action of all three compounds increased with time. The effect was reversed when the drug

Table 1 Effect of diadenosine polyphosphates (19 μM) on catecholamine release evoked by 25 mM K^+ solution

Effector	*% effect*
KCl alone	100 ± 6
+ AP_3A	151 ± 12
+ AP_4A	63 ± 2
+ AP_5A	173 ± 6

Concentrations refer to final concentrations. Figures are mean ± s.e.mean for six cell beds corresponding to three different preparations.

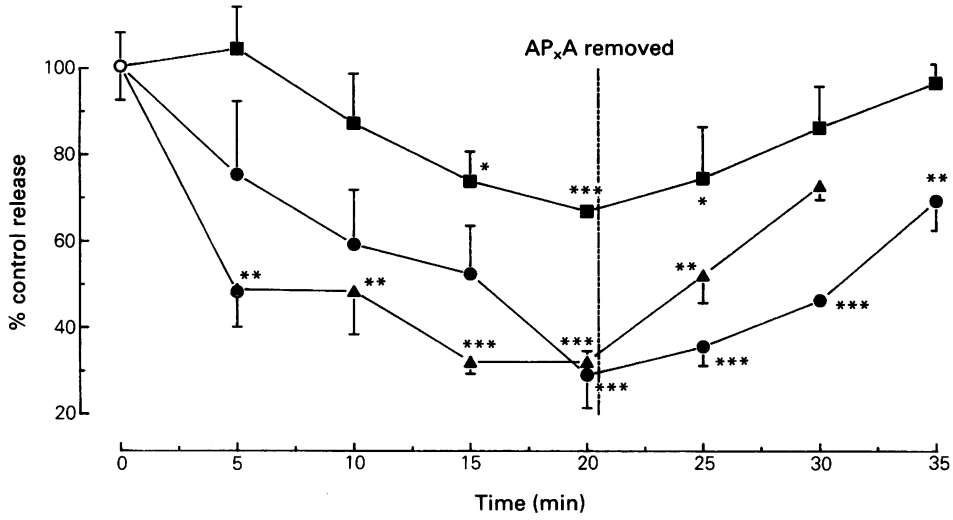

Figure 5 Effect of continuous perfusion of 10 μM diadenosine polyphosphates (AP_xA) on 10 μM (initial concentration) nicotine evoked catecholamine (CA) release with respect to time. Cells were perfused with drug-containing medium. At the vertical line the medium was replaced by a drug-free one. (○) Control release prior to drug perfusion, (●) AP_3A, (▲) AP_4A and (■) AP_5A. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Vertical bars show s.e.mean.

was removed from perfusing medium (Figure 5). The rank order of these inhibitory agents appeared to be $AP_4A \geqslant AP_3A > AP_5A$.

Discussion

The results presented here demonstrate, for the first time, that adenosine polyphosphates can play a role in nicotine-evoked CA release. This novel effect adds to the emerging extracellular actions being shown for these compounds, for instance on macrophage-induced cell growth (Ogilvie & Lüthje, 1987) and platelet aggregation (Louie *et al.*, 1988). It is important to realize that in our model diadenosine polyphosphates are active in the low μM range, whereas in the other studies high μM or μM concentrations were needed to obtain the effects.

Although chromaffin cells have a low hydrolytic activity, the effects seems to be due to diadenosine polyphosphates themselves, rather than to degradation products. Since in our experimental system drugs are in contact with the cell bed only for a few seconds, the possible degradation would be negligible (<1% per hour in cultured cells, M.T. Miras-Portugal, unpublished results). In these conditions ATP, ADP, AMP or adenosine concentrations resulting from diadenosine phosphate degradation must be far below the μM range. Since the K_d values for ATP and adenosine at purinoceptors (Reilly & Burnstock, 1987) and the known effects of adenosine on CA release (Chern *et al.*, 1988) are produced at concentrations in the order of 100 μM, an action through these degradation products is probably precluded.

The action of these effectors, especially that of AP_4A and AP_5A, may be physiologically important in the local control of CA release, since they are stored at an intragranular concentration of about 6 mM in chromaffin cells (Rodriguez del Castillo *et al.*, 1988), and are released together with CAs in the exocytotic process. The finding that the lower the concentration of nicotine used to stimulate the cells, the higher the modulator potential, is concordant with results on other systems, such as phorbol ester modulation of glutamate release from synaptosomes (Diaz-Guerra *et al.*, 1988). This behaviour points to a predominant role of these compounds on the basal/sub-maximal levels of secretion. In fact Malhotra & Wakade (1987) have shown that in the adrenal medulla *in situ* acetylcholine is not the major component of splanchnic nerve stimulation input to chromaffin cells.

The results with AP_3A and AP_5A seem confusing, since they are inhibitors of nicotine-evoked release, but are activators when CA secretion is evoked by high K^+. However, similar results have been obtained with substance P, which modulates nicotine-evoked release but has no action on K^+-evoked release (Livett *et al.*, 1983). Similar opposing effects of different diadenosine polyphosphates on the same response have been found previously (Chao & Zamecnik, 1984; Lüthje *et al.*, 1985). CA secretion from chromaffin cells is triggered by a fast rise in cytosolic calcium concentration, $[Ca^{2+}]_i$ (Kao & Schneider, 1986), but there is growing evidence to support the idea that nicotine and nicotinic agonists activate an alternative second messenger system, in addition to the rise in free cytosolic $[Ca^{2+}]$ (Cobbold *et al.*, 1987; Minenko *et al.*, 1987). It may be possible that the inhibition of nicotine-evoked release produced by these compounds could be mediated through this alternative pathway. Enhancement of K^+-evoked release could be related to their own secretory action on basal output.

We have shown that AP_3A, AP_4A and AP_5A could play a physiological role in the modulation of basal and evoked release of CA from chromaffin cells, though the assay method does not permit any conclusion to be drawn concerning any differential effects these compounds may have on the output of adrenaline and noradrenaline. These compounds could serve as valuable tools for the study of secondary and later steps in exocytosis and offer a new direction for the development of pharmacologically active drugs.

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$GABA_B$ receptor-mediated inhibition of the neurogenic vasopressor response in the pithed rat[1]

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1 The effects of γ-aminobutyric acid (GABA) and related drugs on the vasopressor response induced by electrical stimulation (single pulse of 30 V and 1 ms) of the preganglionic sympathetic nerve fibres or by injection of noradrenaline 0.3 nmol kg^{-1} were studied in the pithed rat.

2 The electrically-induced increase in diastolic blood pressure was inhibited by GABA and the $GABA_B$-receptor agonist **R**-(−)-baclofen but was not affected by its **S**-(+)-enantiomer and by the $GABA_A$-receptor agonists muscimol and 3-aminopropane sulphonic acid.

3 The dose-response curve of **R**-(−)-baclofen for its inhibitory effect on the electrically-induced vasopressor response was shifted to the right by the $GABA_B$-receptor antagonist 2-hydroxysaclofen, but was not affected by the $GABA_A$-receptor antagonist bicuculline. 2-Hydroxysaclofen and bicuculline by themselves did not affect the electrically-induced vasopressor response.

4 The increase in diastolic blood pressure induced by exogenous noradrenaline was not affected by the GABA-related drugs, which also had no (or very slight) effects on the basal diastolic blood pressure.

5 It is concluded that GABA inhibits catecholamine release in the resistance vessels of the rat via $GABA_B$-receptors, probably located presynaptically on the postganglionic sympathetic nerve fibres.

Introduction

γ-Aminobutyric acid (GABA) is involved in the central regulation of cardiovascular functions. Thus, intracerebral or intracisternal administration of GABA, GABA receptor agonists and antagonists produces alterations of blood pressure and heart rate (for review, see DeFeudis, 1983; Antonaccio, 1984; Bousquet *et al.*, 1985). In addition, GABA is also capable of relaxing vascular smooth muscle by activation of postsynaptic $GABA_A$- receptors located on the vessels themselves, and locally formed GABA might play a role *in vivo* in the cerebral vascular bed at least under pathophysiological conditions (for review, see Krause, 1986). In some isolated vessels of the rabbit (Starke & Weitzell, 1980; Anwar & Mason, 1982; Manzini *et al.*, 1985) and in middle cerebral arteries of the goat (Miranda *et al.*, 1989), presynaptic $GABA_B$-receptors could be identified, activation of which produces an inhibition of the stimulation-evoked noradrenaline release and/or contraction. Miranda *et al.* (1989) could also show that, in the anaesthetized goat, the decrease in cerebral blood flow induced by electrical stimulation of the cervical sympathetic nerves was diminished by GABA and baclofen via $GABA_B$-receptors.

The question of whether presynaptic $GABA_B$-receptors are also present in vessels of the rat and, more importantly, whether peripherally administered GABA might influence blood pressure via $GABA_B$-receptors in the resistance vessels has not been addressed. Therefore, we examined the effects of GABA and related drugs on the neurogenic vasopressor response in the pithed rat.

Methods

Male Wistar rats weighing 170–420 g were anaesthetized with methohexitone 300 μmol kg^{-1} i.p. and then injected i.p. with atropine 2 μmol kg^{-1}. Following cannulation of the trachea, the animals were pithed and artificially respired with air (60 strokes min^{-1}). Both vagi were cut. Arterial blood pressure was measured from the right carotid artery via a Statham P 23 ID pressure transducer (Statham Instruments, Puerto Rico) and was recorded on a Hellige Servomed (Hellige, Freiburg, F.R.G.). The left jugular vein was cannulated for i.v. injections of drugs. Body temperature was kept constant via a thermostatically controlled heating table.

Subsequent to i.v. injection of (+)-tubocurarine 1.3 μmol kg^{-1}, an increase in blood pressure was induced four times (S_1, S_2, S_3, S_4) either by an i.v. injection of noradrenaline 0.3 nmol kg^{-1} or by a single electrical pulse (30 V, 1 ms; delivered from a Stimulator T; Hugo Sachs, March-Hugstetten, F.R.G.) generated between the pithing rod and an indifferent electrode placed dorsally (according to Gillespie & Muir, 1967). GABA-receptor antagonists, their vehicle (water) or saline (other experiments) were administered 5 min after injection of (+)-tubocurarine and 5 min before S_1. GABA-receptor agonists (or their vehicles) were injected i.v. in increasing doses 5 min before S_2, S_3 and S_4. An interval of 7 min elapsed between two subsequent injections of noradrenaline or electrical stimuli.

In the series described in the last paragraph of Results, 30 s periods of electrical stimulation (2 Hz, 30 V, 1 ms) instead of single electrical pulses were administered.

Calculations and statistics

Results are given as means ± s.e.mean throughout the paper (n: number of rats). In order to quantify the effects of drugs on the rise in diastolic blood pressure induced electrically or by injection of noradrenaline, the ratios S_2/S_1, S_3/S_1 and S_4/S_1 were determined. For quantification of drug-induced effects on the basal blood pressure the ratio of the basal blood pressure immediately before S_2 (t_2), S_3 (t_3) or S_4 (t_4) over that immediately before S_1 (t_1) was determined (t_2/t_1, t_3/t_1 and t_4/t_1). Ratios were expressed as percentages of the corresponding ratios obtained from animals which received the vehicle instead of the drug. For statistical comparison of the corresponding S_n/S_1 and t_n/t_1 values from drug- and vehicle-treated animals, Student's t test was used. If two or more experimental series were compared to the same control series, the t test was subjected to Bonferroni's procedure.

Drugs used

3-Aminopropane sulphonic acid (sodium salt), γ-aminobutyric acid (GABA), atropine sulphate, muscimol, (−)-noradrenaline bitartrate, (+)-tubocurarine chloride (Sigma, Munich, F.R.G.); **R**-(−)-, **S**-(+)-baclofen hydro-

[1] Dedicated to M. Göthert on the occasion of his 50th birthday

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Table 1 Effects of bicuculline and 2-hydroxysaclofen on basal diastolic blood pressure and on the electrically- and noradrenaline-induced rise in diastolic blood pressure (BP) in pithed rats

	Control	*Bicuculline* ($10\,\mu mol\,kg^{-1}$)	*2-Hydroxysaclofen* ($50\,\mu mol\,kg^{-1}$)
Basal BP (mmHg)	55.2 ± 1.5	51.3 ± 1.4	49.0 ± 1.0*
Rise in BP (mmHg) induced by exogenous noradrenaline	18.5 ± 1.4	17.8 ± 1.9	16.7 ± 1.8
Electrically-induced rise in BP (mmHg)	15.3 ± 1.1	13.1 ± 1.1	15.7 ± 1.3

Pithed rats received an i.v. injection of bicuculline, 2-hydroxysaclofen or water (vehicle for the antagonists; control). Five min later, noradrenaline $0.3\,nmol\,kg^{-1}$ was injected i.v. or a single electrical pulse of 30 V and 1 ms was administered. Basal blood pressure was determined immediately before the injection of noradrenaline or the administration of the electrical stimulus. Data shown are means ± s.e.mean of 8–18 experiments.
* $P < 0.01$ compared to the control.

chloride (CIBA-Geigy, Basle, Switzerland); (+)-bicuculline methiodide (Bioscience Products, Emmenbrücke, Switzerland or Sigma, Munich, F.R.G.; according to Simonyi *et al.* (1989), the correct chemical name of this drug is (−)-bicuculline methiodide); 2-hydroxysaclofen (Tocris Neuramin, Buckhurst Hill, U.K.); methohexitone sodium (Brevimytal Natrium; Lilly, Giessen, F.R.G.).

Stock solutions of most of the drugs were prepared in saline and diluted with saline to the concentration required. Noradrenaline was dissolved and diluted in saline containing ascorbic acid $6\,mmol\,l^{-1}$. Methohexitone, bicuculline and 2-hydroxysaclofen were dissolved in water; muscimol was dissolved in HCl $0.05\,mol\,l^{-1}$. Drugs were injected in a volume of $0.5\,ml\,kg^{-1}$, $1\,ml\,kg^{-1}$ (atropine, (+)-tubocurarine),

Table 2 Effect of R-(−)-baclofen on basal diastolic blood pressure and on the electrically- and noradrenaline-induced rise in diastolic blood pressure (BP) in pithed rats

Parameter studied	*Experimental conditions*	t_1 or S_1 (mmHg)	t_2/t_1 or S_2/S_1	t_3/t_1 or S_3/S_1	t_4/t_1 or S_4/S_1
Basal BP	Control	48.1 ± 1.3	1.04 ± 0.01	1.02 ± 0.02	1.00 ± 0.03
	R-(−)-Baclofen	49.3 ± 1.5	1.00 ± 0.01	1.02 ± 0.02	1.03 ± 0.03
Rise in BP induced by exogenous noradrenaline	Control	16.5 ± 1.5	1.15 ± 0.10	1.14 ± 0.06	1.10 ± 0.17
	R-(−)-Baclofen	15.0 ± 1.8	1.03 ± 0.06	1.01 ± 0.10	0.97 ± 0.10
Electrically-induced rise in BP	Control	15.8 ± 1.8	1.05 ± 0.05	1.08 ± 0.05	1.10 ± 0.05
	R-(−)-Baclofen	17.7 ± 2.1	0.77 ± 0.03*	0.54 ± 0.03**	0.53 ± 0.04**

Four stimuli (S_1–S_4; i.v. injections of noradrenaline $0.3\,nmol\,kg^{-1}$ or single electrical pulses of 30 V and 1 ms) were administered to pithed rats at intervals of 7 min. R-(−)-Baclofen was injected i.v. in three increasing doses 5 min before S_2 ($1\,\mu mol\,kg^{-1}$), S_3 ($10\,\mu mol\,kg^{-1}$) and S_4 ($100\,\mu mol\,kg^{-1}$), whereas saline was injected 5 min before S_1. In the control series, saline was given 5 min before S_1–S_4. The ratios of the rise in blood pressure evoked by S_2, S_3 or S_4 over that evoked by S_1 were determined. To quantify the effects of R-(−)-baclofen on basal blood pressure, the ratios of the blood pressure immediately before S_2, S_3 or S_4 (t_2, t_3 and t_4) over that before S_1 (t_1) were calculated (t_2/t_1, t_3/t_1 and t_4/t_1). Data shown are means ± s.e.mean of 5–12 experiments.
*$P < 0.005$, **$P < 0.001$ compared to the corresponding control.

Table 3 Effects of GABA receptor agonists on basal diastolic blood pressure and on the noradrenaline-induced rise in diastolic blood pressure (BP) in pithed rats

Parameter studied	*Agonist*	*Dose of agonist* ($\mu mol\,kg^{-1}$) *0.1*	*1*	*10*	*100*	*300*
Basal BP	GABA	—	—	94 ± 1*	93 ± 2	98 ± 2
	R-(−)-Baclofen	90 ± 2*	96 ± 1	100 ± 2	103 ± 3	—
	S-(+)-Baclofen	—	—	99 ± 1	107 ± 3	109 ± 2
	Muscimol	—	96 ± 1	99 ± 2	101 ± 3	—
	3-APS	—	—	97 ± 1	103 ± 2	108 ± 2
Rise in BP induced by exogenous noradrenaline	GABA	—	—	92 ± 6	95 ± 6	116 ± 9
	R-(−)-Baclofen	—	89 ± 5	89 ± 9	88 ± 9	—
	S-(+)-Baclofen	—	—	101 ± 5	112 ± 6	133 ± 8
	Muscimol	—	89 ± 5	93 ± 3	108 ± 3	—
	3-APS	—	—	92 ± 6	109 ± 9	123 ± 15

Pithed rats received four injections of noradrenaline $0.3\,nmol\,kg^{-1}$ (S_1–S_4) at intervals of 7 min. GABA receptor agonists were injected i.v. in three increasing doses 5 min before S_2, S_3 and S_4. In the control series, the vehicle was injected instead (HCl $0.05\,mol\,l^{-1}$ for muscimol; saline for the other drugs). In each series of experiments, saline was injected 5 min before S_1. The ratios of the rise in blood pressure evoked by S_2, S_3 or S_4 over that evoked by S_1 were determined. To quantify the effects of the drugs on basal blood pressure, the ratios of the blood pressure immediately before S_2, S_3 or S_4 (t_2, t_3 and t_4) over that before S_1 (t_1) were determined. Results are given as % of controls; S_n/S_1 and t_n/t_1 values are given in Table 2 (for R-(−)-baclofen and its control) or not shown. 3-APS: 3-aminopropane sulphonic acid. Data shown are means ± s.e.mean of 4–12 experiments.
*$P < 0.05$ compared to the corresponding control (not shown).

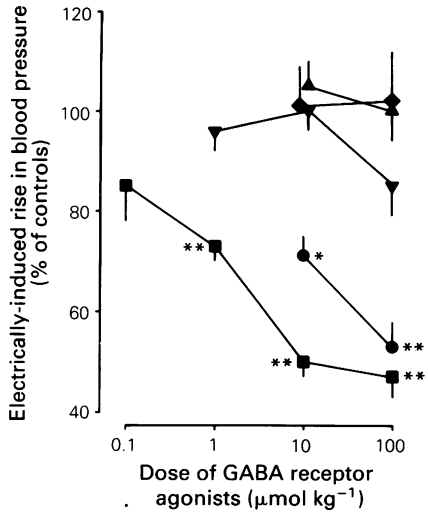

Figure 1 Effect of GABA (●), 3-aminopropane sulphonic acid (▲), muscimol (▼), R-(−)-baclofen (■) and S-(+)-baclofen (◆) on the electrically-induced rise in diastolic blood pressure. Four single electrical pulses of 30 V and 1 ms (S_1–S_4) were administered to pithed and vagotomized rats at intervals of 7 min. Saline was injected 5 min before S_1. The GABA receptor agonist under study was injected i.v. in three increasing doses 5 min before S_2, S_3 and S_4. The ratios of the rise in blood pressure evoked by S_2, S_3 or S_4 over that evoked by S_1 were determined; these ratios were expressed as percentages of the respective S_n/S_1 values in controls (vehicle injected i.v. 5 min before S_2, S_3 and S_4). The S_n/S_1 values obtained in the series with R-(−)-baclofen and in the corresponding control series (which also refers to S-(+)-baclofen, GABA and 3-aminopropane sulphonic acid) are given in Table 1. Points shown are means of 4–7 experiments and vertical lines represent s.e.mean. $*P < 0.02$, $**P < 0.005$ (compared to the corresponding controls).

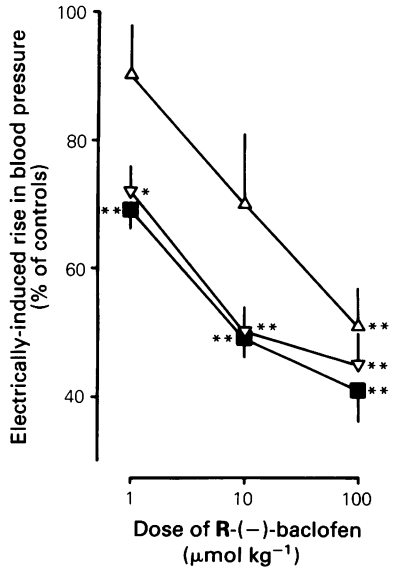

Figure 2 Effect of R-(−)-baclofen on the electrically-induced rise in diastolic blood pressure and interaction with GABA receptor antagonists. Four single electrical pulses of 30 V and 1 ms (S_1–S_4) were administered to pithed and vagotomized rats at intervals of 7 min. Bicuculline 10 μmol kg^{-1} (▽), 2-hydroxysaclofen 50 μmol kg^{-1} (△) or water (■) was injected i.v. 5 min before S_1. R-(−)-baclofen was injected i.v. in three increasing doses 5 min before S_2, S_3 and S_4. The ratios of the rise in blood pressure evoked by S_2, S_3 or S_4 over that evoked by S_1 were determined; these ratios were expressed as percentages of the respective S_n/S_1 values in controls (saline injected i.v. 5 min before S_2, S_3 and S_4). Points shown are means of 4–5 experiments and vertical lines represent s.e.mean. $*P < 0.02$, $**P < 0.005$ (compared to the corresponding controls).

2 ml kg^{-1} (bicuculline, 2-hydroxysaclofen) or 8 ml kg^{-1} (methohexitone).

Results

Basal diastolic blood pressure immediately before S_1 (first injection of noradrenaline or first electrical stimulation) was 47.7 ± 0.6 (n = 78) and 55.2 ± 1.5 (n = 18) in rats which had received saline or water 5 min beforehand, respectively. Basal blood pressure was not affected by the GABA$_A$-receptor antagonist bicuculline 10 μmol kg^{-1}, but slightly decreased by the GABA$_B$-receptor antagonist 2-hydroxysaclofen 50 μmol kg^{-1} (Table 1). GABA and GABA-receptor agonists (injected in increasing doses 5 min before S_2, S_3 and S_4) only slightly affected basal blood pressure (alteration ≤10%) or did not have any effect (Tables 2 and 3).

Noradrenaline 0.3 nmol kg^{-1} increased basal diastolic blood pressure by 7.5–27.5 mmHg. Subsequent injections administered at intervals of 7 min produced similar effects (Table 2 or not shown). The vasopressor response to noradrenaline was not affected by the GABA-receptor antagonists (Table 1) and by the GABA-receptor agonists at doses up to 100 μmol kg^{-1} (Tables 2 and 3). At 300 μmol kg^{-1}, GABA, S-(+)-baclofen and 3-aminopropane sulphonic acid tended to increase the effect of noradrenaline (Table 3).

Administration of a single electrical pulse of 30 V and 1 ms (between the pithing rod and an indifferent electrode placed dorsally) led to an increase in basal blood pressure of 10–30 mmHg in saline-treated rats. Subsequent stimuli administered at intervals of 7 min produced similar effects (Table 2 or not shown).

In the first series of experiments, the effects of GABA and GABA-receptor agonists on the electrically-induced rise in blood pressure were studied. (The effects of GABA, S-(+)-baclofen and 3-aminopropane sulphonic acid, each 300 μmol kg^{-1}, were not further considered since, at this high dose, the drugs interfered with the noradrenaline-induced rise in blood pressure (Table 3)). The electrically-induced vasopressor response was inhibited by GABA 10 and 100 μmol kg^{-1} in a dose-dependent manner (Figure 1). The GABA$_B$-receptor agonist R-(−)-baclofen even produced an inhibitory effect at a ten fold lower dose. The effect was nearly maximal at 10 μmol kg^{-1} (maximum response about 50–55%; Figure 1). Unlike its R-(−)-enantiomer, S-(+)-baclofen failed to inhibit the electrically-induced rise in blood pressure up to 100 μmol kg^{-1}; the same held true for the GABA$_A$-receptor agonist 3-aminopropane sulphonic acid (Figure 1). Another GABA$_A$-receptor agonist, muscimol, had no effect at 1 and 10 μmol kg^{-1} and tended to inhibit the electrically-induced rise in blood pressure at 100 μmol kg^{-1} (Figure 1).

In the second series of experiments, the interaction of R-(−)-baclofen with the GABA-receptor antagonists bicuculline and 2-hydroxysaclofen was studied. The dose-response curve of R-(−)-baclofen for its inhibitory effect on the electrically-induced vasopressor response was not affected by the GABA$_A$-receptor antagonist bicuculline 10 μmol kg^{-1}, but shifted to the right by the GABA$_B$-receptor antagonist 2-hydroxysaclofen 50 μmol kg^{-1} (by a factor of 10; Figure 2). Bicuculline and 2-hydroxysaclofen by themselves did not affect the electrically-induced rise in blood pressure (Table 1).

In the final series of experiments, the effect of R-(−)-baclofen 100 μmol kg^{-1} on the rise in blood pressure induced by a 30 s period of electrical stimulation (2 Hz, 30 V, 1 ms) was examined. The S_2/S_1 values in animals which received R-(−)-baclofen or saline were 0.83 ± 0.01 and 1.08 ± 0.03, respectively (n = 4 each; $P < 0.001$).

Discussion

It was the aim of the present study to examine the effects of GABA and related drugs (for review, see Bowery, 1989) on

the sympathetic nerves supplying the resistance vessels in the pithed rat. Both the effects of the drugs on the electrically-induced vasopressor response and their interaction with exogenously added noradrenaline were studied. The vasopressor response induced by a single electrical pulse appears to be solely due to release of catecholamines, since it was almost fully suppressed by prior administration of adrenoceptor antagonists at doses abolishing the vasopressor response to exogenously added noradrenaline without affecting that to ATP (unpublished results; under different schedules of electrical stimulation, ATP does contribute to the electrically-induced vasopressor response; Bulloch & McGrath, 1988; Schlicker *et al.*, 1989). The lack of effect of GABA and related drugs (at doses up to 100 μmol kg^{-1}) on the vasopressor response induced by exogenous noradrenaline allows us to use the electrically-induced end organ response as a parameter for the electrically-evoked transmitter release from sympathetic nerves.

The missing or very slight effects of the GABA-related drugs on basal blood pressure exclude the possibility that differences between the drugs with respect to their effects on the electrically-induced vasopressor response are related to differences in their effects on basal blood pressure (De Jonge *et al.*, 1983). The missing or very slight effect of **R**-(−)-baclofen on basal blood pressure, observed in the present model, is not discordant with the more marked hypotensive effect of low doses of racemic baclofen obtained in the anaesthetized rat (Chahl & Walker, 1980), since pithing abolishes the centrally driven sympathetic tone involved in the action of this drug (DeFeudis, 1983; Antonaccio, 1984; Bousquet *et al.*, 1985).

GABA decreased the electrically-induced vasopressor response, and its effect was mimicked by the $GABA_B$-receptor agonist **R**-(−)-baclofen, which was even more potent in this respect than GABA itself, whereas its **S**-(+)-enantiomer was inactive. The electrically-induced vasopressor response was not (or hardly) affected by the $GABA_A$-receptor agonists aminopropane sulphonic acid and muscimol. These results suggest the involvement of $GABA_B$-receptors in the depression of the electrically-induced rise in blood pressure. The rank order of potencies for the agonists **R**-(−)-baclofen, GABA and muscimol in the pithed rat is identical to that found under *in vitro* conditions in our previous study (in which $GABA_B$-receptors modulating 5-hydroxytryptamine release from rat brain cortex slices were examined; Schlicker *et al.*, 1984). This is most remarkable since, in the pithed rat, differences between the drugs might be influenced by differences in their pharmacokinetic properties.

Further support for the involvement of $GABA_B$-receptors in the effect of GABA comes from the results obtained with the antagonists; thus, the dose-response curve for **R**-(−)-baclofen was not affected by the $GABA_A$-receptor antagonist bicuculline but shifted to the right by 2-hydroxysaclofen, a recently characterized $GABA_B$ receptor antagonist (Curtis *et al.*, 1988; Kerr *et al.*, 1988), which is relatively potent and selective (no effect on, e.g., α-adrenoceptors, $GABA_A$ or muscarinic receptors; Kerr *et al.*, 1988; present study).

An inhibitory effect of **R**-(−)-baclofen on the neurogenic vasopressor response was also obtained when a 30 s period of electrical stimulation was administered at a frequency of 2 Hz. In this case, the extent of inhibition was lower than under the standard condition (single pulse). The difference in the degree of inhibition was not unexpected, since receptor-mediated modulation of transmitter release is highly dependent on the conditions used for stimulation (Starke, 1977). The differential inhibitory effect of **R**-(−)-baclofen is compatible with the view that the $GABA_B$-receptors considered here are located presynaptically on the postganglionic sympathetic nerve fibres, although a presynaptic location on the preganglionic sympathetic nerve fibres is conceivable as well.

The finding that the inhibitory effect of **R**-(−)-baclofen is detectable at 2 Hz (i.e. at a frequency normally occurring in the autonomic nerves) provides evidence that the $GABA_B$-receptors might be activated under physiological conditions. Although GABAergic neurones have so far not been detected in the vascular wall (unlike in the myenteric plexus of the guinea-pig, Jessen *et al.*, 1983), glutamic acid decarboxylase, the enzyme that synthesizes GABA, has been shown to occur in various blood vessels (Krause, 1986).

Even if the $GABA_B$-receptors under investigation should not have a physiological role, they might be of clinical relevance, provided they are also detectable in human resistance vessels. Firstly, the decrease in blood pressure which sometimes occurs as a side-effect on treatment with baclofen might be related to the activation of presynaptic $GABA_B$-receptors in the resistance vessels, although there is much evidence that central $GABA_B$-receptors are involved in this side-effect (Sill *et al.*, 1986; see also above). Secondly, the characterized $GABA_B$-receptors might become the target for future antihypertensive drugs. For further investigation of both points, it would be interesting to examine the blood pressure effects of $GABA_B$-receptor agonists which, unlike baclofen, do not cross the blood-brain barrier.

In conclusion, the present study shows that $GABA_B$-receptors are also detectable in vessels of the rat and, in particular, that they occur in resistance vessels. These $GABA_B$-receptors, which most probably are located presynaptically on the postganglionic sympathetic nerve fibres, might be involved in the modulation of blood pressure and might become another target for antihypertensive drugs.

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Antagonism by nifedipine of contraction and Ca^{2+}-influx evoked by ATP in guinea-pig urinary bladder

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1 The effects of Ca^{2+}-antagonists, especially nifedipine, on contraction and increase of intracellular Ca^{2+} (Fura-2/AM method) evoked by ATP were evaluated in a thin outer layer segment of guinea-pig urinary bladder.

2 The ATP-evoked contraction was markedly inhibited by dihydropyridine-type Ca^{2+}-antagonists, such as nifedipine and nitrendipine, but not by D-600, ω-conotoxin and tetramethrin.

3 This antagonism by nifedipine of ATP-evoked contractions was competitive from the Schild plot analysis, the pA_2 value being 8.23. The reduction of ATP-evoked contraction by nifedipine (0.1 μM) was fully reversed by administration of Bay K 8644 (0.1 μM).

4 ATP (100 μM) caused an increase of fluorescence brightness after loading Fura-2/AM, which was coupled with a contraction of the bladder. Both the contraction and the elevation of intracellular Ca^{2+} evoked by the nucleotide were completely antagonized by nifedipine.

5 These results suggest that ATP may activate the dihydropyridine-sensitive, voltage-dependent Ca^{2+}-channels in a direct or indirect fashion and, thereby, elicit a contraction of the bladder.

Introduction

It has been shown that adenosine 5'-triphosphate (ATP) coexists with noradrenaline (Lagercrantz & Stjarne, 1974) or acetylcholine (ACh) (Dowdall *et al.*, 1974) in synaptic vesicles in peripheral autonomic nerves and may function as a neurotransmitter (Katsuragi & Furukawa, 1985; Burnstock, 1986; Lew & White, 1987; Katsuragi *et al.*, 1988). This nucleotide is capable of producing a contractile response of a variety of smooth muscles including the guinea-pig vas deferens (Sneddon & Burnstock, 1984; Sneddon & Westfall, 1984) and guinea-pig urinary bladder (Burnstock *et al.*, 1972; Kasakov & Burnstock, 1983; Westfall *et al.*, 1983; Katsuragi *et al.*, 1986) and blood vessels of the rabbit and dog (Von Kugelgen & Starke, 1985; Muramatsu, 1986). In addition, depolarization by ATP of the cell membranes from guinea-pig vas deferens (Wakui & Inomata, 1985), rat vas deferens (Nakazawa & Matsuki, 1987) and rat basilar artery (Byrne & Large, 1986) have been observed with a double sucrose gap method. Furthermore, miniature endplate potentials (m.e.p.ps) and excitatory junction potentials (e.j.ps) in guinea-pig vas deferens seem to be mediated not by noradrenaline but by ATP, because these m.e.p.ps and e.j.ps were largely blocked by α,β-methylene ATP, a desensitizer of P_{2x}-purinoceptors, and were not affected by prazosin (Sneddon & Burnstock, 1984; Stjärne & Åstrand, 1984).

To date there is no specific antagonist for postsynaptic P_{2x}-receptors (ATP-sensitive receptors). Further characterization of ATP as a neurotransmitter awaits the development of specific P_{2x}-purinoceptor antagonists. Early studies with rat vas deferens suggest that the nature of the contractile response to ATP, but not to noradrenaline, is similar to that of electrical stimulation in that it is antagonized by nifedipine, a Ca^{2+}-channel blocker (Stone, 1981). Blakeley and his colleagues (1981) showed that the electrically-evoked initial phase of the mechanical response in guinea-pig vas deferens was abolished by nifedipine, but the electrically-evoked late phase of the mechanical response was selectively inhibited by prazosin, an α_1-adrenoceptor antagonist, but not by nifedipine.

Therefore, in the present study we attempted to clarify the nature of the antagonism by Ca^{2+}-antagonists (especially nifedipine) of the ATP-evoked contraction and intracellular Ca^{2+} elevation in the guinea-pig urinary bladder.

[1] Author for corresondence.

Methods

Measurement of tension development

Male guinea-pigs weighing 250–350 g were stunned and bled. A muscle segment (about 1 mm in width and 1 mm in length) of the thin outer layer of the urinary bladder was dissected longitudinally as described previously (Usune *et al.*, 1986) and suspended in a bath (about 0.1 ml). The segment was superfused at 1.8 ml min^{-1} via a peristaltic pump (Gilson, HP-1) with a modified Krebs solution (pH 7.4) bubbled with 97% O_2 and 3% CO_2. The composition of the modified Krebs solution was as follows (mM): NaCl 121.4, KCl 5.9, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 15.5 and glucose 11.5. The solution was kept at 32°C to inhibit spontaneous contraction. Contractions were recorded isometrically with a strain-gauge transducer (Toyo-Baldwin, T7-8-240). After each preparation had been loaded with 100 mg wt it was allowed to equilibrate for 60 min before the start of the experiment. When the contractions evoked by administration of ATP at 20 min intervals became constant (usually three administrations were needed), test Ca^{2+} antagonists, which were dissolved in either 0.1% ethanol or 0.1% dimethylsulphoxide, were introduced to the superfusate.

Measurement of fluorescence intensity

A thin outer layer muscle segment (about 2 mm width and 7 mm length) of the urinary bladder was dissected and suspended in a bath (about 0.7 ml), and loaded with Krebs solution containing 5 μM Fura-2/AM with 0.01% pluronic F-127 (a non-cytotoxic detergent, gift from Dojin, Japan) for 180 min at room temperature (23–25°C). After being loaded, the strip was superfused at 3.6 ml min^{-1} with the modified Krebs solution. To minimize movement, the segment was stretched and fixed at 200 μm intervals on a silicon board by fine insect pins, but one end of the tissue (about 1 mm length) remained free for the measurement of force development. Contractions of the tissue were recorded isometrically with a strain-gauge

transducer (HBM, WO.5T). The fluorescence signals and contractions were measured simultaneously in the same preparation.

As described previously (Usune *et al.*, 1989), fluorescence signals were measured by a single beam fluorometer (Farrand, Ratio fluorometer-2) which was equipped with a mercury arc lamp of 85 W. After exposure to an excitation wavelength of either 340 or 380 nm, fluorescence signals from the tissue were measured at an emission wavelength of 511 nm, by a photomultiplier tube (Hamamatsu photonics, R1527 SEL), and the output voltage was further amplified (16 Hz cut-off frequency). The excitation light was passed through a slit (about 1 mm width and 3 mm length) before reaching the tissue. It is well documented (Himpens & Somlyo, 1988) that during exposure to 340 nm light, stimulation of the tissue produces an increased fluorescence (511 nm) from the strip which is proportional to the concentration of intracellular free Ca^{2+} combined with Fura-2. In contrast, a decrease in fluorescence after excitation at 380 nm, reflects a reduction of intracellular free Fura-2 concentration following formation of a Fura-2-Ca^{2+} complex. Hence, after it had been confirmed that the fluorescence from a strip excited at a wavelength of 380 nm was decreased by ATP (300 μM), the present fluorescence measurement was carried out with a single beam excitation light of 340 nm (not by the fluorescence ratio method, Usune *et al.*, 1989). Addition of ATP or nifedipine in the absence of Fura-2 caused no autofluorescence with excitation and emission wavelengths at 340 and 511 nm, respectively (data not shown).

Drugs

The drugs used here were: ATP benzeneacetonitrile hydrochloride (Boeringer Mannheim), nifedipine, nitrendipine (Sigma), D-600 (benzene acetonitrile hydrochloride; gift from Knoll), ω-conotoxin (Peptide Inst.), tetramethrin (gift from Sumitomo Chem.), Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate; gift from Bayer), tetrodotoxin (Sankyo) and Fura-2/AM (Dojin).

Statistical analysis

Values are expressed as mean ± s.e. and n indicates the number of experiments. The statistical significance was assessed by paired t test for values from the fluorescence experiment and by Dunnett's t test for multiple comparisons between several values obtained with different concentrations of an antagonist and a control value. Differences were considered to be significant when $P < 0.05$.

Results

Effects of Ca^{2+}-antagonists on the ATP-evoked contraction

ATP administered at 20 min intervals, produced phasic contractions of guinea-pig urinary bladder in a concentration-dependent manner, in the range of 1.0 to 1000 μM (Figure 1). The ATP-evoked contraction (100 μM) was virtually unaffected by exposure to tetrodotoxin (0.3 μM) for 40 min (data not shown). However, this ATP-evoked contraction was completely abolished by exposure to Ca^{2+}-free medium containing 10 μM EGTA for 20 min (data not shown). In the presence of Mn^{2+} at concentrations of 100 and 300 μM, the ATP-evoked contractions were reduced to 83.2 ± 8.3% ($n = 8$) and 56.9 ± 5.2% ($n = 6$, $P < 0.05$), respectively. The effects of various organic Ca^{2+}-channel blockers in concentrations ranging from 0.1 to 1 μM on ATP (100 μM)-evoked contraction (100%) are expressed as alterations of % contraction. The inhibition by nifedipine (0.3 μM) was most potent. Nitrendipine exhibited a moderate blockade of this ATP-evoked contraction. However, the Ca^{2+}-antagonists ω-conotoxin, D-600 and tetramethrin did not inhibit the ATP-evoked contraction even at a concentration of 1 μM (Figure 2).

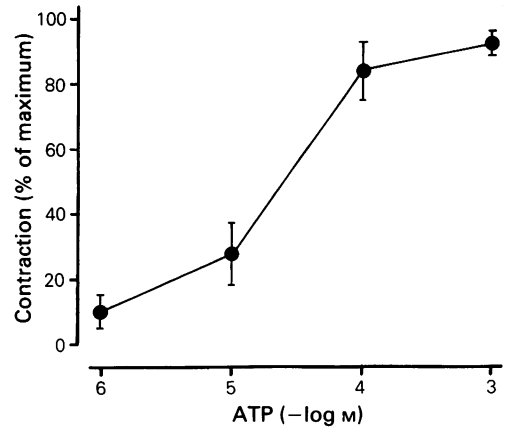

Figure 1 The concentration-response curve for the contraction induced by ATP in guinea-pig urinary bladder. A series of ATP concentrations was administered at 20 min intervals to the perfusate. Contractions were expressed as % of the maximum response in each preparation. Each point indicates mean and vertical lines show s.e. ($n = 4$). The maximum ATP (1000 μM)-induced contraction was 2.07 ± 0.04 mN.

Antagonism by nifedipine of the ATP-evoked contraction

Concentration-response curves for ATP shifted to the right in the presence of nifedipine (0.03 to 0.3 μM) (data not shown). In order to analyse the mode of inhibition by nifedipine from the data, Schild plots were made by plotting the log

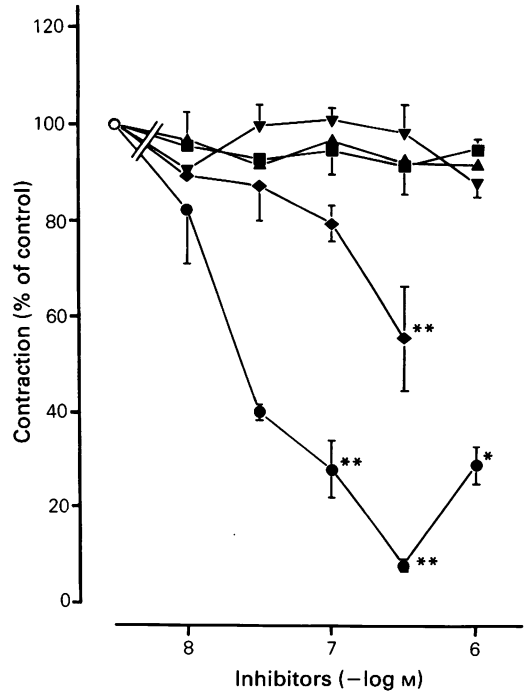

Figure 2 Effects of organic Ca^{2+}-antagonists on ATP-evoked contraction. Contractions are expressed as a percentage of the control contraction induced by ATP (100 μM) without antagonists. (○) Control; (■) ω-conotoxin; (▲) D-600; (▼) tetramethrin; (◆) nitrendipine; (●) nifedipine. After the contractions evoked by repeated administrations of ATP at 20 min intervals had attained a constant amplitude, various concentrations of the respective Ca^{2+}-antagonist were introduced to the perfusate and their effects on the ATP-evoked contractions were examined in the same preparation. Each point indicates the mean and vertical lines show s.e. ($n = 4$ to 10). * $P < 0.05$; ** $P < 0.01$ from the control (Dunnett's t test).

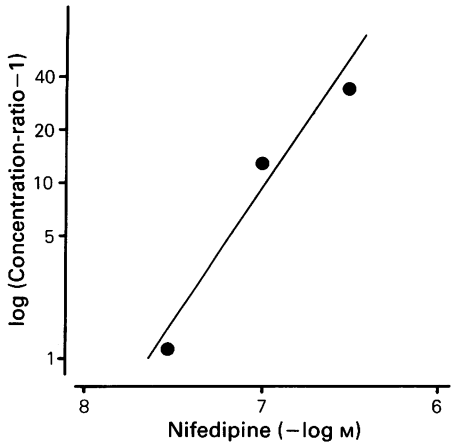

Figure 3 Schild plot of antagonism by nifedipine of ATP-evoked contraction. Concentration-ratio of ATP denotes the ratio of the 50% responses elicited by the agonist in the presence and absence of various concentrations of nifedipine. Each point indicates the mean value from ten experiments.

(concentration-ratio − 1) of ATP against −log molar concentration of nifedipine (Figure 3). The apparent pA_2 value of the Ca^{2+} antagonist for ATP obtained from this plot was 8.23 and the slope 0.977 (correlation coefficient, $r = 100$), suggesting a competitive antagonism.

In the presence of nifedipine (0.3 μM), the ATP (100 μM)-evoked contraction was almost abolished. However, after subsequent introduction of Bay K 8644 (0.3 μM), the contraction of the bladder evoked by ATP reappeared. A recording from a single experiment is shown in Figure 4.

Effect of nifedipine on the ATP-evoked contraction and the fluorescence signals

The thin outer layer preparation of guinea-pig urinary bladder previously exposed to Krebs solution containing 5 μM Fura-2/AM for 180 min was used for this experiment. ATP (100 μM) produced a contraction coupled with an increased fluorescence (340 nm/511 nm), indicating an increased intracellular Ca^{2+}-concentration. Typical recordings of the contractions and the fluorescence signals (340 or 380 nm/511 nm) evoked by ATP administered at 20 min intervals in the presence or absence of nifedipine are shown in Figure 5. When the fluorescence intensities at 340 nm (excitation) and 511 nm (emission) caused by the first application of ATP to the tissue after loading with Fura-2/AM were expressed as 100%, the intensities produced by the second application of the nucleotide after 20 min with and without nifedipine (0.1 μM) amounted to 28.2 ± 3.4% ($n = 4$) and 80.0 ± 7.7% ($n = 4$), respectively. The difference of the values between the second applications was statistically significant ($P < 0.05$ by paired t test).

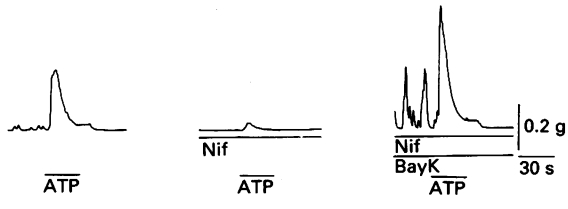

Figure 4 Effects of Bay K 8644 on the antagonism by nifedipine of the ATP-evoked contraction. Nifedipine (Nif, 0.3 μM) alone and in combination with Bay K 8644 (Bay K, 0.3 μM) was added to the perfusate 20 min before administration of ATP (100 μM) in the same preparation. This single experiment was chosen from four experiments showing similar results.

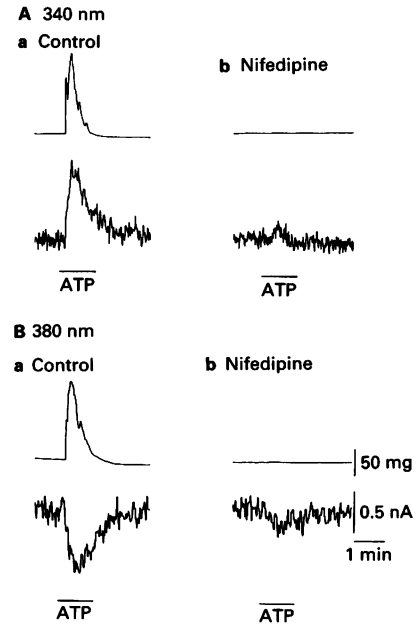

Figure 5 Typical traces of contractions and intracellular Ca^{2+} elevation evoked by ATP (100 μM) in the presence or absence of nifedipine (0.1 μM). (A) 340 nm (excitation); 511 nm (emission). (B) 380 nm (excitation); 511 nm (emission). (A) and (B) were taken from the same preparation. Upper traces; contractions. Lower traces; fluorescence signals. Repeated administrations of ATP were made at 20 min intervals to the perfusate. Nifedipine was introduced to the perfusate 20 min before ATP.

Discussion

When electrical stimulation is applied to the nerves of guinea-pig or rat urinary bladder, an atropine-resistant twitch contraction occurs (Dahlen & Hedqvist, 1980). ATP administered exogenously to this tissue also evokes a transient contraction. α,β-Methylene ATP, a desensitizer of P_{2x}-purinoceptors, is able to inhibit both the ATP-evoked contraction and the atropine-resistant component of the nerve-evoked contraction (Kasakov & Burnstock, 1983; Katsuragi *et al.*, 1986). From such facts, Burnstock and his coworkers (1978) have postulated that the non-cholinergic innervation of this tissue may be 'purinergic'. Recent double sucrose gap studies provided further evidence that electrically evoked e.j.ps in guinea-pig, rabbit or pig urinary bladder were abolished by desensitization of P_{2x}-purinoceptors with α,β-methylene ATP, but not by atropine, (Fujii, 1988). In the present work, we verified that contraction of the guinea-pig bladder could be evoked by ATP in a concentration-dependent fashion. This ATP-evoked contraction was inhibited by L-type Ca^{2+}-channel blockers such as nitrendipine and nifedipine, the latter being a more potent and competitive antagonist for the ATP evoked-contraction. On the other hand, ω-conotoxin, (L- and N-type Ca^{2+}-channel blocker, McCleskey *et al.*, 1987), tetramethrin, (T-type Ca^{2+}-channel blocker, Hagiwara *et al.*, 1988), and D-600 (L-type blocker) did not display such antagonism.

According to Triggle *et al.* (1979), the potencies of nifedipine for inhibition of muscarinic agonist- and KCl-evoked phasic contractions in guinea-pig ileum were 50 and 4 times, respectively, more than those of D-600. Thus, our finding that nifedipine was about 10 times more potent than D-600, suggests that the comparative potencies of inhibition vary with the different stimulants used to evoke a given response.

In an earlier study with rat vas deferens, French & Scott (1981) first presented the possibility that nifedipine blocked selectively the electrically-evoked non-adrenergic contraction from the prostatic end of the rat vas deferens without affecting the adrenergic contraction. From a series of studies on this nucleotide, it has been proposed that this non-adrenergic con-

traction is mediated by ATP. In the guinea-pig vas deferens, the initial and late phases of the mechanical response to nerve stimulation were selectively inhibited in the presence of nifedipine and prazosin, respectively (Blakeley *et al.*, 1981). The study by Stone (1981) showed that contractions of guinea-pig vas deferens evoked by ATP and nerve stimulation were inhibited with a similar time-course after addition of nifedipine (5 μM). It has also been found that nifedipine blocks the purinergic rather than the adrenergic nerve-mediated vasopressor responses in the pithed rat (Bulloch & McGrath, 1988). In the guinea-pig urinary bladder, we found that the ATP-evoked contraction was competitively antagonized by nifedipine with a pA_2 value of 8.23 from the Schild plot analysis. In addition, the inhibition of the ATP-evoked contraction by nifedipine was reversed by administration of Bay K 8644, a Ca^{2+}-agonist. As well as causing this contraction, ATP simultaneously produced an increased fluorescence intensity of the tissue due to a rise in the intracellular Fura-2-Ca^{2+} complex. This evoked fluorescence brightness was also antagonized by nifedipine (0.3 μM).

It has been shown that exogenously applied ATP depolarizes the vas deferens of the rat (Nakazawa & Matsuki, 1987) or guinea-pig (Wakui & Inomata, 1985) and single cells (Benham *et al.*, 1987), as well as intact preparations (Miyahara & Suzuki, 1987), from the rabbit ear artery. Patch-clamp studies have demonstrated ATP-sensitive K^+-channels on guinea-pig or rabbit cardiac cells (Noma, 1983). Similarly, in guinea-pig myenteric neurones. ATP seems to induce a membrane hyperpolarization in S-neurones and depolarization in AH-neurones which are due to activation and inactivation of a Ca^{2+}-sensitive K^+-conductance, respectively (Katayama & Morita, 1989). Accordingly, the membrane depolarization elicited by ATP might result from a decrease of K^+-outward current. On the other hand, ATP seems to open several types of channel directly. Benham & Tsien (1987) have shown that a receptor gated non-selective cation channel of smooth muscle cells from rabbit ear artery can be activated by ATP and can mediate inward Ca^{2+} current. In single vascular smooth muscle cells isolated from guinea-pig mesenteric artery, intracellularly applied ATP activated the slow Ca^{2+}-channels, but not the fast Ca^{2+}-channels, in a concentration-related manner (Ohya & Sperelakis, 1989). A fluorescence study with Indo-I indicated that ATP stimulates transsarcolemmal influx of Ca^{2+} in rat cardiac myocytes (Danziger *et al.*, 1988).

From these findings, the following possible mechanism may account for the nifedipine-sensitive fluorescence brightness developed by ATP. ATP could first depolarize the cell membrane of guinea-pig urinary bladder via the opening of a non-selective cation channel or by inactivating Ca^{2+}-sensitive potassium conductance. Subsequently, the voltage-gated Ca^{2+} channels must be opened by this membrane depolarization and these Ca^{2+}-channels are primarily nifedipine-sensitive. There is also the possibility that an increase in Ca^{2+}-influx by ATP is brought about directly by the alteration of an active site on the molecule of the voltage-gated Ca^{2+}-channels (Ohya & Sperelakis, 1989), or through activation of a G-protein as shown with adenosine, a breakdown metabolite of ATP (Kurachi *et al.*, 1986). Further patch-clamp studies are needed to define the precise mode of action of ATP involved in the facilitation of the Ca^{2+}-influx.

However, the lack of sensitivity of the response to D-600, which is thought to have a similar effect on L-type Ca^{2+} channels to nifedipine, leaves open the possibility that nifedipine may be a competitive antagonist for the P_{2x}-purinoceptors. This question remains to be solved.

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Glibenclamide is a competitive antagonist of the thromboxane A_2 receptor in dog coronary artery *in vitro*

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1 Glibenclamide, a sulphonylurea oral hypoglycaemic agent is a widely used antagonist of cromakalim-activated K^+ channels in smooth muscle.

2 In isolated ring segments of the large circumflex coronary artery from the dog, glibenclamide (1–30 μM) caused a concentration-dependent reduction in both spontaneous isometric force and contractions induced by U46619, a thromboxane A_2-mimetic.

3 Glibenclamide behaved as a competitive antagonist of U46619 with an estimated pK_B ($-\log K_B$) value of 6.2 by Schild regression analysis (slope 1.07).

4 Glibenclamide (30 μM) was apparently selective since it had no effect on the concentration-contraction curves to endothelin-1, noradrenaline or KCl.

5 We suggest that this additional property of glibenclamide should be considered in any smooth muscle study where active force is raised by either the exogenous application or endogenous generation of thromboxane A_2.

Introduction

The oral hypoglycaemic drug, glibenclamide, stimulates release of insulin from pancreatic acinar cells, probably by blocking an ATP-sensitive K^+ channel located in the plasma membrane (Schmid-Antomarchi *et al.*, 1987). It is also one of the most potent inhibitors of the cromakalim-activated K^+ channel in smooth muscle (Quast & Cook, 1988; Buckingham *et al.*, 1989; Cavero *et al.*, 1989; Eltze 1989a,b; Winquist *et al.*, 1989). Here we show that glibenclamide is also a competitive antagonist of the stable thromboxane A_2-mimetic, U46619, in dog isolated coronary artery. U46619 is a constrictor agent commonly used to induce active force in isolated blood vessels when studying vasorelaxation responses. Our results show that this competitive interaction of glibenclamide with U46619 is not shared with other constrictors such as endothelin-1, noradrenaline and KCl. This additional property of glibenclamide at the thromboxane A_2 receptor should be considered in any studies relating to smooth muscle reactivity where exogenous or endogenous thromboxane A_2-like compounds are present.

Methods

Coronary artery assay

Greyhound dogs (20–30 kg) of either sex were killed by an overdose of sodium pentobarbitone (80 mg kg^{-1}, i.v.). The heart was then removed and the circumflex coronary artery dissected from adhering connective tissue and fat. Ring segments of artery 3 mm long were mounted on stainless steel wires in 25 ml jacketed organ baths containing a modified Krebs solution of the following composition (mM): Na^+ 144, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 128.7, HCO_3^- 25, SO_4^{2-} 1.2, $H_2PO_4^-$ 1.2 and glucose 11, aerated with a gas mixture of 95% O_2 and 5% CO_2 and then allowed to equilibrate at 37°C for 30 min. Endothelium was removed from some rings by abrading the luminal surface with a tapered filter paper (Whatman No. 1, see Cocks & Angus, 1983). To examine direct relaxation effects of glibenclamide, some rings were stretched initially to 4 g and adjusted if necessary to 4 g, 30 min later (see Cocks & Angus, 1983). These rings were then allowed to equilibrate for 30 min before an EC_{80} concentration of U46619 (30 nM) or vehicle was added. Glibenclamide (3–30 μM) was then added when the spontaneous or U46619-induced contractions reached a plateau. For construction of cumulative concentration-contraction curves, all rings were stretched to an optimised resting circumference which corresponded to an equivalent transmural pressure of 100 mmHg. This normalisation procedure for large arteries has been published previously (see Angus *et al.*, 1986). After a further 60 min equilibration period, either glibenclamide or its vehicle (methanol) was added. A further 40 min was then allowed before cumulative concentration-contraction curves to U46619, noradrenaline, endothelin-1 or KCl were obtained. The noradrenaline curves were obtained in the presence of propranolol (1 μM) in endothelium-denuded rings. Only one concentration-contraction curve was obtained in any one ring.

Statistical methods

All contraction responses were normalised as percentages of the maximal contraction (F_{max}) to 75 mM KCl, which was added in the presence of the final concentration of each agonist. Each normalised contraction curve was then computer fitted with a logistic equation which gave estimates of the concentrations of the agent necessary to give EC_{10-90} of its maximum response. Full details have been published elsewhere (see Nakashima *et al.*, 1982; Angus *et al.*, 1986). The fitted EC_{50} value (expressed as the negative logarithm of the molar concentration) from each curve to U46619 was used to calculate the concentration ratio (CR) at the different concentrations of glibenclamide on any one day. Linear regression of the log (CR − 1) against glibenclamide concentration (log B), the 95% confidence limits for the line, the slope (b) and the x intercept (pK_B) were then determined (Snedecor & Cochran, 1967) from this Schild analysis (Arunlakshana & Schild, 1959). Unpaired *t* tests were used to test for statistical significance for data between rings and significance was accepted at the $P < 0.05$ level.

Preliminary experiments showed that equilibration of the arteries with glibenclamide before construction of the cumulative concentration-response curve to U46619 often caused a fall in resting force. This may have been due to antagonism of endogenous thromboxane A_2. If this was indeed correct, the control (zero glibenclamide) U46619 curve would be lower in sensitivity and the estimates of dose-ratios for the Schild analysis would also be low. A similar phenomenon has been discussed and analysed in detail regarding basal endogenous

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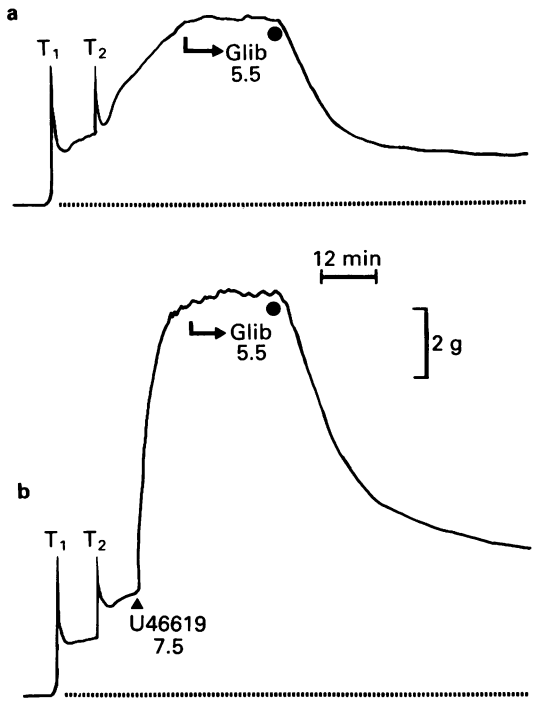

Figure 1 Original chart recordings showing the effect of glibenclamide (Glib) on spontaneously developed force (a) and U46619-induced force (b) in the greyhound circumflex coronary artery. Drug concentrations are given as $-\log$ M. T_1 and T_2 represent stretches of the tissues to 4 g force. Before the arrow the time calibration bar represents 40 min.

histamine release and acid secretion in the mouse stomach (Angus *et al.*, 1980). We therefore used a second analytical technique that does not rely on dose-ratios to estimate the pK_B value. These techniques and their advantages have been published elsewhere (Stone & Angus, 1978; Angus *et al.*, 1980). The entire data set of 313 data points was fitted with a reciprocal logistic model utilising the weighting facility of GLIM3 (General Linear Interactive Modelling; Stone & Angus, 1978). Concentration-response lines were fitted with a common slope and individual slopes to test for parallelism before applying the interactive, non-linear least-square curve

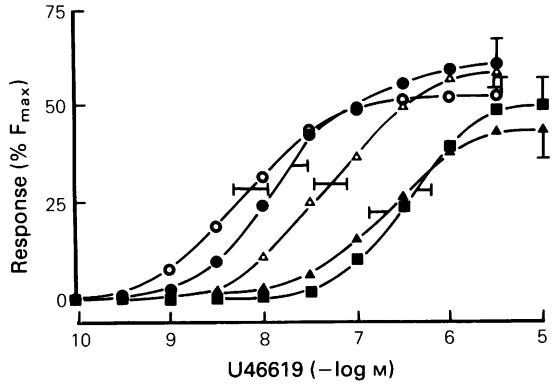

Figure 2 Cumulative concentration-contraction curves for U46619 in the greyhound circumflex coronary artery in the absence (○) and presence of 1 (●), 3 (△), 10 (▲) and 30 (■) μM glibenclamide. Values represent means of 6–7 experiments expressed as the percentages of F_{max} for each tissue. The vertical bars are 1 s.e.mean of the maximal response. The horizontal bars represent 1 s.e.mean of the EC_{50} ($-\log$ M) values at the point of intersection of each curve as determined from logistic curve fitting (see Methods). The EC_{50} values for U46619 were, respectively, 8.13 ± 0.18 ($n = 7$), 7.71 ± 0.18 ($n = 7$), 7.28 ± 0.18 ($n = 6$), 6.70 ± 0.17 ($n = 6$) and 6.35 ± 0.15 ($n = 6$) for 0, 1, 3, 10 and 30 μM glibenclamide. The maximum responses to U46619 (% F_{max}) for each concentration of glibenclamide were not significantly different from the control value, which was 51.8 ± 5.8%.

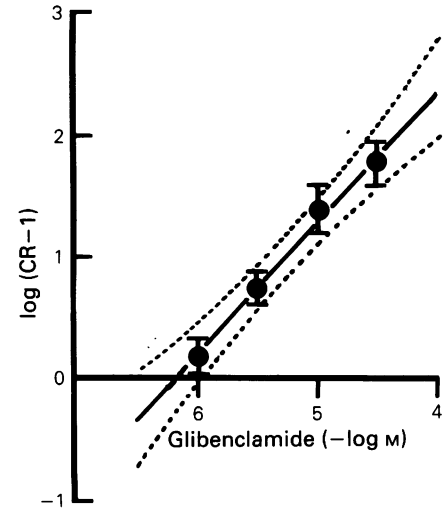

Figure 3 Graphical display of the antagonism of U46619 by glibenclamide according to Schild regression analysis. The solid line is the regression of the individual values from 6–7 experiments at each concentration of glibenclamide. The dotted lines represent the 95% confidence limits of the linear regression. The symbols represent the mean with 1 s.e.mean (vertical bars) values at each concentration of glibenclamide. Ordinate scale: concentration-ratio -1 (log scale). Slope = 1.07; $pK_B = 6.18$.

fitting approach of Waud (1975) to estimate the antagonist dissociation constant (K_B) and the parameter, n, equivalent to the slope in the Schild plot as a test of the competitive model. Finally, we used the 'Clark' plot (Stone & Angus, 1978; Stone, 1980) as a graphical display of how well the rightward displacement of the concentration-response curves conformed to the model of simple competitive antagonism.

Drugs

Drugs used and their sources were: U46619 (1,5,5-hydroxy-11,9-(epoxymethano)prosta-5Z,13E-dienoic acid, Upjohn: Kalamazoo, U.S.A.), endothelin-1 (Auspep: Melbourne, Australia), (−)-noradrenaline bitartrate, indomethacin, 5-hydroxytryptamine creatinine sulphate, acetylcholine bromide, (±)-propranolol hydrochloride (all from Sigma, U.S.A.); glibenclamide (Daonil) and tolbutamide (Hoechst: Australia). All drugs were freshly prepared, and except for indomethacin and

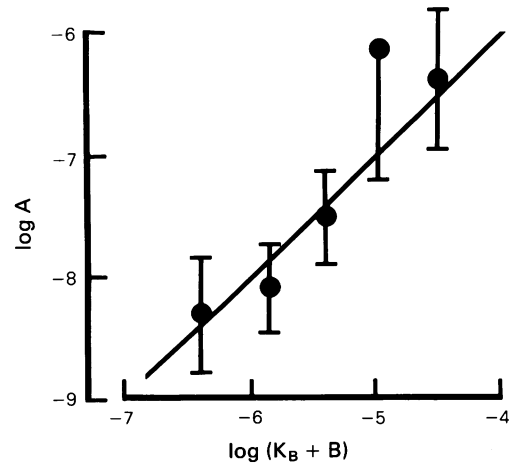

Figure 4 Graphical display ('Clark' plot) of the relationship between the level of agonist (A) (U46619, log M) and the level of antagonist (B + K_B) (glibenclamide, log M). Points shown display the degree of displacement of the mean concentration-response curves from the model of simple competitivity indicated by the solid line. The error bars are the 95% confidence limits. Note that the K_B was estimated by an interactive, non-linear least squares fitting approach using the entire data set (see Methods).

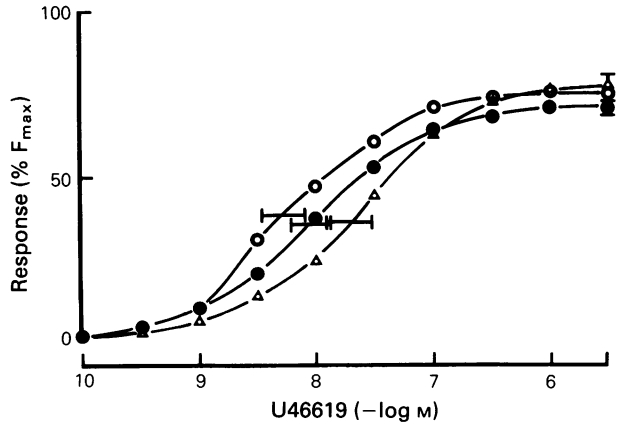

Figure 5 Concentration-contraction curves for U46619 in the absence (○) and presence of 3 (●) and 30 (△) μM tolbutamide. Data are expressed as described in Figure 2. The control EC_{50} (−log M) and maximum response (% F_{max}) were, respectively, 8.27 ± 0.18 and 74.5 ± 3.3 (n = 5). The EC_{50} and maximum response values for 3 and 30 μM tolbutamide were not significantly different from the control values.

glibenclamide, were dissolved in distilled water. Indomethacin was made up as a 0.1 M stock in 1 M Na_2CO_3 and then diluted in distilled water. Glibenclamide and tolbutamide were dissolved in 100% methanol to a final concentration of 0.01 M.

Results

Endothelium-intact rings developed spontaneous, active force ('tone', range: 1–7 g) within the 60 min equilibration period after their internal circumference had been set. By contrast, rings without endothelium usually gave either no increase in active force over the same time, or developed less than 1 g initially and then returned to baseline within approximately 60 min. In endothelium-intact arteries, glibenclamide (3–30 μM) caused concentration-dependent relaxation of the developed spontaneous tone with total relaxation in 3 out of 7 experiments (Figure 1a). In two more experiments in which rings also developed spontaneous force, glibenclamide up to 30 μM failed to cause any relaxation, whilst in the remaining two experiments this concentration of glibenclamide caused less than 50% relaxation. Glibenclamide (3–30 μM) completely relaxed both endothelium-intact and -denuded rings of artery precontracted with U46619 (Figure 1b). The vehicle control (methanol) did not relax spontaneous force in any of these experiments.

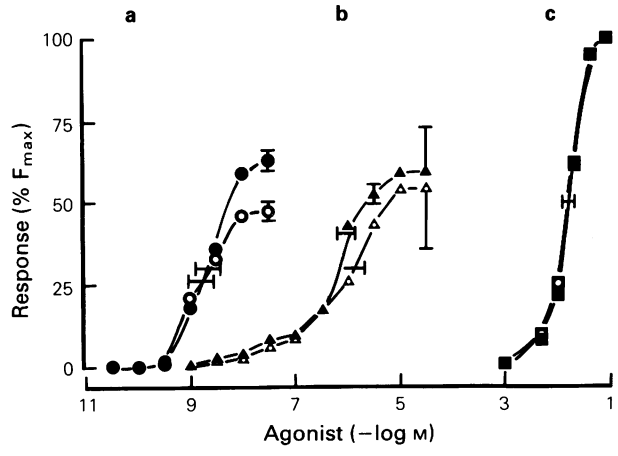

Figure 6 Concentration-contraction curves for (a) endothelin-1, (b) noradrenaline and (c) KCl in the absence (open symbols) and presence (closed symbols) of 30 μM glibenclamide. The noradrenaline curves were constructed in the presence of propranolol (1 μM) in endothelium-denuded rings. The data are expressed as described in Figure 2. The control EC_{50} values (−log M) for endothelin-1, noradrenaline and KCl were, respectively, 8.80 ± 0.24, 5.89 ± 0.19 and 1.80 ± 0.09 (n = 3). The corresponding maximum responses (% F_{max}) for endothelin-1 and noradrenaline were, respectively, 48 ± 7 and 55 ± 19. The corresponding maximum response (g) for KCl was 26 ± 5. Glibenclamide (30 μM) did not significantly alter either the EC_{50} or the maximum response for any of the agonists.

The effect of glibenclamide on the cumulative concentration-contraction curve to U46619 is shown in Figure 2. Increasing concentrations of glibenclamide (1–30 μM) caused a progressive decrease in the sensitivity (EC_{50}) of U46619 but did not significantly alter the maximum response (F_{max}). Schild regression analysis of the effect of glibenclamide on U46619 gave a pK_B of 6.18 (95% confidence limits 5.42 and 7.08) with a slope of the regression line of 1.07 (95% confidence limits 0.78 and 1.34, Figure 3). With the interactive technique, the reciprocal logistic model could be fitted with parallel slopes (F for individual slopes = 0.37, d.f. 4, 219) giving a pK_B of 6.39 (95% confidence limits 6.25, 6.60) with no significant departure from competitivity (n = 1.003, where the parameter n is equivalent to the slope in the Schild plot; see Stone & Angus, 1978). The 'Clark' plot of log A against log (B + K_B) (Figure 4) indicated that all points lay within the 95% confidence limits of the competitive model (indicated by the error bars). Further, there was no evidence of any significant departure of the control point from the competitive model indicating that the direct effect of glibenclamide on resting tone did not interfere with the estimation of the pK_B.

Another sulphonylurea compound, tolbutamide (3 and 30 μM), structurally related to glibenclamide, caused a small but not significant ($P > 0.05$, unpaired t test) decrease in sensitivity to U46619 without affecting the maximal contraction (Figure 5). Glibenclamide (30 μM) had no significant effect on either the EC_{50} or maximal contraction for endothelin-1, noradrenaline or KCl (Figure 6).

Discussion

The hypoglycaemic drug glibenclamide has been reported to be one of the most potent inhibitors of cromakalim-activated K^+ channels in a variety of smooth muscle preparations (see Introduction for references). It is not known whether the same mechanism of action is involved in both this smooth muscle response and the inhibition of ATP-activated K^+ channels involved in the hypoglycaemic response. There is, however, a marked discrepancy between the antagonist potencies for glibenclamide at the cromakalim-activated ATP-regulated K^+ channels in the pancreatic acinar cell (Schmid-Antomarchi *et al.*, 1987; Zünkler *et al.*, 1988; but see Matthews & Shotton, 1984) and the relaxation response to cromakalim in smooth muscle (Buckingham *et al.*, 1989; Cavero *et al.*, 1989; Eltze, 1989a,b; Winquist *et al.*, 1989). Regardless of this discrepancy, our data indicate another, previously unreported, property of glibenclamide. The results demonstrate that glibenclamide competitively inhibits the contractile response to a thromboxane A_2 analogue, U46619, in the dog isolated coronary artery. Our estimates of the pK_B for glibenclamide at this vascular thromboxane A_2 receptor were 6.18 and 6.39 by Schild analysis and interactive analysis respectively.

The concern that any glibenclamide-sensitive endogenous thromboxane A_2 may have interfered with the pK_B estimate by the Schild regression method was not supported by the result from the interactive method of analysis. Presumably, basal thromboxane A_2 generation did not occur in all tissues in this assay, a conclusion supported by the preliminary tests with glibenclamide on spontaneously developed force. We have observed similar variability in the effect of a specific thromboxane A_2 receptor antagonist, GR32191 (Lumley *et al.*, 1989) on spontaneously developed force in the dog coronary artery (Cocks & Angus, unpublished data).

In the rat portal vein, glibenclamide (3 μM) caused a 10 fold rightward shift of the cromakalim concentration-relaxation

curve (Winquist *et al.*, 1989) to give an estimated pK_B (assuming competitivity) of 6.48, very similar to our estimate at the thromboxane receptor. Other reports of pK_B values of glibenclamide at the 'cromakalim' receptor in the rat aorta were 6.95 (Cavero *et al.*, 1989) and approximately 7 (calculated from data of Buckingham *et al.*, 1989). In the guinea-pig isolated pulmonary artery, Eltze (1989b) reported similar pK_B values of 7.2 for glibenclamide against three vasorelaxants, cromakalim, pinacidil and RP 49356. In each case the inhibition was competitive. He also demonstrated competitive antagonism by glibenclamide ($pK_B = 7.2$) of the cromakalim-mediated inhibition of twitch contractions in the rabbit vas deferens (Eltze, 1989a). Taken together, these studies clearly indicate that concentrations of glibenclamide in the range 0.1–10 μM that are necessary to antagonize the cromakalim-induced relaxation of smooth muscle fall within the range of the antagonist activity of glibenclamide at the thromboxane A_2 receptor.

In conclusion, we have shown that glibenclamide has an additional novel property of being a relatively potent and selective antagonist of thromboxane A_2 receptors. This action of glibenclamide should be considered in studies on smooth muscle K^+ channels, particularly if a contracting agonist like U46619 is used or if there is spontaneous tone induced presumably by endogenous thromboxane A_2.

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Evaluation of the effect of azapropazone on neutrophil migration in regional myocardial ischaemia/reperfusion injury in rabbits

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1 The purpose of the present study was to determine the myocardial cytoprotective efficacy of azapropazone (AZA) and its potential site of action on neutrophil infiltration into reperfused/ischaemic myocardium with or without *in vivo* activation of neutrophils in rabbits.

2 AZA, 100 mg kg^{-1}, was administered i.v. 10 min after occlusion of the left circumflex (LCX) artery in rabbits with and without pretreatment with phorbol myristate acetate ester (PMA). The LCX occlusion was then released at 10 min after AZA administration. Haemodynamic parameters (heart rate, LV pressure, mean arterial blood pressure and *dp/dt*) were monitored throughout the experiment. After 60 min reperfusion, the area at risk was delineated and the heart was then excised and divided into epi- and endocardial pieces for analysis of myeloperoxidase activity.

3 AZA inhibited neutrophil infiltration into the reperfused/ischaemic rabbit myocardium with and without PMA treatment. The inhibition of neutrophil infiltration was more apparent in the epicardium than in the endocardium. Additionally, AZA inhibited to a similar extent the *in vivo* PMA-stimulated neutrophil migration into the epicardium and endocardium area at risk. AZA had no significant effect on the haemodynamic parameters as compared to control.

4 AZA administered in an anaesthetized rabbit model of LCX occlusion/reperfusion resulted in the reduction of infarct size.

5 It is concluded that AZA has significant inhibitory effects on neutrophil migration which might contribute to its myocardial cytoprotective effect.

Introduction

It has been reported that tissue injury can occur either upon blood flow restriction (occlusion or ischaemia-induced damage) or upon reperfusion of a preoccluded artery (reperfusion-induced damage) (Farber *et al.*, 1982; Braunwald & Kloner, 1985). Occlusion-induced damage results from a series of events including hypoxia, tissue acidosis, activation of proteolytic enzymes and the release of endogenous vasoactive mediators (Heyndrickx *et al.*, 1975; DeBoer *et al.*, 1980; Cobbe & Poole-Wilson, 1980; Reimer *et al.*, 1981). Further, numerous reports have implicated xanthine oxidase and neutrophil derived oxygen free radicals in reperfusion-induced myocardial damage (Freeman & Crapo, 1982; Hess & Manson, 1984; Rowe *et al.*, 1984; McCord, 1985).

Azapropazone is a nonsteroidal anti-inflammatory agent with uricosuric properties (anti-gout). *In vitro* studies demonstrated that azapropazone is a xanthine oxidase inhibitor with an IC_{50} of 70–140 μg ml^{-1} and an inhibitor of a variety of neutrophil function (migration, aggregation, superoxide production) at 40–400 μg ml^{-1} (Rakich & Marshall, 1986; Jahn & Thiele, 1988). For these reasons, it has been postulated that azapropazone (AZA) should exert a beneficial effect in myocardial infarction due to ischaemia and reperfusion (Rakich & Marshall, 1986). Subsequent studies in rats and dogs have supported these suggestions (Montor *et al.*, 1987; Knabb *et al.*, 1988; Mousa *et al.*, 1989b).

Since it has been found that the distribution of xanthine oxidase is species-dependent and that the human heart is devoid of xanthine oxidase (Al-Khalidi & Chaglassian, 1965; Eddy *et al.*, 1987; de Jong *et al.*, 1989), it was of interest to explore the *in vivo* myocardial cytoprotective efficacy of AZA against myocardial ischaemic/reperfusion injury in an animal which is devoid of xanthine oxidase. The present study was therefore undertaken to determine the myocardial cytoprotective efficacy of AZA and its potential site of action on the neutrophil migration into ischaemic rabbit myocardium which is known to be devoid of xanthine oxidase (Eddy *et al.*, 1987; de Jong *et al.*, 1989).

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Methods

Experimental preparations

Male, New Zealand white rabbits (3–4 kg) were used in the study ($n = 22$ for experiment No. 1 and $n = 29$ for experiment No. 2). Animals were anaesthetized with pentobarbitone sodium (25–30 mg kg^{-1}, i.v.) and placed on a respiratory pump (Model 665; Harvard Apparatus, South Natick, MA, U.S.A.). A left thoracotomy in the fourth intercostal space was performed. The epicardium was incised and the left circumflex coronary artery (LCX) was totally occluded (fixed ligation) approximately at a point midway between the apex and base. The left femoral artery and vein were cannulated for the monitoring of mean arterial blood pressure/heart rate and for the intravenous administration of AZA and/or phorbol myristate acetate (PMA), respectively. Arterial blood pH, P_{O_2} and P_{CO_2} were monitored at various times during the experiment by use of an acid-base analyzer, (ABL 30; Radiometer, Copenhagen). The respiratory pump rate and volume were adjusted to keep the arterial pH at 7.35 ± 0.15, the P_{CO_2} at 30 ± 5 mmHg and the P_{O_2} at 105 ± 15 mmHg. Throughout the experiments, the animals were kept on heated operating tables to maintain rectal temperature around 37°C.

Experiment No. 1

The first marginal branch of the LCX was ligated at a point midway between the apex and the base. After 30 min of complete occlusion, the ligation was released for 5 h. Thereafter, the animal was killed with an i.v. dose of KCl until asystole;

Table 1 Summarized haemodynamics in anaesthetized rabbits subjected to coronary artery occlusion followed by reperfusion: effects of azapropazone (AZA)

Haemodynamic[a] *parameters*	*Treatment group*	*Baseline*	*Occlusion* (10 min)	*Minutes post-reperfusion* 5	10	20	40	60
Heart rate (beats min^{-1})	Vehicle ($n = 5$)	96 ± 4	104 ± 5	93 ± 4	91 ± 5	95 ± 5	99 ± 5	99 ± 5
	AZA ($n = 6$)	101 ± 6	102 ± 4	113 ± 7	109 ± 6	107 ± 7	106 ± 6	104 ± 6
	PMA ($n = 6$)	98 ± 2	99 ± 4	101 ± 3	100 ± 3	101 ± 3	102 ± 2	104 ± 3
	AZA/PMA ($n = 6$)	94 ± 5	95 ± 5	98 ± 6	97 ± 6	96 ± 6	97 ± 6	95 ± 5
Mean arterial blood pressure (mmHg)	Vehicle ($n = 5$)	78 ± 3	74 ± 5	68 ± 4	68 ± 4	69 ± 4	69 ± 4	70 ± 4
	AZA ($n = 6$)	76 ± 6	76 ± 6	79 ± 5	80 ± 4	84 ± 5	84 ± 6	83 ± 6
	PMA ($n = 6$)	77 ± 6	79 ± 5	73 ± 6	73 ± 6	75 ± 6	75 ± 6	76 ± 7
	AZA/PMA ($n = 6$)	70 ± 3	77 ± 4	75 ± 5	69 ± 5	72 ± 4	72 ± 3	71 ± 4
Left ventricular pressure (mmHg)	Vehicle ($n = 5$)	101 ± 6	102 ± 4	93 ± 8	92 ± 8	95 ± 9	98 ± 6	98 ± 6
	AZA ($n = 6$)	101 ± 6	107 ± 8	108 ± 6	110 ± 7	111 ± 6	113 ± 7	113 ± 8
	PMA ($n = 6$)	100 ± 6	109 ± 7	95 ± 9	103 ± 7	102 ± 9	101 ± 9	105 ± 10
	AZA/PMA ($n = 6$)	96 ± 4	98 ± 4	97 ± 5	97 ± 5	97 ± 4	98 ± 4	97 ± 5
$+dp/dt$ ($mmHg\,s^{-1}$)	Vehicle ($n = 5$)	2400 ± 200	2200 ± 100	1900 ± 200	2000 ± 200	2000 ± 200	2200 ± 200	2200 ± 200
	AZA ($n = 6$)	2400 ± 200	2500 ± 200	2500 ± 100	2600 ± 200	2700 ± 200	2700 ± 200	2700 ± 200
	PMA ($n = 6$)	2100 ± 100	2400 ± 200	2100 ± 200	2200 ± 200	2300 ± 200	2300 ± 200	2400 ± 200
	AZA/PMA ($n = 6$)	2000 ± 100	2500 ± 100	2200 ± 200	2200 ± 100	2200 ± 100	2200 ± 200	2300 ± 200

[a] All values represent mean ± s.e. PMA = phorbal myristate acetate.

the heart was then excised and the coronary ostia were perfused via the aorta with 1.5% triphenylterrazolium chloride (TTC) in phosphate buffer to delineate infarcted tissue from normal myocardium. The heart was then fixed in a large volume of 10% buffered formalin for at least 48 h. After fixation, the heart was sliced from apex to base in parallel transverse slices of 2 mm thickness. The outlines of the infarcted and normal myocardium were traced on clear acetate and overlay areas were determined with an IBM image analyzer. Infarct size was calculated as percentage of left ventricular mass.

Treatment groups: Group I: control ($n = 10$), vehicle, $1\,ml\,kg^{-1}$ i.v. 1 h before occlusion, $0.5\,ml\,kg^{-1}$, 5 min before reperfusion and $0.5\,ml\,kg^{-1}$, 2.5 h after reperfusion. Group II: AZA ($n = 12$), $100\,mg\,kg^{-1}$ i.v. 1 h before occlusion, $50\,mg\,kg^{-1}$, 5 min before reperfusion and $50\,mg\,kg^{-1}$ 2–5 h after reperfusion.

Experiment No. 2:

AZA was administered ($100\,mg\,kg^{-1}$, i.v.) 10 min after the LCX occlusion. In another group of animals PMA was infused i.v. ($50\,\mu g\,kg^{-1}$ over 5 min) just before the release of

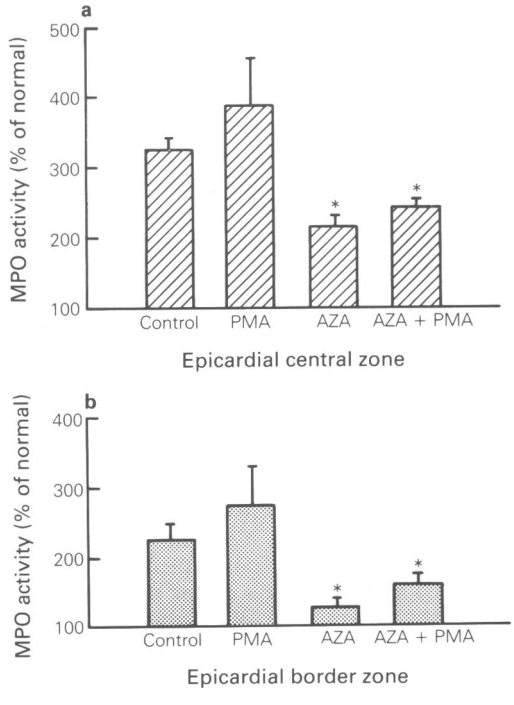

Figure 1 Effects of azapropazone (AZA) on neutrophil migration as evident from changes in tissue myeloperozidase (MPO) activity in rabbit myocardium (epicardium) subjected to 20 min of left circumflex artery occlusion followed by 60 min of reperfusion. Columns represent mean MPO activity (% of normal or non-ischaemic myocardium) with s.e. shown by vertical bars. AZA ($100\,mg\,kg^{-1}$, i.v. at 10 min post occlusion) produced a significant inhibition (*$P < 0.01$) of neutrophil migration (MPO activity) into epicardial central (a) or border (b) zones within areas at risk in either phorbol myristate acetate (PMA)-treated or non-PMA-treated groups.

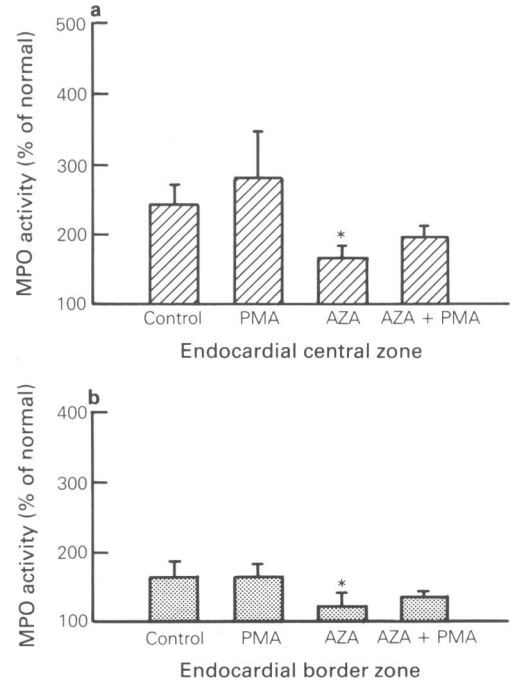

Figure 2 Effects of azapropazone (AZA) on neutrophil migration myeloperoxidase (MPO) activity) in rabbit myocardium (endocardium) subjected to 20 min of left circumflex artery occlusion followed by 60 min of reperfusion. Columns represent mean MPO activity (% of normal or non-ischaemic myocardium) with s.e. shown by vertical bars. AZA produced a significant inhibition (*$P < 0.01$) of neutrophil migration into endocardial central (a) and border (b) zones within areas at risk in the non-phorbol myristate acetate (PMA) treated group.

the LCX occlusion. After 20 min, the LCX was released and the artery was reperfused for 60 min. Haemodynamic parameters were monitored throughout the study. At the end of the study, the area at risk was delineated by infusing monastral blue dye into the left ventricle. This was followed by an i.v. dose of KCl until asystole was observed. The heart was then excised, divided into a mapped epi- and endocardial pieces, central and border zones, for the biochemical determination of tissue myeloperoxidase (MPO) activity.

Treatment groups: Group I: control ($n = 5$): vehicle 1 ml kg^{-1}, i.v. 10 min post-LCX occlusion. Group II: AZA alone ($n = 6$): AZA 100 mg kg^{-1}, i.v. 10 min post-LCX occlusion. Group III: PMA alone ($n = 6$): PMA 50 μg kg^{-1} 5 min^{-1} infused i.v. at 15 min post-LCX occlusion for 5 min. Group IV: PMA plus AZA ($n = 6$): AZA 100 mg kg^{-1}, i.v. followed by PMA, 50 μg kg^{-1} 5 min^{-1} as described above.

Myeloperoxidase (MPO) assay

Myeloperoxidase is a neutrophil-specific enzyme (Bradley *et al.*, 1982). MPO was assayed in cardiac tissues (normal area, area at risk including central and border zones from epi- and endocardial pieces) for the determination of the extent of migrated or activated neutrophil upon myocardial ischaemia/reperfusion injury as previously described by Mullane *et al.* (1985). The tissues were extensively washed from blood in cold saline and then homogenized in 50 mM phosphate buffer containing 0.5% hexadecyl-trimethyl ammonium bromide to solubilize MPO. The homogenates were then centrifuged at 40,000 *g* for 15 min. The supernatant which contain the MPO enzyme was decanted. To a 100 μl of the tissue supernatant, 2.9 ml of 50 mM phosphate buffer (pH 6.0) containing 0.167 mg ml^{-1} of O-dianisidine HCl plus 0.003% H_2O_2 was added. The change in absorbance at 460 nm at 25°C was monitored for 2–3 min. Data were expressed as a percentage of normal or nonischaemic tissue.

Chemicals

Azapropazone (Dupont, Wilmington, DE) 100 mg ml^{-1} was dissolved in saline, bubbled with N_2 and the pH was adjusted to 9.0–9.5. Monastral blue dye, hexadecyltrimethyl ammonium bromide, O-diansidine HCl, 30% hydrogen peroxide, triphenyltetrazolium chloride (TTC) and phorbol myristate acetate (PMA) were obtained from Sigma Chemical Co. (St Louis, MO. U.S.A.).

Statistical analysis

The experimental results are given as the mean ± s.e.mean. The statistical significance of the difference between AZA-treated groups and respective control groups was determined by Student's *t* test. Differences were considered statistically significant if $P < 0.05$.

Results

Effect of azapropazone on infarct size

Infarct size amounted to 28.9 ± 3.8% of the left ventricle in the control group ($n = 10$), while in the AZA group ($n = 12$) infarct size was significantly reduced (20.4 ± 1.2% of left ventricular mass, $P < 0.05$).

Effects on haemodynamics

The effects of AZA on the different haemodynamic parameters (heart rate, mean arterial blood pressure, LV pressure, LVEDP and LV*dp*/*dt*) were examined in the LCX ligated rabbit model of regional myocardial ischaemia/reperfusion injury. Each parameter was monitored before LCX occlusion, before the different treatment schedules, during the LCX occlusion period (20 min) and during the reperfusion period (60 min). AZA had no significant effect on any of the recorded parameters as compared to the control groups (Table 1). The lack of effect on heart rate, mean arterial blood pressure of their product suggested that AZA has no effect on myocardial oxygen consumption.

Areas at risk

Occlusion of the LCX resulted in anatomical risk areas comprising 32.2% ± 2.3 and 36.4% ± 2.5 of left ventricle (LV) for epi- and endocardium respectively in the control group. The area at risk was not statistically different among the four different groups. The area at risk amounted to 36.0% ± 4.9 and 36.8% ± 6.1; 29.1% ± 2.8 and 30.9% ± 4.4; 33.7% ± 5.4 and 33.8% ± 4.8 of the LV for epi- and endocardium in the AZA; PMA; and AZA/PMA groups respectively.

Effects on neutrophil infiltration

AZA 100 mg kg^{-1}, i.v. administered during the LCX occlusion, resulted in a significant inhibition of neutrophil infiltration into the border zones of ischaemic/reperfused (I/R) rabbit myocardium either in PMA-treated group (44.6 ± 3.5% inhibition in I/R epi- and 24.4 ± 2.9% inhibition in I/R endocardial border zones) or non-PMA-treated group (46.8 ± 2.5% inhibition I/R epi- and 28.6 ± 2.5% inhibition in I/R endocardial border zones) (Figures 1 and 2). Similarly, azapropazone inhibited neutrophil migration into I/R rabbit myocardium (central zone) either in the PMA-treated group (40.3 ± 3.4% inhibition in epi- and 26.4 ± 3.1% inhibition in endocardium) or the non-PMA treated group (36.5 ± 1.1% inhibition in epi- and 34.8 ± 2.8% inhibition in endocardium) (Figures 1 and 2). The neutrophil migration was much more apparent in the epicardium (227–325% of normal epicardium) than in the endocardium (166–244% of normal endocardium) and in the central zone (244–325% of normal myocardium) compared to the border zone (166–227% of normal myocardium) of either the epi- or the endocardium (Figures 1 and 2). Likewise, the effect of AZA was greatest in the epicardium (46.8 ± 2.5 and 44.6 ± 3.5%) versus (28.6 ± 2.5 and 24.4 ± 2.9%) inhibition in the endocardium.

PMA tended to enhance the neutrophil migration (1.2–1.5 times the control levels) into reperfused/ischaemic rabbit myocardium. AZA produced a similar degree of inhibition in neutrophil migration in the PMA-treated group as compared to the non-PMA-treated group.

Discussion

It has been previously reported that either the depletion of neutrophil or the inhibition of neutrophil functions produced a reduction of myocardial infarct size in a regional ischaemia/reperfusion model (Lucchesi & Mullane, 1986). In addition, the infiltration of neutrophils into injured myocardial tissue upon ischaemia/reperfusion has been linked to myocardial dysfunction (Rowe *et al.*, 1984). Therefore, the anti-ischaemic/cytoprotective effect of AZA may be due to an inhibition of neutrophil migration following reperfusion of an occluded coronary artery. Furthermore, AZA was shown to be equipotent either in the presence or the absence of the *in vivo* activator (PMA) of the neutrophil. These data are consistent with the previously reported *in vitro* effects of AZA on PMA-stimulated neutrophil migration (Rakich & Marshall, 1986) and are in agreement with recent studies with AZA in a topically inflamed swine's skin model in which a multiblister suction technique was used (Mousa *et al.*, 1989a).

In our study, the i.v. infusion of PMA (50 μg kg^{-1} 5 min^{-1}) resulted only in a very slight (insignificant) increase in neutrophil migration into I/R myocardium without any significant changes in the different haemodynamic parameters. This is different from earlier studies by Rowe *et al.* (1984) in which

PMA infusion was shown to result in the activation of neutrophils with a significant change in haemodynamics which was inhibited by the combination of superoxide dismutase and catalase and/or neutrophil depletion. It seems that the cardiovascular effects to PMA are associated with the activation of the neutrophil and this may explain why we were not able to obtain either effect in our study. The lack of PMA effects may be explained on the basis of the inadequate PMA infusion dose, the responsiveness of the animals or the possiblity that the neutrophil might be already maximally activated due to the I/R protocol used in the study.

The present results provide direct evidence of an altered functional response of neutrophils *in vivo* by AZA at a dose that reduces myocardial infarct size (Montor *et al.*, 1987; Knabb *et al.*, 1988; Mousa *et al.*, 1989b). Additionally, since dermal neutrophil migration in an *in vivo* swine multiblister suction technique model has also been shown to be reduced (Mousa *et al.*, 1989a), it seems likely that the decreased neutrophil infiltration into the reperfused ischaemic myocardium is a direct effect of AZA on neutrophil function.

Earlier work in various models of regional ischaemia-reperfusion injury demonstrated a reduction of infarct size in the AZA-treated groups as compared to the respective control group (Montor *et al.*, 1987; Mousa *et al.*, 1989b). The presented inhibitory efficacy of AZA on the infiltration of neutrophils into reperfused/ischaemic rabbit myocardium might explain the cytoprotective efficacy of AZA in the rabbit heart model which is devoid of the enzyme xanthine oxidase (i.e., the reduction of infarct size). It should be noted, however, that because of the differences in the protocols used (1 and 5 h periods of reperfusion in the neutrophil migration and infarct size studies, respectively) a direct link between these two effects cannot be made. Moreover, in the present study AZA was given prior to the coronary artery occlusion. A previous study has demonstrated an equivalent effect to AZA in limiting infarct size when given either prior to occlusion, post occlusion or even prior to reperfusion (Knabb *et al.*, 1988).

It is concluded that AZA is an effective inhibitor of neutrophil migration into ischaemia/reperfused injured myocardium in rabbits. Furthermore, the present study suggests that the potential myocardial cytoprotective/anti-ischaemic efficacy of AZA is not limited to the inhibition of xanthine oxidase.

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Mechanism of endothelin-induced contraction in guinea-pig trachea: comparison with rat aorta

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1 Endothelin (1 nM–0.3 μM) produced a concentration-dependent contraction of guinea-pig epithelium-containing (intact) trachea (EC_{50} = 30.9 nM). Endothelin was a less potent agonist than leukotriene D_4 (LTD_4; EC_{50} = 0.77 nM), but was more potent than carbachol (EC_{50} = 0.15 μM) or substance P (EC_{50} = 1.4 μM). Endothelin was a more potent contractile agent in rat endothelium-denuded aorta (EC_{50} = 2.1 nM) than in guinea-pig trachea.

2 Endothelin-induced contraction in guinea-pig trachea was unaffected by mepyramine (10 μM), atropine (1 μM), SK&F 104353 (10 μM), a leukotriene receptor antgonist, or SQ 29,548 (1 μM), a thromboxane receptor antagonist. The contraction produced by 0.3 μM endothelin was potentiated by cyclo-oxygenase inhibition with 5 μM indomethacin.

3 Nicardipine (0.01 or 0.1 μM) or incubation in calcium-free medium +0.1 mM EGTA for 30 min had a relatively minor or no effect on endothelin concentration-response curves in guinea-pig intact trachea, but markedly inhibited responses produced by endothelin in endothelium-denuded aorta of the rat. Increasing the EGTA concentration in calcium-free medium to 1 mM abolished endothelin-induced contraction in guinea-pig trachea.

4 In guinea-pig trachea, ryanodine (10 μM) produced a 2.1 fold shift to the right of endothelin concentration-response curves and reduced the maximum response elicited by 0.3 μM endothelin.

5 Staurosporine (0.01 μM and 0.1 μM), a protein kinase C inhibitor, was without effect on endothelin- or carbachol-induced contraction in guinea-pig trachea, but markedly inhibited the response produced by endothelin in rat aorta.

6 Endothelin (3 nM–0.3 μM) produced a concentration-dependent stimulation of phosphatidylinositol (PI) turnover in guinea-pig intact trachea, with an EC_{50} value of 45.9 nM.

7 Removal of the epithilium markedly potentiated endothelin-induced contraction in guinea-pig trachea, producing a 4.7 fold leftward shift in endothelin concentration-response curves and an increase in the contractile response elicited by 0.3 μM endothelin.

8 These data indicate that endothelin is a potent agonist in guinea-pig trachea whose response is markedly enhanced by removal of the airway epithelium. Endothelin-induced contraction is not mediated to a marked extent by calcium influx via dihydropyridine-sensitive calcium channels and does not involve the release of histamine, acetylcholine, leukotrienes or thromboxane. Rather, endothelin appears to produce contraction of guinea-pig trachea via a direct action which involves stimulation of PI turnover and utilization of calcium from intracellular stores and, also, calcium influx via a pathway that is not sensitive to dihydropyridine calcium channel inhibitors. Endothelin-induced contraction of rat aorta was more sensitive to the effects of incubation in Ca^{2+}-free medium, nicardipine or staurosporine, suggesting that differences exist in the relative mechanisms whereby endothelin produces contraction in different tissues.

Introduction

Endothelin is a recently discovered potent vasoconstrictor peptide isolated from porcine cultured endothelial cells (Yanagisawa *et al.*, 1988). This 21-amino acid peptide bears a close structural homology with a group of snake venom toxins, the sarafotoxins (Lee & Chiappinelli, 1988), and there is evidence that they may possess similar mechanisms of action (Kloog *et al.*, 1988; Van Renterghem *et al.*, 1988; Resink *et al.*, 1988; Ohlstein *et al.*, 1989). In a preliminary communication endothelin was shown to be an extremely potent bronchoconstrictor agent in guinea-pig trachea, possessing substantially greater potency than leukotriene D_4 (LTD_4) or neurokinin A (Uchida *et al.*, 1988).

Based on results of experiments in which the effects of calcium-free medium and a dihydropyridine calcium channel antagonist, nicardipine, were examined, it was hypothesized that endothelin produced contraction of vascular and airway smooth muscle by interacting with and activating voltage-dependent calcium channels (Yanagisawa *et al.*, 1988; Uchida *et al.*, 1988). Although this postulate is supported by a preliminary study in human vascular tissue (Hughes *et al.* 1988), several recent publications provide strong evidence that endothelin acts by a mechanism other than an interaction with dihydropyridine-sensitive calcium channels and stimulation of extracellular calcium influx (Van Renterghem *et al.*, 1988; Resink *et al.*, 1988; Hirata *et al.*, 1988; Miasiro *et al.*, 1988; Auguet *et al.*, 1988). These studies suggest that endothelin produces contraction of smooth muscle by interaction with a specific receptor and subsequent release of intracellular calcium as a consequence of stimulation of phosphatidylinositol (PI) turnover.

In the present study the mechanism(s) of the contractile effect of endothelin in guinea-pig trachea was investigated and a comparison was made with endothelin-induced contraction in rat aorta. A preliminary account of the results has been presented previously (Hay, 1989b).

Methods

Tissue preparation

Tracheae were removed from male Hartley guinea-pigs (Hazelton Research Animals, Denver, PA, U.S.A.; 450–650 g body weight) and placed in modified Krebs-Henseleit solution.

The composition of the Krebs-Henseleit solution, which was gassed with 95% O_2 : 5% CO_2 and maintained at 37°C, was (mM): NaCl 113.0, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 5.5. The calcium-free physiological buffer used in some studies was of the same composition as Krebs-Henseleit solution except that $CaCl_2$ was omitted and 0.1 mM or 1 mM EGTA added. Following careful removal of adherent fat and connective tissue, the trachea was cut open along its longitudinal axis, directly opposite the smooth muscle, and strips consisting of two adjacent cartilage rings were prepared. Thoratic aortae were removed from male Sprague-Dawley rats (Charles River Breeding Laboratories, Raleigh, NC, U.S.A.; 250–350 g body weight) and cleaned of connective tissue and adherent fat. The isolated arteries were placed round 21 g syringe needles and cut into strips of approximately 15 mm length and 2 mm width; endothelium was removed from aorta by rotation of the tissue several times round the syringe needle. The tissue preparations were then placed in 10 ml water-jacketed organ baths containing Krebs-Henseleit solution and connected via silk suture to Grass FTO3C force-displacement transducers. Mechanical responses were recorded isometrically by multi-channel polygraphs. Tissues were equilibrated, under 1.5 g and 1 g resting load for trachea and aorta, respectively, for at least 1 h, and washed every 15 min with fresh Krebs-Henseleit solution, before the start of each experiment.

In some experiments, the epithelium was removed mechanically from alternate strips of trachea (permitting paired analysis) by gently rubbing the luminal surface with a cotton-tipped applicator. It has been demonstrated previously that this procedure effectively removes the epithelium from guinea-pig trachea without producing obvious damage to the underlying mucosal and smooth muscle layers, and does not alter the basic mechanical properties of the tissue (Hay *et al.*, 1986).

Concentration-response curves

Concentration-response curves for agonists were generally obtained by their cumulative addition to the organ bath in either 3 or 10 fold increments according to the technique of Van Rossum (1963). Each drug concentration was left in contact with the preparation until the response reached a plateau before addition of the subsequent agonist concentration. In experiments examining the effects of antagonists, tissues were exposed to these agents for 30 min before addition of contractile agonists. In some experiments the influence of extracellular calcium on agonist-induced contractions was examined by incubation of tissues with calcium-free Krebs-Henseleit solution +EGTA (0.1 or 1 mM) for 30 min before addition of agonist; during the incubation period in calcium-free Krebs-Henseleit solution tissues were washed several times with physiological buffer. Studies in which the influence of ryanodine on agonist-induced contractions was examined were conducted in calcium-free Krebs-Henseleit solution +0.1 mM EGTA. Tissues were exposed to calcium-free Krebs-Henseleit solution +0.1 mM EGTA containing ryanodine for 30 min before construction of agonist concentration-response curves; tissues were washed several times during the incubation period. Most experiments were conducted in the presence of 5 μM indomethacin, which was present throughout the study. LTD_4 concentration-response curves were performed in the presence of 3 mM L-cysteine, which inhibits the conversion of LTD_4 to LTE_4 (Snyder *et al.*, 1984). Studies in which the effects of KCl in guinea-pig trachea or rat aorta were examined were generally conducted in the presence of atropine (1 μM) or phentolamine (1 μM), respectively.

After the equilibration period, and before construction of concentration-response curves, tissues were exposed to either 10 μM carbachol for guinea-pig trachea or 60 mM KCl for rat aorta. Following plateau of this reference contraction, tissues were washed several times over 15–30 min until the tension returned to baseline level. The preparations were then left for at least 30 min before the start of the experiment.

Phosphatidylinositol turnover studies

PI turnover in guinea-pig trachea was measured by a modification of the method outlined by Berridge *et al.* (1983). Tracheae, from which the cartilage was removed, were incubated for 4 h with 10 μCi ml^{-1} [^{3}H]-myo-inositol (specific activity = 83.2 Ci mmol^{-1}) in 3 ml Krebs-Henseleit solution maintained at 37°C and gassed with 95% O_2: 5% CO_2. Following the loading period tissues were washed three times with 3 ml Krebs-Henseleit solution containing 10 mM Li^+ and 1 mM myo-inositol; buffer was removed by aspiration. Tissues were placed in individual test-tubes containing 1 ml of Krebs-Henseleit solution +10 mM Li^+, which prevents the metabolism of inositol monophosphate (IP_1) to inositol (Allison & Blisner, 1976) and increases the accumulation and recovery of inositol phosphates (Berridge *et al.*, 1982). Following incubation with the appropriate concentration of endothelin for 60 min the reaction was stopped by addition of 3 ml of a mixture of chloroform: methanol: hydrochloric acid in a ratio of 2 : 1 : 0.1 (Best *et al.*, 1985). The test-tubes were vortexed vigorously and then centrifuged for 10 min at 150 *g* to separate the aqueous and chloroform phases. The upper aqueous phase was removed, diluted with 4 ml of distilled H_2O and then added to columns containing 1 ml of Dowex AG 1-X8 (100–200 mesh, formate form) anion exchange resin to permit separation and extraction of the inositol phosphates. The inositol phosphates were eluted by the following procedure: 5 ml of distilled H_2O was added to columns three times to elute free [^{3}H]-myo-inositol, followed by three washes with 5 ml of 60 mM sodium formate: 5 mM sodium tetraborate which elutes glycerophosphoinositol. Total inositol phosphates were then eluted by three 5 ml additions of 1 M ammonium formate: 0.1 M formic acid. Radioactivity, measured by a TM Analytic counter (Elk Grove Village, IL) at an efficiency of 30%, was determined in a 5 ml aliquot of the combined eluate samples to which 2 ml distilled H_2O and 10 ml of Aquasol liquid scintillation cocktail (New England Nuclear, Boston, MA) had been added. Individual tissues were dried at room temperature before being weighed and data were expressed as d.p.m. per mg tissue weight. In addition, in some experiments radioactiity present in the chloroform phase was measured to determine [^{3}H]-myo-inositol incorporation into phospholipids; 200 μl of the lower phase was dried overnight at room temperature and 10 ml of Aquasol was added before radioactivity was counted.

Analysis of data

Agonist-induced responses for each tissue were expressed as a percentage of the reference contraction or as absolute developed tension (in g). Geometric mean EC_{50} values were calculated from linear regression analyses of probit-transformed data. All data are given as mean ± s.e.mean. Statistical analysis was conducted by ANOVA or Student's *t* test for paired or unpaired samples where appropriate; a probability value less than 0.05 was regarded as significant.

Drugs

The following drugs were used: endothelin (human, porcine) was purchased from Peninsula Laboratories (Belmont, CA, U.S.A.) or Calbiochem Corp. (La Jolla, CA, U.S.A.). Staurosporine was obtained from Eli Lilly Co. (Indianapolis, IN, U.S.A.). SK&F 104353 (2(S)-hydroxy-3(R)-(2-carboxyethylthio)-3-[2-(8-phenyloctyl)phenyl]-propanoic acid) was synthesized at SmithKline Beecham Pharmaceuticals (King of Prussia, PA, U.S.A.). Ryanodine and H-7 (1-(5-isoquinolinesulphonyl)-2-methylpiperazine) were obtained from Calbiochem Corp. (La Jolla, CA, U.S.A.). Carbachol, atropine, EGTA, mepyramine and nicardipine were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 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 Squibb Institute of Medical Research (Princeton, NJ, U.S.A.).

Results

Characteristics of endothelin-induced contraction

In intact isolated trachea of the guinea-pig endothelin produced a concentration-dependent contractile response. The threshold concentration of endothelin for eliciting contraction was 1 nM–10 nM. Endothelin did not appear to be a very efficacious agonist, with 0.3 μM endothelin eliciting a response of only 0.52 ± 0.11 g (n = 10), which represents only 57.3 ± 8.2% of the reference contraction to 10 μM carbachol. However, due to lack of availability, concentrations of endothelin greater than 0.3 μM could not be examined in this study. Therefore, although the data would suggest that contraction elicited by 0.3 μM endothelin is close to the maximum response produced by endothelin, a true comparison between the maximum contractile responses produced by carbachol and endothelin could not be ascertained. Indomethacin (5 μM) markedly potentiated contraction produced by higher concentrations of endothelin (0.03–0.3 μM), e.g., response to 0.3 μM endothelin was 1.24 ± 0.09 g (n = 32; $P > 0.0005$ compared to response in the absence of indomethacin), which corresponds to 77.7 ± 3.7% of the response to 10 μM carbachol. In view of the marked enhancement of endothelin-induced contraction by indomethacin, unless stated otherwise, the subsequent experiments with contractile agonists were performed in the presence of 5 μM indomethacin, to inhibit the release of prostanoids.

Contraction produced by endothelin in guinea-pig intact trachea was slow to develop with a time to 50% of peak response of 3.2 ± 0.2 min (n = 4) with 0.1 μM endothelin; contraction was initiated approximately 15 s after addition of endothelin. In addition, the response to endothelin was slow to washout: time to 50% relaxation was 28.1 ± 9.8 min for 0.1 μM endothelin; (n = 4). There was no difference in the concentration-response curves to endothelin (1 nM–0.1 μM) in normal and siliconized organ baths (data not shown).

In the presence of indomethacin, endothelin (1 nM–0.3 μM) produced a concentration-dependent contractile response with an EC_{50} value = 30.9 nM (n = 32) (Figure 1; Table 1). A comparison was made between the concentration-response curves produced by endothelin, LTD_4, substance P and carbachol in intact guinea-pig trachea (Figure 1 and Table 1); quantities of endothelin and substance P sufficient to produce a maximum response were not available. LTD_4 ws the most potent of the contractile agonists, followed by endothelin, carbachol and finally, substance P.

Atropine (1 μM), mepyramine (1 or 10 μM), SK&F 104353 (1 or 10 μM), a leukotriene receptor antagonist (Hay *et al.*, 1987) or SQ 29,548 (0.1 or 1 μM), a thromboxane receptor antagonist

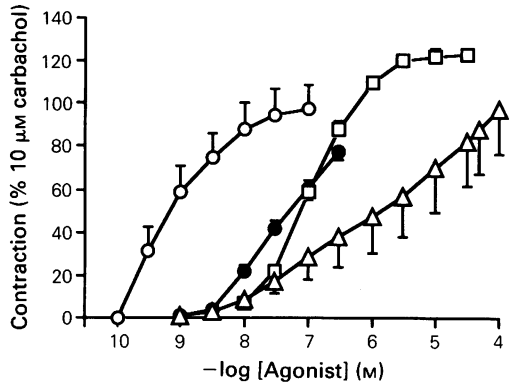

Figure 1 Concentration-response curves to endothelin (●), carbachol (□), leukotriene D_4 (LTD_4) (○) and substance P (△) in guinea-pig intact trachea. Results are expressed as a percentage of the reference contraction to 10 μM carbachol and are the mean with vertical lines indicating s.e.mean; endothelin, n = 32; carbachol, n = 20; LTD_4, n = 4; substance P, n = 4. Experiments were conducted in the presence of 5 μM indomethacin.

(Ogletree *et al.*, 1985) were without effect on endothelin-induced contraction (Figure 2).

Removal of the epithelium increased the responsiveness of guinea-pig trachea to the contractile effects of endothelin, as reflected by a 4.7 fold decrease in the EC_{50} and an increase in the contractile response produced by 0.3 μM endothelin (Figure 3); EC_{50} (−log M): +epithelium = 7.50 ± 0.07 (n = 23); −epithelium = 7.84 ± 0.06 (n = 23, $P < 0.0005$ compared to +epithelium); contractile response with 0.3 μM endothelin (% of that to 10 μM carbachol): +epithelium = 87.0 ± 5.4 (n = 23); −epithelium = 108.5 ± 6.5 (n = 23; $P < 0.005$ compared to +epithelium).

Endothelin was a more potent agonist in endothelium-denuded aorta of the rat than in guinea-pig trachea, with an EC_{50} value = 2.1 nM (−log M = 8.68 ± 0.17, n = 8; $P < 0.0005$ compared to guinea-pig intact or denuded trachea; Table 1).

Mechanism of endothelin-induced contraction

Extracellular calcium A series of experiments was conducted to determine the relative extracellular and intracellular calcium sources responsible for the endothelin-induced contraction in guinea-pig trachea and rat aorta.

In guinea-pig trachea, incubation for 30 min in calcium-free Krebs +0.1 mM EGTA was without effect on endothelin concentration-response curves (Figure 4a). Calcium-deprivation for 30 min had a significant inhibitory effect against contractions induced by carbachol (Figure 4c) or KCl (Figure 4e). Increasing the concentration of EGTA in calcium-free medium to 1 mM abolished contractions produced by endothelin (Figure 4b) or KCl (Figure 4f). Further inhibition

Table 1 Comparison of potencies and efficacies of endothelin, substance P, leukotriene D_4 (LTD_4) and carbachol in guinea-pig intact isolated trachea and contraction to endothelin in rat endothelium-denuded aorta

			Maximum contraction	
Agonist	n	EC_{50} (−log M)	*% reference contraction*	*g tension*
Guinea-pig trachea				
Endothelin	32	7.51 ± 0.06	77.7 ± 3.7	1.24 ± 0.09
LTD_4	4	9.12 ± 0.19**	97.9 ± 10.7*	1.39 ± 0.42
Carbachol	20	6.84 ± 0.15**	122.6 ± 3.4**	2.81 ± 0.21**
Substance P	4	5.85 ± 0.20**	97.5 ± 21.2	1.63 ± 0.36
Rat aorta				
Endothelin	8	8.86 ± 0.17**	197.4 ± 14.7	0.81 ± 0.15*

Results are expressed as EC_{50} values (−log M) and contractile response (% reference contraction or g tension) and are the means ± s.e.mean. Studies were performed in the presence of 5 μM indomethacin.
Significantly different from endothelin in guinea-pig trachea, *$P < 0.05$, **$P < 0.0005$.
[a] Reference contraction was 10 μM carbachol for guinea-pig trachea and 60 mM KCl for rat aorta.

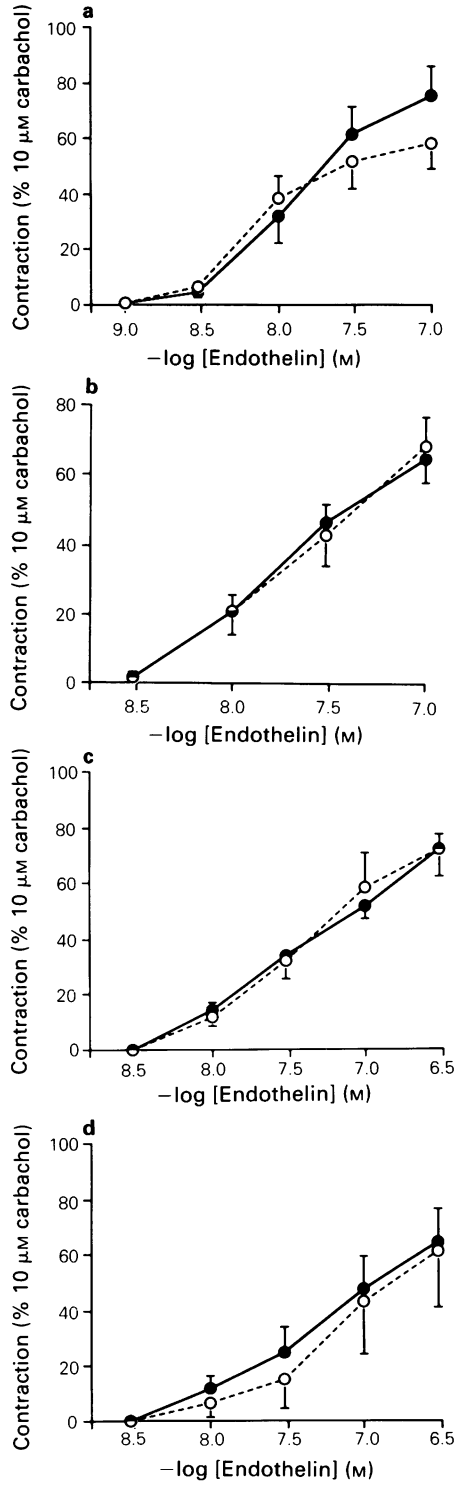

Figure 2 Effects of (a) atropine (1 μM); (b) mepyramine (10 μM); (c) SK&F 104353 (10 μM) and (d) SQ 29,548 (1 μM) on endothelin concentration-response curves in guinea-pig intact trachea. Results are expressed as a percentage of the reference contraction to 10 μM carbachol and are the mean, with vertical lines indicating s.e.mean (●—●) Control tissues; (O---O) treated tissues; (a) $n = 6$; (b) $n = 7$; (c) $n = 4$; (d) $n = 4$. Experiments in which the effects of atropine, mepyramine and SK&F 104353 were examined, but not those with SQ 29,548, were conducted in the presence of 5 μM indomethacin.

of carbachol concentration-response curves was also produced by incubation for 30 min in calcium-free medium + 1 mM EGTA, although an appreciable contractile response persisted (Figure 4d). Addition of 2.5 mM $CaCl_2$ at the end of the experiment returned endothelin-induced contraction to control value (Figure 4b) and partially restored responses produced

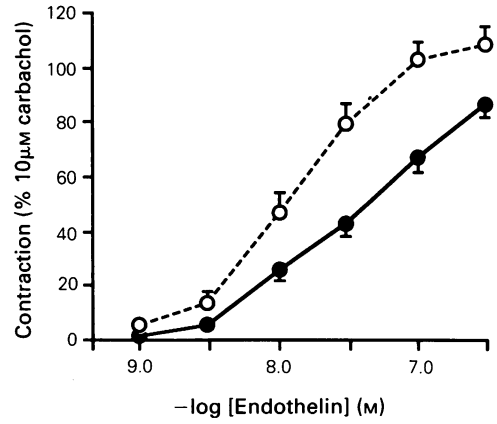

Figure 3 Effects of removal of the epithelium on endothelin concentration-response curves in guinea-pig isolated trachea. Results are expressed as a percentage of the reference contraction to 10 μM carbachol and are the mean of 23 tissues; vertical lines show s.e.mean. (●——●) + Epithelium; (O---O) − epithelium. Experiments were conducted in the presence of 5 μM indomethacin.

by carbachol (Figure 4d) or KCl (Figure 4f); addition of 2.5 mM $CaCl_2$ did not appreciably affect the level of tone in control tissues.

Nicardipine (0.01 μM) had no effect on endothelin concentration-response curves ($n = 3$; data not shown). Increasing the concentration of nicardipine to 0.1 μM produced significant inhibition of contractions produced by 0.03 and 0.1 μM endothelin but had no effect on responses produced by lower endothelin concentrations (Figure 5a). Nicardipine (0.1 μM) inhibited contractions produced by low concentrations of carbachol (0.01–0.1 μM) but had no effect on responses produced by higher carbachol concentrations (Figure 5b). KCl-induced contractions were inhibited to a greater extent by nicardipine (0.1 μM) than those produced by endothelin and, especially, carbachol (Figure 5c).

In contrast to guinea-pig trachea, incubation of rat aorta in calcium-free medium + 0.1 mM EGTA for 30 min produced substantial inhibition of endothelin-induced contraction (Figure 6a). At the end of the experiment, addition of 2.5 mM $CaCl_2$ to tissues exposed to 0.1 μM endothelin increased the contractile response to the same level as that observed in control tissues; addition of 2.5 mM $CaCl_2$ had no effect in control tissues. Nicardipine (0.01 μM) produced appreciable inhibition of endothelin-induced contraction in rat aorta (Figure 6b). Increasing the concentration of nicardipine to 0.1 μM did not elicit significant further inhibition of contraction produced by endothelin (Figure 6c). Incubation for 30 min in calcium-free Krebs + 0.1 mM EGTA produced marked inhibition and 0.1 μM nicardipine essentially abolished contraction produced by KCl in rat aorta (data not shown).

Intracellular calcium The effects of ryanodine (10 μM) on responses elicited by endothelin, carbachol and KCl in intact guinea-pig trachea are summarized in Figure 7. These experiments were conducted in the presence of calcium-free medium + 0.1 mM EGTA. Ryanodine (10 μM) produced significant inhibition of endothelin-induced contraction (Figure 7a). Ryanodine produced a small but significant 2 fold shift to the right of carbachol concentration-response curves, without affecting the maximum contractile response (Figure 7b), but had no effect against KCl-induced responses (Figure 7c).

Protein kinase C Staurosporine, a purported protein kinase C inhibitor, at concentrations of 0.01 μM (data not shown) or 0.1 μM (Figure 8a) did not inhibit endothelin-induced contraction. Responses to KCl were inhibited to a small extent by 0.1 μM staurosporine whereas those produced by carbachol were unaffected (data not shown). Another purported protein kinase C inhibitor, H-7, at concentrations of 3 μM or 10 μM was without effect on endothelin-induced contraction. A

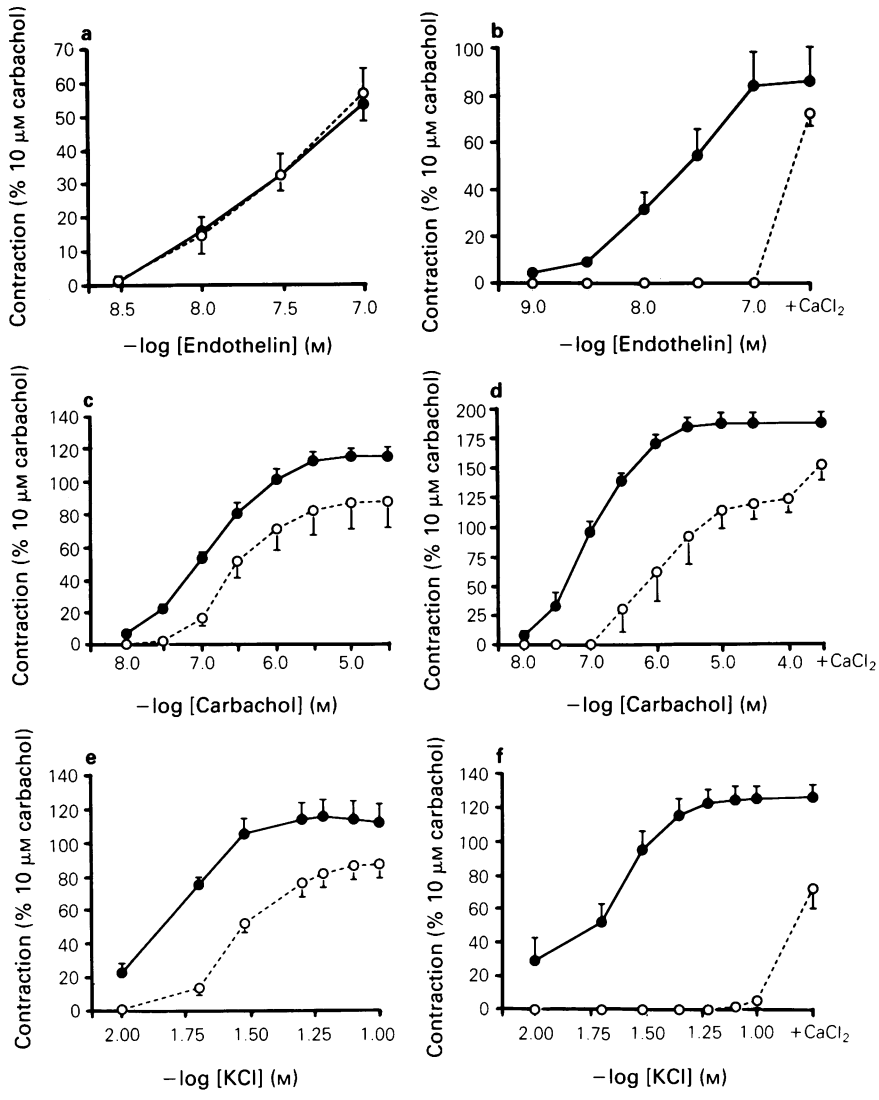

Figure 4 Effects of 30 min incubation in calcium-free Krebs +0.1 mM EGTA (a, c, e) or +1 mM EGTA (b, d, f) on concentration-response curves produced by endothelin (a, b), carbachol (c, d) or KCl (e, f) in guinea-pig intact trachea. Results are expressed as a percentage of the reference contraction to 10 μM carbachol and are the mean with vertical lines indicating s.e.mean. (●——●) Control tissues; (○---○) treated tissues; (a) $n = 4$; (b) $n = 4$; (c) $n = 8$; (d) $n = 4$; (e) $n = 7$; (f) $n = 4$. Studies examining KCl-induced contractions were conducted in the presence of 1 μM atropine. Re-addition of 2.5 mM $CaCl_2$ to tissues is indicated by $+CaCl_2$. Experiments were conducted in the presence of 5 μM indomethacin.

higher concentration of H-7, 30 μM, produced substantial inhibition of endothelin concentration-response curves (data not shown).

In rat aorta, 0.01 μM staurosporine produced marked inhibition (Figure 8b) and 0.1 μM staurosporine abolished endothelin-induced contractions (Figure 8c). Staurosporine (0.1 μM) also abolished the contraction produced by KCl (data not shown).

Phosphatidylinositol turnover in guinea-pig trachea In the presence of 5 μM indomethacin, endothelin (3 nM–0.3 μM) produced a concentration-dependent stimulation of PI turnover, measured as total water-soluble inositol phosphates, in epithelium-containing guinea-pig trachea from which the cartilage had been removed (Figure 9). The calculated EC_{50} ($-\log$ M) was 7.34 ± 0.20, ($n = 8$), which is in close agreement with the EC_{50} value for producing contraction (7.51 ± 0.06; Table 1). Endothelin (0.3 μM) did not stimulate [^{3}H]-myo-inositol incorporation into glycerophosphoinositol or the phospholipid (chloroform) phase (data not shown).

Discussion

In a preliminary communication by Uchida and co-workers (1988) endothelin was observed to be a potent contractile agonist in guinea-pig isolated trachea, with a potency of more than 2 log units greater than neurokinin A or LTD_4. In the present study it was confirmed that endothelin is a potent constrictor of guinea-pig isolated trachea. However, endothelin was approximately 60 fold less potent in this series of experiments (EC_{50} = 30.9 nM) than was previously determined (EC_{50} = 0.53 nM) (Uchida *et al.*, 1988). The reason(s) for this dicrepancy is unknown but may be related to different sources of endothelin, differences in the integrity of the airway epithelium, in addition to different strains of guinea-pigs used. It should be noted that the potency of endothelin in this study is similar to that presented in a recent publication also using guinea-pig trachea (Maggi *et al.*, 1989). In the present set of experiments endothelin was a more potent agonist in rat aorta than guinea-pig trachea.

Endothelin-induced contraction appears to involve a direct action on smooth muscle. Thus, the response was unaffected by mepyramine, atropine, SK&F 104353, a leukotriene receptor antagonist (Hay *et al.*, 1987) or SQ 29,548, a thromboxane receptor antagonist (Ogletree *et al.*, 1985), indicating that it does not involve the release of histamine, acetylcholine, leukotrienes or thromboxane. Indomethacin potentiated contractions produced by high concentrations of endothelin (0.03–0.3 μM), suggesting that, as is the case with several other bronchoconstrictor agonists (Orehek *et al.*, 1975; Saad &

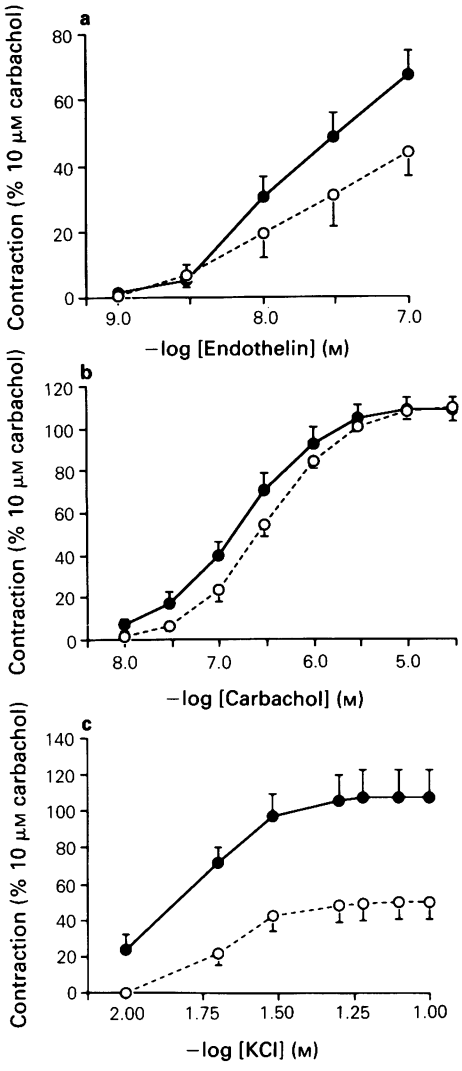

Figure 5 Effects of nicardipine (0.1 μM) on concentration-response curves produced by (a) endothelin, (b) carbachol and (c) KCl in guinea-pig intact trachea. Results are expressed as a percentage of the reference contraction to 10 μM carbachol and are the mean with vertical lines indicating s.e.mean. (●——●) Control tissues; (○---○) treated tissues; (a) $n = 7$; (b) $n = 4$; (c) $n = 4$. Studies examining KCl-induced contractions were conducted in the presence of 1 μM atropine. Experiments were conducted in the presence of 5 μM indomethacin.

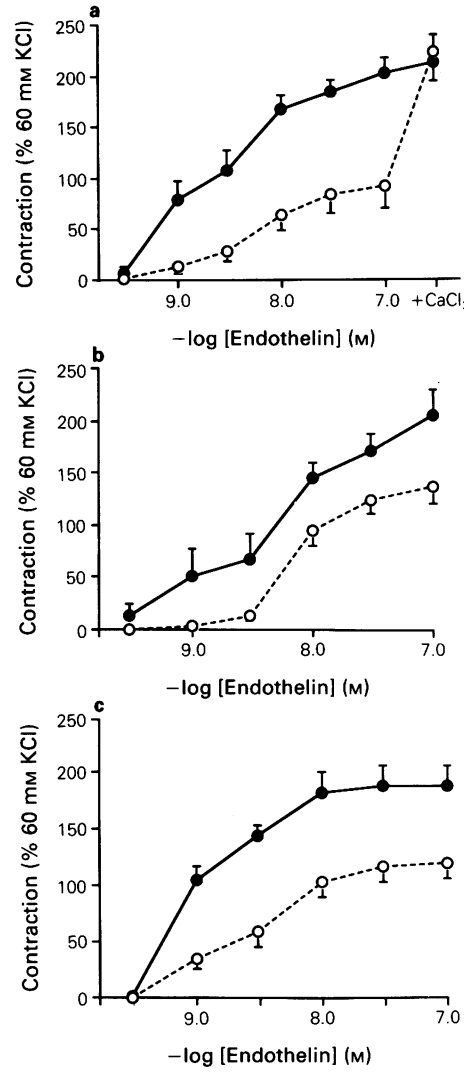

Figure 6 Effects of (a) 30 min incubation in calcium-free Krebs +0.1 mM EGTA, (b) 0.01 μM nicardipine or (c) 0.1 μM nicardipine on endothelin concentration-response curves in rat endothelium-denuded aorta. Results are expressed as a percentage of the reference contraction to 60 mM KCl and are the mean with vertical lines indicating s.e.mean. (●——●) Control tissues; (○---○) treated tissues; (a) $n = 8$; (b) $n = 4$; (c) $n = 4$. Re-addition of 2.5 mM $CaCl_2$ to tissues is indicated by $+CaCl_2$. Experiments were conducted in the presence of 5 μM indomethacin.

Burka, 1983), responses produced by endothelin in guinea-pig trachea are normally attenuated by a bronchodilator prostanoid(s), presumably prostaglandin E_2 (PGE_2). This finding is in contrast to the effects of indomethacin against bronchoconstriction produced by administration of endothelin *in vivo*. Thus, indomethacin abolished bronchospasm induced by intravenous administration of endothelin (Payne & Whittle, 1988) and significantly inhibited bronchoconstriction produced by aerosol administration of endothelin in guinea-pigs (Lagente *et al.*, 1989). This suggests that administration of endothelin *in vivo*, especially via the intravenous route, produced bronchospasm in guinea-pigs indirectly via the release of contrictor cyclo-oxygenase products of arachidonic acid. One likely candidate is thromboxane, a potent bronchoconstrictor in the guinea-pig (Svensson *et al.*, 1977), as it has been shown that endothelin releases eicosanoids, including thromboxane, from guinea-pig isolated lung (De Nucci *et al.*, 1988. However, this mechanism is not involved in endothelin-induced contraction in guinea-pig isolated trachea, as reflected by the lack of inhibitory effect of indomethacin, or a specific thromboxane receptor antagonist SQ 29,548 (Ogletree *et al.*, 1985), in concentrations that produced substantial inhibition of contractions elicited by the thromboxane mimetic, U-44069 (data not shown).

Although the data indicate that endothelin produces contraction of guinea-pig isolated trachea by a direct action, the response is influenced by the epithelium. Removal of the epithelium increased the responsiveness of the tracheal smooth muscle to the contractile effects of endothelin, similar to the observations obtained for other bronchoconstrictor and bronchorelaxant agents in airways from several species including man (Fedan *et al.*, 1988). The mechanism(s) responsible for this modulatory influence of the epithelium remains controversial, although postulates for which there is relatively convincing evidence include: (i) the epithelium is a source of prostanoid and non-prostanoid factors (Flavahan *et al.*, 1985; Hay *et al.*, 1986; Tschirhart *et al.*, 1987; Vanhoutte, 1988; Braunstein *et al.*, 1988) and (ii) the epithelium is a site of metabolism for some agents including catecholamines (Farmer *et al.*, 1986) and neurokinins (Devillier *et al.*, 1988; Frossard *et al.*, 1989). There is preliminary evidence to suggest that the epithelium inhibits contraction produced by endothelin in guinea-pig isolated trachea by acting as a locus for its meta-

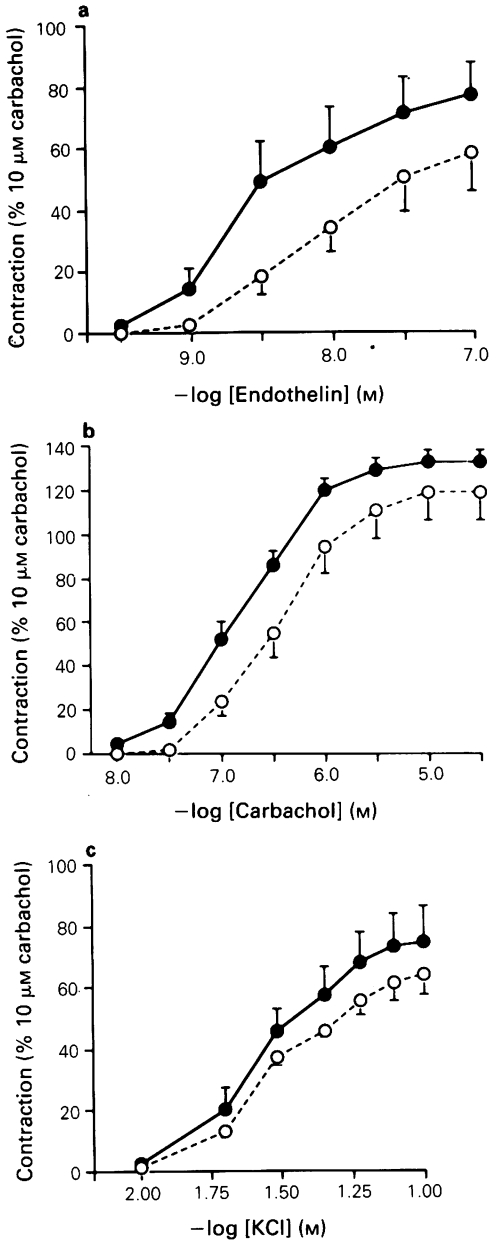

Figure 7 Effects of 10 μM ryanodine on concentration-response curves produced by (a) endothelin, (b) carbachol and (c) KCl in guinea-pig intact trachea. Results are expressed as a percentage of the reference contraction to 10 μM carbachol and are the mean with vertical lines indicating s.e.mean. (●——●) Control tissues; (○---○) treated tissues; (a) $n = 8$; (b) $n = 4$; (c) $n = 4$. Studies examining KCl-induced contractions were conducted in the presence of 1 μM atropine. These studies were performed in calcium-free Krebs +0.1 mM EGTA (30 min pretreatment). Experiments were conducted in the presence of 5 μM indomethacin.

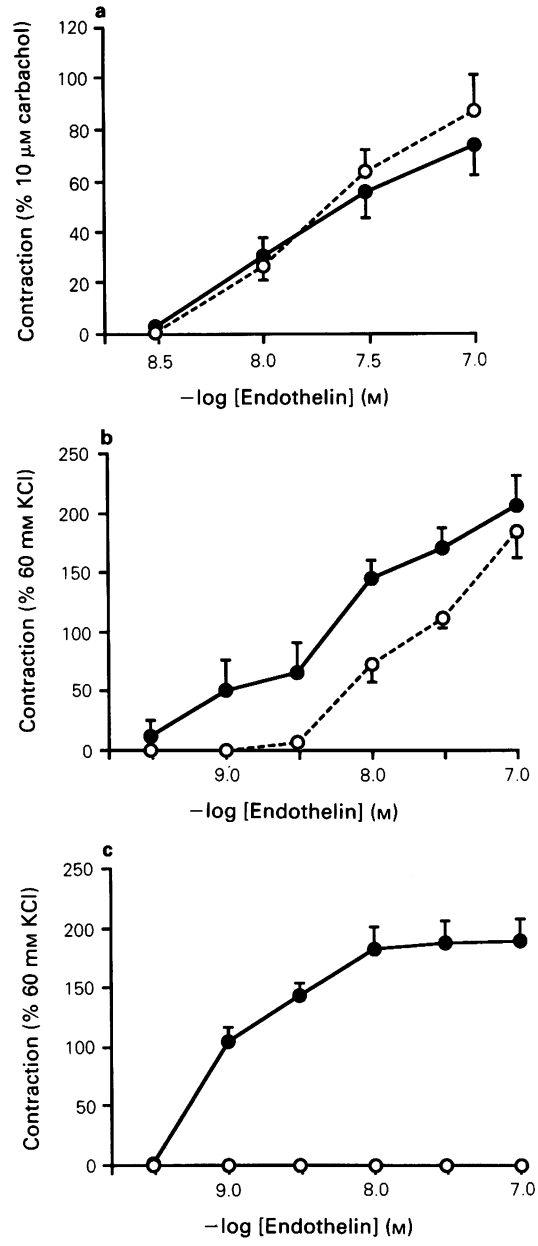

Figure 8 Effects of staurosporine (0.01 μM or 0.1 μM) on concentration-response curves produced by endothelin in (a) guinea-pig intact trachea or (b, c) rat endothelium-denuded aorta. Results are expressed as a percentage of the reference contraction to (a) 10 μM carbachol or (b, c) 60 mM KCl and are the mean with vertical lines indicating s.e.mean. (●——●) Control tissues; (○---○) treated tissues; (a) $n = 9$; (b) $n = 4$; (c) $n = 4$. Tissues were exposed to 0.1 μM staurosporine (a and c) or 0.01 μM staurosporine (b) for 30 min before construction of agonist concentration-response curves. Experiments were conducted in the presence of 5 μM indomethacin.

bolism (Hay *et al.*, 1989a). It has recently been shown that the epithelium also attenuates endothelin-induced contraction in the guinea-pig main bronchus (Maggi *et al.*, 1989).

In the first publication on endothelin, contraction of porcine coronary artery was shown to be abolished by incubation in calcium-free medium +1 mM EGTA and substantially inhibited by nicardipine, a dihydropyridine calcium channel inhibitor (Yanagisawa *et al.*, 1988). Based on these findings it was proposed that endothelin elicited contraction by activation of the dihydropyridine-sensitive calcium channels, i.e., by acting as an endogenous calcium channel 'agonist' or activator (Yanagisawa *et al.*, 1988). A similar mechanism was postulated to account for the endothelin-induced contraction of guinea-pig isolated trachea, a response which was partially inhibited by nicardipine (Uchida *et al.*, 1988). However, the majority of studies published subsequent to the original study provide evidence that endothelin acts largely via a mechanism other than activation of dihydropyridine-sensitive channels (see below). In the present study 30 min incubation in calcium-free medium +0.1 mM EGTA had no effect on endothelin-induced contraction of guinea-pig trachea. In addition nicardipine, at a concentration of 0.01 μM, also had no effect on responses produced by endothelin, whereas increasing the concentration of nicardipine to 0.1 μM had a significant inhibitory effect. A high concentration of nifedipine (1 μM) was shown to possess only a small inhibitory effect against response produced by endothelin in guinea-pig isolated trachea (Maggi *et al.*, 1989). These data would suggest that only a small component of the endothelin-induced contraction is mediated by calcium influx via dihydropyridine-sensitive

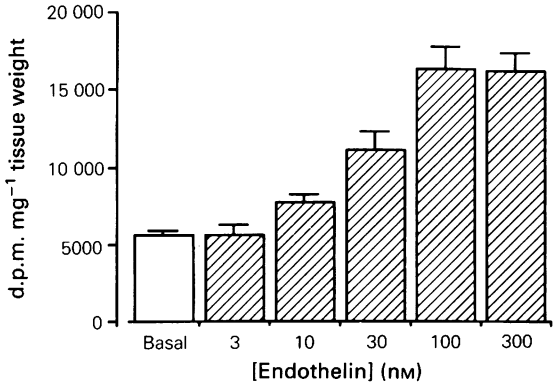

Figure 9 Endothelin-induced stimulation of phosphatidylinositol turnover in epithelium-containing guinea-pig trachea from which the cartilage had been removed. Results are expressed as d.p.m. mg^{-1} tissue dry weight and are the mean with bars showing s.e.mean of 7–8 tissues. Tissues were exposed to endothelin for 60 min and total inositol phosphates were then extracted as outlined in the Methods section. The calculated EC_{50} (−log M) was 7.51 + 0.06. Experiments were conducted in the presence of 5 μM indomethacin.

calcium channels. In fact, the inhibitory effect of these high concentrations of the dihydropyridines may be mediated via a non-specific mechanism other than their recognized action of inhibition of voltage-dependent calcium channels; for example, phosphodiesterase inhibition (Norman *et al.*, 1983).

Increasing the concentration of EGTA in the calcium-free medium to 1 mM abolished endothelin-induced contraction. One postulate is that the contraction of guinea-pig trachea induced by endothelin is mediated by utilization of a high affinity, superficially-bound (i.e., membrane) source of calcium that is removed by high concentrations of EGTA. However, one has to consider that incubation in calcium-free medium + 1 mM EGTA for 30 min may produce attenuation of endothelin-induced contraction by depleting the intracellular calcium stores responsible for the response, rather than by chelation of extracellular or superficially bound calcium (Wheeler & Weiss, 1979; Guan *et al.*, 1988). For example, responses induced by carbachol were further inhibited following incubation for 30 min in calcium-free medium + 1 mM EGTA, suggesting significant depletion of intracellular calcium stores. The differences in the effects of calcium-free medium + 1 mM EGTA on carbachol- and endothelin-induced contraction suggests differences in the mechanism(s) and relative contributions of extracellular and intracellular calcium sources responsible for these responses. Furthermore, the larger inhibitory influence of ryanodine on endothelin-induced contraction compared to those produced by carbachol may indicate differences in the relative efficacies of endothelin and carbachol for stimulating the release of intracellular calcium.

In rabbit aorta, although the endothelin-induced contraction is resistant to calcium channel inhibitors, it is markedly attenuated (approximately 70–80% reduction in the maximum response) by incubation in calcium-free medium + EGTA (Ohlstein *et al.*, 1989; Sugiura *et al.*, 1989) suggesting that it involves utilization of a superficial or membrane-associated pool of calcium that is accessible to EGTA. In contrast, in the present study endothelin-induced contraction in rat aorta was sensitive to inhibition either by nicardipine or by incubation in calcium-free medium + EGTA. This suggests that both tissue and species differences exist in the relative calcium sources responsible for endothelin-induced contraction.

It is clear that the endothelin-induced contraction of guinea-pig isolated trachea is partially resistant to the effects of the dihydropyridine calcium channel inhibitors and also calcium-deprivation with 0.1 mM EGTA, suggesting that the response is mediated to a significant extent by mobilization of calcium from intracellular stores. This proposal is supported by the data from these studies using ryanodine. Ryanodine is a naturally occurring alkaloid that has been shown to affect calcium mobilization specifically from the sarcoplamic reticulum in a variety of tissues including vascular and airway smooth muscles (Ito *et al.*, 1986; Hwang & Van Breemen, 1987; Gerthoffer *et al.*, 1988). In the present study, ryanodine produced more inhibition of the endothelin-induced contraction than of responses produced by carbachol, and did not inhibit KCl-induced responses. These findings are similar to those obtained by Gerthoffer and co-workers (Gerthoffer *et al.*, 1988) who observed that in canine trachealis ryanodine inhibited carbachol-induced contraction—which appears to involve predominantly mobilization of intracellular calcium release—but not KCl- or 5-hydroxytyptamine (5-HT)-induced contractions which are more reliant on the influx of extracellular calcium (Gerthoffer, 1986; Gerthoffer *et al.*, 1988).

Further support for endothelin inducing contraction of guinea-pig trachea partly by release of calcium from intracellular stores is the finding that endothelin stimulates PI turnover which, in a variety of tissues including smooth muscle, has been shown to stimulate release of calcium from the sarcoplasmic reticulum (Streb *et al.*, 1983; Hashimoto *et al.*, 1985; Somolyo *et al.*, 1987). In airway smooth muscle, agonists whose contractile responses are relatively resistant to calcium deprivation, suggesting they involve the mobilization of intracellular calcium, stimulate PI turnover and formation of IP_3 (Baron *et al.*, 1984; Hashimoto *et al.*, 1985) Takuwa *et al.*, 1986; Duncan *et al.*, 1987). In the present study the EC_{50} for endothelin-induced stimulation of PI turnover in guinea-pig intact trachea was comparable to the EC_{50} for producing contraction (45.9 nM vs 30.9 nM, respectively), suggesting that the two parameters may be linked. Endothelin stimulates PI metabolism and increases the levels of the inositol phosphates in cultured vascular smooth muscle cells from a variety of species (Resink *et al.*, 1988; Van Renterghem *et al.*, 1988; Araki *et al.*, 1989; Marsden *et al.*, 1989; Sugiura *et al.*, 1989). In addition, endothelin elevated the intracellular free concentration of Ca^{2+}, measured fluorometrically (Kai *et al.*, 1989; Marsden *et al.*, 1989) and stimulated ^{45}Ca efflux (Miasiro *et al.*, 1988; Marsden *et al.*, 1989).

Although some discrepancies exist, the majority of evidence to date is at odds with the original postulate that endothelin produces contraction of smooth muscle by activating dihydropyridine-sensitive calcium channels (Yanagisawa *et al.*, 1988). Thus, the response appears to involve mobilization of intracellular calcium, probably as a consequence of stimulation of PI turnover. A mechanism of action other than an interaction with dihydropyridine calcium channels is supported by data from binding studies, which indicate that, in rat cardiac membrane fragments, endothelin did not affect the affinity or the number of dihydropyridine binding sites and did not displace ligands for dihydropyridines and two other classes of calcium channel inhibitors (Gu *et al.*, 1989). In skeletal muscle T-tubule membrane endothelin did not affect binding of four different classes of calcium channel inhibitors (Van Renterghem *et al.*, 1988). Furthermore, specific high affinity binding of [^{125}I]-endothelin in rat aortic vascular smooth muscle cells was unaffected by nicardipine, verapamil or dilitiazem (Hirata *et al.*, 1988).

However, there appears to be a component of the response to endothelin that does involve stimulation of extracellular calcium influx via dihydropyridine-senstive membrane channels. The relative contribution of this mechanism to the endothelin-induced response is likely to depend on the tissue. For example, in the present study, endothelin-induced contraction of rat aorta was more susceptible to the effects of removal of extracellular calcium and nicardipine than was the response in the guinea-pig isolated trachea. In porcine coronary artery, endothelin-induced contraction was markedly attenuated by nicardipine (Yanagisawa *et al.*, 1988), whereas in rabbit aorta, a dihydropyridine calcium channel inhibitor had only a slight (Marsden *et al.*, 1989) or no effect (Ohlstein *et al.*, 1989) on the response. In agreement with the present study, Van Renterghem and co-workers (Van Renterghem *et*

al., 1988) observed that endothelin-induced contraction of rat aorta was inhibited markedly by a dihydropyridine calcium channel inhibitor, whereas in another study no effect was observed (Auguet *et al.*, 1988).

The role of diacylglycerol (DAG) and protein kinase C activation in endothelin-induced contraction of smooth muscle remains to be clarified. In the present study, staurosporine (0.01 and 0.1 μM) and H-7 (3 and 10 μM), two purported protein kinase C inhibitors (Hidaka *et al.*, 1984; Inagaki *et al.*, 1985; Tamaoki *et al.*, 1986) had no effect against endothelin-induced contraction of guinea-pig trachea. A higher concentration of H-7, 30 μM, markedly attenuated endothelin-induced contraction. However, the inhibitory effect of this high concentration of H-7 may be due to an action other than inhibition of protein kinase C. Thus, the data from the present study would suggest that protein kinase C is not intimately involved in endothelin-induced contraction of guinea-pig isolated trachea. In contrast, staurosporine produced marked inhibition of response to endothelin in rat aorta suggesting an involvement of activation of protein kinase C in this tissue. This highlights further the apparent differences in the fundamental mechanisms responsible for endothelin-induced contraction in rat aorta and guinea-pig trachea. However, the ability of staurosporine (0.1 μM) to abolish KCl-induced contraction suggests a non-specific mechanism of action of staurosporine, a feature of many purported selective protein kinase C inhibitors (Rüegg & Burgess, 1989). Furthermore, in rabbit aorta staurosporine (Ohlstein *et al.*, 1989) and H-7 (Sugiura *et al.*, 1989) inhibited endothelin-induced contraction. In addition, endothelin stimulated DAG formation and protein kinase C phosphorylation in cultured vascular smooth muscle cells (Griendling *et al.*, 1989; Resink *et al.*, 1989), and it was suggested that this pathway may be involved in mediating endothelin-induced vasoconstriction.

In summary, endothelin is a potent constrictor of guinea-pig isolated trachea, and appears to act predominantly, if not exclusively, by a direct action on the smooth muscle cell. Endothelin-induced contraction is inhibited markedly by the epithelium. A minor component of the response is sensitive to inhibition by a dihydropyridine-sensitive calcium channel inhibitor. However, the endothelin-induced contraction appears to involve, predominantly, a mechanism other than activation of dihydropyridine-sensitive calcium channels, namely stimulation of PI turnover and subsequent release of calcium from intracellular stores, in addition to calcium influx via a pathway that is not blocked by dihydropyridine calcium channel inhibitors but is sensitive to calcium-free medium containing a high concentration of EGTA (1 mM). Endothelin-induced contraction of rat aorta was more sensitive to the effects of incubation in Ca^{2+}-free medium, nicardipine or staurosporine, suggesting that differences exist in the relative mechanisms whereby endothelin produces contraction in different tissues.

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