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OBITUARY

EDITH BÜLBRING FRS

Photograph by Professor Giorgio Gabella

Edith Bülbring was a great personality who was famous internationally for her work on the physiology and pharmacology of smooth muscles. She retained her interest in the field right up to her death on July 5th 1990 at the age of 86 years. Her collaborators came from all parts of the world and almost all of them when they returned to their own countries became eminent in their own right. Many of them trained others in smooth muscle pharmacology and so Edith acquired a generation of scientific 'grandchildren'.

Her father was German and a professor of English first in Groningen (The Netherlands) and later in Bonn. Her mother was Jewish and Dutch and came from a well known family of bankers in The Hague. Edith was the youngest of four children (three sisters and a brother) and was born on 27th December 1903 in Bonn. Edith's Jewish origins were to have profound effects on her later life.

Her family was very scholarly and Edith became fluent in German, Dutch, French and (somewhat later) English. She could also speak tolerable Italian. She developed as an accomplished piano player under the tutelage of a family friend. Her early life was probably rather sheltered but made difficult by the death of her brother killed during the First World War and even more difficult by the death of her father in 1917. However, due to help from her mother's family Edith was able to continue her education which had begun at the Gymnasium and she entered the University of Bonn to study physiology and medicine. During her preclinical studies she worked on nerve staining with the histologist, Boeke, in Utrecht. She studied medicine also in Munich where she was attracted by the work of von Müller, a famous Professor of Medicine. She was by this time in her early twenties and she very much enjoyed the social life in Munich which included operas, dancing, skiing in the winter (which was a very energetic sport in those days) and walking in the mountains in the summer. After Munich, Edith went to Freiburg, drawn by the presence of another famous scientist, Aschoff. There she heard and was inspired by Trendelenburg's lectures on pharmacology. She returned to Bonn to finish her clinical training and her medical degree, studying the histology of phaechromocytoma cells for her doctorate. After this she was a house physician in Berlin before joining Trendelenburg to work in his laboratory in Berlin for two years (1929–1931). She worked as this famous pharmacologist's assistant until his death in 1931; among other topics she studied was the action of cardiac glycosides on the frog heart. It was in this laboratory that she began her lifelong friendship with Marthe Vogt. After Trendelenburg's death she returned to clinical work as a paediatrician in Jena and then in Berlin. However, the rise of antisemitism led to her being dismissed from her post in Berlin. She returned briefly to Bonn before coming to England in 1933.

Edith came to England with her sister Maud initially for a holiday but during this she visited her old mentor Friedemann who was working at Henry Dale's laboratory in Hampstead. In this way she met Henry Dale who introduced her to Professor J.H. Burn. He was then at the laboratories of the Pharmaceutical Society in Bloomsbury Square. So began her life and work in England which became her home. Edith worked at the Pharmaceutical Society Laboratories until 1938 when she followed Burn who had moved to head the Department of Pharmacology, Oxford. She was initially a departmental demonstrator there but became increasingly independent and was appointed successively as University demonstrator (1946), Reader (1960) and eventually to a personal Professorship (in 1967). She was elected to a Professorial Fellowship of Lady Margaret Hall in 1960 and remained a fellow of that college up to her death. Her work before 1951 was on many aspects of physiology and pharmacology. It included a description of the rat phrenic nerve-diaphragm preparation (Bülbring, 1946). She also worked with D. Whitteridge and G.L. Brown as well as publishing extensively with J.H. Burn.

Her interest in smooth muscle began in her late forties after returning from a year in Johns Hopkins University in 1949–50. Her first interest was in the metabolic activity of smooth muscle (Bülbring, 1953) but was soon followed by studies on its electrical activity (Bülbring & Hooton, 1954). It was for her work on the electrical properties and innervation of smooth muscles that she became most famous. Her main contributions were to show that tension in intestinal muscle was correlated with the frequency of spike discharge. Later she went on to show with Tadao Tomita that in smooth muscle, calcium entry was responsible for the action potential rather than sodium entry as in nerve and skeletal muscle. She showed with Hirosi Kuriyama that during the action of excitatory transmitters, such as acetylcholine, the action potential frequency was increased whereas inhibitory hormones, such as adrenaline, decreased spike discharge frequency and hyperpolarized the membrane. Her interest in the metabolism of smooth muscle continued and involved several collaborators notably Gustav Born. Her early work on excitation was done with Mollie Holman and on the actions of neutrotransmitters with Geoff Burnstock. She worked extensively on peristalsis and on the mechanism of α- and β-adrenoceptor effects in intestinal smooth muscle. Perhaps her greatest scientific contribution was to excite the interest of so many talented investigators who continued and expanded her pioneering work on smooth muscles and the actions of hormones and transmitters in laboratories around the world.

In her middle years it is probably fair to say that Edith was

a formidable woman. She pursued excellence with single-minded determination and had little time for the mediocre. In all things she was meticulous, neat, organised, generally exacting and uncompromising. Another side of her was, however, in a way impulsive and generous; she had great warmth and enthusiasm which drew those about her. Her passion for experimental work persisted even after her retirement. Her determination and indomitable spirit triumphed over the disability of losing a leg in her late seventies. Her cheerfulness never left her. Her mind was clear to the end and she never lost her interest in smooth muscle research and scientific ideas – 'Now tell me what exciting discoveries you have made' she used to say as a greeting.

Her house in north Oxford was built in the expectation that her two sisters Maud and Lucie would join her. However Maud died rather early and only Lucie came to live with her. She predeceased Edith by a few years. Well remembered are the weekly meetings of the members of her research group held after work at Edith's house where results of experiments were discussed either in the garden or in the sitting room overlooking it. Visitors were common and subjects were wide ranging – Edith was keen to learn about discoveries in other fields as well as in smooth muscle. Edith loved these informal gatherings which included over the years visitors and collaborators from all parts of the world. Outside of work Edith played the piano solo, in duet, or as an accompanist, although after retirement she gave up playing before an audience. She still enjoyed opera and paintings and had an enviable library on the old masters. Dutch painters, especially Rembrandt, were some of her favourites. She was a remarkable woman who said she had noticed no disadvantages of her gender or nationality in this country.

Edith Bülbring's work brought great credit to British Pharmacology. She became a member of the Society in 1936 and was elected to honorary membership in 1976. In 1983 she was awarded the Wellcome Gold Medal. Her work was also recognised in the numerous other honours she received both in the U.K. and abroad. She was given a personal Professorship at Oxford in 1967, nine years after she was elected Fellow of The Royal Society. She was elected honorary member of the Italian Pharmaceutical Society, Deutsche Physiologische Gesellschaft, The Physiological Society, and received honorary degrees from Universities of Groningen (The Netherlands), Leuven (Belgium), Homburg Saar (Germany) and was awarded the Schmiedeberg-Plakette der Deutschen Pharmakologischen Gesellschaft.

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SPECIAL REPORT

Ethanol inhibition of baroreflex bradycardia: role of brainstem GABA receptors

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Ethanol administered i.v. or into the nucleus tractus solitarii (NTS) of rats anaesthetized with urethane inhibits baroreflex bradycardia elicited by phenylephrine. This effect is prevented or reduced by pretreatment of rats with 3-mercaptopropionic acid, bicuculline, or RO 15-4513. Intra-NTS injection of muscimol also inhibits baroreflex bradycardia and causes a pressor response which is potentiated by intra-NTS ethanol. It is proposed that ethanol inhibits baroreflex bradycardia, at least in part, by potentiating the action of endogenous γ-aminobutyric acid (GABA) at $GABA_A$ receptors in the NTS or its vicinity.

Introduction The main inhibitory and excitatory neurotransmitters in the central nervous system are γ-aminobutyric acid (GABA) and glutamate, respectively. Recent evidence indicates that the effects of ethanol on the nervous system may be mediated through its selective potentiation of $GABA_A$ receptor-mediated events (Suzdak *et al.*, 1986) and selective inhibition of effects mediated by the NMDA-type glutamate receptor (Lovinger *et al.*, 1989). In addition to its well known neurotoxic effects, ethanol also affects cardiovascular functions. Clinical and population studies have demonstrated the hypertensive effect of chronic ethanol consumption (Potter & Beevers, 1984). Although the mechanism underlying this effect is not clear, recent studies in the rat indicate that ethanol is a potent inhibitor of the depressor baroreflex response (Zhang *et al.*, 1989), which could contribute to its hypertensive action. The first synapse of the baroreflex arc is in the medullary nucleus of the solitary tract (NTS). While the nature of the baroreflex transmitter in the NTS has not been unequivocally established, hypothalamic GABA-ergic neurones can exert powerful inhibition of the baroreflex by activating $GABA_A$ receptors in the NTS (Barman & Gebber, 1979). Here we provide evidence that the baroreflex inhibitory effect of ethanol in anaesthetized rats is due, at least in part, to its potentiation of $GABA_A$ receptor effects in the area of the NTS.

Methods Male Sprague-Dawley rats weighing 300–350 g were anaesthetized with urethane, $0.3\,g\,kg^{-1}$ i.p. plus $0.8\,g\,kg^{-1}$ i.v. The femoral vein was cannulated for drug injections and a cannula in the femoral artery was connected to a pressure transducer and polygraph for direct measurement of blood pressure and heart rate. For microinjection of drugs into the NTS, the dorsal surface of the medulla was exposed by limited craniotomy and a glass microcannula was inserted into the NTS according to published coordinates (Mastrianni *et al.*, 1989). Drugs or ethanol were microinjected slowly in volumes of 50–100 nl. Baroreflex bradycardia was elicited by the i.v. injection of graded doses of phenylephrine (5, 10, 20, $40\,\mu g\,kg^{-1}$). Peak increases in blood pressure were plotted against the corresponding peak increases in pulse period, and the slope of the regression was taken as an indicator of baroreflex sensitivity.

Results Intravenous injection of $1\,g\,kg^{-1}$ ethanol into rats anaesthetized with urethane produced blood ethanol concentrations of 109 ± 10, 95 ± 1, 74 ± 3, and 61 ± 3 mg% at 5, 15, 40 and 60 min postinjection, respectively, as measured by gas chromatography. Basal blood pressure and heart rate were not significantly changed by ethanol. Intravenous injection of phenylephrine elicited a dose-dependent pressor effect and reflex bradycardia. When phenylephrine was injected following ethanol, the reflex bradycardic response was markedly reduced, resulting in a significant reduction of the slope of the baroreflex function curve (Table 1). In agreement with a recent report (Zhang *et al.*, 1989), microinjection of ethanol into the NTS also inhibited baroreflex bradycardia: the degree of inhibition was the same after the unilateral injection of 200 nmol ethanol or the bilateral injection of 25 nmol/side ethanol (Table 1). The inhibition was reversible with normal baroreflex responses restored within 2 h. No inhibition was observed when ethanol was microinjected as little as 0.5 mm lateral or rostral from the site of injection in the NTS.

In order to test whether endogenous GABA is involved in the baroreflex inhibitory effect of ethanol, rats were treated with the GABA depleting agent 3-mercaptopropionic acid at a

Table 1 The effect on ethanol on baroreflex sensitivity

			Ethanol	
		$1\,mg\,kg^{-1}$	NTS, unilat.	NTS, bilat.
Pretreatment	*Control*	i.v.	200 nmol	25 nmol/side
—	0.84 ± 0.10	0.32 ± 0.04*		
—	0.69 ± 0.15			0.37 ± 0.08*
—	0.68 ± 0.10		0.37 ± 0.08*	
3-MP	0.83 ± 0.27	0.97 ± 0.27		
3-MP	0.54 ± 0.12		0.75 ± 0.21	
RO 15-4513	0.81 ± 0.26	0.72 ± 0.15		
Bicuculline	0.71 ± 0.13	0.53 ± 0.12*		

In each animal, baroreflex sensitivity was determined before (control) and after the administration of ethanol, as described in Methods. Numbers represent the slope of the regression in ms $mmHg^{-1}$ (means ± s.e., $n = 4$–6). Asterisks indicate significant difference from the corresponding control value, * $P < 0.005$. 3-MP (3-mercaptopropionic acid, $100\,mg\,kg^{-1}$ i.p.), bicuculline ($2\,mg\,kg^{-1}$ i.p.) or RO 15-4513 ($5\,mg\,kg^{-1}$ i.p.) were given 5 min before the first test dose of phenylephrine in the control period.

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dose of 100 mg kg^{-1} i.p., which causes a substantial decrease in brain GABA content within 15 min which lasts for more than 60 min (Roberts *et al.*, 1978). 3-Mercaptopropionic acid treatment caused a small decrease in basal blood pressure. When ethanol was administered to these rats either i.v. or intra-NTS, it did not inhibit baroreflex bradycardia (Table 1). These results suggest that endogenous GABA localized in the NTS is involved in the baroreflex inhibitory action of ethanol. In other experiments, treatment of rats with the $GABA_A$ receptor antagonist bicuculline, 2 mg kg^{-1} i.p., also attenuated, although did not abolish, the baroreflex inhibitory action of ethanol (Table 1). These findings suggest that activation of $GABA_A$ receptors contributes to the baroreflex inhibitory action of ethanol. Further evidence for this was obtained in experiments with the benzodiazepine inverse agonist, RO 15-4513 (ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo-[1,5α],[1,4]benzodiazepine-3-carboxylate), a compound that inhibits certain actions of ethanol presumably by interfering with the interaction between ethanol and the $GABA_A$ receptor complex (Ticku & Kulkarni, 1988). In rats pretreated with RO 15-4513, 5 mg kg^{-1} i.p., ethanol failed to inhibit baroreflex bradycardia (Table 1).

To test whether ethanol interacts with $GABA_A$ receptors in the NTS, we examined its effect on the response to the $GABA_A$ receptor agonist, muscimol. Muscimol microinjected into the NTS elicited a dose-dependent pressor response with no change in heart rate (Figure 1), and inhibited baroreflex bradycardia (baroreflex slope: 0.89 ± 0.12 vs 0.27 ± 0.08 ms mmHg^{-1} before and after 40 pmol muscimol, respectively, $P < 0.005$). When muscimol was tested after the intra-NTS injection of 200 nmol ethanol, it caused significant tachycardia and its pressor effect was markedly potentiated (Figure 1). The effect of ethanol on the muscimol response was selective: intra-NTS injection of 200 nmol ethanol did not influence the hypotensive and bradycardic response to 1 nmol glutamate microinjected into the same site (not shown).

Discussion The findings presented strongly suggest that potentiation of the effects of endogenous GABA at $GABA_A$ receptors in the NTS contributes to the inhibition of baroreflex bradycardia by ethanol. When ethanol was microinjected into the NTS, it inhibited baroreflex bradycardia, potentiated the effects of similarly administered muscimol and did not affect the response to glutamate. This selectivity and the reversibility of the inhibition of baroreflex bradycardia make it unlikely that the effects of ethanol in the NTS would be caused by non-specific tissue damage. The lack of effect of ethanol on the glutamate response is not in conflict with the reported inhibition by ethanol of NMDA-receptor-mediated responses (Lovinger *et al.*, 1989), as the effects of glutamate in the NTS are not affected by either NMDA or non-NMDA antagonists (Leone & Gordon, 1989).

Because of the close proximity of the NTS to the dorsal vagal nucleus it is possible that intra-NTS ethanol may have reached and interacted with $GABA_A$ receptors at the latter site (Zhang *et al.*, 1989; Mastrianni *et al.*, 1989). Indeed, there is evidence that the baroreflex inhibitory action of both ethanol (Zhang *et al.*, 1989) and GABA (Barman & Gebber, 1979) is primarily due to inhibition of the vagal outflow to the heart. Another possible site where GABA may be involved in the baroreflex inhibitory action of systemically administered ethanol is the caudal ventrolateral medulla where GABA inhibits depressor baroreflex responses through stimulation of $GABA_A$ receptors (Willette *et al.*, 1983). In any case, the present observations provide an example of the selective interaction of ethanol with a specific type of neurotransmitter receptor as the basis for one of ethanol's biological effects.

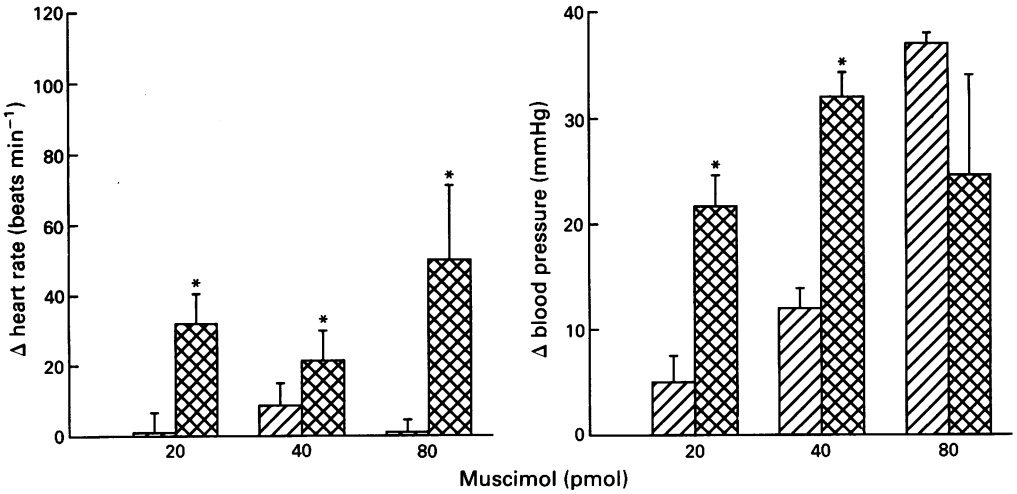

Figure 1 Ethanol potentiates the pressor and tachycardic effects of muscimol. Muscimol was injected bilaterally into the nucleus tractus solitarii (NTS) before (hatched columns) and after the bilateral intra-NTS injection of 200 nmol ethanol (cross-hatched columns). Numbers indicate the dose of muscimol in pmol/side. Vertical bars represent s.e., $n = 3$. Each rat was tested with only one dose of muscimol. * indicates significant difference between corresponding paired control and post-ethanol effects ($P < 0.05$).

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Prevention by the NMDA receptor antagonist, MK801 of neuronal loss produced by tetanus toxin in the rat hippocampus

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1 The behavioural and neuropathological effects of tetanus toxin, microinjected directly into the hippocampus, were studied in rats.

2 A single dose (1000 minimum lethal doses, MLDs) of tetanus toxin, injected unilaterally into the hippocampus produced a time-dependent neuronal loss in the CA1 pyramidal cell layer. In comparison with the contralateral, untreated side these effects became statistically significant ($P < 0.05$) 7 days (22.0 ± 1.1% reduction) and 10 days (29.2 ± 1.7% reduction) after the injection. No significant changes were observed 7 days after treatment with 500 MLDs whereas a reduction of 37.5 ± 3.1% in the CA1 area cell number was produced 4 days after the injection of 2000 MLDs.

3 Behavioural stimulatory effects were also induced by tetanus toxin (1000 MLDs) within 48 h of the injection and these culminated in generalized convulsions 5–7 days later. Convulsions were observed after a shorter period of latency in rats receiving 2000 MLDs tetanus toxin whereas 500 MLDs were ineffective.

4 No behavioural and neuropathological effects were observed in rats treated with neutralized tetanus toxin (1000 MLDs), bovine serum albumin or phosphate buffer.

5 Pretreatment with MK801 (0.3 mg kg^{-1}, i.p., given 1 h before and after the injection with tetanus toxin and then once daily for 4 or 7 days) prevented the behavioural and neuropathological effects induced by tetanus toxin (1000–2000 MLDs). In addition, such treatment fully protected the animals from the lethal effects induced by 1000 MLDs tetanus toxin. By contrast, pretreatment with diazepam (3.0 mg kg^{-1}, i.p.) using the same schedule as for MK801 did not antagonize the effects of tetanus toxin (1000–2000 MLDs).

6 In conclusion, the present experiments have demonstrated that the intrahippocampal injection of tetanus toxin produces in rats a dose- and time-dependent behavioural stimulation and neuronal loss in the CA1 pyramidal cell layer which can be prevented by the non-competitive NMDA antagonist, MK801.

Introduction

Excitatory amino acid receptors have been implicated in the mediation of neuronal degeneration produced by transient ischaemia (Simon *et al.*, 1984; Rothman & Olney, 1986). In particular, activation of the N-methyl-D-aspartate (NMDA) receptor subtype appears to play a major role since antagonists of this receptor complex, e.g. 2-aminophosphonovaleric acid and MK801, prevent ischaemic damage as well as the degeneration produced by administration of NMDA receptor agonists (Simon *et al.*, 1984; Wong *et al.*, 1986; Foster *et al.*, 1987; Gill *et al.*, 1987). In normal brain, neuronal cell activity is maintained by the integrated actions of excitatory and inhibitory afferents. In many brain regions, such as the hippocampus, glutamic acid and γ-aminobutyric acid (GABA) are the neurotransmitters which probably subserve these functions (Fagg & Foster, 1983; Taylor, 1988). Under abnormal conditions when neurodegeneration occurs the synaptic extracellular concentration of glutamate increases (Beneviste *et al.*, 1984) producing neuronal excitation (Suzuki *et al.*, 1983). However, excitation and perhaps degeneration could also arise without any net change in glutamate levels if the GABA-mediated inhibitory input is reduced. We have tested this hypothesis using tetanus toxin which selectively blocks the GABAergic inhibitory tone in the CNS (Davies & Tongroach, 1979) and the release of GABA in rat hippocampal slices (Collingridge *et al.*, 1981).

Methods

Injections and histology

Adult male Wistar rats (280–300 g) were anaesthetized with chloral hydrate (400 mg kg^{-1}, i.p.) and tetanus toxin microinjected unilaterally into the CA1 hippocampal area (coordinates A = −4 mm from the bregma, L = 2 mm from the midline, V = 2.4 mm below the dura mater according to the rat brain atlas of Paxinos & Watson, 1982; the volume of injection was 1 μl min^{-1}) by means of a Hamilton microsyringe (5 μl) mounted on a stereotaxic frame. Animals injected with bovine serum albumin (BSA), phosphate buffer (pH = 7.0; used to dissolve tetanus toxin) and neutralized tetanus toxin (the neutralization was carried out with a F(ab)' fragment of the native IgG antitetanus toxin as previously described by Gawade *et al.*, 1985) were used as controls. After 1, 4, 7, 10 days the animals were anaesthetized and perfused – fixed by intracardiac administration of 100 ml 0.1% paraformaldehyde in phosphate buffered (pH = 7.0) saline. Brain coronal sections were cut from a 1 mm brain block which included the needle track and every tenth slice for 300 μm either side of the track was stained with cresyl fast violet, toluidine blue or a silver stain procedure described by Gallyas *et al.* (1980). In pilot experiments we have determined that the damage produced by tetanus toxin extended at least 400 μm either side of the injection site. The number of cells was counted in areas of 3700 μm^2 in each of the pyramidal cell layers of CA1, CA2 and CA3 hippocampal regions and granule cell layer of the dentate gyrus, stained with cresyl fast violet. Cell counting was always performed 'blind' at the same location for all slices ($n = 6$ sections per brain; the site of injection was the same for both control and tetanus toxin-treated animals). Only cells showing normal morphological characteristics were included. Pyknotic cells or cells with cytoplasmic vacuolization and swollen membranes were excluded from the count.

The mean number of neurones from each area was pooled and expressed as mean (±s.e.mean) neurones per brain area per treatment group ($n = 3$–6 brains per treatment group; 6 sections per brain). The pooled mean cell number counted in the treated side was compared with the corresponding contralateral area (control) and the statistical difference within each group was evaluated by Student's *t* test.

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Behavioural and survival study

For the behavioural and survival study, 10 rats from each group of treatment were used. Postural, locomotor and lethal effects induced by the treatments were studied twice daily in blind conditions. The mortality rate was stored and is reported as a percentage of survivors.

Drugs

Tetanus toxin (2.5×10^7 mouse minimum lethal doses, MLDs mg^{-1} of protein) and the F(ab)' fragment were kind gifts of Prof. B. Bizzini (Pasteur Institute, Paris) and were dissolved in phosphate buffer (pH = 7.0). Bovine serum albumin (Sigma, U.S.A.) and MK801 (Dizocilpine, ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate, SEMAT, U.K.) were dissolved in twice distilled, pyrogen-free H_2O. The commercially available vials of diazepam (Valium, Roche, Switzerland) were used.

Results

Neuropathological effects

A single dose of tetanus toxin (1000 MLDs; $n = 6$ rats) injected into the right hippocampus failed to produce any neuropathological effects 24 h after the treatment. By contrast, a statistically significant ($P < 0.05$) reduction in the number of cells in the CA1 region was observed after 7 and 10 days ($n = 4$–6 rats respectively). The number of cells was reduced by 22.0 ± 1.1% and 29.2 ± 1.7% at 7 and 10 days, respectively (Table 1). A lower dose of toxin (500 MLDs; $n = 6$ rats) did not produce any apparent reduction in cell number 7 days after treatment whereas a higher dose, 2000 MLDs ($n = 3$ rats) produced a significant ($P < 0.05$) reduction (37.5 ± 2.3%) in the CA1 area cell number. A summary of the quantitative changes produced by these three doses of tetanus toxin at various times is shown in Table 1. The corresponding morphological changes are illustrated in Figure 1 and Figure 2. In rats treated with bovine serum albumin (BSA; 300 ng), phosphate buffer (vehicle for tetanus toxin) or neutralized tetanus toxin (1000 MLDs; by using a 3 fold excess of a non precipitable F(ab)' fragment of the native specific IgG, see Gawade *et al.*, 1985) no morphological or quantitative changes in neuronal cell numbers were observed ($n = 6$ rats per each treatment); (Table 1).

Behavioural effects

Tetanus toxin (1000 MLDs; $n = 10$ rats) produced both postural and locomotor changes characterized by tail rigidity, hunched back, turning and touch and sound-evoked circling, ipsilateral to the site of injection, within 48 h of the treatment. These effects were observed in all of the toxin-treated rats and culminated 5–7 days later in generalized convulsions. Similar effects were evoked by 2000 MLDs tetanus toxin ($n = 10$ rats) although the animals were only observed for a shorter period of time (4 days) due to the high mortality rate (see Figure 3). Minor behavioural changes (i.e. piloerection, tail rigidity and occasionally touch-evoked ipsilateral circling) were produced by 500 MLDs ($n = 10$ rats).

Table 1 Neuronal loss induced by tetanus toxin, directly microinjected into the rat CA1 hippocampal area and its antagonism by MK801

Treatment group	*Number of animals*	CA1		CA2		CA3		DG	
		L	R	L	R	L	R	L	R
Tetanus toxin (1000 MLDs) 1 day after	6	36.5 ± 0.7	32.6 ± 0.6	31.7 ± 0.7	31.0 ± 0.7	16.7 ± 0.5	15.6 ± 0.3	79.2 ± 0.7	78.1 ± 1.1
Tetanus toxin (1000 MLDs) 7 days after	4	44.5 ± 1.7	34.7 ± 1.7*	38.4 ± 1.7	35.9 ± 2.1	20.3 ± 0.5	18.8 ± 0.5	92.0 ± 5.2	95.5 ± 5.7
Tetanus toxin (1000 MLDs) 10 days after	6	37.2 ± 0.9	26.3 ± 0.9*	34.4 ± 0.5	31.5 ± 0.5	19.7 ± 0.5	18.0 ± 0.4	76.1 ± 0.9	74.2 ± 0.7
Neutralized toxin (1000 MLDs) 7 days after	6	44.9 ± 1.8	44.7 ± 1.6	40.3 ± 0.6	39.4 ± 0.5	25.1 ± 0.6	24.6 ± 0.7	82.9 ± 1.8	82.6 ± 1.6
Tetanus toxin (500 MLDs) 7 days after	6	37.7 ± 0.5	35.5 ± 0.4	28.1 ± 0.6	26.1 ± 0.5	22.0 ± 0.4	20.4 ± 0.7	71.7 ± 0.9	68.5 ± 0.6
Tetanus toxin (2000 MLDs) 4 days after	3	39.7 ± 0.7	24.8 ± 1.0*	29.7 ± 0.7	27.4 ± 0.4	24.6 ± 0.5	22.3 ± 0.7	68.9 ± 0.9	65.4 ± 0.9
Tetanus toxin (1000 MLDs) + MK801 (7 days after)	6	39.5 ± 1.0	37.9 ± 0.9	31.9 ± 0.3	30.5 ± 0.4	22.7 ± 0.3	21.7 ± 0.3	76.6 ± 0.3	74.0 ± 0.3
Tetanus toxin (2000 MLDs) + MK801 (7 days after)	3	39.9 ± 1.2	35.8 ± 1.2	35.2 ± 1.2	32.2 ± 1.3	21.2 ± 0.9	19.3 ± 0.6	73.4 ± 2.0	71.6 ± 2.3
Tetanus toxin (2000 MLDs) + Diazepam (4 days after)	3	40.5 ± 0.3	27.9 ± 0.6*	36.7 ± 0.5	34.3 ± 0.6	24.2 ± 0.7	21.3 ± 0.6	78.6 ± 0.8	75.8 ± 0.9

Brain sections (10 μm) of perfused-fixed rats ($n = 3$–6 per treatment group) were analysed under light microscopy (Leitz 40×). Cell counting was performed blind and the analyses concerned the pyramidal layers of CA1, CA2 and CA3 hippocampal areas and the dentate gyrus (DG). Quantitation of the cell numbers (dark degenerating neurones were excluded from the counting) was performed with a 3700 μm^2 box positioned in corresponding areas of control and treated hippocampus within each section ($n = 6$ sections per brain). Data are represented as means ± s.e.mean of cells. Statistically significant changes within the means of treated (R) vs control (L; untreated) side were evaluated by using Student's t test (unpaired data). Due to the interanimal variations in the cell numbers within the same areas, comparison between groups of treatment was not allowed. * denotes $P < 0.05$.

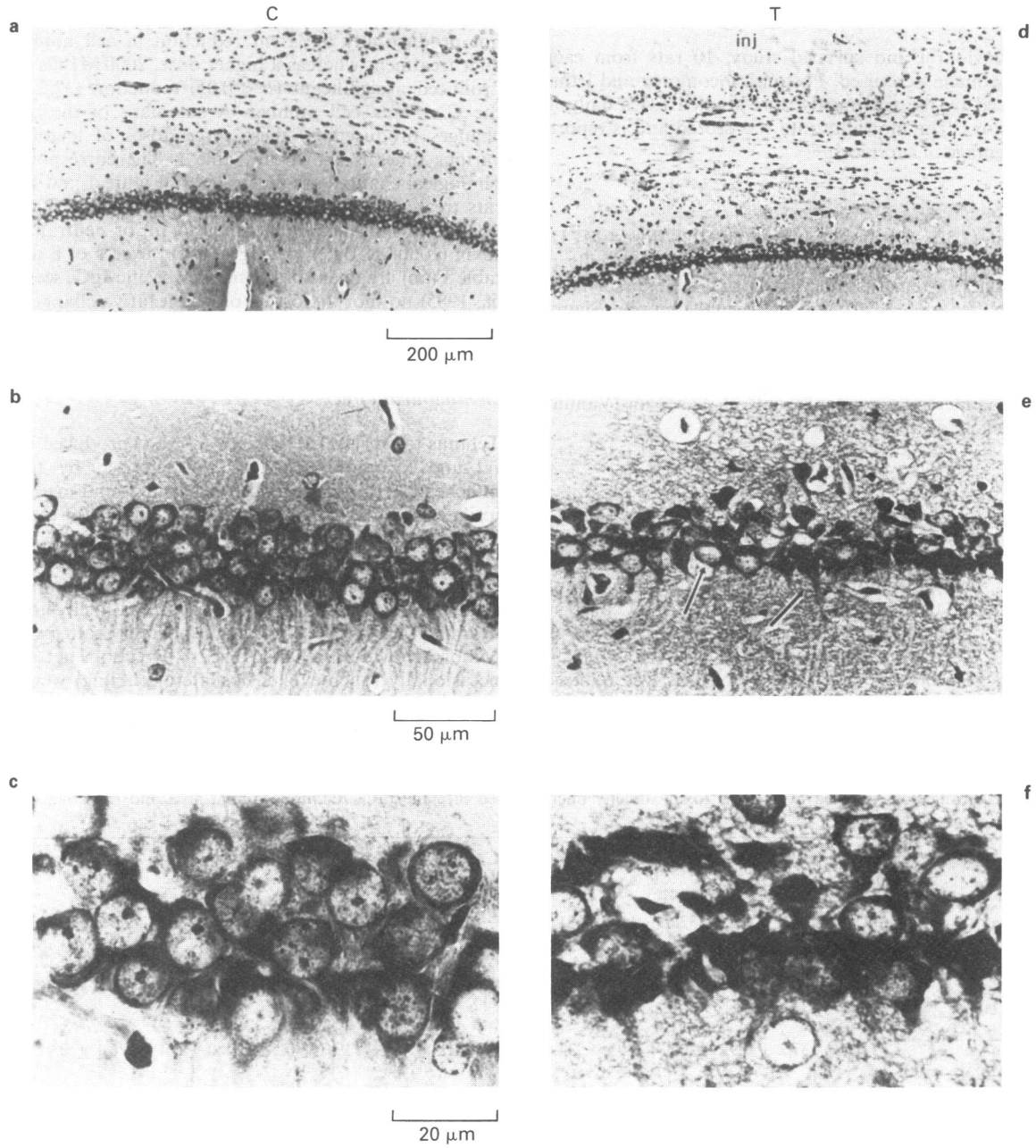

Figure 1 Light photomicrographs of a rat brain coronal section (10 μm; toluidine blue stain) depicting a typical pattern of neuronal damage in the CA1 pyramidal cell layer seven days after a single injection of tetanus toxin (1000 MLDs; d, e and f) compared with the contralateral control (uninjected) side (a, b and c). The same area of damage in (d) (10 × magnification) is also shown at higher magnification in (e) (40 ×) and (f) (100 ×). Note the dark somatodendritic staining (right arrow) and the chromatolytic phase in some neurones (left arrow). T = tetanus toxin-treated side; C = control (untreated) side; Inj = injection tract.

Antagonism study

In an attempt to test our hypothesis that excitatory amino acid receptor activation was responsible for the neuropathological and behavioural response to tetanus toxin, rats were treated with either diazepam at a concentration known to be active in blocking generalized seizures (Goodman & Gillman, 1985) or with MK801, a selective NMDA receptor antagonist (Wong *et al.*, 1986; Kemp *et al.*, 1987) which is able to cross the blood brain barrier after systemic administration (Clineschmidt *et al.*, 1982). Diazepam (3.0 mg kg^{-1}, i.p., given 1 h before and after the toxin injection and once a day for 4 or 7 days after) did not show any protective effect against the neuropathological (Table 1) or behavioural effects induced by 1000 or 2000 MLDs tetanus toxin (n = 10 rats per each dose), respectively. By contrast, MK801 (0.3 mg kg^{-1} i.p., given 1 h before and after the toxin injection and once a day for 4 or 7 days, n = 10 rats per each group of treatment) completely abolished the neuropathological effects of both 1000 and 2000 MLDs tetanus toxin (Table 1 and Figure 4). In neither group was any convulsive behaviour observed. At the end of the study period (day 10) there was a significantly greater survival of animals treated with tetanus toxin and MK801 compared to those treated with tetanus toxin alone ($P < 0.001$ by G-test of independence with Williams correction) (Figure 3).

Discussion

The present results have shown that the injection of tetanus toxin into the rat hippocampus produces potent behavioural stimulation accompanied by neuronal loss in the CA1 pyramidal cell layer. Control experiments carried out with neutralized tetanus toxin indicate that the toxin itself and not contaminants was responsible for the behavioural and neuropathological effects. The behavioural effects of tetanus toxin

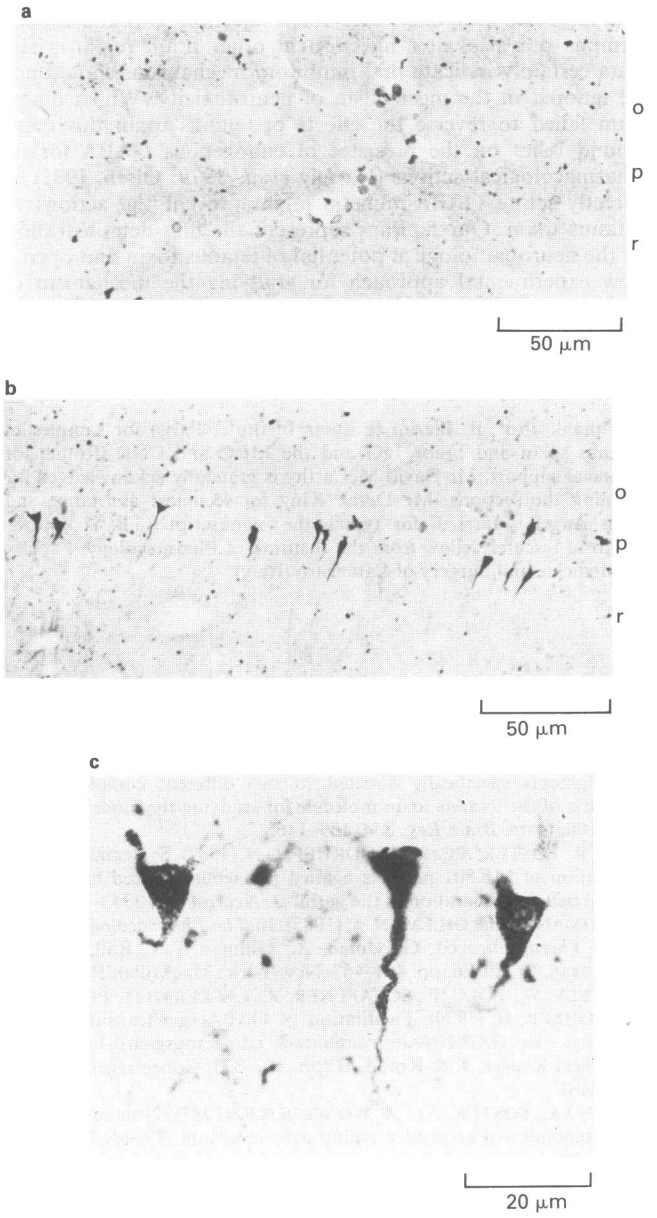

Figure 2 Rat brain section (14 μm) showing the CA1 hippocampal areas, stained by the procedure of Gallyas *et al.* (1980) to illustrate the effect of tetanus toxin (1000 MLDs) injected ten days earlier. In (b) and (c) (40 × and 100 × magnification respectively), tetanus toxin was injected and in this hippocampus argyrophilic neurones were observed in the pyramidal cell layer. In the contralateral side (a) no silver impregnation was observed. ○ = stratum oriens; p = stratum pyramidale; r = stratum radiatum.

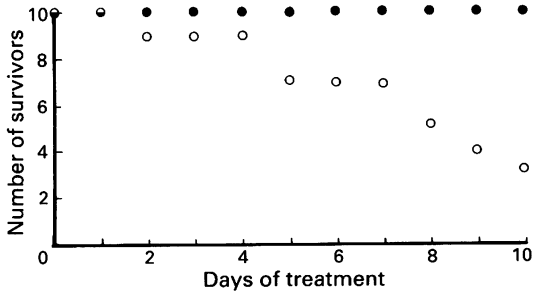

Figure 3 Time course of the lethality induced by tetanus toxin (1000 MLDs; ○) and its antagonism by systemic treatment with MK801 (0.3 mg kg^{-1}, i.p.; ●). Note that MK801 abolished the lethal effects even ten days after tetanus toxin injection. Treatment with diazepam (3.0 mg kg^{-1}), with the same schedule as for MK801, failed to show any protective effects and the lethality was not significantly different from tetanus toxin alone (data not shown).

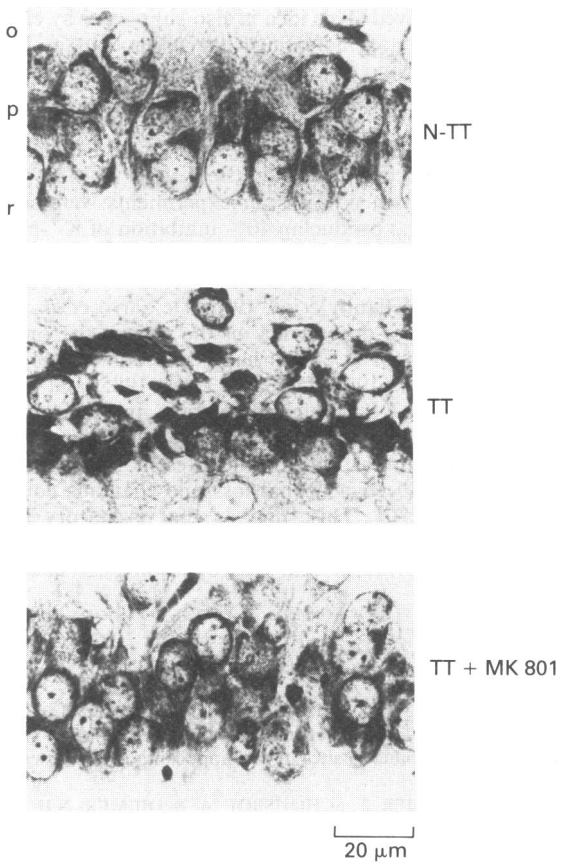

Figure 4 Light micrographs of brain coronal sections from 3 rats showing the protective effect of MK801 (TT + MK801, see text for dosage) against the neuronal damage induced by tetanus toxin (TT; 1000 MLDs). Tetanus toxin was injected seven days earlier and only the regions where the injections were made are shown. In the top panel, neutralized tetanus toxin (N-TT 1000 MLDs equivalent) was injected and no degeneration is evident. ○ = stratum oriens; p = stratum pyramidale; r = stratum radiatum. Magnification 100 ×. Toluidine blue stain.

observed in our study have been described previously by other groups (Mellanby *et al.*, 1977; De Sarro *et al.*, 1985). However, prevention of these effects by an NMDA antagonist has never been demonstrated before. Interestingly the inability of an enhancer of GABAergic function to suppress the behavioural effects has already been described. De Sarro *et al.* (1985) failed to obtain any reversal in behaviour with sodium valproate. The neuropathological effects of tetanus toxin under our experimental conditions were limited to the area close to the injection site which confirms the poor penetration of tetanus toxin within brain tissue after its focal injection, as described by Mellanby & Thompson (1977) using ^{125}Iodine-labelled toxin. This has also been confirmed by an autoradiographical study in which a significant reduction in $GABA_A$ receptor binding sites has been observed in the CA1 pyramidal cell layer but not in other hippocampal regions, 7 and 10 days after the focal application of tetanus toxin (Bagetta *et al.*, 1990). The neuronal population most vulnerable to the effects of tetanus toxin appeared to be the pyramidal cells but other experimental studies, such as detection of GAD-immunoreactive neurones, are required. The effects of the toxin were time- and dose-dependent although the dose-response curve appeared to be very steep. The minor behavioural effects observed with 500 MLDs indicated that the toxin had exerted some effect but this was insufficient to produce neurotoxicity. If, as we suspect, the neurotoxicity results from an unopposed action of excitatory transmitter, a major reduction in GABA release would be required. Thus partial inhibition might be expected to produce some behavioural changes without allowing the threshold for glutamate

toxicity to be achieved. This idea is also supported by electrophysiological evidence indicating that tetanus toxin blocks inhibitory transmission (mainly GABAergic) at the presynaptic level, leaving unaffected the excitatory input within the CNS (Curtis & De Groat, 1968; Curtis *et al.*, 1973; Davies & Tongroach, 1977; Calabresi *et al.*, 1989) and in foetal mice dissociated spinal cord neurones (Bergey *et al.*, 1987). The doses of tetanus toxin used in our experiments were smaller than that (10^4 LD_{50}) producing 40% inhibition of K^+-evoked GABA release in hippocampal slices (Collingridge *et al.*, 1981) but similar to those used for producing an excitatory focus in the same region (Mellanby *et al.*, 1977; De Sarro *et al.*, 1985). In addition, the dose-dependency of the rate of the onset of the tetanus toxin-evoked effects has been confirmed both *in vivo* (Mellanby *et al.*, 1977; De Sarro *et al.*, 1985) and *in vitro* (Bergey *et al.*, 1987) although differences in the purity of separate batches of toxin may invalidate such comparisons. The most important aspects of the present study are that tetanus toxin produced neuronal loss and that this could be prevented by MK801. This suggests that excitatory amino acid transmission might play an important role in the mediation of the behavioural and neuropathological effects of tetanus toxin not only after microinjection into the hippocampus but also after injection in other brain regions. The data certainly indicate that inhibitory mechanisms should not be ignored in the mechanism of neurotoxicity. Whilst diazepam failed to reverse the effects of tetanus toxin this compound relies on the presence of endogenous GABA for its pharmacological actions (Haefely *et al.*, 1979; Olsen, 1981). A directly-acting GABA-mimetic might prevent the actions of tetanus toxin. Our findings represent the first demonstration of the neuropathological potential of tetanus toxin and open a new experimental approach for studying the mechanism of action of the toxin as well as of neurotoxicity. In addition, the excitatory amino acid receptor antagonism may provide a therapeutic strategy for the treatment of human tetanus.

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Activities of endothelin-1 in the vascular network of the rabbit ear: a microangiographic study

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1 The effects of endothelin-1 on perfusion pressure and on arterial and venous diameters were examined simultaneously in a rabbit isolated ear preparation perfused with physiological buffer. The effects of hypoxia and inhibition of endothelium-derived relaxant factor (EDRF) activity on vascular responses to endothelin-1 were also investigated.

2 Endothelin-1 was potent at increasing perfusion pressure (ED_{50} = 46.7 ± 11.0 pmol; R_{max} = 85.3 ± 5.3 mmHg). The potency and maximum reactivity were not significantly affected by hypoxia, inhibition of EDRF activity with 50 μM N-nitro-L-arginine methyl ester (NAME) or a combination of hypoxia and NAME.

3 Endothelin-1 caused equipotent dose-dependent constrictions of the first four generations of arterial branch vessels (G_1–G_4) but did not influence the diameter of the central ear artery except at high doses of the peptide when 'paradoxical dilatation' was observed. The peptide was also equipotent at causing constriction of the smaller venous vessels (V_1–V_4) but did not affect the large veins (V_0).

4 Under conditions of hypoxia the potency of endothelin-1 was reduced in G_2 and G_3, was unaffected in G_4 and the peptide did not significantly constrict either G_0 or G_1. Hypoxia reduced the potency of endothelin-1 in the smaller venous vessels (V_1–V_4), but conversely unmasked a marked constriction of the large veins (V_0), which was not observed under normoxic conditions.

5 NAME 50 μM abolished the vasodilator effects of acetylcholine in this preparation. Inhibition of EDRF activity with NAME under normoxic conditions did not influence the constrictor activity of endothelin-1 on the arterial or venous branch vessels. However, inhibition of EDRF activity under hypoxic conditions prevented the reduction of potency of endothelin-1 as a constrictor of arterial and venous branch vessels which occurred in hypoxia. In the presence of NAME endothelin-1 constricted V_0 in both normoxia and hypoxia with equipotency but the maximum effect was greatest in hypoxia.

6 In conclusion, endothelin-1 is a powerful vasoconstrictor which acts with greater potency in veins than arteries in the rabbit isolated ear. Although hypoxia does not influence pressor responses it nevertheless alters the spatial pattern of vasoconstriction. In particular hypoxia unmasks constriction of the large veins by endothelin-1. Constriction of these veins was also observed in the absence of EDRF in normoxia, but to a much lesser degree so that the effect of hypoxia may only be partially due to reduced EDRF activity. Hypoxia may therefore directly or indirectly increase the sensitivity of the main veins to endothelin-1.

Introduction

Endothelin-1 was the first characterized member of a family of 21 amino acid vasoactive peptides, termed the endothelins (Yanagisawa *et al.*, 1988). Endothelin-1 is a potent vasoconstrictor, not only does it produce prolonged pressor responses in the ganglion blocked rat (Yanagisawa *et al.*, 1988) but also it contracts isolated arterial and venous segments (D'Orleans-Juste *et al.*, 1988). Similarly the peptide increases perfusion pressure in both isolated and *in situ* perfused resistance beds (Hiley *et al.*, 1989; Randall *et al.*, 1989). Both Brain (1989) and Fortes *et al.* (1989) have demonstrated that endothelin-1 constricts microvessels (<40 μm). Endothelin-1 also constricts larger pial arterioles (ca. 160 μm) in piglets (Armstead *et al.*, 1989). In the rat mesentery the peptide constricts both arterioles and venules (Fortes *et al.*, 1989), moreover, in this vascular bed Warner (1990) has recently shown that endothelin-1 is more potent, in terms of pressor responses, on the venous compared to the arterial side. In contrast, in the hamster cheek pouch the vasoconstriction to endothelin-1 is entirely arterial (Brain, 1989). Using the technique of X-ray microangiography (Griffith *et al.*, 1987) we have examined simultaneously the actions of endothelin-1 on both large and small arterial and venous vessels in the rabbit ear.

The activities of endothelin-1 are known to be influenced by various factors. Warner *et al.* (1989) demonstrated that endothelin-1 causes vasodilatation which is likely to be mediated by endothelium-derived relaxing factor (EDRF). Thus inhibition of EDRF activity or destruction of the endothelium is associated with increased reactivity of the peptide in isolated arterial resistance vessels (Randall *et al.*, 1989) which may be due to the loss of tonic EDRF or that released by endothelin-1 itself. However, in the conscious rat pressor responses to endothelin-1 are not augmented by treatment with N^G-monomethyl-L-arginine (Gardiner *et al.*, 1989), suggesting that *in vivo* other mechanisms may also operate. In the present study we describe the actions of endothelin-1 in the presence of N-nitro-L-arginine methyl ester (NAME), an inhibitor of the synthesis of the nitric oxide component of endothelium-derived relaxing factor (EDRF) (Moore *et al.*, 1990).

The activities of a variety of vasoconstrictors are known to be augmented under conditions of hypoxia (Van Neuten & Vanhoutte, 1980). Under such conditions the release of a diffusible endothelium-derived vasoconstrictor has been demonstrated (Rubanyi & Vanhoutte, 1985; Kwan *et al.*, 1989). In the pithed rat MacLean *et al.* (1989) have shown that the potency of endothelin-1 as a vasoconstrictor in the intact mesenteric arterial bed is doubled in hypoxia produced by lowering the ventilation volume. Also, Liu *et al.* (1989; 1990) have demonstrated that global cardiac ischaemia, but not hypoxia, increases the proportion of endothelin-1 binding sites in the cardiac plasma membrane due to the movement of receptors from the cytosol. In view of the possibility of hypoxic facilitation of vascular responses to endothelin-1 the activity of the peptide was studied in the rabbit ear under hypoxic conditions. The effects of hypoxia on vasomotor tone were also considered in the resting preparation.

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Methods

Preparation of the rabbit ear vascular bed

Male New Zealand White rabbits (2–2.5 kg) were killed by a blow to the back of the head. An ear was removed and the central artery cannulated (Portex PP50 tubing). The preparation was perfused at a rate of 2 ml min^{-1} with Holman's solution (composition in mmol l^{-1}: NaCl 120, KCl 5, $CaCl_2$ 2.5, NaH_2PO_4 1.3, $NaHCO_3$ 25, sucrose 10 and D-glucose 11). The physiological buffer also contained 5% dextran (MW: 80000) and was gassed with either 95% O_2/5% CO_2 (normoxia) or 95% N_2/5% CO_2 (hypoxia). The perfusion fluid was maintained at 35°C.

Preparation of isolated central ear artery

The vascular bed was prepared as above. The connective tissue and skin were dissected from the central artery by cutting with a scalpel blade leaving less than 3 mm of tissue on either side. A cut was made at the apex of the ear to allow outflow of the perfusate. The ear was perfused with gassed Holman's solution and experiments were performed in the same manner as those on the intact preparation.

Experimental protocol

The ear was positioned vertically in front of a 4 μm microfocal X-ray microscope. Following an equilibration period (1 h) microangiographs were taken of the resting preparations. The exposure time was 15 s at a kilovoltage of 30 kv. Radiographic contrast was achieved by a 35 s pulse of Omnipaque (iohexol, 300 mg iodine ml^{-1}) diluted 3 fold in the perfusion fluid. Use of this agent is without significant influence on either vasomotor tone or vascular responses during the period of X-ray exposure (Griffith *et al.*, 1988).

Endothelin-1 (0.1 pmol–1000 pmol) was injected into the perfusion fluid in volumes of less than 30 μl. The perfusion pressure in the bed was measured by means of a pressure transducer placed close to the inflow cannula. The pressor response was maximal 110 s after injection of the peptide, at which time a microangiograph was taken as described above. The plateau of the response was maintained for >5 min and was unaffected by the radiographic contrast medium.

In two sets of experiments the preparations were perfused with gassed Holman's solution containing 50 μM NAME throughout the experiments. In the experiments involving hypoxia the perfusate was gassed with 95% N_2/5% CO_2 for the entire experiment. As in the normoxia experiments perfusate was removed via a syringe and oxygen tension was determined by means of a blood-gas analyser.

In order to study the effects of NAME or hypoxia on the endothelium-dependent vasodilatation to acetylcholine in the whole ear preparation tone was established by addition of 5-hydroxytryptamine and histamine to the perfusate to achieve individual concentrations of 1 μM. The vasodilator properties of acetylcholine were assessed by bolus injections of the agent, in volumes less than 30 μl, in to the perfusion system. The possibility that low doses of endothelin-1 caused inhibition of established tone was examined in a similar manner. The effects of NAME on responses to acetylcholine were examined by addition of the agent after tone had been established, but 30 min before experimental determinations. Following these additions of acetylcholine, the possibility that 10 mM L-arginine reverses the effects of NAME was investigated by addition of this amino acid to the perfusate 30 min before further doses of acetylcholine. The effects of hypoxia on relaxation to acetylcholine were tested in a similar manner except the perfusate was gassed with 95% N_2/5% CO_2 throughout the experiment.

Quantitation

The microradiographs were analysed by a semi-interactive image analysis system as previously described (Griffith *et al.*, 1987; 1988; 1989). A gold calibration grid was placed on the surface of the ear to determine the magnification factor and the radiographs were further magnified by means of an electronic image analysis system. The diameter of each vessel studied was measured 4 times and the results averaged. The vessels were classed sequentially according to their generation of branching (arterial vessels: G_0, the central artery, and branch vessels G_1, G_2, G_3 to G_4, the smallest observed vessels; venous vessels: V_0, a large vein, V_1, V_2, V_3 to V_4, the smallest vessel) (Figure 1).

Data and statistical analysis

All data are given as the mean ± s.e.mean. Dose-response curves for the pressor responses were analysed by fitting the logistic equation:

$$R = \frac{R_{max} \cdot A^{n_H}}{ED_{50}{}^{n_H} + A^{n_H}}$$

where R is the increase in perfusion pressure, A the dose of peptide, R_{max} the maximum pressor response, n_H the slope function and ED_{50} the dose of peptide giving the half maximal response.

A modified Marquardt procedure, as implemented in the Harwell routine VB01A on a mainframe computer, was used to carry out the curve fitting (Aceves *et al.*, 1985).

The potency of endothelin-1 at changing vessel diameters is expressed as the $ED_{50(MP)}$, which is defined as dose of the peptide giving the half maximal reduction in diameter. The maximum diameter change was taken to have occurred at the

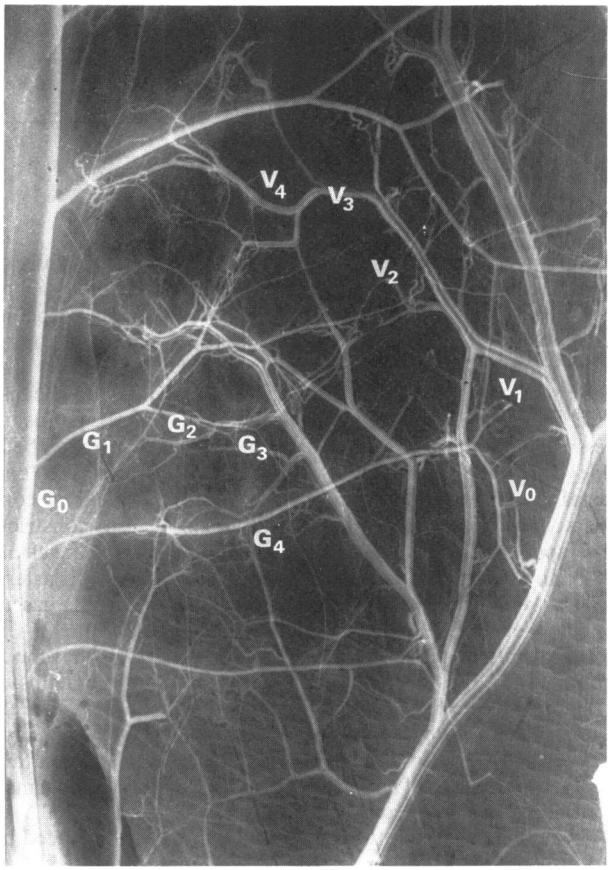

Figure 1 A high resolution angiograph of the resting rabbit ear perfused with oxygenated Holman's solution containing radiographic contrast medium (Omnipaque). The arterial and venous vessels are labelled according to their generation of branching.

dose giving the maximum pressor (MP) response in the intact ear. $ED_{50(MP)}$ values for the effects of the peptide on vessel diameter were determined for each generation in each preparation. The computer derived parameters, the $ED_{50(MP)}$ values for the diameter changes, the absolute diameter changes and perfusion pressures were compared by one way analysis of variance.

Drugs

All of the solutions, except those of endothelin-1 were prepared on the day of the experiment. N-nitro-L-arginine methyl ester, L-arginine hydrochloride, histamine dihydrochloride, and 5-hydroxytryptamine as creatine sulphate complex (all from Sigma Chemical Company, Poole, U.K.) were dissolved in saline and then diluted to the required concentrations in the Holman's solution. Omnipaque (Nycomed, U.K.) was diluted in the perfusion fluid. Acetylcholine chloride (Sigma) was dissolved in saline.

Endothelin-1 was obtained from the Peptide Institute, Osaka, Japan, via Scientific Marketing Associates, London and was initially dissolved in distilled water to give 100 μM solution. The 100 μM stock solutions were stored as 100 μl aliquots at −20°C until the day of the experiment when it was diluted to the required concentration and kept on ice.

Results

Pressor responses to endothelin-1 in the isolated perfused ear vascular bed of the rabbit

In control preparations endothelin-1 (0.1–300 pmol) produced dose-related increases in perfusion pressure (Figure 2a) which lasted for 10–30 min before returning to baseline (n = 6–11). The dose-response curve was described by an ED_{50} of 46.7 ± 11.0 pmol, the maximum calculated increase in perfusion pressure was 85.3 ± 5.3 mmHg and the slope function was 1.2 ± 0.1 (n = 6–11). Throughout the experiment the resting perfusion pressure was 35.8 ± 3.5 mmHg.

Effects of endothelin-1 on arterial and venous vessels diameters

In the intact preparation endothelin-1 (0.1–300 pmol) did not alter the diameter of the central artery (G_0) except at doses above 60 pmol where a dilatation was observed (Figure 3a). The maximum diameter observed (916 ± 81 μm) occurred at 100 pmol and was significantly ($P < 0.05$) greater than the resting diameter (662 ± 47 μm).

In the isolated central artery preparation the diameter of G_0 was not significantly altered by the peptide (0.3–300 pmol; n = 5–7). The resting diameter was 486 ± 16 μm and was not significantly different from that at the highest dose (563 ± 84 μm).

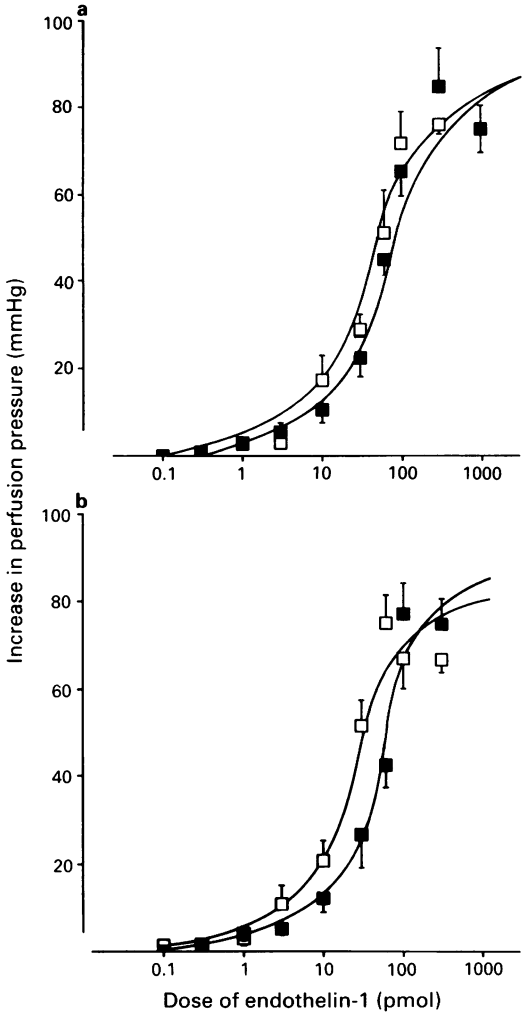

Figure 2 Pressor responses to endothelin-1 in the isolated perfused ear preparation of the rabbit under normoxic or hypoxic conditions in the absence (a) and presence (b) of N-nitro-L-arginine methyl ester (NAME). (a) The control responses to endothelin-1 under normoxic conditions are shown by (□; n = 6–11). (■) Represents the responses to the peptide under hypoxic conditions (n = 9–19). (b) (□) Show the responses to endothelin-1 in the presence of NAME under normoxic conditions (n = 7–8) and (■) represent the responses in the presence of NAME under hypoxic conditions (n = 4–10). The dose-response curves were fitted as described in the Methods. The points show the mean and the vertical lines show the s.e.mean.

In all of the arterial branch generations studied endothelin-1 (0.1–300 pmol; n = 6–11) caused dose-related reductions in diameter (Figure 3b). Table 1 shows that endothelin-1 was statistically equipotent as a vasoconstrictor in each arterial generation.

Table 1 $ED_{50(MP)}$ values for the reductions in arterial diameter in response to endothelin-1 in the rabbit isolated ear

Generation	*Normoxia*	*Hypoxia*	*Normoxia +50 μM NAME*	*Hypoxia +50 μM NAME*
G_1	39.3 ± 4.2 (10–30)	—	40.9 ± 10.0 (60–100)	56.8 ± 9.9 (10–30)
G_2	46.5 ± 12.7 (10–30)	214 ± 59††† (30–60)	19.1 ± 5.5 (10–30)	53.3 ± 10.1 (10–30)
G_3	30.9 ± 8.1 (10–30)	135 ± 38†† (10–30)	21.7 ± 4.5 (3–10)	57.3 ± 14.5 (10–30)
G_4	16.5 ± 6.1 (3–10)	34.2 ± 12.0** (10–30)	17.7 ± 7.7 (1–3)	30.9 ± 6.3 (3–10)

The values in parentheses indicate the thresholds for significant ($P < 0.05$) constriction. The values are in pmol and the $ED_{50(MP)}$ are given as mean ± s.e.mean, the n values for each $ED_{50(MP)}$ values are contained in Figure 3. ** Indicate significant intergeneration differences ($P < 0.01$) in the $ED_{50(MP)}$ value under the same experimental conditions; †† ($P < 0.01$) and ††† ($P < 0.001$) indicate significant differences in the four experimental groups for the $ED_{50(MP)}$ value in any given group. NAME = N-nitro-L-arginine methyl ester.

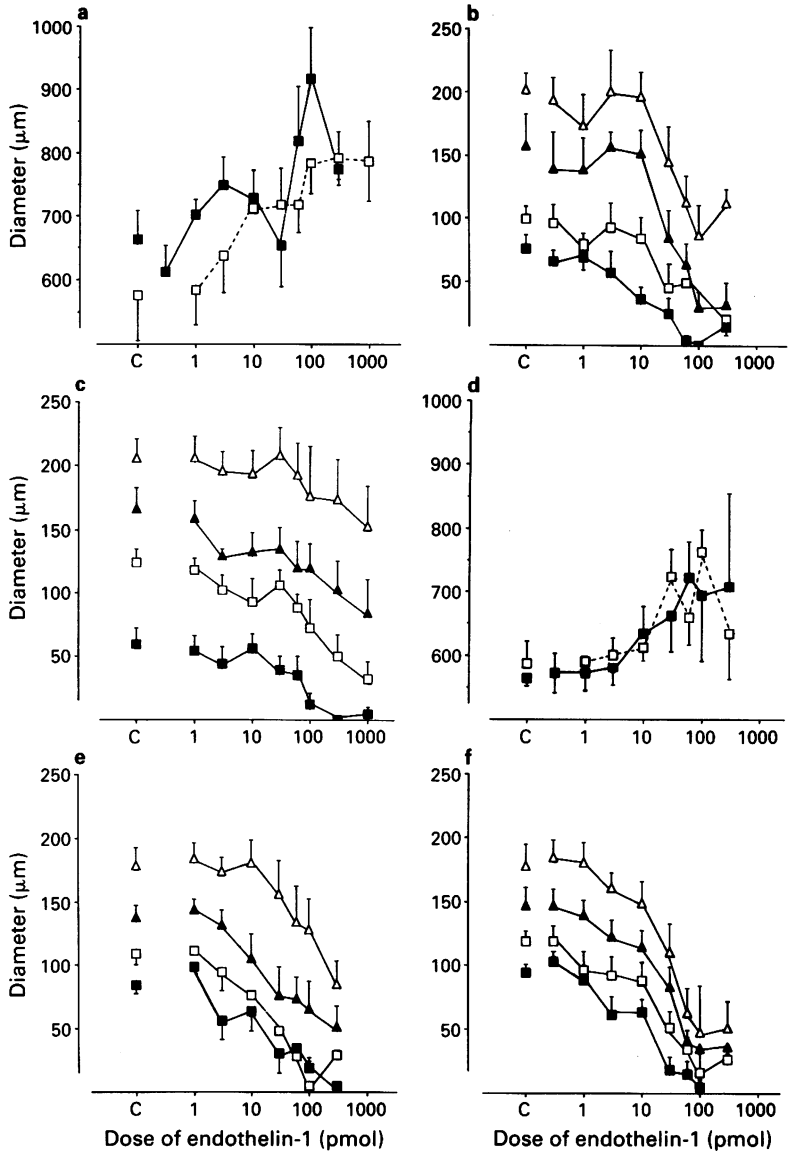

Figure 3 The arterial diameter changes induced by endothelin-1 in the rabbit ear preparation under (a) conditions of normoxia (■; $n = 6$) and hypoxia (□, $n = 6$–10) in G_0, (b) normoxic conditions in arterial branch vessels ($n = 6$–11), (c) hypoxic conditions in arterial branch vessels ($n = 6$–8), (d) normoxic (□; $n = 6$–8) and hypoxic (■; $n = 7$–10) conditions in the presence of 50 μM N-nitro-L-arginine methyl ester (NAME) in G_0, (e) normoxic conditions in the presence of 50 μM NAME in the arterial branch generations ($n = 6$–8) and (f) hypoxic conditions in the presence of 50 μM NAME for the arterial branch vessels ($n = 7$–10). In (b, c, e and f) the branch generations are represented by (△) G_1, (▲) G_2, (□) G_3 and (■) G_4. The C on the abscissa scales indicates the position of resting vessel diameter. The points show the mean diameters and the vertical lines the s.e.mean.

Figure 4a shows that on the venous vessels endothelin-1 (0.1–300 pmol) did not alter the diameter of the large veins (V_0) but did cause dose-related constrictions in the smaller veins (V_1–V_4). The $ED_{50(MP)}$ values for these actions are given in Table 2 and it can be seen that the peptide was equipotent in each generation.

From the spatially averaged data for the actions of endothelin-1 on the arterial and venous vessels the peptide was significantly ($P < 0.05$) more potent as a constrictor in the venous bed; $ED_{50(MP)}$ values 33.3 ± 7.7 pmol (arterial) and 15.1 ± 4.4 pmol (venous).

Effects of hypoxia and 50 μM NAME on endothelium-dependent relaxations to acetylcholine

In normoxic control preparation the combination of 1 μM 5-hydroxytryptamine and 1 μM histamine increased perfusion pressure by 103 ± 21 mmHg. Acetylcholine (0.55–550 pmol) caused dose-related relaxations of the established tone (Figure 5a). The dose-response curve for the relaxation was described by an $ED_{50} = 66.6 \pm 13.8$ pmol and an $R_{max} = 71.8 \pm 5.1\%$ ($n = 5$). Under these conditions endothelin-1 (0.1–10 pmol) did not cause any relaxations of established tone ($n = 4$). Higher doses of the peptide caused increases in perfusion pressure which were not preceeded by dilator responses.

Under normoxic conditions inclusion of 50 μM NAME in the perfusion fluid increased the established tone by 39.0 ± 13.6 mmHg and Figure 5a shows that relaxations to acetylcholine (0.55–550 pmol; $n = 5$) were completely abolished. Inclusion of 10 mM L-arginine in the perfusate appeared to reverse the effects of NAME on the established tone by causing a decrease in tone of 32.8 ± 8.2 mmHg, but did not reverse the effects of the agent on the relaxations to acetylcholine (Figure 5a; $n = 5$).

Under conditions of moderate hypoxia (Pa_{O_2} ca. 60 mmHg) the increase in tone induced by the combination of 5-hydroxytryptamine and histamine was 59.6 ± 5.3 mmHg. Under these conditions the potency of acetylcholine (0.55 pmol–165 nmol) was significantly ($P < 0.001$) reduced compared to normoxia and was described by an ED_{50} of

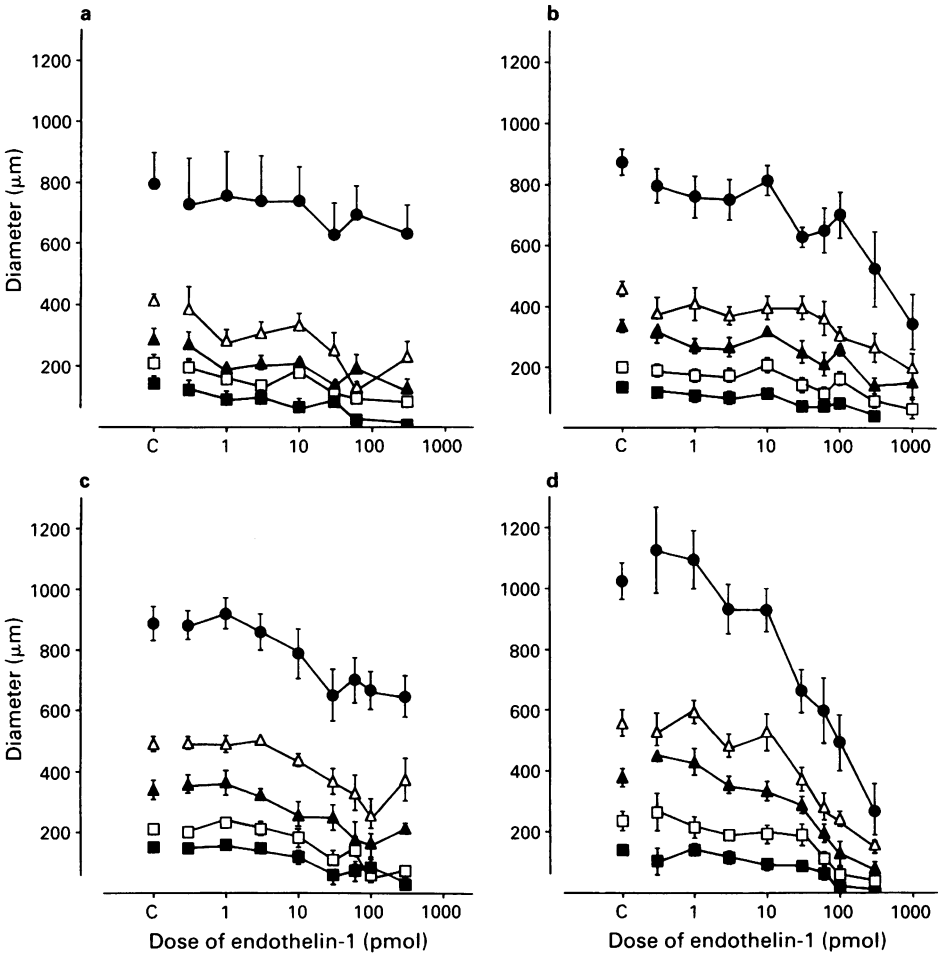

Figure 4 The venous diameter changes induced by endothelin-1 in the rabbit ear preparation under (a) normoxic conditions ($n = 5$–8), (b) hypoxic conditions ($n = 4$–15), (c) normoxic conditions in the presence of 50 μM N-nitro-L-arginine methyl ester (NAME) ($n = 5$–8) and (d) hypoxic conditions in the presence of NAME ($n = 7$–10). In each graph V_0 is represented by (●) and the branch generations; (△) V_1, (▲) V_2, (□) V_3 and (■) V_4. The C on each abscissa scale indicates the position of resting vessel diameter. The points show the mean diameters and the vertical lines the s.e.mean.

1.68 ± 0.26 nmol ($n = 5$) (Figure 5b). The R_{max} was 82.5 ± 6.4% and did not differ significantly from the value obtained in normoxia.

Vascular responses to endothelin-1 in hypoxia

The resting arterial and venous calibres in hypoxia (Pao_2 ca. 60 mmHg) did not differ significantly from those in normoxia (Pao_2 500–600 mmHg) (Figures 3a,b,c and 4a,b). The basal perfusion pressure in the intact bed was 42.6 ± 3.1 mmHg and Figure 2a shows that the pressor responses to endothelin-1 (0.1–1000 pmol; $n = 9$–19) did not differ significantly from those in normoxia and the dose-response curve was described by the following parameters: $ED_{50} = 54.8 \pm 16.9$ pmol, $R_{max} = 85.1 \pm 8.7$ mmHg and $n_H = 1.0 \pm 0.2$.

Compared to normoxia the potency of the peptide (0.3–1000 pmol) at causing constriction of the arterial branch vessels was significantly reduced in G_2 and G_3, unaltered in

Table 2 $ED_{50(MP)}$ values for the reductions in venous diameters in response to endothelin-1 in the rabbit isolated ear

Generation	*Normoxia*	*Hypoxia*	*Normoxia + 50 μM NAME*	*Hypoxia + 50 μM NAME*
V_0	—	73.8 ± 21.1 (10–30)	39.3 ± 13.4 (10–30)	32.5 ± 9.3 (10–30)
V_1	26.3 ± 9.5 (0.3–1)	83.2 ± 17.8†† (1–3)	36.3 ± 11.9 (10–30)	26.7 ± 4.5 (10–30)
V_2	12.4 ± 3.5** (0.3–1)	65.4 ± 13.5†† (30–60)	33.6 ± 8.8 (30–60)	40.4 ± 5.6 (10–30)
V_3	15.3 ± 3.2* (1–3)	88.4 ± 31.8† (30–60)	35.1 ± 12.7 (10–30)	43.9 ± 8.5 (30–60)
V_4	6.5 ± 1.6*** (3–10)	137 ± 42† (30–60)	11.8 ± 2.5 (10–30)	40.3 ± 6.6 (10–30)

The values in parentheses indicate the thresholds for significant ($P < 0.05$) constriction. The values are in pmol and the $ED_{50(MP)}$ are given as mean ± s.e.mean; the n values for each $ED_{50(MP)}$ value are contained in Figure 4. † ($P < 0.05$), †† ($P < 0.01$) and ††† ($P < 0.001$) represent significant differences in an $ED_{50(MP)}$ value in one generation compared to the value in the same generation under different experimental conditions. * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$) indicate significant differences between the $ED_{50(MP)}$ value in any given generation in normoxia compared to the value obtained either in normoxia plus N-nitro-L-arginine methyl ester (NAME) or hypoxia plus NAME.

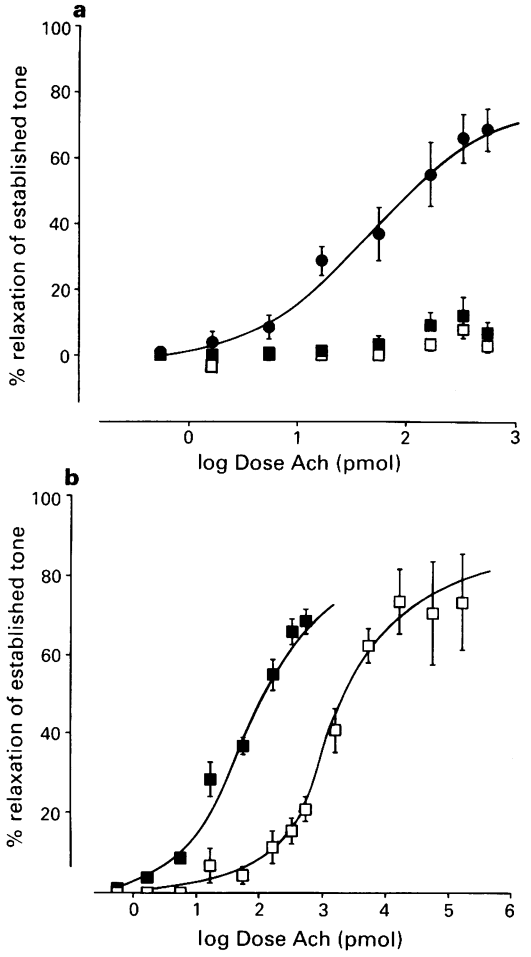

Figure 5 The relaxation of established tone of acetylcholine (ACh) in the isolated perfused ear of the rabbit. (a) Shows the relaxation to ACh in the absence of N-nitro-L-arginine methyl ester (NAME) (●), in the presence of 50 μM NAME (□) and in the presence of 50 μM NAME plus 10 mM L-arginine (■), $n = 5$ in each group. (b) Shows the relaxation to ACh in normoxia (■; data taken from (a)) and hypoxia (□), $n = 5$ for both groups. The dose-response curves were computer fitted as described in the Methods. The points show the mean and the vertical lines the s.e.mean.

G_4, while in G_1 endothelin-1 did not cause significant reductions in diameter (Figure 3b; Table 1). However, Figure 4b shows that in the venous circulation endothelin-1 caused a marked and significant ($P < 0.01$) reduction in the diameter of the large veins (V_0) during hypoxia (from a resting value of 873 ± 42 μm to 343 ± 98 μm at the highest dose) which was not observed in normoxia. While the peptide acted with equipotency in all venous generations the $ED_{50(MP)}$ values were significantly increased in V_1 ($P < 0.01$), V_2 ($P < 0.01$), V_3 ($P < 0.05$) and V_4 ($P < 0.05$) compared to normoxia ($n = 4$–15).

Effects of 50 μM NAME on vascular responses to endothelin-1

Inclusion of 50 μM NAME in the perfusion fluid did not influence either the resting vessel diameters (Figures 3a,d,e, and 4a,c) or the basal perfusion pressure in the intact bed (39.7 ± 3.9 mmHg). The pressor responses to endothelin (0.1–300 pmol) were also unaffected by this treatment ($n = 7$–8), the dose-response curve being described by 31.0 ± 19.9 pmol (ED_{50}), 100 ± 26 mmHg (R_{max}) and 1.0 ± 0.2 (n_H) and is shown in Figure 2b.

In the presence of NAME endothelin-1 (1–300 pmol) constricted G_1–G_4 ($n = 6$–8) with equipotency but caused dilatation of G_0 (Figure 3c). On the venous side the peptide (0.3–300 pmol; $n = 5$–8) constricted all of the generations including V_0 with equipotency (Figure 4c). Under these conditions the peptide caused a modest but significant ($P < 0.050$) reduction in the diameter of V_0 from a resting value of 887 ± 56 μm to 648 ± 69 μm at 300 pmol endothelin-1. Tables 1 and 2 show that the $ED_{50(MP)}$ values for each vessel, except V_0, did not differ from those found in the normoxic controls.

Effects of 50 μM NAME and hypoxia on vascular responses to endothelin-1

Hypoxia combined with inhibition of nitric oxide synthesis did not influence the basal perfusion pressure (38.8 ± 8.3 mmHg) or the resting arterial and venous diameters (Figures 3a,d,f and 4a,d) compared to normoxic controls. The pressor responses to endothelin-1 (0.1–300 pmol) were similarly unaffected by the treatment, and the dose-response curve (Figure 2b) was described by an ED_{50} = 56.8 ± 21.7 pmol, R_{max} = 94.2 ± 12.5 mmHg and n_H = 1.0 ± 0.1 ($n = 4$–10). None of the parameters differed from those obtained under control conditions.

Under these conditions endothelin-1 brought about reductions in the diameters of G_1, G_2, G_3 and G_4 ($n = 7$–10), acting with equipotency in each generation (Figure 3f; Table 1), and these values did not differ significantly from those obtained under control conditions. Once again a dilatation was observed in the central artery at the higher doses (Figure 3d). In the venous network the peptide constricted all of the branch generations (Figure 4d; Table 2; $n = 7$–10). There was also a significant ($P < 0.001$) constriction of V_0 from a resting diameter of 1026 ± 60 μm to 264 ± 93 μm at 300 pmol endothelin-1. However, the potencies of the peptide, relative to those found under normoxic control conditions, were significantly reduced in V_2 ($P < 0.01$), V_3 ($P < 0.05$) and V_4 ($P < 0.001$).

Discussion

The results presented in this paper clearly demonstrate that endothelin-1 is a potent vasoconstrictor in the rabbit isolated ear, producing not only arterial but also venous constriction.

The technique of microangiography has allowed the first quantitative, simultaneous examination of vascular responses to endothelin-1 in arterial and venous vessels both large and small. In the arterial side the peptide constricted all generations studied except G_0 where 'paradoxical dilatation' was observed at high concentrations of endothelin-1. The peptide was equipotent at causing vasoconstriction in all subsequent arterial branch generations, although there was a trend for the potency to increase with diminishing vessel size. This trend became significant in hypoxia when the potency increased progressively in consecutive generations from G_2 to G_4. This pattern of constrictor activity in all branch generations is unusual for a vasoconstrictor in this preparation, as it has previously been shown that reactivities to 1 μM 5-hydroxytryptamine (Griffith *et al.*, 1989) and 1 μM noradrenaline (Griffith & Edwards, unpublished observations) are greatest in G_0 and diminish progressively with vessel size ($G_0 > G_1 > G_2 > G_3$). The activity of endothelin-1 on the venous network described here may also be considered unusual as both 5-hydroxytryptamine and noradrenaline apparently act only on arterial vessels in the bed (Randall, Edwards & Griffith, unpublished observations). In the isolated central artery preparation endothelin-1 was without activity so that the 'paradoxical dilatation' observed in the G_0 in the intact preparation is likely to occur secondary to overriding vasoconstriction of 'downstream' branch vessels, which would lead to increased intraluminal pressure and passive distension of the central artery. It is of interest that the dilatation was observed in the presence of NAME and thus occurs independently of EDRF activity. This phenomenon has also been shown to occur in this preparation in response to other constrictor stimuli such as haemoglobin (Griffith *et al.*, 1987;

1989), in the rat kidney in response to the α_1-adrenoceptor agonist methoxamine (Burton *et al.*, 1989) and magnetic resonance imaging has shown that, *in vivo*, phenylephrine causes passive distension of the rat carotid artery (Behling *et al.*, 1989). The lack of activity of endothelin-1 in the central artery is unusual as endothelin-1 is known to constrict a variety of large conduit vessels (Yanagisawa *et al.*, 1988; D'Orleans-Juste *et al.*, 1988). The activity of endothelin-1 in the smallest branches studied (G_3–G_4) confirms previous studies in which endothelin-1 has been shown to be a potent constrictor of smaller arterioles (Brain, 1989; Fortes *et al.*, 1989).

In addition to arterial constriction endothelin-1 constricted all of the venous vessels studied except V_0. Indeed the $ED_{50(MP)}$ values, when spatially averaged for the arterial and venous sides, demonstrate that the peptide was twice as potent on the venous side. This agrees with studies on isolated segments of vascular tissue (D'Orleans-Juste *et al.*, 1988) and in the superior mesenteric bed of the rat (Warner, 1990). In this latter preparation the maximum responses of the isolated arterial bed to endothelin-1 are appreciably smaller than those in the intact bed (ca. 20 mmHg compared to ca. 80 mmHg; Randall *et al.*, 1989), and this is likely to reflect the appreciable activity of the peptide on the smaller arterioles ($<60\,\mu m$) and venous vessels which are present only in the intact preparation. Although endothelin-1 would appear more potent on the venous vessels in the rabbit ear, the fractional diameter changes were generally larger on the arterial side. However, it should be noted that in this preparation the veins are elliptical in shape with the long axis lying in the plane of the ear so that constrictor responses may in fact be greater than are actually implied by the diameter changes observed (Griffith *et al.*, 1988).

Recent studies have shown that, in common with other vasoconstrictors, the activity of endothelin-1 is enhanced in hypoxia (MacLean *et al.*, 1989). In the present study the pressor activity of the peptide in the intact bed was unaffected by reducing the oxygen tension of the perfusate. However, analysis of the diameter changes revealed that the actions of the peptide are in fact influenced by oxygen tension. In hypoxia the potency of endothelin-1 is markedly reduced in all of the arterial branch vessels except G_4 and also venous vessels. However, in the venous network the peptide caused a profound constriction of the large veins (V_0) which was not apparent in normoxia, so that at the highest doses of endothelin-1 there is a halving of diameter. Clearly this will lead to increased pressure in the more proximal parts of the venous network and the arterial network. This effect may override vasoconstriction in the arterial vessels and thus account for the accompanying reduction in the potency of the peptide in G_1–G_3. The ability of venoconstriction to influence arterial vasomotor tone is shown by the constrictor pattern of angiotension II in this preparation. Angiotensin II has been found to constrict all of the venous vessels in the intact ear without any apparent effect on arterial diameters. However, when the larger venous vessels are removed from this preparation angiotensin II is able to cause pronounced arterial constriction (Randall & Griffith, unpublished observations). Similarly, Warner (1990) demonstrated that in an isolated mesenteric venous preparation the pressor responses to angiotensin II, a selective venoconstrictor without dilator properties, were significantly greater than those produced when injected into the intact mesenteric circulation. Moreover, Warner showed that in the intact preparation, the pressor responses to endothelin-1 were less than the arithmetical sum of responses in the isolated arterial and venous preparations. In the ear preparation the hydraulic coupling of the venous to the arterial circulation may be further enhanced by the presence of numerous arterio-venous shunts (Bellman, 1953). The possibility that hydraulic effects 'downstream' may influence 'upstream' vasoconstricton was shown by Gore (1972) who found that maximum constrictor responses depended on optimal wall stress. Hence pronounced venoconstriction in the ear may alter wall stress in more proximal vessels in such a way as to attenuate constriction. In the present context, the enhanced reactivity of V_0 towards endothelin-1 in hypoxia would thus appear to have important consequences for the overall vascular responses of the bed.

In other vessels such hypoxic facilitation has been attributed to the production of a constrictor substance (Rubanyi & Vanhoutte, 1985; Kwan *et al.*, 1989). In the present study the release of significant quantities of a direct constrictor in response to hypoxia would appear doubtful as neither resting vessels diameters nor basal perfusion pressures differed between the conditions. It has also been shown that there is impaired release of both agonist and tonically released EDRF in hypoxia (Furchgott & Zawadzki, 1980; DeMey & Vanhoutte, 1983; Warren *et al.*, 1989; Johns *et al.*, 1989). The possible link between hypoxia and impaired EDRF release has previously been demonstrated by Griffith *et al.* (1986) who showed that inhibitors of mitochondrial respiration inhibit EDRF synthesis or release. In the present study moderate hypoxia caused a marked rightward shift in the dose-response curve for the endothelium-dependent vasodilator effects of acetylcholine. Other workers have shown that oxygen tensions of 42 mmHg can reduce endothelium-dependent relaxations in isolated blood vessels (Johns *et al.*, 1989) while Po_2 values of 15 mmHg inhibit the release of EDRF from cultured endothelial cells (Warren *et al.*, 1989). These findings are consistent with hypoxia impairing either the synthesis/release of EDRF and/or the responsiveness of the vascular smooth muscle to EDRF.

The impaired activity of acetylcholine as an endothelium-dependent vasodilator in hypoxia confirms that the reduced oxygen tension in hypoxia may reduce EDRF activity. It is possible that this interference may have reduced the modulator effect of tonic EDRF on vasoconstriction and thus enabled endothelin-1 to constrict V_0 in hypoxia. However, in the presence of NAME and thus in the absence of EDRF activity the constriction of V_0 was much less than found in hypoxia alone, so that the augmented reactivity found in hypoxia cannot be entirely secondary to impairment of tonic EDRF. Therefore it seems likely that the greater constriction found in hypoxia is largely due to increased sensitivity of the vascular smooth muscle to endothelin-1, or due to the facilitatory effects of a factor released by hypoxia. The former mechanism is known to occur in the heart where ischaemia causes an increase of endothelin-1 receptors in the plasma membrane (Liu *et al.*, 1989; 1990).

The influence of tonic EDRF in the main veins, as shown by the modest facilitation of constrictor responses in V_0 in the presence of NAME, is surprising in view of early studies demonstrating that EDRF release was small in veins compared to arteries (DeMey & Vanhoutte, 1982). However, in sharp contrast to this, McGrath *et al.* (1990) have recently shown in the rabbit that both the tonic release of EDRF, as assessed by endothelium-dependent modulation of constrictor responses to α-adrenoceptor agonists and EDRF release in response to acetylcholine, are greater in veins than the corresponding arteries.

In isolated mesenteric arterial preparations inhibition of EDRF activity results in enhancement of pressor responses to endothelins (Warner *et al.*, 1989; Randall *et al.*, 1989). This is thought to be due to the loss of tonically released EDRF, which may modulate vasoconstriction, or due to the loss of EDRF release stimulated by endothelin-1 itself. However, in the intact mesenteric circulation *in vivo* (Randall *et al.*, 1989) and in the conscious rat (Gardiner *et al.*, 1989) inhibition of EDRF does not augment pressor responses to endothelin-1. In the ear preparation low doses of endothelin-1 did not cause any relaxation of established tone and it would therefore appear that in the ear endothelin-1 does not stimulate EDRF release directly. Inhibition of EDRF in the ear did not augment the pressor responses to endothelin-1 but did alter the pattern of vasoconstriction and this is likely to reflect the loss of tonic EDRF. It is of interest that NAME augmented the tone established by 5-hydroxytryptamine and histamine

which suggests that tonically released EDRF modulates constriction due to these agents. Furthermore, this apparent inhibitory effect of NAME on tonically released EDRF was reversed by L-arginine, whereas the effects of NAME on the stimulated release of EDRF were not. This finding may suggest differences in the synthetic mechanisms for tonic and stimulated EDRF.

In conclusion, endothelin-1 is a potent vasoconstrictor of both large and small branch arterial and venous vessels in the rabbit ear. In this respect the peptide is more potent on the venous compared to the arterial vessels. In the arterial vessels endothelin-1 is equipotent as a vasoconstrictor in all branch generations. Under conditions of hypoxia the net pressor responses of the bed are unaltered, but the patterns of constriction of the arterial and venous vessels are greatly changed. In particular hypoxia augments constriction of the large veins. This effect cannot be fully explained by hypoxia-induced impairment of EDRF activity as complete inhibition of EDRF had only a marginal effect on vasoconstriction. The enhanced activity of endothelin-1 in V_0 in hypoxia may thus reflect a direct or an indirect increase in the sensitivity of the vascular smooth muscle to endothelin-1 as a consequence of the reduced oxygen tension.

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Negative inotropic effects of disopyramide on guinea-pig papillary muscles

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1 The inhibitory effects of disopyramide on electromechanical responses were investigated in guinea-pig papillary muscles driven by electrical stimuli. Disopyramide up to 10^{-5} M did not cause a negative inotropic effect, while the maximum upstroke velocity of the action potential (dV/dt_{max}) was significantly decreased.

2 At higher concentrations, this drug dose-dependently inhibited the contraction, and dV/dt_{max} was further decreased. This inhibition of contraction was accompanied by a depression of the slow action potential in partially depolarized preparations by increasing $[K^+]_o$ (26 mM).

3 In preparations pretreated with nifedipine (10^{-6} M) and ryanodine (10^{-6} M), the contraction was almost completely inhibited. In such preparations, ouabain (2×10^{-6} M) markedly increased the contraction, probably through the Na^+-Ca^{2+} exchange mechanism. This contraction was inhibited by disopyramide above 10^{-8} M, and an almost complete inhibition was caused at 3×10^{-5} M.

4 A similar inhibitory effect was observed on the contraction increased by the lowering of $[Na^+]_o$ (36 mM).

5 These results suggest that disopyramide at high concentrations inhibits Ca influx through slow Ca^{2+} channels and at low concentrations, it reduces the contraction increased through the Na^+-Ca^{2+} exchange mechanism. Disopyramide had a greater effect on cardiac contractility mediated by the Na^+-Ca^{2+} exchange mechanism.

Introduction

Disopyramide is a widely used class 1A antiarrhythmic drug with a pharmacological profile of action similar to that of quinidine and procainamide (Campbell, 1986). Disopyramide decreases the rate of diastolic depolarization during phase 4 of the action potential decreases the upstroke velocity of phase 0 of the action potential and prolongs the duration of the action potential and the refractory period (phases 2 and 3). In addition, a negative inotropic effect of disopyramide has been identified in animal experiments (Nayler, 1979; Walsh & Horwitz, 1979; Abdollah *et al.*, 1984; Beltrame *et al.*, 1984) and has been detected by echocardiography (Martin *et al.*, 1980; Pollick *et al.*, 1982; Holt *et al.*, 1983), radionuclide angiography (Wisenberg *et al.*, 1984) and during cardiac catheterisation (Thadani *et al.*, 1981) in normal volunteers. In some haemodynamic studies on patient groups, the effects of disopyramide on myocardial performance have been minimal (Marrott *et al.*, 1976; Sutton, 1976), but in patients with pre-existing left ventricular function abnormalities, negative inotropic effects are more marked (Hills *et al.*, 1976; Jensen *et al.*, 1976; Sutton, 1976; Davies *et al.*, 1979; Hulting & Rosenhamer, 1979; Naqui *et al.*, 1979; Scheinman *et al.*, 1980; Gottdiener *et al.*, 1983; Greene *et al.*, 1983; Marrott *et al.*, 1983; Cameron *et al.*, 1984). However, the mechanisms of the negative inotropic effects of disopyramide have not been clarified. The present study was thus undertaken to assess the electromechanical effects of disopyramide on guinea-pig cardiac muscle driven by electrical stimuli in order to clarify the mode of its negative inotropic action.

Methods

Guinea-pigs weighing 250–300 g were killed following cervical dislocation. The hearts were quickly removed and papillary muscles, 2 to 3 mm in length and less than 1 mm in diameter, were isolated from the right ventricles. These preparations were then mounted in a tissue bath and superfused continuously with Krebs-Ringer solution at 30°C, equilibrated with 95% O_2 and 5% CO_2. The composition of the Krebs-Ringer solution was as follows (mM): NaCl 120.3, KCl 4.8, $CaCl_2$ 1.2, $MgSO_4 \cdot 7H_2O$ 1.3, KH_2PO_4 1.2, $NaHCO_3$ 24.2 and glucose 5.5 (pH 7.4). In some experiments the K^+ concentration of the solution was raised to 26 mM by substituting KCl for NaCl on an equimolar basis to inactivate the fast Na^+ channel, and then $[Ca^{2+}]_o$ was also increased to 3.6 mM to induce a sufficient contraction at a driving frequency of 0.2 Hz. In a low Na^+ medium, the Na^+ concentration was reduced to 36 mM by replacing the NaCl by choline chloride and atropine at 10^{-4} M was added. The preparations were driven constantly at 1 Hz unless otherwise specified. Pulses employed for stimulation were 1.5 ms in duration and three times the diastolic threshold in intensity. Membrane action potentials were recorded through glass microelectrodes filled with 3 M KCl (10–30 MΩ), and displayed on a storage oscilloscope (Tektronix 7613). The contractile force was measured isometrically through a force displacement transducer and displayed simultaneously with the action potential.

The following parameters were measured to assess the electromechanical performance of ventricular muscles: amplitude of action potential (APA), maximum diastolic potential (MDP), maximum upstroke velocity of action potential (dV/dt_{max}), duration of action potential from upstroke to 10, 30, 80% repolarization (APD_{10}, APD_{30} and APD_{80}), peak developed tension (DT), and time to peak developed tension (tPT).

Values were expressed as the mean ± s.e. Statistical analysis of measured parameters was performed by a paired t test, and P values of less than 0.05 were considered to indicate a significant difference. More details on each procedure are given under Results.

Results

Electromechanical effects of disopyramide

Effects of disopyramide on the contraction and membrane action potential were examined in preparations driven at 1 Hz

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(Figures 1 and 2). Disopyramide at lower concentrations (10^{-7} M to 10^{-5} M) had no apparent effect on the contraction, whereas at higher concentrations (10^{-4} M and 10^{-3} M), it inhibited the contraction in a dose-dependent manner (Figure 1, open symbols).

Disopyramide at 10^{-6} M did not affect membrane action potential. At 10^{-5} M, this drug significantly decreased the maximum upstroke velocity of the action potential (dV/dt_{max}) without affecting other parameters of the action potential, such as action potential amplitude (APA), maximum diastolic potential (MDP) and action potential duration from the upstroke to 10, 30 and 80% of repolarization (APD_{10}, APD_{30} and APD_{80}) or the developed tension (DT) (Figure 2, Table 1). At a higher concentration (10^{-4} M), disopyramide caused a negative inotropic effect and a shortening of time to peak tension (tPT), while the parameters of action potential except for dV/dt_{max} were not affected (Figure 2, Table 1). At the highest concentration used in the present experiments (10^{-3} M), the effects of disopyramide on DT, tPT and dV/dt_{max} were greater. A marked depression of the plateau phase of the action potential which is characterized by a decrease in APA and a shortening of APD_{30} and APD_{80} was caused (Figure 2, Table 1).

Electromechanical effects of disopyramide on high K^+-depolarized preparations

The effects of disopyramide were evaluated on preparations depolarized by increasing $[K^+]_o$ to inactivate Na^+ channels.

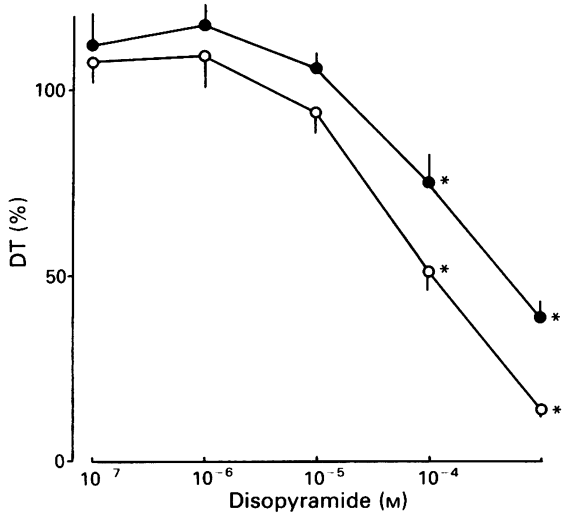

Figure 1 The effects of disopyramide on the contractile force (DT) of guinea-pig papillary muscles. The experiments were performed under two different conditions: (○) indicates the effect on preparations driven at 1 Hz in a normal medium; (●) indicates that on a partially depolarized preparation driven at 0.2 Hz in high K^+ and high Ca^{2+} containing medium (26 mM K^+ and 3.6 mM Ca^{2+}). Disopyramide was applied for 15 min. Each value is the mean of 6 experiments, vertical lines show s.e. * Significantly different from control values (100%) at $P < 0.05$.

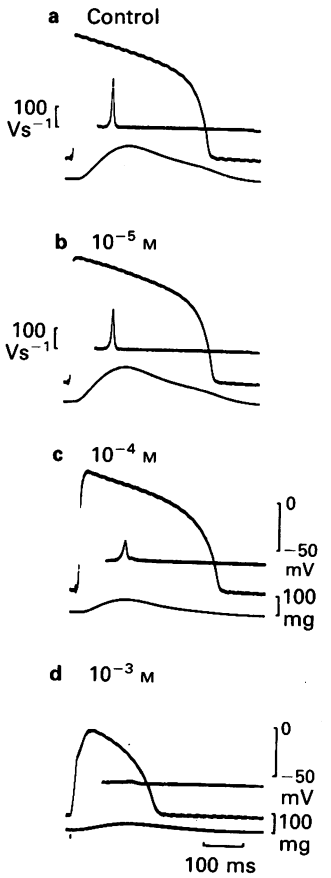

Figure 2 Electromechanical effects of disopyramide on papillary muscles driven at 1 Hz in normal medium. Top trace shows dV/dt_{max}, middle trace shows the action potential and bottom trace shows developed tension. (a) Control; (b), (c) and (d) disopyramide at 10^{-5}, 10^{-4} and 10^{-3} M was applied for 15 min, respectively.

In high K^+-containing medium (26 mM), $[Ca^{2+}]_o$ was also increased to 3.6 mM to induce an apparent mechanical response at a driving frequency of 0.2 Hz (Figure 3). In such preparations, disopyramide at 10^{-6} M caused a slight but significant increase in contraction (118 ± 4.4% of control, $n = 5$) (Figures 1 and 3). At higher concentrations (10^{-4} M, 10^{-3} M) however, the inhibitory effects of disopyramide were observed. The present dose-dependence of its inhibitory effects was similar to that observed in normal medium, although the inhibitory effects of this drug were reduced in the modified medium (Figure 1, open and closed symbols).

Under similar conditions (3.6 mM $[Ca^{2+}]_o$ and 26 mM $[K^+]_o$), the membrane resting potential was depolarized to nearly −50 mV (Figure 3). In this preparation, the electrical stimuli induced a slow action potential having a low upstroke velocity (10–15 V s^{-1}), indicating nearly complete inactivation of the fast Na^+ channels (Figure 3). Under the same conditions, disopyramide at 10^{-6} M prolonged the duration of this

Table 1 Effects of disopyramide on membrane action potential and the contraction of guinea-pig papillary muscles

	tPT (ms)	*APA* (mV)	*MDP* (mV)	dV/dt_{max} (Vs^{-1})	APD_{10} (ms)	APD_{30} (ms)	APD_{80} (ms)
Control	146 ± 1.4	122 ± 3.3	87.2 ± 1.1	286 ± 22	83.3 ± 13	238 ± 208	311 ± 24
Disopyramide 10^{-5} M	140 ± 1.4	121 ± 2.7	88.3 ± 0.6	229 ± 10*	104 ± 14	253 ± 25	331 ± 30
Disopyramide 10^{-4} M	126 ± 4.2*	112 ± 5.8	87.8 ± 0.6	117 ± 10*	95.8 ± 14	227 ± 25	317 ± 30
Disopyramide 10^{-3} M	125 ± 7.0*	88.3 ± 1.9*	86.7 ± .97	23.6 ± 8*	63.9 ± 4.1	135 ± 11*	201 ± 16*

tPT: time to peak tension, APA: action potential amplitude, MDP: maximum diastolic potential, dV/dt_{max}: maximum upstroke velocity of action potential, APD_{10}, APD_{30}, and APD_{80}: action potential duration from the upstroke to 10, 30, and 80% repolarization. Values are mean ± s.e. ($n = 5$) before and 10 min after application of disopyramide. * Significantly different from the values of control at $P < 0.05$.

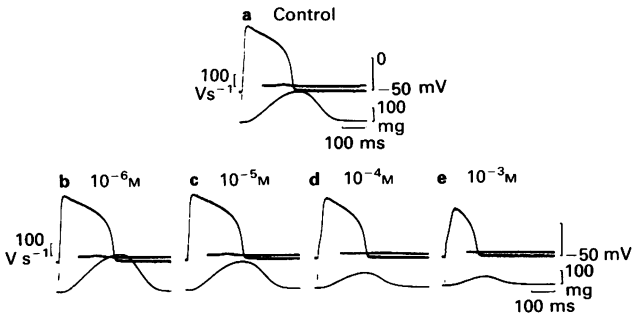

Figure 3 Electromechanical effects of disopyramide on partially depolarized papillary muscles driven at 0.2 Hz. The preparations were depolarized in high K^+ (26 mM) and high Ca^{2+} (3.6 mM) containing medium. (a) Control in this medium; (b), (c), (d) and (e) disopyramide at 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M was applied for 15 min, respectively.

slow action potential with a slight increase in DT. Higher concentrations (10^{-4} M, 10^{-3} M) of disopyramide dose-dependently inhibited the slow action potential and DT (Figure 3).

Effects of disopyramide on preparations pretreated with ouabain or low $[Na]_o$ medium in the presence of nifedipine and ryanodine

After blocking Ca^{2+} influx through slow Ca^{2+} channels and internal Ca^{2+} release by treatment with nifedipine and ryanodine, ouabain and lowering $[Na]_o$ increased the contraction. In such preparations, the effects of disopyramide were examined. The combined treatment with nifedipine (10^{-6} M) and ryanodine (10^{-6} M) markedly decreased DT (15.4 ± 2.7% of control, $n = 10$). In such preparations, ouabain (2×10^{-6} M) increased DT to 84.7 ± 14.7% of control ($n = 5$). Under these conditions, an additional application of disopyramide at 10^{-8} M caused a significant decrease in DT (76.8 ± 4.1% of DT before application of disopyramide), and at higher concentrations (10^{-7} M–3×10^{-5} M), this drug dose-dependently inhibited DT (Figure 4, open symbols). At 3×10^{-5} M, DT which was increased by ouabain was almost completely inhibited. Similarly, the inhibitory effects of disopyramide (10^{-6} M, 3×10^{-5} M) were observed on DT increased by lowering $[Na^+]_o$ to 36 mM in the presence of atropine (10^{-4} M), nifedipine and ryanodine at a driving frequency of 0.2 Hz (Figure 4, closed symbols).

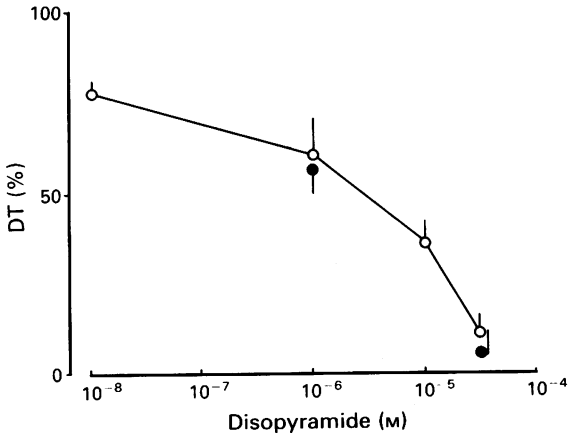

Figure 4 Dose-response curve for disopyramide under the influence of ouabain (○) or low $[Na^+]_o$ (●) in the presence of nifedipine and ryanodine. In preparations pretreated with nifedipine (10^{-6} M) and ryanodine (10^{-6} M) for 30 min, ouabain (2×10^{-6} M) and a low Na^+ medium (36 mM) containing atropine (10^{-4} M) were applied for 40 and 10 min, respectively. Under these conditions, disopyramide was applied for 15 min. The developed tension (DT) before the application of disopyramide is expressed as 100%. Each value is the mean of 5 experiments; vertical lines indicate s.e.

Discussion

In the present experiments, disopyramide caused a negative inotropic effect in a relatively high concentration range (above 10^{-4} M). This negative inotropic effect of disopyramide was accompanied by a depression of the slow action potential in partially depolarized preparations. At the highest concentration used in the present study (10^{-3} M), the action potential plateau was markedly decreased with an almost complete inhibition of contraction. These electrical responses inhibited by disopyramide are mediated through the slow Ca^{2+} inward current. Therefore, the negative inotropic effect of this drug at high concentrations may be attributed to the inhibition of Ca^{2+} influx through slow Ca^{2+} channels. This is consistent with a previous study on guinea-pig atrial preparations (Hashimoto *et al.*, 1979).

Disopyramide at much lower concentrations (10^{-8} M–3×10^{-5} M) inhibited the contraction which was augmented by ouabain in the presence of nifedipine and ryanodine. Since in this experiment, Ca^{2+} influx through slow Ca^{2+} channels was already blocked by nifedipine, this negative inotropic effect is not due to inhibition of slow Ca^{2+} channels by disopyramide. The involvement of the internal Ca^{2+} release mechanism was also eliminated because this mechanism was blocked by ryanodine. These results indicate that disopyramide more effectively inhibits the nifedipine- and ryanodine-insensitive contraction augmented by ouabain.

Ouabain has been thought to increase the contraction through a Na^+-Ca^{2+} exchange mechanism (Reuter & Seitz, 1968; Sheu & Fozzard, 1982; Wasserstrom *et al.*, 1983; Eisner *et al.*, 1984; Vassalle & Lee, 1984; Wier & Hess, 1984; Grupp *et al.*, 1985). Ouabain inhibits Na^+-K^+ ATPase and results in an accumulation of $[Na^+]_i$. The increased $[Na^+]_i$ enhances $[Ca^{2+}]_i$ through the Na^+-Ca^{2+} exchange mechanism and contributes to an increase in the contraction. Furthermore, under the present experimental conditions, Ca^{2+} influx for activation of contraction was directly mediated through the Na^+-Ca^{2+} exchange mechanism, since other sources of Ca^{2+} for activation of contraction (Ca^{2+} influx through slow Ca^{2+} channels and internal Ca^{2+} release) were blocked by pretreatment with nifedipine and ryanodine. A similar result has been reported by Bers *et al.* (1988). These findings suggest that disopyramide effects the contraction activated through the Na^+-Ca^{2+} exchange mechanism.

This suggestion was also supported by the present experiments in a low Na^+ medium containing nifedipine and ryanodine. A reduction of the Na^+ gradient across the cell membrane by lowering $[Na^+]_o$ produces an increase in contraction through the Na^+-Ca^{2+} exchange mechanism (Chapman, 1979; Sheu & Fozzard, 1982). The contraction increased under these conditions was effectively inhibited by disopyramide in much the same concentration-range as observed in that increased by ouabain. Thus, a similar inhibitory action of disopyramide was observed on the contraction increased by two different procedures through the Na^+-Ca^{2+} exchange mechanism, suggesting that the present inhibitory action is also a common feature of the mechanical responses through this exchange mechanism.

Disopyramide above 10^{-5} M inhibited dV/dt_{max}, indicating that this drug inhibited the fast Na^+ channels, as reported previously (Kojima, 1981; Campbell, 1986). However, this action may not play a major role in the genesis of the present negative inotropic effects of disopyramide for the following reasons: (1) The concentration of disopyramide as an inhibitor of the contraction increased by both ouabain and a low Na^+ medium, was markedly different from that for decreasing dV/dt_{max} as an indicator of Na^+ channel activity; (2) even after a decrease in dV/dt_{max} produced by replacing NaCl with choline chloride, an inhibition of contraction by disopyramide was observed; (3) the negative inotropic effect of this drug was not substantially affected by complete inactivation of Na^+ channels by depolarizing the resting potential in a high K^+ containing medium.

The present results suggest that disopyramide caused the negative inotropic effects mediated through two different mechanisms. In normal medium, disopyramide decreased the contraction by inhibiting Ca^{2+} influx through slow Ca^{2+} channels at high concentrations (above 10^{-4} M), while at low concentrations (10^{-8}–3×10^{-5} M), this drug inhibited the contraction which was increased through the Na^{+}-Ca^{2+} exchange mechanism by ouabain and a low Na^{+} medium. Thus, disopyramide is more useful in suppressing contraction which is dependent on the Na^{+}-Ca^{2+} exchange mechanism. Since triggered automaticity mediated by increased $[Ca^{2+}]_i$ has recently received considerable attention as a possible mechanism of human arrhythmias (Rosen & Feder, 1981), it is assumed that disopyramide is a valuable agent for such arrhythmias under these conditions of Ca^{2+} overload through the Na^{+}-Ca^{2+} exchange mechanism. Furthermore, this inhibitory action of disopyramide may also be involved in more marked negative inotropic effects reported in patients with pre-existing left ventricular abnormalities (Hills *et al.*, 1976; Jensen *et al.*, 1976; Sutton, 1976; Davies *et al.*, 1979; Hulting & Rosenhamer, 1979; Naqui *et al.*, 1979; Scheinman *et al.*, 1980; Gottdiener *et al.*, 1983; Greene *et al.*, 1983; Marrott *et al.*, 1983; Cameron *et al.*, 1984).

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Facilitation by 3,4-diaminopyridine of regenerative acetylcholine release from mouse motor nerve

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1 Effects of 3,4-diaminopyridine (DAP) on endplate potentials (e.p.ps) were studied in mouse phrenic nerve-hemidiaphragms.

2 In cut muscle preparations, low concentrations of DAP (2–20 μM) increased the amplitude of e.p.ps and shifted the curve relating Ca^{2+} concentration to e.p.p. amplitude leftward.

3 High concentrations of DAP (40–4000 μM) prolonged the duration of e.p.ps dose-dependently up to one hundred fold (ca. 200 ms), yielding, in addition to the normal phasic e.p.p., a prolonged plateau depolarization component which was often preceded by an upstroke depolarization. During the plateau depolarization, nerve stimulations did not evoke any e.p.p.

4 The plateau component of prolonged e.p.ps was suppressed by tubocurarine, verapamil, nifedipine, Mn^{2+} and Cd^{2+} (but not by atropine) at low concentrations that had negligible effect on the amplitude of miniature e.p.ps or the phasic component of e.p.ps. Abolition of the plateau component by these agents restored the capability of the nerve terminal to evoke e.p.ps on nerve stimulation.

5 Low concentrations of neostigmine (0.01–0.02 μM) markedly lengthened DAP-prolonged e.p.ps. However, the regenerative endplate depolarization evoked in the presence of high concentrations of neostigmine (0.3–0.5 μM) was not prolonged by DAP.

6 Tetraethylammonium (1 mM) did not provoke prolonged e.p.ps but acted cooperatively with DAP to prolong the duration of plateau depolarization. At a high concentration (3 mM), tetraethylammonium depressed the amplitude of miniature e.p.ps and abolished DAP-prolonged e.p.ps.

7 In uncut muscle preparations, DAP apparently did not modify the time course and amplitude of miniature e.p.ps. Upon direct stimulation by current injection at endplate, DAP increased the muscle action potentials by only about 30%, but induced no prolonged depolarization.

8 These results suggest that the prolonged e.p.ps induced in the presence of DAP are due to a regenerative release of acetylcholine from motor nerve and the induction probably involves a presynaptic Ca^{2+} channel different from that for normal e.p.ps. It may be inferred that the regenerative acetylcholine release is recruited by Ca^{2+} channels modulated by nicotinic receptors and K^+ channels.

Introduction

Aminopyridines are known to increase the amplitude of endplate potential (e.p.p.) and the effect is attributed to an increase in the number of transmitter quanta released by each presynaptic action potential (Lundh, 1979; Molgo *et al.*, 1980). This facilitatory effect of aminopyridines on transmitter release is considered to result from an enhanced Ca^{2+} entry into nerve terminals (Lundh, 1978; Horn *et al.*, 1979; Simmons & Dun, 1984), either due to a direct enhancement of Ca^{2+} influx (Illes & Thesleff, 1978; Lundh, 1978; Rogawski & Barker, 1983) or to an effect secondary to blockage of voltage-dependent K^+ channels (Llinas *et al.*, 1976; Yeh *et al.*, 1976; Bostock *et al.*, 1981).

In the vertebrate neuromuscular junction (Lundh, 1978; Katz & Miledi, 1979; Molgo *et al.*, 1980) and the *Torpedo* electric organ (Corthay *et al.*, 1982; Muller, 1986), aminopyridines markedly potentiate and prolong the release of acetylcholine (ACh) evoked either by a focal electrical depolarization of nerve terminals or by field stimulation. Histological experiments reveal that transmissions of nerve impulses in the presence of aminopyridines are accompanied by the occurrence of endoexocytotic images in the presynaptic membrane (Heuser *et al.*, 1979) or by the appearance of large presynaptic intramembrane particles (Garcia-Segura *et al.*, 1986). Perineural recordings from mammalian motor nerve terminals also disclose that nerve stimulation leads to production of Ca^{2+} spike with prolonged profile when K^+ channels are suppressed by aminopyridine and tetraethylammonium (Brigant & Mallart, 1982; Penner & Dreyer, 1986). Yet, the ACh release consequent to the provoked Ca^{2+} spike and the resulting postsynaptic responses have not been well correlated. The purposes of the present experiments were to explore the presynaptic effect of a wide concentration range of 3,4-diaminopyridine (DAP), the most potent analogue of aminopyridines, in mouse neuromuscular junction and to investigate the effects of Ca^{2+} channel blockers and cholinoceptor antagonists on the synaptic facilitation produced by DAP in order to shed more light on the role of presynaptic cholinoceptors and the Ca^{2+} channel in modulating the release of ACh. The blocking agents have been shown, at low concentrations, to antagonize the regenerative release of ACh evoked after inhibition of acetylcholinesterase (Hong & Chang, 1989).

Methods

Phrenic nerve-hemidiaphragm preparations were isolated from ICR mice (20–25 g) of either sex, bathed in Tyrode solution (composition in mM: NaCl 137, KCl 2.8, $CaCl_2$ 0.1–5.4, $MgCl_2$ 1.1, NaH_2PO_4 0.33, $NaHCO_3$ 11.9 and dextrose 11.2), oxygenated with 95% O_2 plus 5% CO_2 and maintained at 37 ± 0.5°C. The phrenic nerve was stimulated with rectangular pulses of 0.03 ms width at frequencies as indicated to evoke single or trains of endplate potentials (e.p.ps).

Intracellular recordings of miniature e.p.ps (m.e.p.ps) and muscle action potentials in uncut preparations and evoked e.p.ps in cut muscle preparations were carried out by conventional techniques by use of glass microelectrodes filled with 3 M KCl (resistance 3–10 MΩ). The potentials were registered with a high impedance amplifier in d.c.-coupled mode and

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hardcopied with an electrostatic or a waveform recorder (GOULD). Muscle action potentials were evoked by injections of currents into the endplate and non-endplate areas for 5 ms by use of a current clamp amplifier with capacitance compensation (DAGAN).

All data are expressed as mean ± s.e.mean of recordings obtained from more than 15 endplates from at least 3 preparations. Differences between group means were analysed by Student's t test. Atropine, neostigmine bromide, tubocurarine chloride, tetraethylammonium chloride (TEA), verapamil hydrochloride, nifedipine, $MnCl_2$ and $CdCl_2$ were purchased from Sigma (U.S.A.). DAP was supplied by Koch-Light Laboratory (U.K.). All these chemicals, alone or in combination did not change muscle resting membrane potentials by more than 3 mV. Nifedipine was dissolved in dimethylsulphoxide and protected from light. The final concentration of dimethylsulphoxide in the organ bath was kept below 0.1% in order to avoid an effect of the vehicle.

Results

Effects of 3,4-diaminopyridine on amplitude of e.p.ps

In Tyrode solution containing normal Ca^{2+} concentration (1.8 mM), DAP at 5 μM increased e.p.p. amplitudes by about 80% and a nearly maximal (125%) augmentation was obtained after treatment with 40 μM DAP (Table 1). Increasing DAP up to 4000 μM did not further increase the e.p.p. amplitude. This augmentation of e.p.p. amplitude was observed at Ca^{2+} concentrations ranging from 0.1 to 5.4 mM (Figure 1). Upon repetitive stimulation at 50 Hz or higher, the amplitudes of all successive e.p.ps were increased and well maintained in the presence of low concentrations (5–20 μM) of DAP (Figure 2). The average amplitude of the 21st to 40th e.p.ps relative to that of the first e.p.p. in the same train was 83 ± 3% in control while that after 5 μM DAP was 70 ± 4% ($P < 0.05$). The mean amplitude of these e.p.ps, compared with that of control, was increased by 58 ± 5%, suggesting that in the presence of DAP mobilization of neurotransmitter was elevated. However, with higher concentrations of DAP the pattern of a train of e.p.ps differed markedly from that of control as described in the next section.

Effects of 3,4-diaminopyridine on duration of e.p.ps

The duration of e.p.ps was not significantly changed by DAP at concentrations lower than 20 μM. On increasing the concentration of DAP to 20–4000 μM, endplate depolarizations in response to single pulses to the phrenic nerve were prolonged in a dose-dependent manner (Table 1). At 4000 μM DAP, the highest concentration tested, the 70% duration of the e.p.p. was about 200 ms, being one hundred times the normal e.p.ps. However, the prolonged e.p.ps had a shape different from normal e.p.ps and appeared to be composed of three components: a phasic peak depolarization like normal e.p.ps, followed by a plateau or rebound depolarization component which might be preceded with an upstroke depolarization (Figures 3 and 4). The duration and magnitude of the plateau were a function of DAP concentrations. During the plateau,

Table 1 Effects of 3,4-diaminopyridine (DAP) on the amplitude and duration of endplate potentials (e.p.ps) in cut diaphragm preparations

Treatment (μM)	*E.p.ps* *Amplitude* (mV)	70% *duration* (ms)
Control	8.9 ± 1.0	2.5 ± 0.3
DAP		
2	11.2 ± 1.3	2.4 ± 0.2
5	16.2 ± 1.2	3.4 ± 0.3
20	17.7 ± 1.3	6.0 ± 0.9
40	18.4 ± 1.2	20 ± 4
200	19.2 ± 1.5	46 ± 8
400	19.6 ± 1.3	59 ± 7
4000	20.7 ± 1.9	216 ± 35
TEA		
1000	12.3 ± 1.1	2.9 ± 0.4
3000	11.3 ± 1.3	4.1 ± 0.9

E.p.ps were elicited with single pulses. The amplitude of e.p.ps was the mean from 3 preparations each with 8–10 endplates. The resting membrane potentials were in the range of −40 to −46 mV.

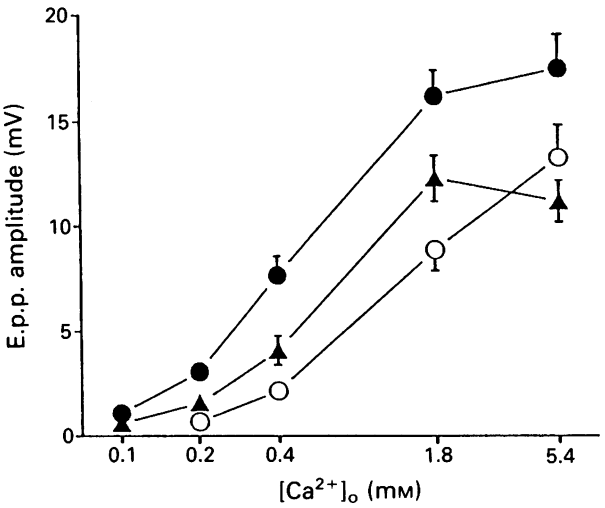

Figure 1 Effects of 3,4-diaminopyridine (DAP) and tetraethylammonium (TEA) on the amplitude of endplate potentials (e.p.ps) of cut diaphragm preparations. E.p.ps were evoked at 0.66 Hz in the absence (○) or presence of DAP (5 μM, ●) or TEA (1 mM, ▲). No e.p.p. was evoked at 0.1 mM $[Ca^{2+}]_o$ if preparations were not treated with drugs. Vertical lines show s.e.mean.

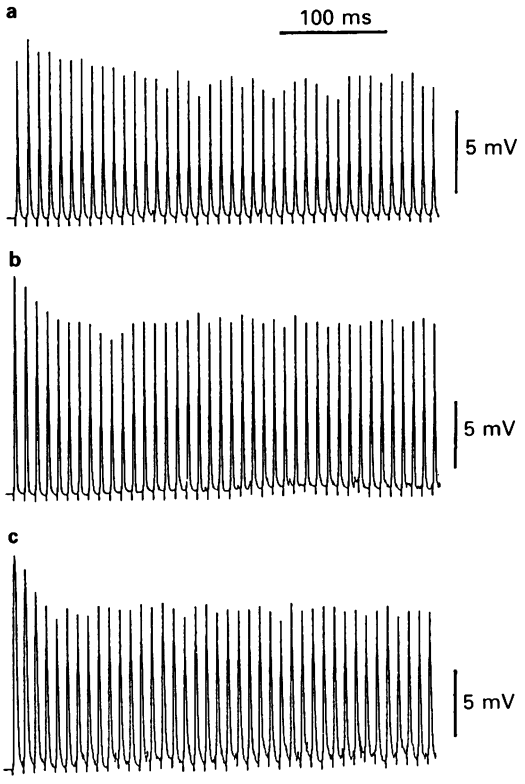

Figure 2 Effects of 3,4-diaminopyridine (DAP) on the patterns of e.p.ps elicited by a train of pulses. E.p.ps were evoked from the same endplate of −46 mV at 100 Hz in the absence (a) or presence of 5 (b) or 20 (c) μM DAP

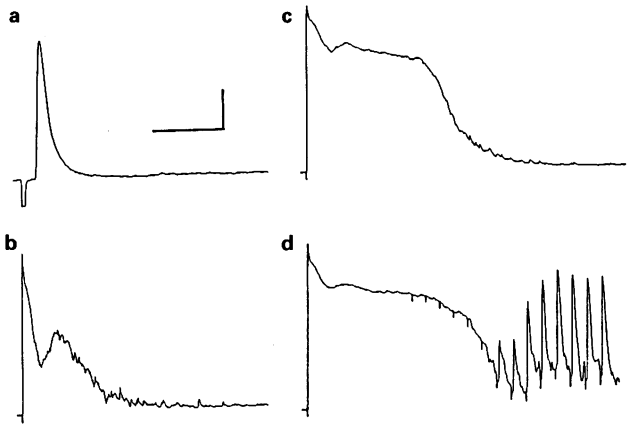

Figure 3 Effects of high concentrations of 3,4-diaminopyridine (DAP) on the duration of endplate potentials (e.p.ps). E.p.ps were evoked from the same endplate of −44 mV by single pulses (a,b,c) or, in addition, 150 ms later by a train of 14 pulses at 50 Hz (d) in the absence (a) or presence of 400 (b) or 4000 (c,d) μM DAP. Note the failure and reappearance of e.p.ps in (d). Calibrations were 2.5 mV and 10 ms (a) or 5 mV and 100 ms (b–d).

nerve stimulations were no longer able to convey e.p.p. which recovered as the membrane potential repolarized (Figure 3d). These prolonged depolarizations were non-propagative and could only be registered within the endplate area. In addition, during the decay phase of prolonged depolarizations, the recorded membrane potential showed increased noise. Sometimes spontaneous repetitive e.p.ps with reduced duration were triggered by a single stimulation when the concentration of DAP reached 200 μM (Figure 4). These repetitive depolarizations could persist for several minutes and might not be accompanied by a plateau component if the discharge rate was greater than $2\,s^{-1}$. They were not abolished by tubocurarine (1 μM) or Mn^{2+} (1.5 mM), though both agents suppressed the peak amplitude and restored the prolonged e.p.ps to a normal time course (see below), suggesting that the repetitive discharges are presynaptic in origin and not subordinate to plateau depolarization.

Effects of 3,4-diaminopyridine on m.e.p.ps and muscle action potentials

Within the concentration range of DAP tested, neither the amplitude nor the 70% duration of m.e.p.ps was significantly altered. The amplitudes before and after treatment with 4000 μM DAP were 1.4 ± 0.3 and 1.6 ± 0.4 mV, and the 70% durations 1.8 ± 0.3 and 2.1 ± 0.3 ms, respectively. The frequency of m.e.p.ps was approximately doubled by this high concentration of DAP (3.1 ± 0.5 vs $1.4 \pm 0.3\,s^{-1}$). The duration of muscle action potentials, elicited directly by current injections, was not altered after 40 μM DAP, and was increased by only 31 ± 6% after 4000 μM DAP (0.83 ± 0.08 vs 0.63 ± 0.06 ms). These results indicate that the prolonged e.p.ps cannot be due solely to a direct postsynaptic effect produced by DAP on K^+, Ca^{2+} or ACh ion channels.

Effects of cholinoceptor antagonists on prolonged e.p.ps

The nicotinic receptor antagonist tubocurarine at very low concentrations (0.025–0.075 μM), which were only 1/10–1/4 of its dissociation constant, dose-dependently shortened the duration of DAP-prolonged e.p.ps with no apparent effect on the peak amplitude (Table 2). The 70% duration of prolonged e.p.ps was decreased 75% by 0.075 μM tubocurarine, whereas the associated membrane noises were only slightly suppressed (Figure 5). Shortening of prolonged e.p.ps by tubocurarine was due mostly to deletion of the plateau component. The upstroke component appeared more resistant to tubocurarine. On repetitive stimulation, e.p.ps with normal duration could be elicited in the presence of tubocurarine as soon as the prolonged e.p.p. faded (Figure 5c). This result together with that in Figure 3d indicate that nerve action potential is unable to provoke transmitter release during the plateau depolarization.

Atropine, at a concentration (1–3 μM) sufficient for the blockade of muscarinic receptors, did not change the amplitude or duration of either normal e.p.ps or prolonged e.p.ps.

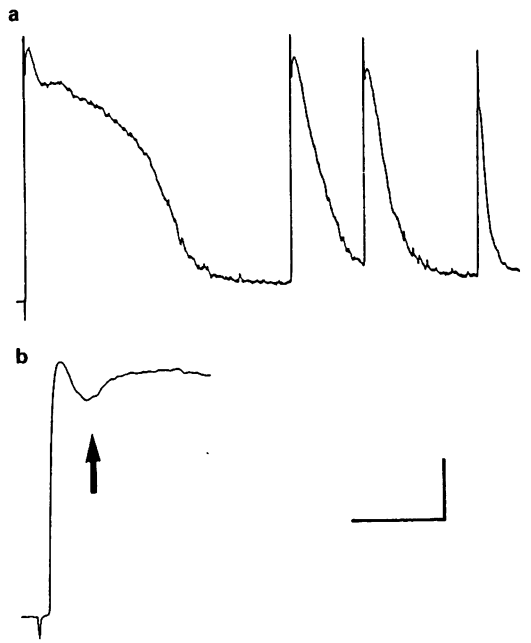

Figure 4 Effects of 3,4-diaminopyridine (DAP) on the spontaneously generated endplate potentials (e.p.ps). The phrenic nerve was stimulated only once in the presence of 2000 μM DAP. In (b) the time scale was expanded to illustrate the upstroke (↑) of the pulse-evoked prolonged e.p.p. The resting membrane potential was −42 mV. Calibrations were 5 mV and 100 (a) or 5 (b) ms.

Table 2 Effects of tubocurarine, verapamil, Mn^{2+} and tetraethylammonium (TEA) on the amplitude and duration of endplate potentials (e.p.ps) evoked in the presence of 3,4-diaminopyridine (DAP)

Treatment (μM)	*E.p.ps* *Amplitude* (mV)	*70% duration* (%)
DAP 4000	20.7 ± 1.9	100
+ Tubocurarine		
0.01	21.3 ± 1.9	82 ± 4*
0.025	19.6 ± 1.6	58 ± 4*
0.05	20.6 ± 1.7	33 ± 4*
0.075	19.8 ± 2.3	24 ± 4*
+ Verapamil		
1	19.2 ± 1.5	53 ± 3*
3	20.4 ± 2.0	46 ± 3*
10	18.5 ± 1.6	27 ± 1*
+ Mn^{2+}		
250	19.3 ± 1.7	57 ± 5*
500	17.8 ± 5.7	44 ± 4*
750	16.9 ± 1.5*	30 ± 3*
+ TEA		
1000	19.1 ± 2.0	188 ± 8*
3000	18.2 ± 1.4	34 ± 3*

E.p.ps were elicited with single pulses. The duration of e.p.ps is presented as % of that in the presence of 4000 μM DAP alone which was 207 ± 37 ms. The resting membrane potentials of the lowest and highest groups were 43.1 ± 1.4 and 46.2 ± 2.2 mV, respectively.
* $P < 0.05$ vs DAP control.

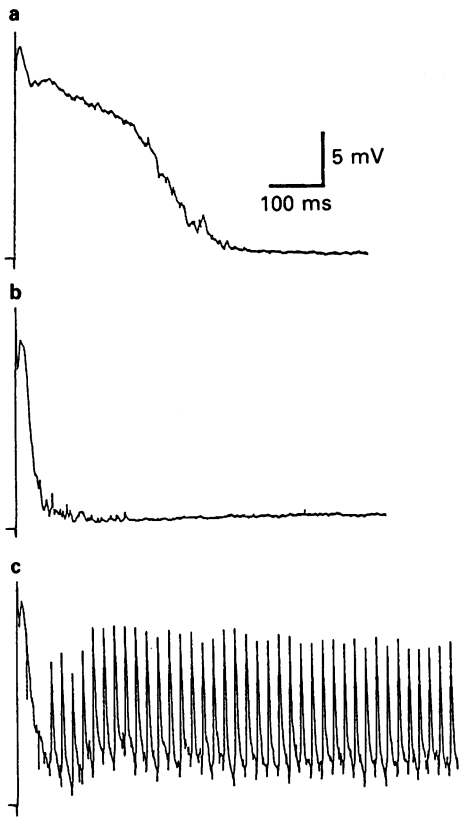

Figure 5 Effects of a low concentration of tubocurarine on 3,4-diaminopyridine (DAP)-induced prolonged e.p.ps. E.p.ps were evoked from the same endplate of −44 mV by single pulses (a,b) or by a train of pulses at 50 Hz (c) in the presence of 4000 μM DAP alone (a) or, in addition, with 0.1 μM tubocurarine (b,c).

Hence, participation of muscarinic receptors in the generation of prolonged e.p.ps seems unlikely. At higher concentrations, tubocurarine (0.2–2 μM) and atropine (30–100 μM) depressed the amplitude of e.p.ps and abolished the plateau component of prolonged e.p.ps.

Table 3 Effects of neostigmine and 3,4-diaminopyridine (DAP) on the amplitude and duration of endplate potentials (e.p.ps)

Treatment (μM)	*Pulse mode*	*E.p.ps* *Amplitude* (mV)	*70% duration* (ms)
DAP			
0	S	8.9 ± 1.0	2.5 ± 0.3
200	S	19.2 ± 1.5	46 ± 8
4000	S	20.7 ± 1.9	216 ± 35
Neostigmine			
0.01–0.02	S,T	11.9 ± 1.1	4.1 ± 0.6
0.3–0.5	S	14.5 ± 1.3	14.9 ± 2.3
	T	19.8 ± 2.1[a]	945 ± 69[a]
DAP 200 + Neostigmine			
0.01–0.02	S	19.9 ± 2.0	491 ± 53
0.3–0.5	S	21.3 ± 1.9	1042 ± 85
DAP 4000 + Neostigmine			
0.01–0.02	S	20.4 ± 2.3	807 ± 68
0.3–0.5	S	21.2 ± 2.2	1153 ± 96

E.p.ps were evoked either with single pulses (S) or at 100 Hz with a train of 3–10 pulses (T). The resting membrane potentials was 45.2 ± 1.9 mV (without DAP) and 45.7 ± 2.1 mV (with DAP).

[a] Values from regenerative depolarizations induced by a train of 5–10 pulses at 100 Hz.

Effects of Ca^{2+} channel blockers on prolonged e.p.ps

Verapamil, an organic Ca^{2+} channel blocker, at low concentrations (1–10 μM) which exerted no obvious depressant effect on e.p.p. amplitudes either in the absence or presence of DAP, shortened DAP-prolonged e.p.ps dose-dependently (Table 2). As in the case of tubocurarine, shortening was due to abbreviation of the plateau component, which could be abolished by high concentrations of verapamil (30–100 μM). The effect of nifedipine, a dihydropyridine analogue of L-type Ca^{2+} channel blockers, on prolonged e.p.ps was similar to that of verapamil but it was about 3 times less potent.

Manganous ion, an inorganic Ca^{2+} channel blocker, shortened prolonged e.p.ps concentration-dependently at 250–750 μM (Table 2). These concentrations of Mn^{2+}, in contrast to the aforementioned blocking agents, also depressed the amplitude of normal e.p.ps by 15–60%. However, the phasic component of DAP-prolonged e.p.ps was less affected by Mn^{2+} (Table 2). When the concentration of Mn^{2+} was raised to 1.5 mM, prolonged e.p.ps could no longer be elicited even after repetitive nerve stimulations. These effects of Mn^{2+} were not due to the block of cholinoceptors since the amplitude of m.e.p.ps was not changed (1.7 ± 0.3 vs 1.3 ± 0.3 mV). Cd^{2+} had a similar effect to Mn^{2+} on prolonged e.p.ps but was about 10 times more potent.

Interaction between 3,4-diaminopyridine and tetraethylammonium

Since DAP blocks certain types of K^+ channels (Cook, 1988), another K^+ channel blocker, TEA was investigated to see if the prolonged e.p.p. was a generalized phenomenon after blockade of K^+ channels. TEA 0.3–3 mM increased the amplitude of the e.p.p. and shifted the curve relating $[Ca^{2+}]_o$ to e.p.p. amplitude leftward (Figure 1). In this respect, TEA was less potent and less efficacious than DAP. At 3 mM, TEA slightly increased the duration of muscle action potential to 0.85 ± 0.09 ms, an effect comparable to that after 4 mM DAP. However, the duration of the e.p.p. was increased at the most from 2.5 ± 0.3 to 4.1 ± 0.9 ms (Table 1) and no sign of prolonged endplate depolarization, similar to that produced after DAP, was observed even with repetitive nerve stimulation at 20–200 Hz. Since TEA at 3 mM exhibited a significant cholinoceptor blocking action, as reflected by about a 35% suppression of m.e.p.p. amplitudes (0.9 ± 0.2 vs 1.3 ± 0.2 mV), higher concentrations of TEA were not studied. Although TEA itself did not provoke a prolonged e.p.p., TEA at 1 mM acted cooperatively with DAP to prolong e.p.ps (Table 2). At a higher concentration (3 mM), TEA abolished the plateau component of DAP-prolonged e.p.ps. The amplitude of single e.p.ps in the presence of DAP was not augmented by TEA (Table 2).

Interaction of 3,4-diaminopyridine with neostigmine

Previously, we had shown that a regenerative ACh release could be elicited in mouse phrenic nerve by a train of pulses (not single pulses) when endplate acetylcholinesterase was inactivated (Chang & Hong, 1986; Hong & Chang, 1989). The 70% duration of endplate depolarizations resulting from this regenerative ACh release lasted for about 1000 ms. To study the effect of enzyme inhibition on DAP-prolonged e.p.ps, preparations were first treated with a low concentration (0.01–0.02 μM) of neostigmine, which increased the duration of single e.p.ps from 2.5 ± 0.3 to 4.1 ± 0.6 ms but provoked no regenerative ACh release even with 250 Hz of repetitive pulses. After this pretreatment, the duration of DAP-induced prolongation of e.p.ps was remarkably increased by 3 to 10 fold, whereas the peak amplitude was not increased (Table 3). This synergistic effect between neostigmine and DAP on the e.p.p. duration appeared to saturate at high concentrations of neostigmine (0.3–0.5 μM), after which DAP did not further prolong the e.p.p. duration (Table 3).

Discussion

In addition to confirming the presynaptic facilitatory effect of diaminopyridines on transmitter release (Thesleff, 1980), the present experiments disclosed that DAP caused, dose-dependently, a prolonged plateau depolarization following the regular phasic e.p.ps. Since DAP does not alter cholinesterase activity (Bowman *et al.*, 1977), postsynaptic cholinoceptor sensitivity (Harvey & Marshall, 1977) and the m.e.p.p. amplitude, the prolonged e.p.ps in the presence of DAP may be regarded as a postsynaptic expression of sustained release of ACh triggered by single pulses. That the prolonged e.p.ps are not the result of inhibition of muscle K^+ channel and/or activation of muscle Ca^{2+} channel is evident, since the muscle action potentials elicited by intracellular current injections were not associated with such prolonged depolarization. Moreover, the prolonged e.p.ps had a quick induction time (less than 10 ms) compared with the slow rise time (150 ms) of muscle Ca^{2+} action potentials or currents that were revealed after blockade of K^+ channels (Chiarandini & Stefani, 1983; Cognard *et al.*, 1988). This short induction time also precludes a dramatic build up of K^+ in the synaptic cleft as a cause of sustained ACh release. The finding that shortening the prolonged e.p.ps by tubocurarine at very low concentrations, did not suppress m.e.p.ps indicates that prolonged e.p.ps are due neither to cumulative K^+ efflux from the postsynaptic site nor to an artifact of possible local endplate contraction. On the other hand, the prolonged e.p.ps cannot be accounted for by repetitive spontaneous neuronal discharges (Lundh, 1978; Heuser *et al.*, 1979; Riker *et al.*, 1985; Perreault & Avoli, 1989), since in the present experiments the spontaneous discharges were resistant to inhibition by tubocurarine, verapamil and Mn^{2+} while the prolonged e.p.ps were very sensitive. It has been shown that tetrodotoxin does not interfere with the induction of prolonged postsynaptic potentials observed in frog nerve-muscle junction (Katz & Miledi, 1979; Molgo *et al.*, 1980) and in *Torpedo* electric organ (Muller, 1986), suggesting that Na^+ channel activation is not involved.

There are many similarities between the prolonged e.p.ps induced by DAP and the regenerative ACh release induced after inhibition of acetylcholinesterase (Chang & Hong, 1986; Hong & Chang, 1989). An upstroke after the phasic endplate depolarization and the prolonged plateau are the two distinctive characteristics. In both cases, additional nerve stimulation did not further prolong the duration and evoked no 'e.p.p.' during the plateau component of prolonged depolarization. Moreover, the prolonged e.p.p. as well as the regenerative release were shortened by low concentrations of nicotinic (but not muscarinic) receptor antagonists and organic Ca^{2+} channel blockers; these concentrations did not suppress the amplitudes of e.p.ps and m.e.p.ps. The prolonged e.p.ps and regenerative release of ACh may share some common mechanism(s) in causing sustained release of ACh. Since the duration of prolonged e.p.ps induced after DAP lasted for a shorter time (200 vs 1000 ms) and was more vulnerable to the inhibitory action of Mn^{2+} than that induced after inhibition of acetylcholinesterase, some differences may exist between the two regenerative release processes in triggering or regulating Ca^{2+} influx into nerve terminals. The longer duration of prolonged e.p.ps in the presence of neostigmine may be due partly to the buffered diffusion of released ACh from the synaptic cleft (Magleby & Terrar, 1975).

Pharmacological studies have revealed abundant K^+ and Ca^{2+} channels on mouse motor nerve terminals to which the release of neurotransmitter was intimately related (Brigant & Mallart, 1982; Penner & Dreyer, 1986; Rowan & Harvey, 1988). Presynaptic nicotinic receptors are also thought to be involved in the positive and negative feedback modulation of transmitter release (Bowman *et al.*, 1986; Chang *et al.*, 1988). The present results suggest that, following administration of high concentrations of DAP, the enhanced evoked-ACh release, in addition to reduction of K^+ current, may allow a prolonged entry of Ca^{2+} into nerve terminals leading to a regenerative release of ACh. Graded inhibitions of prolonged e.p.ps by verapamil, nifedipine, Mn^{2+} and Cd^{2+} are in line with the involvement of L-type Ca^{2+} channels (Lee & Tsien, 1983). The enhancement of DAP action on the duration of e.p.ps by another K^+ channel blocker TEA may suggest that a variety of K^+ channels exist at the nerve terminal for modulation of Ca^{2+} influx. The marked lengthening and shortening of DAP-prolonged e.p.ps, respectively, by neostigmine and tubocurarine at very low concentrations are an indication that the feedback activation of presynaptic nicotinic receptors by released ACh plays a substantial role in activating Ca^{2+} channels. The Ca^{2+} channels involved in the phasic ACh release (as in regular e.p.ps) and in the prolonged e.p.p. are apparently different as judged from the different effective concentrations of DAP (Table 1) and from the different blocking concentrations of various antagonists on these two components.

On comparison of the nerve terminal currents (Penner & Dreyer, 1986; Rowan & Harvey, 1988) with the present prolonged e.p.p. responses, several inconsistencies are evident. For the extracellular perineural currents, TEA alone (10–30 mM) enhanced the slow component of Ca^{2+} spike and DAP displayed maximal effects at 250 μM. A combination of both agents augmented the slow component many times. All these results were obtained from tubocurarine (10 μM at least) -treated preparations to prevent muscle movements. In contrast, prolonged e.p.ps were absent in preparations treated solely with TEA and were abolished by tubocurarine 0.2 μM and by TEA 3 mM. These comparisons suggest that there is probably no sustained evoked ACh release corresponding to the prolonged slow component of terminal Ca^{2+} spike recorded by Mallart (1985) and Penner & Dreyer (1986). Because TEA reduced amplitudes of m.e.p.ps, this nicotinic receptor blocking property could explain the depressant action of TEA on prolonged e.p.ps.

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Enhancement of the endothelial production of prostacyclin by inhibitors of protein synthesis

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1 Pretreatment of bovine aortic endothelial cells with cycloheximide enhanced their capacity to release prostacyclin in response to adenosine 5'-triphosphate (ATP) and bradykinin.

2 The action of cycloheximide was time-dependent; it became detectable after a 1 h exposure to the cells and was maximal after 3 h.

3 Puromycin mimicked the effect of cycloheximide. For these two agents, the enhancement of prostacyclin release was obtained at concentrations producing a partial inhibition of protein synthesis.

4 Cycloheximide increased the mobilization of free arachidonic acid induced by ATP in bovine aortic endothelial cells.

5 In conclusion, the synthesis of new proteins is not involved in the stimulatory action of ATP and bradykinin on prostacyclin production by bovine aortic endothelial cells. Despite the short half-life of prostaglandin H synthase in endothelial cells, cycloheximide and puromycin enhanced the release of prostacyclin induced by agonists. Our data suggest that this release might be under the control of rapidly turning-over phospholipase inhibitory proteins.

Introduction

In rat skeletal muscle, inhibitors of protein synthesis cause a rapid ($t_{1/2} < 10$ min) block in prostaglandin production at the level of prostaglandin H (PGH) synthase (Fagan & Goldberg, 1986). This block is consistent with a rapid turn-over of the enzyme and may account for the antipyretic action of cycloheximide. The turn-over rate of PGH synthase has been measured directly in human umbilical vein endothelial cells, by pulse-chase experiments with [^{35}S]-methionine and detection by reverse immunoblotting: the half-life of the enzyme was less than 10 min (Wu *et al.*, 1988). In this present study, we have investigated whether, as a consequence of this rapid turn-over, inhibitors of protein synthesis decrease the capacity of vascular endothelial cells to release prostacyclin (PGI_2) in response to agonists such as adenosine 5'-triphosphate (ATP) and bradykinin.

Methods

Preparation, culture and incubation of endothelial cells

Bovine aortic endothelial cells (BAEC) were obtained by collagenase digestion of the aorta excised from a freshly slaughtered cow, as previously described (Booyse *et al.*, 1975; Van Coevorden & Boeynaems, 1984). The cells were seeded on 100 mm Petri dishes and incubated at 37°C under an atmosphere of 5% CO_2/95% air in the following medium: MEM D-valine (80%, v/v), foetal calf serum (20%; v/v), glutamine 2 mM, penicillin 100 u ml^{-1}, streptomycin, 100 μg ml^{-1}, amphotericin B 2.5 μg ml^{-1}). The medium was changed after 24 h and then every 3 days. After 4 of 5 days, the primary cultures formed confluent monolayers and could be subcultured. The cells were detached by a 5 min incubation in a Ca^{2+}- and Mg^{2+}-free Hanks buffer containing trypsin (10 mg dl^{-1}) and EDTA (1 mM). Thereafter, they were seeded on 16 mm diameter wells of 24-well plastic trays and the culture was continued in the following medium: DMEM (Dulbecco's modification of Eagle's medium) (60%, v/v), Ham's F_{12} (20%, v/v), foetal calf serum (20%, v/v) with the same concentrations of penicillin, streptomycin and amphotericin B. Experiments were performed with confluent monolayers, between passage 2 and 5. Cycloheximide and puromycin were present during the last part of the culture period (30 min to 5 h). Then, the medium was removed and, after rinsing twice with DMEM, the cells were incubated for 20 min in 0.5 ml DMEM containing the test agents (ATP or bradykinin).

Prostaglandin radioimmunoassay (RIA)

The production of PGI_2 was measured by the RIA of its stable degradation product, prostaglandin 6-keto-$PGF_{1\alpha}$, performed directly in the incubation medium, without extraction and chromatography. A rabbit antiserum was raised against 6-keto-$PGF_{1\alpha}$ coupled to bovine serum albumin (BSA), as described by Maclouf (1981). The limit of detection was 16 pg and the cross-reactivity was 1.2% with $PGF_{2\alpha}$, 0.3% with PGE_2 and <0.1% with thromboxane B_2. Aliquots (100 μl) of incubation medium [^{3}H]-6-keto-$PGF_{1\alpha}$ (11,000 d.p.m.), anti-6-keto-$PGF_{1\alpha}$ antiserum (final dilution 1:10,000) and bovine gamma globulins (2.5 mg ml^{-1}) in Tris buffer (50 mM, pH 7.4) were incubated in a total volume of 0.4 ml for 60 min at room temperature. Then 0.4 ml of a cold 25% (w/w) solution of polyethylene glycol was added to separate bound and free antigen.

Assay of free arachidonic acid release

Arachidonic acid was measured by gas liquid chromatography (g.l.c.) with electron capture detection (ECD), as described previously (Boeynaems *et al.*, 1985). Incubations were performed in a medium containing BSA (1 mg ml^{-1}) and indomethacin (1 μg ml^{-1}). After addition of 1 μg docosahexaenoic acid as an internal standard, the incubation medium was extracted with 1 volume of ethyl acetate. The free fatty acids were converted into pentafluorobenzyl esters by a modification of the method of Wickramasinghe *et al.* (1973). To the dry residue of the extract were added 290 μg pentafluorobenzylbromide (in 5 μl acetonitrile) and 44 μg diisopropylethylamine (in 5 μl acetonitrile). After 5 min at 40°C and evaporation of the solvent under nitrogen, the samples were redissolved in 50 μl hexane. The 20 fold reduction in the amounts of reagents used as compared with the original procedure made it possible to analyze the samples directly without prior purification. Analysis was performed in a Varian model 3700 chromatograph (Varian Associates, Palo Alto, CA, U.S.A.) equipped

with a ^{63}Ni ECD and a 2 m column of 3% OV-1 on Gas chrom Q (Applied Science Laboratories) operated isothermally at 235°C.

Measurement of protein synthesis

BAEC were cultured on 35 mm Petri dishes, in the same conditions as described above. Experiments were started by removing the culture medium and replacing it with methionine-free MEM containing either cycloheximide or puromycin. Ten min later, [^{35}S]-L-methionine (50 μCi/dish) was added. After a 2 h incubation, the medium was removed and 400 μl of the following lysis buffer was added: 62 mM Tris-HCl pH 6.8, 10% (w/v) glycerol, 5% (v/v) β-mercaptoethanol, 23% (w/v) sodium dodecyl sulphate. The radioactivity incorporated into proteins was determined according to the procedure of Siekevitz (1952).

Measurement of cytotoxicity

BAEC were labelled with [^{3}H]-thymidine (5 μCi ml^{-1}) for the last 24 h of the culture period. The medium was then removed and replaced by fresh culture medium containing various concentrations of cycloheximide. Aliquots of this medium were collected at various times for liquid scintillation counting.

Statistical analysis

In every experiment, each incubation was evaluated in triplicate and each experiment was performed at least 3 times. The figures represent the mean ± s.e.mean of all results. The analysis of variance was used to identify significant differences between control cells and cells exposed to cycloheximide or puromycin.

Materials

DMEM, Ham's F_{12}, MEM-D-valine, foetal calf serum, penicillin, streptomycin, amphotericin B and glutamine were obtained from Gibco. Trypsin and methionine-free MEM were purchased from Flow Laboratories. Collagenase type I was from Cooper (Worthington). ATP, bradykinin, cycloheximide and puromycin were obtained from Sigma Chem Co, BSA (fatty acid-poor) from Calbiochem-Behring and 6-keto-$PGF_{1\alpha}$ from Upjohn Diagnostics. [^{3}H]-6-keto-$PGF_{1\alpha}$, [^{35}S]-L-methionine and [^{3}H]-thymidine were purchased from Amersham Corp.

Results

Pretreatment of BAEC with cycloheximide enhanced their production of PGI_2 in response to ATP. In cycloheximide-treated cells, the ATP-induced release of PGI_2 was increased to 311% ± 48% of the control value (mean ± s.e.mean of 29 experiments; range 116%–1336%). This action of cycloheximide was time-dependent (Figure 1): it became detectable after a 1 h exposure to the drug, reached a maximum after 3 h and was maintained up to 5 h. The stimulatory effect of bradykinin on the release of PGI_2 was potentiated by cycloheximide with a similar magnitude to that observed on stimulation with ATP (Figure 2).

Another inhibitor of protein synthesis, puromycin, mimicked the effect of cycloheximide (Figure 3): in puromycin-treated cells, the ATP-induced release of PGI_2 was increased to 235% ± 29% of the control value (8 experiments; range: 146%–350%).

The enhancement of PGI_2 release was obtained at submicromolar concentrations of cycloheximide (Figure 3), which produced only a partial inhibition of protein synthesis (Figure 4): the ED_{50} was estimated to be approximately 0.1 μM for the enhancement of PGI_2 release, whereas the ID_{50} for inhibition of protein synthesis was 0.7 μM.

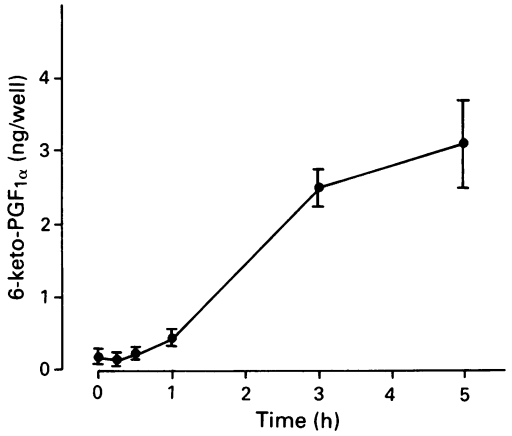

Figure 1 Enhancement by cycloheximide of the ATP-induced release of prostacyclin (PGI_2) from endothelial cells: time course. Bovine aortic endothelial cells were exposed for various periods (15, 60, 180, 300 min) to cycloheximide (2 μM). Then, the culture medium was removed and they were incubated for 20 min in the presence of ATP (5 μM). Results represent the amount of 6-keto-$PGF_{1\alpha}$ accumulated in the medium at the end of this incubation (mean of 3 experiments, in which each condition was tested in triplicate; s.e.mean shown by vertical bars: $P < 0.05$ at 1, 3 and 5 h).

Cycloheximide amplified the mobilization of free arachidonic acid produced by ATP in BAEC (Figure 5). Thus, in cells exposed to cycloheximide, the level of free arachidonate reached under stimulation by ATP was increased to 198% ± 19% of the control value (9 experiments; range 124%–280%).

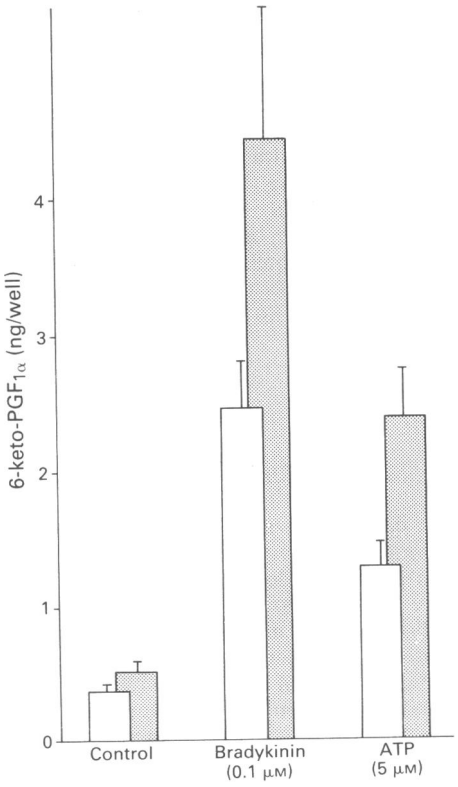

Figure 2 Enhancement by cycloheximide of prostacyclin (PGI_2) release from endothelial cells: comparison between ATP and bradykinin. Bovine aortic endothelial cells were exposed to cycloheximide (2 μM) for 3 h. The culture medium was then removed and the cells were incubated for 20 min, in the presence of either ATP (5 μM) or bradykinin (0.1 μM). Results represent the amount of 6-keto-$PGF_{1\alpha}$ accumulated in the medium at the end of this incubation (mean of 3 experiments, in which each condition was tested in triplicate; s.e.mean shown by vertical bars: $P < 0.05$ for the bradykinin- and ATP-stimulated cells). Open columns, control; stippled columns, cycloheximide (2 μM).

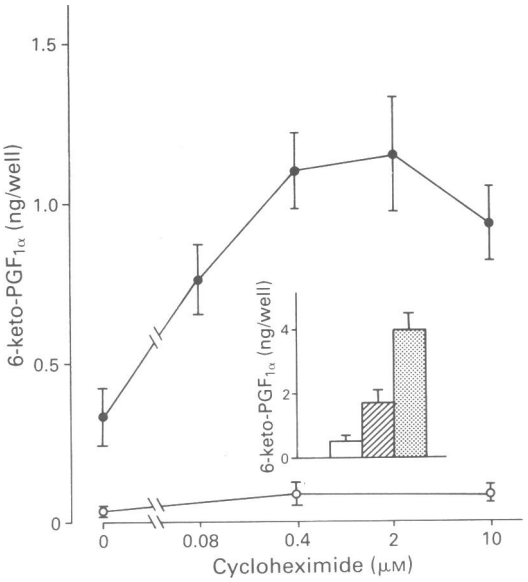

Figure 3 Enhancement by cycloheximide and puromycin of the ATP-induced release of prostacyclin (PGI_2) from endothelial cells. Bovine aortic endothelial cells were exposed for 3 h to increasing concentrations of cycloheximide. After removal of the culture medium, the cells were incubated for 20 min in the presence of ATP (5 μM). Results represent the amount of 6-keto-$PGF_{1\alpha}$ accumulated in the medium at the end of this incubation (mean of 3 experiments, in which each condition was tested in triplicate; vertical bars show s.e.mean). (○) Control; (●) ATP (5 μM). Inset: Cells were exposed for 3 h to puromycin (10 μM), before the test-incubation with ATP (5 μM). Results are the mean of 3 experiments; vertical bars show s.e.mean. Open columns, control; hatched columns, ATP; stippled columns, puromycin then ATP. For ATP-stimulated cells, $P < 0.05$ at all concentrations of cycloheximide and puromycin.

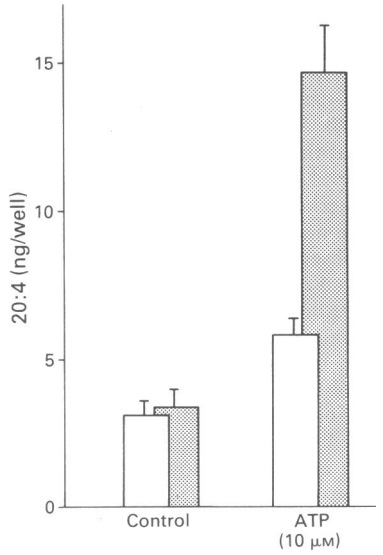

Figure 5 Enhancement of free arachidonate mobilization in cycloheximide-treated endothelial cells. Bovine aortic endothelial cells were cultured for 3 h with (stippled columns) or without (open columns) cycloheximide (2 μM). After removal of the culture medium, the cells were incubated for 30 min in the presence of ATP (10 μM). Results represent the amount of arachidonic acid accumulated in the medium at the end of this incubation, (mean of 3 experiments, in which each condition was tested in triplicate; vertical bars show s.e.mean; $P < 0.05$ for ATP-stimulated cells).

Cytotoxicity was evaluated by the release of [^{3}H]-thymidine from prelabelled cells. No cytotoxic effect of cycloheximide (2 μM) was observed during a 3 h incubation. However, a slight but significant cytotoxic effect of this drug became detectable at 4 and 5 h (Figure 6).

Discussion

In rat skeletal muscle, the short half-life of PGH synthase results in a rapid block of prostaglandin production by inhibitors of protein synthesis (Fagan & Goldberg, 1986). A direct measurement of PGH synthase turn-over has shown that the half-life of this enzyme is also very short in vascular endothelial cells (Wu *et al.*, 1988). Our observation that inhibitors of protein synthesis enhance rather than inhibit the release of PGI_2 from endothelial cells may therefore appear paradoxical. However, Wu *et al.* (1988) noticed that the turn-over of PGH synthase in endothelial cells is characterized by a two-exponential time course, with a rapid phase of degradation followed by a much slower one. Willems *et al.* (1982) have

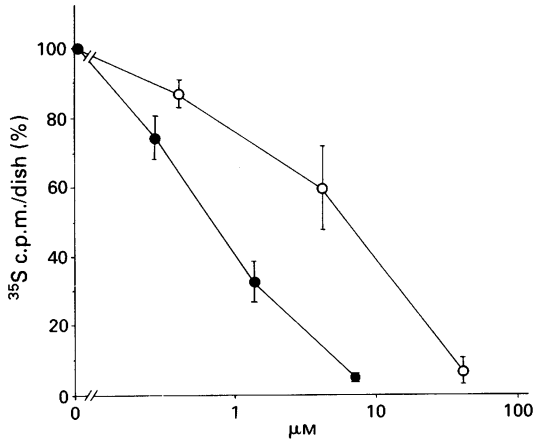

Figure 4 Inhibition of endothelial cells protein synthesis by cycloheximide and puromycin. Bovine aortic endothelial cells were incubated for 2 h in methionine-free MEM containing [^{35}S]-L-methionine (50 μCi/dish) and various concentrations of either cycloheximide (●) or puromycin (○). Results represent the incorporation of radioactivity into cellular proteins, determined by the procedure of Siekevitz (1952) in % of the control value: mean of 3 experiments in which each condition was tested in triplicate; s.e.mean shown by vertical bars.

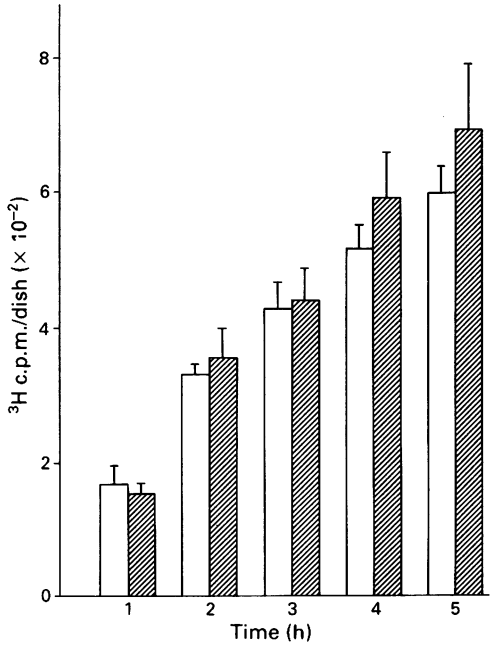

Figure 6 Evaluation of the cytotoxic action of cycloheximide on endothelial cells. At time 0, the culture medium was replaced by fresh medium with (hatched columns) or without (open columns) cycloheximide (2 μM). Aliquots were collected at various times for liquid scintillation counting. Results represent the cumulative radioactivity released in the medium: mean of 3 experiments, in which each condition was tested in triplicate; vertical bars show s.e.mean; ($P < 0.05$ at 4 and 5 h).

provided evidence that two pools of PGH synthase coexist in human umbilical vein endothelial cells; one with a rapid turnover for exogenous arachidonic acid, the other one with a much longer half-life involved in the metabolism of endogenous substrate. In agreement with these observations, our study suggests that the capacity of endothelial cells to produce PGI_2 in response to agonists might be preserved despite the loss of a major fraction of PGH synthase.

Our results are not in agreement with those of Clark *et al.* (1986a) who observed that cycloheximide inhibits the bradykinin-induced release of PGI_2 from a bovine endothelial cell line. These authors speculated that bradykinin, as well as leukotrienes C_4 and D_4, would increase the synthesis of PGI_2 via the induction of a phospholipase A_2-stimulatory protein (Clark *et al.*, 1986b). This mechanism appears unlikely since in endothelial cells, bradykinin induces a very rapid accumulation of inositol 1,4,5-trisphosphate (Derian & Moskowitz, 1986; Lambert *et al.*, 1986), followed by a rise of cytoplasmic Ca^{2+} (Morgan-Boyd *et al.*, 1987; Colden-Stanfield *et al.*, 1987) which is sufficient to activate directly a Ca^{2+}-sensitive phospholipase A_2 (Carter *et al.*, 1988). The discrepancy between our results and those of Clark *et al.* (1986) might be due to their use of very high concentrations of cycloheximide (35 μM) or to peculiarities of their endothelial cell line. From our studies, we also conclude that the enhancement of PGI_2 release is not a mere consequence of cytotoxicity of cycloheximide.

The enhancement of PGI_2 production by cycloheximide is likely to involve inhibition of protein synthesis. Indeed this action was time-dependent, occurred in the same range of concentrations as the inhibition of protein synthesis and was mimicked by puromycin. Another superinductive effect of cycloheximide on endothelial cells has been reported previously: the increase in T lymphocytes adhesion (Cavender *et al.*, 1987). Several inhibitory proteins play a role in the regulation of arachidonic acid metabolism. Lipocortins have been detected in vascular endothelial cells (Hullin *et al.*, 1989), but their role as physiological inhibitors of phospholipase A_2 has become controversial (Davidson *et al.*, 1987). More recently, Raz *et al.* (1989) have shown that the down-regulation of PGH synthase by glucocorticoids involves the induction of a new protein.

In conclusion, our data suggest that the production of PGI_2 by vascular endothelial cells is controlled *inter alia* by (a) rapidly turning-over inhibitory protein(s), which remain(s) to be identified.

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Electrophysiological effects of AFD-21 and AFD-19, new antiarrythmic compounds on papillary muscles and single ventricular myocytes isolated from guinea-pig hearts

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1 The effects of AFD-21, a newly synthesized antiarrhythmic compound, and AFD-19, its active metabolite, on transmembrane action potentials were examined in right ventricular papillary muscles and single ventricular myocytes isolated from guinea-pig hearts.

2 In papillary muscles, AFD-21 10^{-5} M caused a slight prolongation of action potential duration (APD), while AFD-19 above 10^{-6} M shortened APD in a dose-dependent manner.

3 Both AFD-21 and AFD-19 above 10^{-6} M caused a significant and dose-dependent decrease in the maximum upstroke velocity ($\dot{V}_{max}$) of the action potential without affecting the resting membrane potential.

4 In the presence of AFD-21 or AFD-19, trains of stimuli at rates ≥ 0.2 Hz led to an exponential decline in $\dot{V}_{max}$. This use-dependent block was enhanced at higher stimulation frequencies. A time constant for the recovery of $\dot{V}_{max}$ from the use-dependent block was 2.9 s for AFD-21 and 3.6 s for AFD-19.

5 The curves relating membrane potential and $\dot{V}_{max}$ were shifted by AFD-21 (10^{-5} M) or AFD-19 (10^{-5} M) to the direction of more negative potentials by 5.3 mV and 5.1 mV respectively.

6 In single ventricular myocytes treated with AFD-21 (10^{-5} M) or AFD-19 (10^{-5} M), $\dot{V}_{max}$ of test action potentials preceded by conditioning clamp pulses to 0 mV was decreased progressively as the clamp pulse duration was prolonged.

7 These findings suggest that both AFD-21 and AFD-19 have use- and voltage-dependent inhibitory action on the sodium channel by binding to the channel during its inactivated state, and that the unbinding rate is comparable to that of Class I antiarrhythmic drugs with intermediate kinetics.

Introduction

In the early 1970s, Mandel *et al.* (1971, 1972) showed that diphenidol, an antiemetic drug was effective for the treatment of arrhythmias associated with digitalis administration. However, a potent anticholinergic action of the drug prevented its further wide clinical use. AFD-21 (4-diisobutylamino-1,1-diphenyl-1-butanol maleate) is a compound derived from diphenidol, and AFD-19 (4-isobutylamino-1,1-diphenyl-1-butanol maleate) is an active metabolite of AFD-21. These substances have minimal anticholinergic action. *In vivo* studies in mice and dogs have demonstrated that AFD-21 and AFD-19 given orally or parenterally have a potent inhibitory action against ventricular arrhythmias induced by aconitine, digoxin, or coronary ligation (unpublished data). *In vitro* experiments in guinea-pig isolated ventricular muscles have shown that AFD-21 and AFD-19 reduce the maximum upstroke velocity ($\dot{V}_{max}$) of action potential without affecting resting membrane potential (Kojima & Ban, 1989). These findings suggest that primary electrophysiological effects of AFD-21 and AFD-19 are similar to those of local anaesthetic-type (Class I) antiarrhythmic drugs: inhibition of the sodium channel. However, the precise mode of action of these compounds on the cardiac sodium channel in relation to its antiarrhythmic activity remains to be elucidated.

In the present study, the effects of AFD-21 and AFD-19 on the transmembrane action potential were investigated in right ventricular papillary muscles, as well as in single ventricular myocytes isolated from guinea-pig hearts. The modulation of drug-induced $\dot{V}_{max}$ inhibition by stimulation frequencies or by membrane potential level was studied extensively, in order to compare the characteristics of the sodium channel blocking actions of AFD-21 and AFD-19 with other Class I drugs.

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Methods

Papillary muscles

Guinea-pigs of either sex weighing 200 to 250 g were killed by a blow on the head and hearts were quickly removed. Papillary muscles 2 to 3 mm in length and 0.3 to 0.4 mm in diameter were dissected from the right ventricle. The preparation was mounted in the tissue bath and superfused continuously with Krebs-Ringer solution kept at 34°C and gassed with 95% O_2 and 5% CO_2. The composition of the solution was as follows (in mM): NaCl 120.3, KCl 4.0, $CaCl_2$ 1.2, $MgSO_4$ 1.3, $NaHCO_3$ 25.2 and glucose 5.5 (pH 7.4). Equipment for stimulation and for recording transmembrane action potential was the same as described by us previously (Kodama *et al.*, 1985; Toyama *et al.*, 1987).

To study the use-dependent effects of AFD-21 and AFD-19 on the maximum upstroke velocity ($\dot{V}_{max}$) of action potentials, the preparation was stimulated repetitively at varying rates ranging from 0.2 to 2.5 Hz. Rest periods of 60 s, which were sufficient to ensure full recovery from the rate-dependent decrease in $\dot{V}_{max}$, were interposed between the trains of stimuli. This experimental protocol is able to detect the existence of two types of $\dot{V}_{max}$ inhibition by the drugs, tonic and use-dependent block. The former is defined by the decrease of $\dot{V}_{max}$ of the first action potential preceded by the rest period, and the latter is the decrease of $\dot{V}_{max}$ during the train (from the value of first action potential to the new steady state level).

The recovery of $\dot{V}_{max}$ from the use-dependent block was studied by applying a single test stimulus at various coupling intervals following a stimulation train for 20 s at 1.0 Hz. The intensity of the test stimuli was adjusted to obtain a constant latency from the stimulus artifact to the initiation of the action potential upstroke.

To determine the relationship between membrane potential and $\dot{V}_{max}$, the papillary muscle was stimulated at an interstimulus interval of 30 s and the resting potential was made less negative by adding KCl to the medium in 1 to 2 mM steps up to a final K^+ concentration of 20 mM. After an equilibration period of 7 to 8 min for each K^+ concentration, an action potential was recorded to measure $\dot{V}_{max}$.

Single ventricular myocytes

Single ventricular myocytes were isolated enzymatically from guinea-pig hearts by the same procedure as described in our previous study (Kodama *et al.*, 1990). A few drops of cell suspension were placed in a recording chamber attached to an inverted microscope. The chamber was perfused at a rate of 2 ml min^{-1} with normal Krebs solution of the following composition (mM): NaCl 136.9, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.33, HEPES 5.0 and glucose 5.0; pH was adjusted to 7.4 by adding NaOH and the solution was equilibrated by 100% O_2. The temperature was maintained at 35°C.

Following the increase in calcium concentration of the medium to 1.8 mM (normal Krebs solution), 30 to 40% of myocytes deteriorated into round-shaped cells due to irreversible contracture. The remaining cells were tolerant to calcium; their intact rod-shape was maintained without spontaneous beating, and the experiments were carried out with these myocytes.

The single-pipette, whole cell clamp method was employed to control and record membrane potential using single cell/single channel amplifier (List-Medical, L/M-EPC7). The pipettes were heat polished and filled with internal solution to have a resistance ranging from 2 to 3 MΩ. The pipette solution consisted of (mM): KCl 120.0, NaH_2PO_4 10.0, EGTA 1.0, MgATP 5.0 and HEPES 10.0; pH was adjusted to 7.2 by adding KOH. Action potentials were recorded by the current-clamp mode by passing a short stimulus current (2 to 4 ms in duration) through the electrodes. Transition from the voltage-clamp mode to the current-clamp mode was regulated by a pulse generator through an electronic relay. Details of the experimental protocols are given in the Results section.

Drugs and data analysis

AFD-21 and AFD-19 (Nihon Shinyaku Co. Ltd., Osaka, Japan) were dissolved in deionized water and diluted with superfusate (Krebs solution) to achieve the final concentration required. Values are presented as means ± s.e. unless otherwise stated. Data were analysed by *t* test, analysis of variance, Dunnett's test and regression analysis. Differences were considered significant at $P < 0.05$.

Results

Action potentials of papillary muscle

Effects of AFD-21 (3×10^{-7} M to 10^{-5} M) and AFD-19 (3×10^{-7} M to 10^{-5} M) on the membrane action potential configuration were examined in each of six papillary muscles constantly stimulated at 1.0 Hz (Figure 1). Control values of action potential parameters before drug application were as follows: resting membrane potential (RP), −91.3 ± 0.2 mV; maximum upstroke velocity ($\dot{V}_{max}$), 190.2 ± 7.1 V s^{-1}; action potential duration at 30% repolarization (APD_{30}), 204 ± 6 ms; at 90% repolarization (APD_{90}), 271 ± 7 ms ($n = 12$).

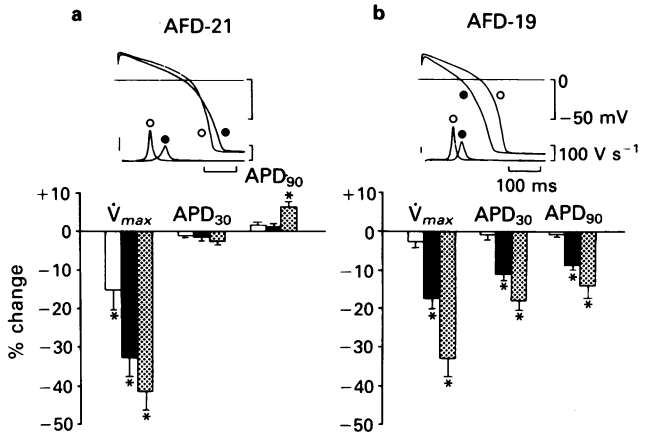

Figure 1 Effects of AFD-21 and AFD-19 on transmembrane action potentials of papillary muscles. Upper panels are superimposed records of membrane action potential and its differentiated upstroke spikes ($\dot{V}_{max}$) before (○) and 30 min after (●) application of AFD-21 (10^{-5} M) or AFD-19 (10^{-5} M). The preparation was constantly stimulated at 1.0 Hz. Lower graphs summarize the change of action potential parameters. APD_{30} and APD_{90} = action potential duration from the upstroke to 30% and 90% repolarization. Values are presented as mean % change from control and bars show s.e.mean ($n = 6$). Effect of AFD-21 or AFD-19 10^{-6} M (open columns), 3×10^{-6} M (solid columns) and 10^{-5} M (stippled columns) are shown. * Significantly different from control at $P < 0.05$.

After exposure to AFD-21 or AFD-19 3×10^{-7} M for 30 min, no significant change in the action potential configuration was observed. AFD-21 10^{-6} M caused a significant decrease in $\dot{V}_{max}$. $\dot{V}_{max}$ was further decreased at the higher concentrations of AFD-21. At 10^{-5} M, APD_{90} was slightly prolonged, while RP and APD_{30} were still unaffected. AFD-19 above 10^{-6} M also caused dose-dependent $\dot{V}_{max}$ reduction without affecting RP. Unlike AFD-21 both APD_{30} and APD_{90} were shortened in a dose-dependent manner by AFD-19 at concentrations ranging from 3×10^{-6} M to 10^{-5} M. IC_{20} of $\dot{V}_{max}$ inhibition induced by AFD-21 and AFD-19, which was obtained by interpolation in a graph of log molar drug concentration versus response, was 1.5×10^{-6} M and 4.3×10^{-6} M respectively.

Use-dependent effects on $\dot{V}_{max}$

The effect of AFD-21 and AFD-19 on $\dot{V}_{max}$ was examined at different rates of stimulation trains separated from each other by a 60 s rest period. In untreated control preparations, the value of $\dot{V}_{max}$ was almost unchanged at rates of stimulation (trains) from 0.2 to 2.0 Hz. After treatment with AFD-21 or AFD-19, $\dot{V}_{max}$ of the first action potential in each train was slightly decreased indicating a minimal tonic block (Table 1).

Table 1 Tonic and use-dependent block of $\dot{V}_{max}$ by AFD-21 and AFD-19

	n	*Tonic block* %	*Onset rate of use-dependent block* 1.0 Hz AP^{-1}	2.0 Hz AP^{-1}
AFD-21				
3×10^{-6} M	(6)	6.2 ± 0.6	0.43 ± 0.02	0.33 ± 0.01
10^{-5} M	(6)	10.4 ± 0.5	0.54 ± 0.03	0.36 ± 0.04
AFD-19				
3×10^{-6} M	(5)	5.4 ± 0.5	0.22 ± 0.02*	0.14 ± 0.01*
10^{-5} M	(5)	14.3 ± 2.7	0.27 ± 0.01*	0.21 ± 0.04*

Values are means ± s.e. n = number of preparations. * Significantly different from the value of AFD-21 at the same concentration ($P < 0.05$).

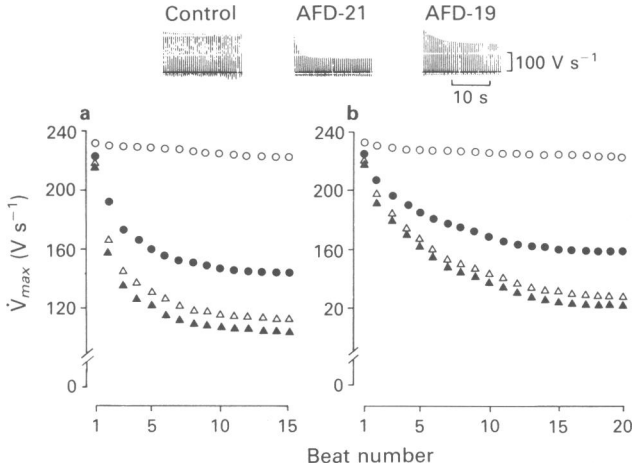

Figure 2 Rate-dependent decrease of the maximum upstroke velocity ($\dot{V}_{max}$). Upper panel: differentiated upstroke spikes of action potentials during stimulation trains at 2.0 Hz in previously quiescent tissue. The records were obtained before, and 30 min after application of AFD-21 3×10^{-6} M or AFD-19 3×10^{-6} M. Lower graphs: beat-to-beat change in $\dot{V}_{max}$ at onset of stimulation trains. Ordinate scales: $\dot{V}_{max}$. Abscissa scales: number of beats (action potentials) from initiation of stimulation train. Frequencies of stimulation were 2.0 Hz under control conditions (○), and 1.0 Hz (●), 2.0 Hz (△) and 2.5 Hz (▲) in the presence of (a) AFD-21 (3×10^{-6} M) or (b) AFD-19 (3×10^{-6} M).

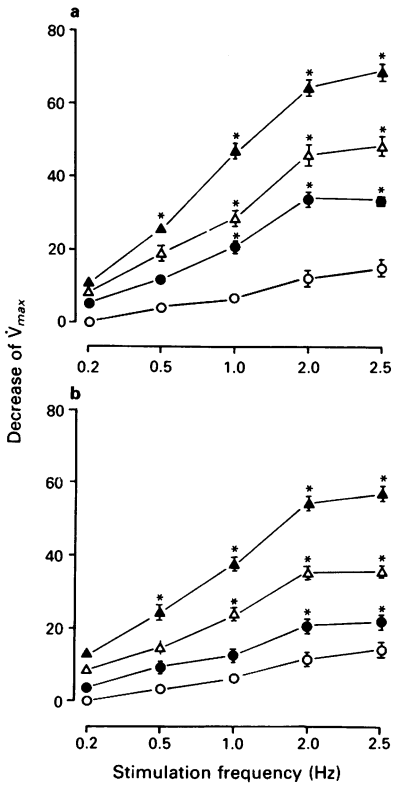

Figure 3 Relationship between stimulation frequency and intensity of the use-dependent block. Ordinate scales: % decrease of maximum upstroke velocity of action potential ($\dot{V}_{max}$) from first action potential of stimulation trains to new steady level. Abscissa scales: stimulation frequency. Data were obtained before (○) and 30 min after the application of (a) AFD-21 or (b) AFD-19 at 10^{-6} M (●), 3×10^{-6} (△) and 10^{-5} M (▲). Values are means and vertical lines show s.e. of the six preparations for AFD-21 and five for AFD-19. * Change was statistically significant from control at $P < 0.05$.

Further decline of $\dot{V}_{max}$ during the repetitive activity (use-dependent block) was dependent on the stimulation frequency; the higher the frequency, the greater the block (Figure 2).

Figure 3 summarizes the percentage decrease of $\dot{V}_{max}$ from the first action potential to the new steady-state level, which was attained at around the 10th to 15th action potentials for AFD-21 and 15th to 20th action potentials for AFD-19. The use-dependent block was larger at higher drug concentrations.

The beat-to-beat decline of $\dot{V}_{max}$ fitted a single exponential curve well (Figure 2), so the onset rate per action potential (AP^{-1}) at which $\dot{V}_{max}$ fell to the new steady-state level could be calculated in each experiment (Table 1). The rate of onset with AFD-21 was faster than that with AFD-19 when compared at the same drug concentration and at the same stimulation frequency (Table 1).

The recovery of $\dot{V}_{max}$ from the use-dependent block was studied by applying a single test stimulus at various coupling intervals following a stimulation train for 20 s at 1.0 Hz. Before the application of the drugs, $\dot{V}_{max}$ of test action potentials recovered almost completely within 100 ms of diastolic interval (the interval from the end of the last action potential to the beginning of the test action potential). After treatment with AFD-21 (10^{-5} M) or with AFD-19 (10^{-5} M), much slower $\dot{V}_{max}$ recovery was observed. Representative results are shown in Figure 4, where fractional reduction of $\dot{V}_{max}$ of test action potentials was plotted against the diastolic interval in a semi-logarithmic graph. In the presence of AFD-21, the recovery time course of $\dot{V}_{max}$ with a diastolic interval longer than 100 ms was approximated by a single exponential function. The average time constant was 2.9 ± 0.1 s ($n = 5$). Similar exponential recovery of $\dot{V}_{max}$ was also observed in papillary muscles treated with AFD-19 at a time constant of 3.6 ± 0.1 s ($n = 5$).

Voltage-dependent effects on $\dot{V}_{max}$

The effects of AFD-21 and ADF-19 on the relationship between $\dot{V}_{max}$ and the resting membrane potential from which the action potential originated were examined in papillary muscles driven at an interstimulus interval of 30 s. The slow rate of stimulation was used to eliminate the use-dependent depression of $\dot{V}_{max}$ by the drugs. The membrane potential was

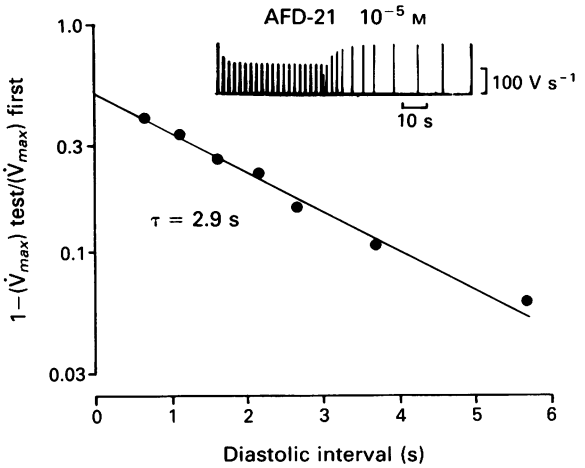

Figure 4 Offset of the use-dependent block of $\dot{V}_{max}$ by AFD-21 in papillary muscles. Inset shows superimposed records of differentiated upstroke spikes of action potentials 30 min after application of AFD-21 (10^{-5} M). The preparation was stimulated at 1.0 Hz for 20 s after a rest period of 60 s. After cessation of the stimulation train, a single test stimulus was applied with various coupling intervals. The graph indicates recovery process of $\dot{V}_{max}$ of test action potential. Ordinate scale: fractional $\dot{V}_{max}$ reduction of the test action potential as compared with $\dot{V}_{max}$ of the first action potential. Abscissa scale: diastolic interval, which was measured from the end (at 95% repolarization) of the last action potential induced by the stimulation train to the upstroke of the test action potential. The time course was approximated by single exponential function with a time constant (τ) of 2.9 s.

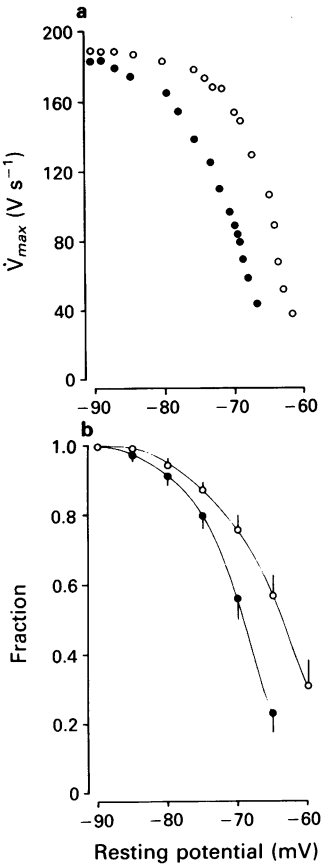

Figure 5 Effects of AFD-21 and AFD-19 on the relationship between resting membrane potential and $\dot{V}_{max}$ in papillary muscles driven with an interstimulus interval of 30 s. Values were obtained before (○) and 30 to 40 min after application of AFD-21 or AFD-19 10^{-5} M (●). Absolute values in one experiment are shown in (a). Means of the normalized values ($n = 5$) are illustrated in (b); vertical lines indicate s.e.mean.

depolarized in steps from the original resting level at 4 mM $[K^+]_0$ to about −60 mV, by increasing the K^+ concentration in the medium. The decrease of $\dot{V}_{max}$ by AFD-21 (10^{-5} M) was more pronounced at less negative membrane potentials (Figure 5). A fraction of $\dot{V}_{max}$ was calculated in each experiment by normalizing the data with the value at 4 mM $[K^+]_0$, and means ± s.e. were obtained. The average curve after application of AFD-21 was shifted by 5.3 mV at a level of 50% reduction along with the voltage axis in the direction of hyperpolarization. AFD-19 caused a similar hyperpolarizing shift of the curve by 5.1 mV (not shown in the figure).

$\dot{V}_{max}$ of single ventricular myocytes

In single ventricular myocytes, the effects of a conditioning clamp pulse on $\dot{V}_{max}$ of the subsequent test action potential were examined, in order to determine whether the use-dependence of the inhibition of $\dot{V}_{max}$ by AFD-21 and AFD-19 is due to the blockade of an activated or an inactivated sodium channel. The baseline characteristics of action potential elicited in the cells at a long interstimulus interval (30 s) were as follows: RP, −83.1 ± 0.3 mV; $\dot{V}_{max}$, 437 ± 13 V s^{-1}; APD_{90}, 170 ± 18 ms ($n = 9$). There were no significant differences in any of these parameters between the subgroups of cells exposed to AFD-21 and AFD-19.

Representative experiments are shown in Figure 6. Following a rest period of 60 s, the membrane potential was clamped up from the resting level (holding potential of −83 mV) to 0 mV for 10 to 2,000 ms. At the end of the conditioning clamp pulse, the membrane potential was clamped back to the holding voltage for 100 ms, which is long enough for a drug-free channel to reactivate fully (Carmeliet & Vereecke, 1979; Ebihara & Johnson, 1980), but short enough so that only partial dissociation of the drug from the blocked channel occurs (Grant *et al.*, 1984). The voltage-clamp was then released, and a stimulus was applied to elicit a test action potential.

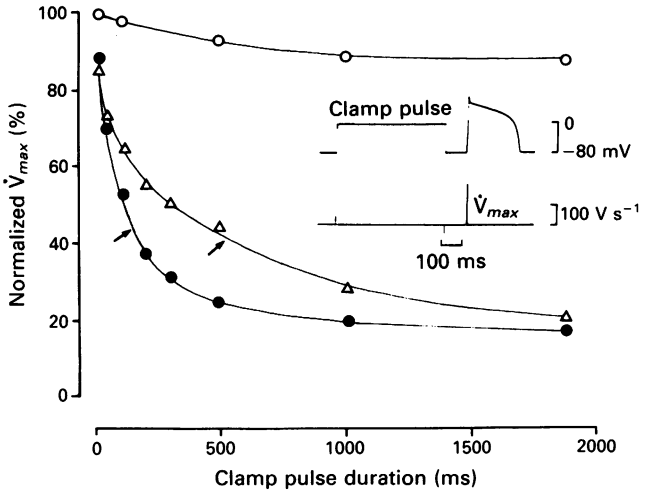

Figure 6 Effects of 0 mV conditioning clamp pulse on the $\dot{V}_{max}$ inhibition induced by AFD-21 and AFD-19 in single ventricular myocytes. Ordinate scale: $\dot{V}_{max}$ of test action potential normalized by the value of action potential without clamp pulse (referenced at the tonic block subtracted value). Abscissa scale: duration of the conditioning 0 mV clamp. Data were obtained before (○) and 10 min after application of AFD-21 10^{-5} M (●) or AFD-19 10^{-5} M (△). Arrows indicate time constant for the exponential decay; 157 ms for 10^{-5} M AFD-21 and 513 ms for 10^{-5} M AFD-19.

In untreated control myocytes, such a clamp pulse with a duration less than 500 ms had no significant effect on the $\dot{V}_{max}$ of the test action potential. However, further prolongation of clamp pulse duration resulted in a slight but significant decrease in $\dot{V}_{max}$. A clamp pulse of 2,000 ms in duration decreased $\dot{V}_{max}$ by 13.0 ± 2.2% ($n = 4$) from the value of action potentials without conditioning clamp pulse (reference level).

Treatment of the myocytes with AFD-21 (10^{-5} M) or with AFD-19 (10^{-5} M) for 15 min did not affect the resting potential (RP). $\dot{V}_{max}$ of the reference action potential was slightly (8 to 13%) decreased compared with the value before drug application. In such myocytes, a conditioning clamp pulse caused a progressive decrease in $\dot{V}_{max}$ of the test action potential as the clamp pulse duration was prolonged (Figure 6). In the presence of AFD-21, the decrease of $\dot{V}_{max}$ from the reference level was minimal (8.6 ± 2.1%, $n = 5$) at 10 ms clamp pulse, but it reached 78.3 ± 6.4% ($n = 5$) at 1000 ms. The $\dot{V}_{max}$ decay with prolongation of clamp pulse duration in each experiment fitted well to a single exponential function. The average time constant was 152 ± 16 ms ($n = 5$). Qualitatively similar results were obtained in experiments with AFD-19; the decrease of $\dot{V}_{max}$ from the reference level was 10.2 ± 3.1% at 10 ms and 69.1 ± 8.0% at 1000 ms clamp pulse ($n = 4$). The time constant of $\dot{V}_{max}$ decay with prolongation of the clamp pulse duration for AFD-19 was 520 ± 21 ms ($n = 4$). The value was significantly longer than that of AFD-21.

Discussion

The present experiments revealed that AFD-21 above 10^{-6} M and AFD-19 above 3×10^{-6} M cause a concentration-dependent decrease in $\dot{V}_{max}$ of papillary muscles. APD was slightly prolonged by AFD-21 at the highest concentration tested (10^{-5} M), while it was shortened appreciably by AFD-19 above 3×10^{-6} M. These findings are partially in agreement with those obtained by Kojima & Ban (1989). They showed that AFD-21 and AFD-19 caused similar dose-dependent decreases in $\dot{V}_{max}$ of guinea-pig papillary muscles at concentrations above 2×10^{-6} M. They observed no significant

change in APD by these compounds. The discrepancies between the present study and their results on the potency of $\dot{V}_{max}$ inhibition and on APD might be attributed to different experimental conditions.

Since the entire tissue was excited simultaneously and there was no conduction within the preparation under the present experimental condition, the decrease of $\dot{V}_{max}$ by AFD-21 or AFD-19 without any accompanying change in RP may reflect an inhibitory effect of these compounds on the fast sodium inward current, I_{Na} (Gintant *et al.*, 1983; Grant *et al.*, 1984). The potency of $\dot{V}_{max}$ inhibition by AFD-21 in guinea-pig papillary muscles constantly driven at 1.0 Hz ($IC_{20} = 1.5 \times 10^{-6}$ M) is higher than that of quinidine and diisopyramide, while that of AFD-19 ($IC_{20} = 4.3 \times 10^{-6}$ M) is comparable to these two compounds (Campbell, 1983a).

The inhibition of $\dot{V}_{max}$ by AFD-21 and AFD-19 was enhanced by a higher stimulation frequency. The frequency-dependence or use-dependence of this effect can be interpreted within the framework of the 'modulated receptor hypothesis', proposed by Hondeghem & Katzung (1977; 1980) to explain the interaction between local anaesthetic type (Class I) antiarrhythmic drugs and cardiac sodium channels. According to this hypothesis, the reduction of I_{Na} is due to the accumulation of drug-associated non-conducting channels (blocked channels). If AFD-21 and AFD-19, like most Class I antiarrhythmic drugs (Grant *et al.*, 1984; Hondeghem & Katzung, 1984), has a higher affinity for the receptor of an activated or inactivated channel than for that of a resting channel, an accumulation of blocked channels during the stimulation train leading to a use-dependent inhibition of $\dot{V}_{max}$ would be expected. The small tonic block of $\dot{V}_{max}$ by AFD-21 and AFD-19 in normally polarized papillary muscles is consistent with such an assumption. The use-dependent block of $\dot{V}_{max}$ by AFD-21 and AFD-19 was observed during stimulation trains at rates ≥ 0.2 Hz, and its onset kinetics were relatively slow. The onset rate at 2.0 Hz (0.33 AP^{-1} at 3×10^{-6} M AFD-21 and 0.14 AP^{-1} at 3×10^{-6} M AFD-19) was comparable to those of quinidine and procainamide, which have been proposed as intermediate kinetic Class I drugs by Campbell (1980, 1983a,b).

Both AFD-21 and AFD-19 caused a slow phase of $\dot{V}_{max}$ recovery following stimulation trains, reflecting the dissociation of the drug from the inactivated or the resting sodium channels. The recovery time constants of AFD-21 (2.9 s) and of AFD-19 (3.6 s) were similar to those obtained for quinidine, procainamide (Campbell, 1983a; Vaughan Williams, 1984) and aprindine (Toyama *et al.*, 1987).

The relationship between $\dot{V}_{max}$ and membrane potential was investigated in papillary muscles stimulated with an interstimulus interval of 30 s. Under such experimental conditions, a decrease in $\dot{V}_{max}$ may reflect only the tonic block by AFD-21 and AFD-19, while the use-dependent block by these compounds may be negligible. The present results have revealed that the decrease in $\dot{V}_{max}$ as a result of tonic block by AFD-21 or by AFD-19 is more pronounced at less negative membrane potentials. This effect is most likely explained by a high affinity of these drugs for inactivated sodium channels (Grant *et al.*, 1984).

Our recent studies have shown that Class I antiarrhythmic drugs can be subdivided into two groups in terms of their sodium channel blocking phase during the conditioning clamp pulse to 0 mV; one 'transient' and one 'maintained' (Kodama *et al.*, 1987; 1989; Courtney, 1988). The former group of drugs (quinidine, disopyramide) may block the sodium channel mainly during its activated state corresponding to the upstroke phase of action potential, while the latter group (lidocaine, mexiletine, tocainide and aprindine) may do so predominantly during the inactivated state which corresponds to the plateau phase of action potential. In the present experiments with AFD-21 and AFD-19, we tested such a 'state-dependency' of sodium channel block in single ventricular myocytes by using similar protocols to those employed in our previous experiments (Kodama *et al.*, 1990). In the presence of AFD-21 (10^{-5} M) or AFD-19 (10^{-5} M), the $\dot{V}_{max}$ of the test action potential decreased progressively as the preceding clamp pulse duration was prolonged. The approximate extent of activated channel block (ACB), which was defined by a percentage decrease of $\dot{V}_{max}$ by a 10 ms clamp pulse from the reference level (tonic block subtracted value), was estimated to be 8.6% for AFD-21 (10^{-5} M) and 10.2% for AFD-19 (10^{-5} M) on average. The approximate extent of inactivated channel block (ICB), which was defined by an additional percentage decrease in $\dot{V}_{max}$ when the clamp pulse duration was prolonged from 10 ms to 500 ms, was 72.3% for AFD-21 (10^{-5} M) and 49.0% for AFD-19 (10^{-5} M). The ratio of ICB/ACB with AFD-21 and AFD-19 was 8.9 and 4.9, respectively. These values are comparable to that with inactivated channel blockers such as mexiletine, tocainide and lidocaine (4.7 to 6.4), but much less than that with activated channel blockers such as quinidine and diisopyramide (1.5 to 1.6) (Kodama *et al.*, 1990). These findings may indicate that both AFD-21 and AFD-19 block the sodium channel primarily when it is in the inactivated state.

Kojima & Ban (1989) demonstrated that APD shortening by nicorandil (an agent to increase K^+ conductance) attenuated the use-dependent block of $\dot{V}_{max}$ with AFD-21, but had no significant effect on that with AFD-19. They, therefore speculated that AFD-21 may preferentially block inactivated sodium channels and AFD-19 activated sodium channels. However, the present data have revealed that there is no such difference in the state-dependency of sodium channel block between the two compounds. The different response to nicorandil can be explained by faster blocking rate of inactivated sodium channels by AFD-21 than AFD-19 during the plateau phase of action potential. A similar hyperpolarizing shift of the curve for the $\dot{V}_{max}$ resting membrane potential relationship by AFD-21 and AFD-19 (Figure 5) may also lend support to the high affinity of the two compounds to the inactivated state of sodium channels.

The *in vitro* concentrations of AFD-21 and AFD-19 used in the present study to decrease $\dot{V}_{max}$ of action potential were within the effective i.v. dose in dog experiments (unpublished data). Accordingly, it seems reasonable to conclude that the primary electrophysiological effect of these compounds as antiarrhythmic agents is an inhibition of fast sodium channels (Class I action). From the change in APD, AFD-21 belongs to Class Ic or Ia drugs, while AFD-19 to Class Ib drugs. From the onset and offset kinetics of the use-dependent $\dot{V}_{max}$ inhibition, they should be classified as intermediate kinetic drugs. From the state-dependency of sodium channel block they belong to inactivated channel blockers. The combination of these aspects for the parent drug (AFD-21) and its metabolite (AFD-19) would provide their unique antiarrhythmic activities, which are different from other antiarrhythmic drugs currently available.

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Receptors involved in mechanical responses to catecholamines in the circular muscle of guinea-pig stomach treated with meclofenamate

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1 In circular muscle strips of the fundus and corpus of guinea-pig stomach, mechanical responses to catecholamines were studied mainly in the presence of a prostaglandin biosynthesis inhibitor, meclofenamate.

2 Normal preparations developed considerable muscle tone, and adrenaline (10–100 μM) in the presence of 3–5 μM propranolol produced a multiphasic response, generally consisting of transient relaxation and contraction, followed by slow relaxation and then contraction. Responses to phenylephrine were similar to those of adrenaline.

3 Meclofenamate (0.3 μM) nearly abolished the muscle tone and under this condition, both adrenaline and phenylephrine produced a simple contraction. This response was strongly inhibited by prazosin, but only weakly by yohimbine.

4 When muscle tone was maintained by prostaglandin E_2 (10 nM) in the presence of meclofenamate, phenylephrine (30 μM) produced transient relaxation followed by slow contraction in most preparations. These were strongly inhibited by prazosin. Adrenaline produced a similar response, but the relaxation was only partially reduced by prazosin. The remaining relaxation was more dominant in the middle fundic region and this was considered to be mediated through β-adrenoceptors.

5 It is concluded that in the circular muscle of the fundic region of guinea-pig stomach, endogenous prostaglandins are involved in maintaining muscle tone and in modifying the response to catecholamines and that both contraction and relaxation are mediated by α_1-adrenoceptors.

Introduction

In the circular muscle of the guinea-pig stomach, catecholamines produce multiphasic mechanical responses through activation of α-adrenoceptors. The relative contribution of contraction and relaxation varies in different regions of the stomach wall and with endogenous muscle tone (Guimaraes, 1969; Bailey, 1971; Haffner, 1971; 1972; Yamaguchi & Tomita, 1974). It has been shown that in the corpus, noradrenaline produces contraction at low concentrations through activation of α_2-adrenoceptors, but relaxation at high concentrations through α_1-adrenoceptors (Sahyoun *et al.*, 1982a,b). On the other hand, activation of α_1-receptors is also considered to be responsible for the contractile response in the guinea-pig stomach (Chihara & Tomita, 1987).

Since the response to α-adrenoceptor activation is composed of contraction and relaxation and their pattern is affected by muscle tone, analysis of receptor types involved in the response is difficult. In some tissues, spontaneous development of muscle tone may be due to endogenous prostaglandins, because it is reduced by an inhibitor of prostaglandin synthesis (meclofenamate or indomethacin; Parekh *et al.*, 1989). Examples include the canine (Milenov & Golenhofen, 1982) and rat stomach fundus (Frankhuizen & Bonta, 1975). In guinea-pig stomach muscles, phospholipase A_2, purified from snake venom, produces mechanical responses very similar to those caused by α-adrenoceptor activation (unpublished observations). It is possible that stimulation of α-adrenoceptors increases endogenous production of prostaglandins, as found in the rabbit vas deferens (Trachte, 1987) and this modifies the direct mechanical response to catecholamines. Thus, in the present experiments, the effects of receptor blocking agents on catecholamine-induced responses were studied in the presence of meclofenamate.

Methods

Hartley guinea-pigs (250–350 g) of either sex were killed by stunning and bleeding. The stomach was removed and opened by cutting the wall along the greater curvature, and the mucosa was completely removed under a binocular microscope. We defined the stomach wall as fundus, corpus and antrum by dividing it into nearly equal parts from the rostral to caudal direction. Four muscle strips (approximately 1 mm width, 7 mm length) were dissected in the direction of the circular muscle fibres between the middle fundus and the rostral side of corpus of the ventral wall of stomach.

Preparations were suspended vertically in a small tube (1 ml in capacity) and superfused with a physiological solution at a rate of 2.5 ml min^{-1} at 35°C. Mechanical responses were measured with an isometric strain gauge and recorded on a potentiometric pen recorder. The experiments were started after the preparation had been equilibrated for at least 1 h to allow full development of muscle tone. Drugs were applied to the superfusing solution. The physiological solution contained (mM): NaCl 129, $KHCO_3$ 6, $CaCl_2$ 2.4, $MgCl_2$ 1.2, glucose 12, Tris-HCl 7.5, the pH being adjusted to 7.4 at 35°C with HCl (ungassed). Experiments were carried out in the presence of propranolol (5 μM) to inhibit β-adrenoceptors, except for β-receptor analysis. When the contribution of β-adrenoceptors was studied, preparations were treated with phenoxybenzamine (50 μM for 30 min followed by a 20 min period of washing) to block α-adrenoceptors, neuronal and extraneuronal catecholamine uptakes (O'Donnell & Wanstall, 1976).

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To obtain dose-response curves, responses to agonists were calculated as a percentage of the maximum contraction obtained with 100 μM adrenaline or phenylephrine, or of the maximum relaxation caused by 10 μM isoprenaline, and were plotted against log concentration of agonist, applied cumulatively with a contact time of 4 min. The concentration producing 50% of the maximum response, EC_{50}, was then interpolated. The dissociation constant (K_D) of antagonists was obtained by a Schild plot (Arunlakshana & Schild, 1959). Numerical values were expressed as mean ± s.d. with the number of preparations in parentheses.

Drugs used were (±)-adrenaline, (±)-isoprenaline, (±)-propranolol, prazosin, yohimbine (all HCl salts from Sigma), indomethacin (Sigma), clonidine and BHT-920 (2-amino-6-allyl-5,6,7,8-tetrahydro-4H-thiazolo-(4,5-d)-azepine) (a gift from Boehringer), sodium meclofenamate monohydrate (a gift from Parke-Davis) and prostaglandin E_2 (a gift from Ono Pharmaceutical Co.). Adrenaline (10 mM) was dissolved in diluted HCl solution (pH about 3) as stock solution and renewed every week.

Results

Circular muscle strips gradually developed muscle tone, after they had been mounted in the chamber, which reached a more or less steady state in about 1 h. Figure 1 shows examples of the adrenaline response in two preparations before and after treatment with meclofenamate (0.1 μM). Adrenaline produced a complicated response depending on the concentration and the prevalent muscle tone, as previously found (Yamaguchi & Tomita, 1974). Furthermore, the response changed during the course of repeated applications of adrenaline at 20–30 min intervals. When the adrenaline concentration was low (about 1 μM), only a slow contraction usually appeared. However, when the concentration was increased, the pattern varied greatly from preparation to preparation. The general pattern was an early transient relaxation, a transient contraction followed by a slow relaxation and a late slow contraction lasting after washout. In 22% of the preparations, the response to the third application of 30 μM adrenaline was mainly contraction (as shown in Figure 1b,c), in 38% it was mainly relaxation (as in Figure 1h,i) and in 40% an intermediate pattern (a complicated mixture of contraction and relaxation) was seen (n = 104), although clear discrimination of the pattern was sometimes difficult. There was a tendency for the transient contraction to increase when adrenaline was applied repeatedly at 20 min intervals.

Meclofenamate (0.1–0.3 μM) strongly reduced the muscle tone and converted the adrenaline response to a simple contraction, independent of the pattern before meclofenamate. The tone decreased to a minimum within 20 min and remained at this level in most of the preparations, but the rate of decrease varied in different preparations. When the inhibition of muscle tone was incomplete, as judged by Ca^{2+} removal, the small early transient relaxation to adrenaline remained (24 in 280 preparations).

Carbachol produced a phasic rhythmic activity on top of a slow contraction (Figure 2). In contrast to the adrenaline response, the pattern of contractions produced by carbachol (50–100 nM) was not much affected by meclofenamate (0.1–0.3 μM) or indomethacin (0.5–1 μM). The small response caused by a low carbachol concentration (10–50 nM) was slightly reduced (86 ± 7% of the control, n = 8) by 0.3 μM meclofenamate.

The responses to phenylephrine, an agonist relatively specific to α_1-adrenoceptors, were similar to those to adrenaline, both before and after meclofenamate application (compare Figure 3a,d with c,f). Small differences were that phenylephrine produced less relaxation in the absence of meclofenamate and faster recovery from contraction in the presence of meclofenamate compared with adrenaline. On the other hand, clonidine, an α_2-adrenoceptor specific agonist, produced a very small and prolonged contraction at the high concentration of 30 μM, compared with phenylephrine and adrenaline at the same concentration (Figure 3b,e). The responses to BHT-920, another α_2-adrenoceptor agonist, were slightly larger and faster than those to clonidine at the same concentration (30 μM). For both BHT-920 (Flavahan et al., 1984) and clonidine (Agrawall et al., 1984) this high concentration gave a maximum contraction in vascular muscles. In the present

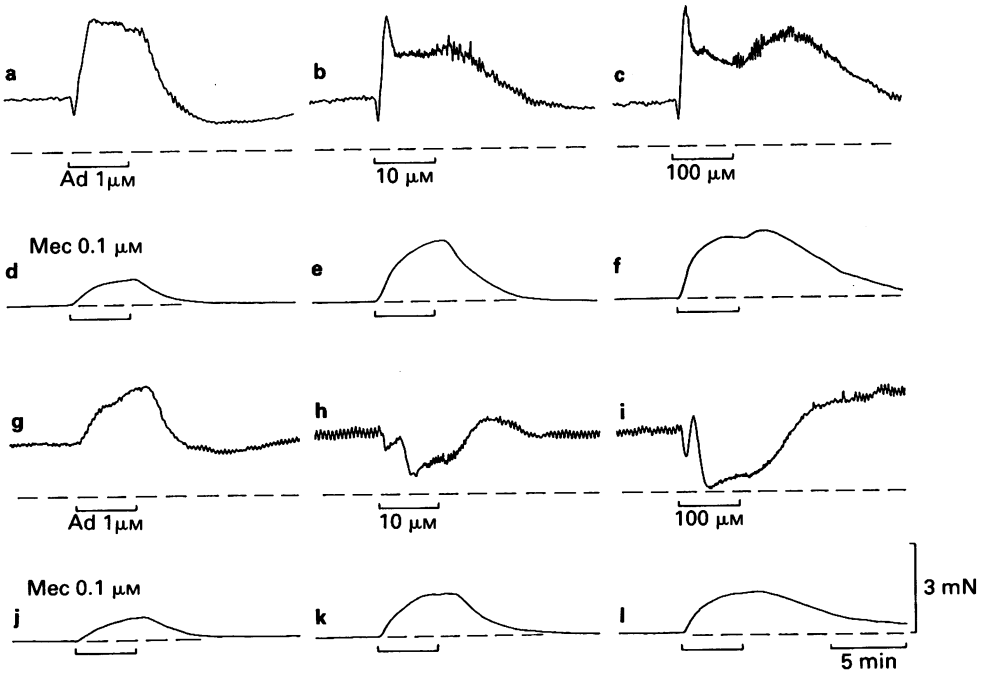

Figure 1 Mechanical responses to adrenaline before and after meclofenamate application in circular muscle strips of guinea-pig stomach fundus. Three different concentrations (1, 10, 100 μM) of adrenaline (Ad) were applied for 4 min, as indicated by horizontal bars, at intervals of 20 min. After recording (c) and (i), meclofenamate (Mec, 0.1 μM) was applied continuously and 30 min later adrenaline application was started. Propranolol (3 μM) was present throughout the experiments. Dotted lines indicate the lowest level of muscle tone. Records, (a–f) and (g–l), are from different preparations. See text for further explanation.

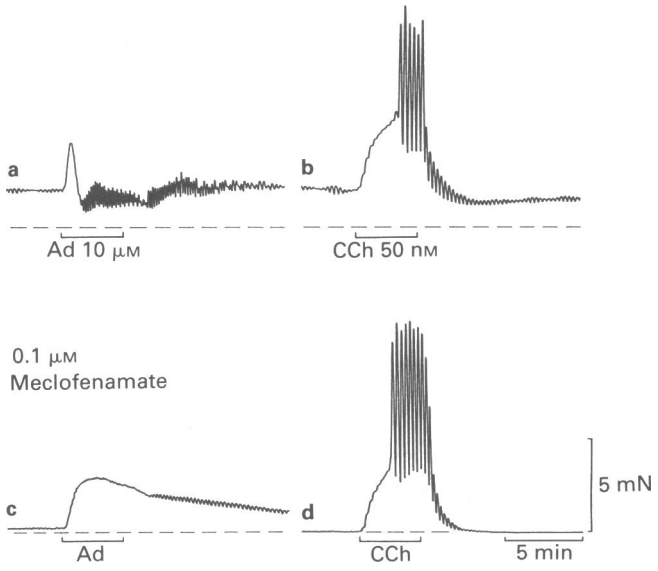

Figure 2 Comparison of the effects of meclofenamate on adrenaline- and carbachol-induced responses. (a and b) Control responses to adrenaline (Ad, 10 μM) and carbachol (CCh, 50 nM). The interval between drug applications was 20 min. (c and d) Responses to the same concentration of the drugs in the presence of meclofenamate (0.1 μM) which was applied after recording (b). Propranolol (3 μM) was present throughout.

experiments, neither produced relaxation between 1 and 30 μM ($n = 8$).

Figure 3 also shows the strong inhibitory effects of prazosin, a specific α_1-adrenoceptor blocker, on responses to three different agonists (30 μM) (g–i). Prazosin (1 μM) almost completely blocked not only the contraction produced by phenylephrine (g) but also that by clonidine (h). The adrenaline response was slightly less susceptible to prazosin (i).

Figure 4 shows the effects of prazosin on the dose-response curve to adrenaline in the presence of meclofenamate (0.3 μM). Prazosin (0.001–0.1 μM) strongly inhibited the response. The slope of the Schild plot was 0.84 ± 0.11 ($n = 6$) which was significantly different ($P < 0.01$) from unity. The apparent pA_2 of prazosin was 9.94 ± 0.12. Yohimbine (0.1–1 μM) had only a weak effect on the size of contraction. The contraction produced by 30 μM adrenaline was reduced to $97 \pm 7\%$ with 0.1 μM and $84 \pm 9\%$ of the control with 1 μM yohimbine ($n = 6$). On the other hand, yohimbine increased the rate of relaxation of contractions produced by adrenaline. The duration of contraction produced by a 4 min application of 30 μM adrenaline, measured at 50% amplitude was 13.5 ± 3.7 min

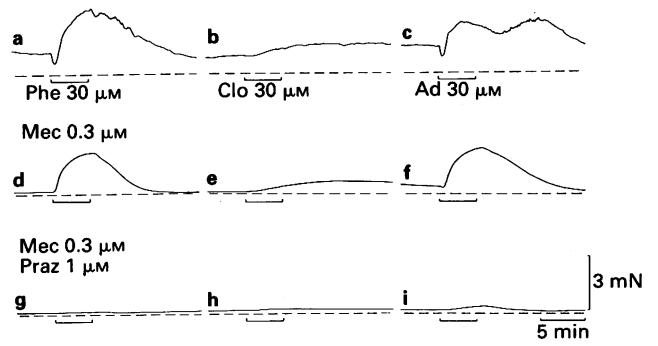

Figure 3 Effects of meclofenamate and prazosin on responses to phenylephrine (Phe, a,d,g), clonidine (Clo, b,e,h), and adrenaline (Ad, c,f,i), each at a concentration of 30 μM. (a–c) Control responses, (d–f) in the presence of meclofenamate (Mec, 0.3 μM), and (g–i) in the presence of meclofenamate (0.3 μM) and prazosin (Praz, 1 μM). These represent successive recordings. Meclofenamate and prazosin were applied 30 min before (d) and (g), respectively. Propranolol (3 μM) was present throughout.

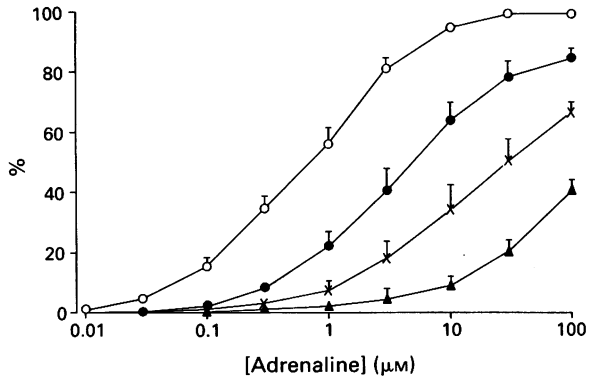

Figure 4 Effects of prazosin (0.001–0.1 μM) on dose-response curves to adrenaline in the presence of 0.3 μM meclofenamate and 3 μM propranolol. Adrenaline was cumulatively applied at each concentration for 4 min, 20 min after preincubation with prazosin and the maximum tension caused by 100 μM adrenaline in the absence of prazosin was taken as 100%. (○) Responses in absence of prazosin; responses in presence of (●) 0.001 μM, (×) 0.01 μM and (▲) 0.1 μM prazosin. Vertical lines show s.d. ($n = 6$).

($n = 14$) and this was reduced to 11.8 ± 3.5 and 7.4 ± 1.5 min by 0.1 and 1 μM yohimbine, respectively.

In order to study the relaxation caused by catecholamines, muscle tone was raised with prostaglandin E_2 (10 nM) to a level similar to that before meclofenamate treatment. As shown in Figure 5, BHT-920, an α_2-adrenoceptor agonist, phenylephrine and adrenaline all produced a simple contraction in the presence of meclofenamate, but the response to BHT-920 was smaller and slower, as with clonidine, compared to other catecholamines. When these catecholamines were applied during sustained prostaglandin application, phenylephrine and adrenaline produced a transient relaxation followed by a slow contraction (e,f), but BHT-920 (and clonidine) failed to produce any relaxation (d). Prazosin (3 μM) blocked the response to phenylephrine almost completely (h), but only partially blocked the adrenaline response (i).

In the preparation shown in Figure 6 which was obtained from the middle region of the fundus, relaxation was the dominant response to adrenaline in the presence of propranolol (3 μM) (a). This changed to a slow contraction after meclofenamate treatment (b). The adrenaline response in the presence of prostaglandin (10 nM) was also mainly relaxation (c). This response was not significantly affected by yohimbine even at 5 μM (d), but was clearly reduced by prazosin (e). The relaxation observed in the presence of prostaglandin is, therefore, unlikely to be mediated through α_2-adrenoceptors and is partly resistant to prazosin. A possibility that β-receptors might be involved in the relaxation was tested by comparing responses to adrenaline and isoprenaline.

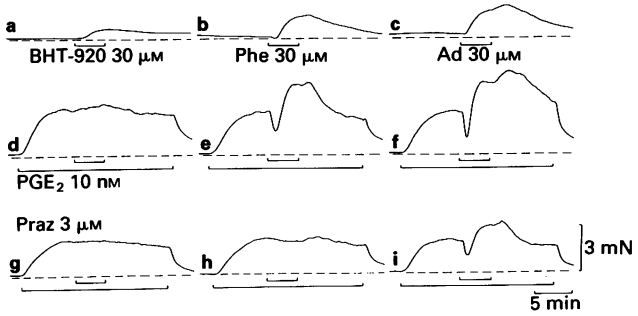

Figure 5 (a,b,c) Responses to three different agonists (30 μM) (BHT-920, phenylephrine, and adrenaline, respectively) in the presence of propranolol (3 μM) and meclofenamate (0.3 μM). (d,e,f) The same agonists were applied during application of prostaglandin E_2 (PGE_2, 10 nM), as indicated by the horizontal bars. (g,h,i) The same as (d,e,f) but in the presence of prazosin (Praz, 3 μM). All records are from the same preparation. See text for further explanation.

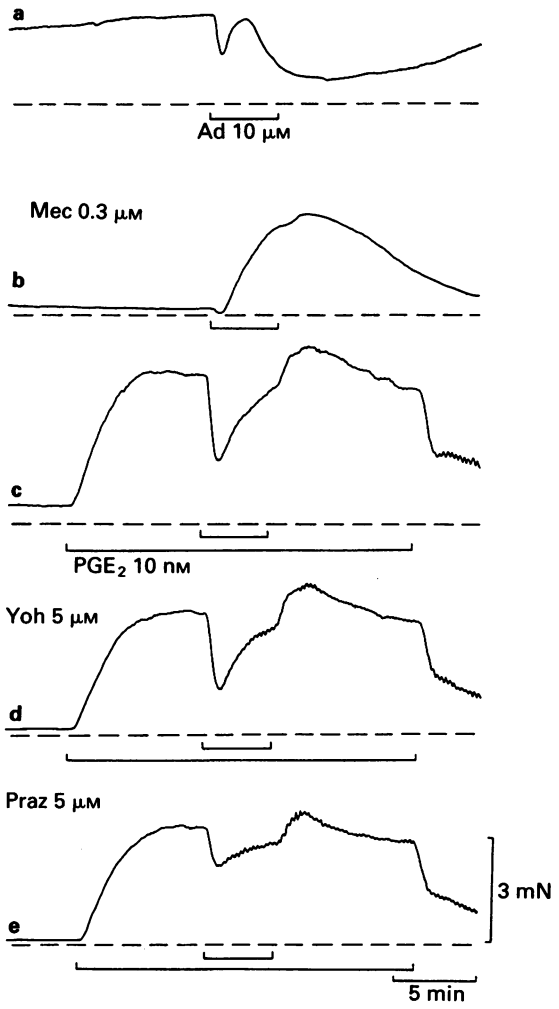

Figure 6 Effects of yohimbine (Yoh) and prazosin on relaxation produced by adrenaline in the presence of meclofenamate (Mec, 0.3 μM), prostaglandin E_2 (PGE_2, 10 nM) and propranolol (3 μM) in a preparation obtained from the middle fundic region. (a) Control response to adrenaline (Ad, 10 μM) before meclofenamate, (b) after meclofenamate and (c) during prostaglandin application. Yohimbine (5 μM) was applied after (c), and 30 min later adrenaline was applied during prostaglandin E_2 (PGE_2)-induced contraction (d). Similarly, prazosin (Praz, 5 μM) was applied between (d) and (e).

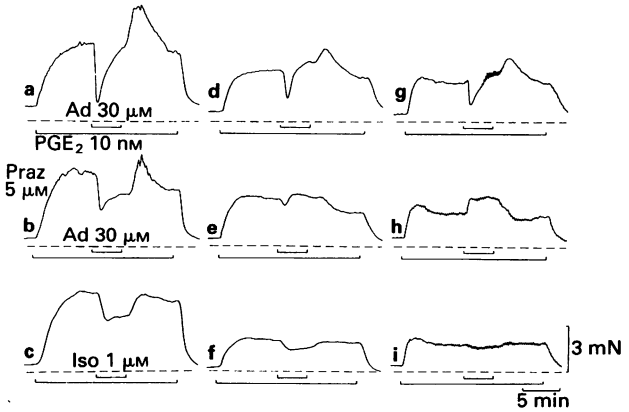

Figure 7 Responses to adrenaline and isoprenaline in the presence of propranolol (5 μM), meclofenamate (0.3 μM) and prostaglandin E_2 (PGE_2, 10 nM) in three different preparations from the same stomach wall; (a–c) middle fundus, (d–f) caudal fundus, and (g–i) rostral corpus. (a,d,g) Responses to adrenaline (Ad, 30 μM) before and (b,e,h) after prazosin (Praz, 5 μM) application. (c,f,i) Responses to isoprenaline (Iso, 1 μM) in the presence of prazosin. See text for further explanation.

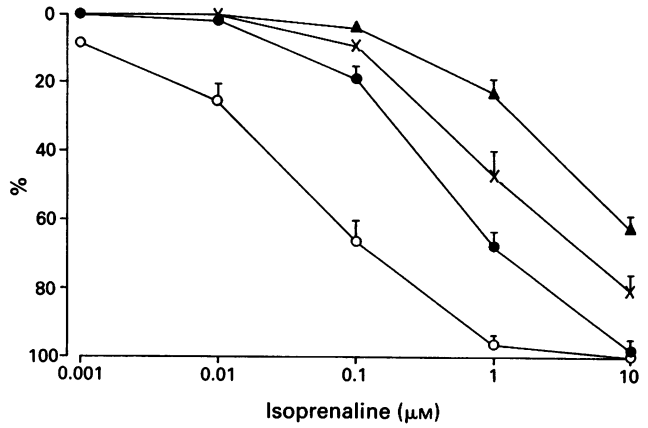

Figure 8 Effects of propranolol on the dose-response curve of isoprenaline-induced relaxation. Preparations (middle fundus) were treated with phenoxybenzamine for 30 min, and isoprenaline was cumulatively applied in the presence of prostaglandin E_2 (10 nM) and meclofenamate (0.3 μM). (○) Control responses in the absence of propranolol; responses in presence of (●) 0.1, (×) 1 and (▲) 5 μM propranolol. Each point represents the mean of 6 preparations with s.d. indicated by vertical bars.

Figure 7 shows responses to adrenaline (30 μM) and isoprenaline (1 μM) in three preparations obtained from the same stomach. When adrenaline was applied in the presence of propranolol (5 μM) and prostaglandin E_2 (10 nM), all preparations produced a transient relaxation (a,d,g). In the preparation obtained from the middle fundus (a–c), the relaxation became smaller but sustained in the presence of prazosin (b), whereas in the preparation from the corpus (g–i), the relaxation was converted to a contraction by prazosin (h). In the muscle strip of the caudal region of fundus (d–f), the adrenaline response was markedly reduced by prazosin (e). Isoprenaline still produced relaxation even in the presence of 5 μM propranolol, but the degree of relaxation decreased in muscle strips taken from the more caudal side of the stomach wall (c to i). This tendency was confirmed in two other experiments. These results suggest that the sustained relaxation caused by adrenaline in the presence of prazosin in the middle fundus (b) is due to activation of β-adrenoceptors.

In Figure 8, inhibition of the isoprenaline-induced relaxation with propranolol (0.1–5 μM) is shown. This result was obtained from 6 preparations of the middle fundic region in the presence of meclofenamate (0.3 μM) and prostaglandin E_2 (10 nM), following treatment with phenoxybenzamine (50 μM). The phenoxybenzamine treatment shifted the dose-response curve of isoprenaline to the left by approximately ten times. Under these conditions, the EC_{50} of isoprenaline was 40 ± 6 nM ($n = 6$). Propranolol, applied 20 min before, dose-dependently inhibited the relaxation by isoprenaline. The slope of the Schild plot was 0.81 ± 0.04 and the apparent dissociation constant of propranolol was 12 ± 5 nM ($n = 6$). Under similar conditions, adrenaline was less effective in producing relaxation, the EC_{50} being 1.6 ± 0.4 μM ($n = 4$). The slope of the Schild plot (0.83 ± 0.03) was similar to that for isoprenaline between 0.1 and 1 μM propranolol. However, increasing the concentration of propranolol from 1 to 5 μM produced only a very small further shift of the dose-response curve. The apparent dissociation constant of propranolol (0.1–1 μM) for adrenaline-induced relaxation was 32 ± 7 nM ($n = 4$).

Discussion

Indomethacin (Smith & Lands, 1971) and meclofenamate (Rome & Lands, 1975) are thought to interfere with prostaglandin biosynthesis mainly by inhibiting the cyclo-oxygenase enzyme, but also by reducing the activity of phospholipase A_2 (Kaplan *et al.*, 1978; Thakkar *et al.*, 1983). Therefore, the reduction of muscle tone and the alteration of the adrenaline

response with meclofenamate and indomethacin observed in the circular muscle of the guinea-pig stomach, suggest that prostaglandins are involved in maintaining the muscle tone and also in modifying the response to adrenaline. Meclofenamate may also act as a blocking agent of prostaglandin receptors (McLean & Gluckman, 1983). This effect was not studied in the present experiments, but since prostaglandin E_2 could induce a clear contraction at less than 10 nM in the presence of 0.3 μM meclofenamate, the receptor blocking action against prostaglandin E_2 in this tissue must be weak.

Since existing muscle tone affects the adrenaline-induced change in muscle tone (Yamaguchi & Tomita, 1974), the change of the adrenaline response into simple contraction in the presence of meclofenamate may result from the decreased muscle tone. However, full recovery of the response pattern was never achieved by raising the muscle tone to the control level with prostaglandin E_2 in the presence of meclofenamate, and the response pattern differed depending on the substance used to increase the muscle tone (e.g., prostaglandin E_2, $F_{2\alpha}$, carbachol) (unpublished observations). This suggests that the change in pattern of the response to catecholamines is not simply due to a fall in tone but to a decrease in production of endogenous prostaglandins or related substances. Catecholamines are known to stimulate prostaglandin synthesis in several tissues. This is probably caused by activation of phospholipase A_2 (Trachte, 1987; Ho & Klein, 1987) or by involvement of diglyceride lipase, following a process mediated by phospholipase C activation (Bell *et al.*, 1979; Irvine, 1982).

In the presence of meclofenamate, the contraction evoked by adrenaline and phenylephrine was strongly inhibited by prazosin, a blocking agent selective for α_1-adrenoceptors. The apparent pA_2 value of prazosin for adrenaline-induced contraction was 9.94, which is similar to that found in those smooth muscles which have predominantly α_1-adrenoceptors (Agrawal *et al.*, 1984). The contractile response to adrenaline was very weakly inhibited by yohimbine (0.1–1 μM). The low value of the slope of Schild plot (0.84) is probably due to inhibitory effects exerted by β-adrenoceptor stimulation at high concentrations of adrenaline, as will be discussed. The idea that α_1-adrenoceptors, rather than α_2-receptors, are responsible for the contraction is supported by the finding that the weak contraction produced by clonidine was blocked by prazosin. Adrenaline may stimulate α_2-receptors to prolong the slow contraction at a high concentration (30 μM), because yohimbine shortens the duration of contraction, but it may be concluded that α_1-receptors are mainly responsible for the contractile response to adrenaline and phenylephrine.

When muscle tone is high, either due to intrinsic generation of prostanoids or in the presence of exogenous prostaglandin, adrenaline and phenylephrine produce relaxation, in addition to the contractile response. Two different mechanisms seem to be involved in the relaxation caused by adrenaline, one involves activation of α_1-adrenoceptors and the other β-adrenoceptors. The contribution of α_1-adrenoceptors is suggested by the observation that phenylephrine, an agonist specific for α_1-adrenoceptors, produced relaxation and that this was blocked by prazosin. Adrenaline-induced relaxation was not affected by yohimbine. Furthermore, clonidine and BHT-920, which have relatively high selectivity for α_2-adrenoceptors, did not produce relaxation. Thus, α_1-adrenoceptors are mainly involved in the relaxation under conditions in which prostaglandin biosynthesis is blocked.

Activation of both α_1- and β-adrenoceptors seems to be responsible for the relaxation caused by adrenaline, particularly in the middle fundic region. The finding that the adrenaline-induced relaxation was more resistant to prazosin than that produced by phenylephrine may be explained if adrenaline at high concentrations (10–100 μM) stimulates β-receptors and that this relaxation is resistant to blockade by propranolol. In the circular muscle of guinea-pig stomach, the ED_{50} value for isoprenaline was 40 nM, and the apparent dissociation constant of propranolol was 12 nM. In other smooth muscles, the ED_{50} of isoprenaline for relaxation has been found to be in the range 6–27 nM, and the dissociation constant of propranolol for this relaxation in the range 0.7–43 nM (guinea-pig myometrium: O'Donnell *et al.*, 1978; guinea-pig trachea: Purdy *et al.*, 1988; rat stomach fundus: Lefebvre *et al.*, 1985; arterial smooth muscles: Purdy *et al.*, 1988; O'Donnell & Wanstall, 1985). Thus, β-adrenoceptors in the circular muscle of guinea-pig stomach fundus seem to be similar to those in other smooth muscles, although the type of β-adrenoceptor was not investigated in the present experiments. The failure of 5 μM propranolol to abolish the adrenaline-induced relaxation is probably due to the high concentration of adrenaline (30–100 μM) used in the present experiments, but the presence of some receptors resistant to propranolol blockade, as found in the guinea-pig ileum (Bond & Clarke, 1988), cannot be excluded.

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Role of nitric oxide in maintaining vascular integrity in endotoxin-induced acute intestinal damage in the rat

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1 The role of endogenous nitric oxide (NO) in maintaining intestinal vascular integrity following acute endotoxin (*E. coli.* lipopolysaccharide) challenge was investigated in the anaesthetized rat by use of N^G-monomethyl-L-arginine (L-NMMA), a selective inhibitor of NO synthesis.

2 L-NMMA (10–50 mg kg^{-1}, i.v.) pretreatment enhanced both the macroscopic and histological intestinal damage and the increases in vascular permeability, measured as the leakage of [^{125}I]-labelled human serum albumen, induced after 15 min by endotoxin (50 mg kg^{-1}, i.v.).

3 The effects of L-NMMA (50 mg kg^{-1}, i.v.) were enantiomer specific, as D-NMMA had no effect. Furthermore, these effects were reversed by L-arginine (300 mg kg^{-1}, i.v.), the precursor of NO synthesis but not by D-arginine (300 mg kg^{-1}, i.v.).

4 L-NMMA (10–50 mg kg^{-1}, i.v.) increased mean systemic arterial blood pressure but this does not appear to be the mechanism by which endotoxin-induced intestinal damage was enhanced, since similar systemic pressor responses induced by phenylephrine (10 μg kg^{-1} min^{-1}, i.v.), had no such effect.

5 The results suggest that synthesis of NO from L-arginine has a role in maintaining the microvascular integrity of the intestinal mucosa following acute endotoxin challenge.

Introduction

Endotoxic shock is characterized by hypotension, intravascular coagulation, increases in vascular permeability, haemoconcentration and gastro-intestinal damage. These effects of endotoxin may result from a direct action of this lipopolysaccharide component of bacterial cell walls on the vascular endothelium (Harlan *et al.*, 1983; Meyrick *et al.*, 1986) or as a consequence of the release of secondary mediators (Parker & Parillo, 1983). In recent studies the vasoactive mediators, platelet activating factor (PAF) and thromboxane A_2 (TXA_2), were found to play key roles in the gastro-intestinal haemorrhagic damage induced by endotoxin in the rat (Wallace *et al.*, 1987; Whittle *et al.*, 1987; Boughton-Smith *et al.*, 1989). In other studies prostacyclin, which is a potent vasodilator and inhibitor of platelet aggregation, attenuated the pathological effects of endotoxin (Lefer *et al.*, 1989; Krausz *et al.*, 1981; Smith *et al.*, 1985; Ditter *et al.*, 1988).

Other endogenous mediators that affect the vasculature may also have a protective role and therefore be involved as a defence mechanism in endotoxic shock. Interest has recently focused on nitric oxide (NO), the labile vasodilator formed from L-arginine by endothelial cells (Palmer *et al.*, 1987; 1988), and which was originally characterized as endothelium-derived relaxing factor or EDRF (Furchgott & Zawadzki, 1980; Furchgott, 1983). In addition to endothelial cells, NO can also be formed by other cells, including macrophages and neutrophils (Hibbs *et al.*, 1988; Marletta *et al.*, 1988; McCall *et al.*, 1989), which may be involved in endotoxic shock. The synthesis of NO by these cells can be selectively inhibited by the L-arginine analogue, N^G-monomethyl-L-arginine (L-NMMA) (Hibbs *et al.*, 1987; Palmer *et al.*, 1988; McCall *et al.*, 1989). Inhibition of endothelial-derived NO by L-NMMA in anaesthetized animals produces an increase in systemic arterial blood pressure and inhibits the hypotensive action of acetylcholine and other endothelium-dependent vasodilators (Rees *et al.*, 1989; Whittle *et al.*, 1989), suggesting that NO may have an important regulatory role on the vasculature *in vivo*. In the present study, therefore, the role of endogenous NO in maintaining vascular integrity in a model of endotoxin-induced acute intestinal damage in the rat was investigated using L-NMMA.

A preliminary account of some of this work has been presented to the British Pharmacological Society (Hutcheson *et al.*, 1990).

Methods

Endotoxin-induced jejunal damage

Male Wistar rats (225–275 g) which had been deprived of food but not water overnight (18–24 h) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and a 25 gauge needle (Butterfly-25, Venisystems) inserted into a tail vein. Lipopolysaccharide from *E. coli* (LPS) was administered as a bolus intravenous injection (4 ml kg^{-1}) at a dose of 50 mg kg^{-1}, which in preliminary dose-range studies gave a moderate degree of intestinal damage. Control animals received isotonic saline (4 ml kg^{-1}) by the same route. After 15 min, a segment of jejunum (6 cm) taken from a region 10–15 cm distal to the pylorus was opened longitudinally and gross macroscopic damage assessed by use of a scoring system of 0 (normal) to 3 (severe damage) based on the degree of hyperaemia and vasocongestion by an observer unaware of the treatment (Boughton-Smith *et al.*, 1989).

Histological damage, in wax-embedded sections (4 μm) of jejunum stained with haemotoxylin and eosin, was assessed under light microscopy in a randomised manner and a scoring system was used where; 0 = normal; 1 = focal regions of vasocongestion; 2 = extensive vasocongestion of the sub-epithelial vessels and congestion to the deeper mucosa; 3 = extensive vasocongestion of the entire depth of the mucosa and submucosal haemorrhage.

Plasma leakage

The vascular permeability and haemorrhage produced by LPS was determined as the gastrointestinal leakage of ^{125}I-labelled human serum albumen ([^{125}I]-HSA, 10 μCi, 37 GBq) administered 20–30 min before LPS or saline as a bolus intravenous injection (150 μl). After a further 15 min, a segment of jejunum (6 cm) was taken as described above, blotted dry, weighed (wet weight) and [^{125}I]-HSA measured with a gamma spectrom-

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eter (Nuclear Enterprises, NE1600). [^{125}I]-HSA was determined in plasma (100 μl) prepared (2 min; 900 g) from blood, drawn from the abdominal aorta and plasma leakage expressed as $\mu l\,g^{-1}$ of tissue.

Blood pressure and haematological analysis

A carotid artery of the anaesthetized rat was cannulated for measurement of systemic arterial blood pressure (Elcomatic EM 750A pressure transducer). In addition, blood samples (100 μl) were collected from the carotid arterial cannula, immediately prior to and 15 min after LPS, for the determination of leukocytes (WBC), erythrocytes (RBC) and haematocrit (HCT) on a Clay Adams Haematology Analyser 5.

Drug treatment

Groups of rats were pretreated with L-NMMA (10, 25 or 50 mg kg^{-1}), its D-enantiometer, D-NMMA (50 mg kg^{-1}) or saline, 15 min prior to LPS injection. The doses of L-NMMA were chosen from previous studies in which they were shown to inhibit endothelium-dependent vasodilatation *in vivo* and the *ex vivo* generation of NO by vascular tissue (Rees *et al.*, 1989; Whittle *et al.*, 1989). In experiments to determine the specificity of L-NMMA, L-arginine (150–300 mg kg^{-1}) or D-arginine (300 mg kg^{-1}) were injected intravenously 5 min after L-NMMA. To determine the importance of the systemic hypertension induced by L-NMMA, control studies were undertaken in which 5 min prior to LPS, the α-adrenoceptor agonist phenylephrine (10 μg kg^{-1} min^{-1}) was administered into a cannulated femoral vein, at a dose that provided a similar increase in blood pressure to that produced by L-NMMA (50 mg kg^{-1}) and continued for 20 min.

Drugs and materials

N^{G}-monomethyl-L-arginine (L-NMMA) and its enantiomer, D-NMMA, were synthesized in the Department of Medicinal Chemistry, WRL by Dr H. Hodson. *E. coli* lipopolysaccharide (0111:B4), L- and D-arginine and phenylephrine were from Sigma Chemical Company and [^{125}I]-labelled human serum albumen was from Amersham International.

Statistical analysis

The data are expressed as the mean ± s.e.mean of (n) rats per experimental group. Statistical comparisons for parametric data were made by Student's t test for unpaired data, except for haematological studies in which a Student's t test for paired data was used. The Mann-Whitney U-test was used for statistical comparisons of non-parametric data. A probability of $P < 0.05$ was considered as statistically significant.

Results

Jejunum damage

As shown in Figure 1, LPS (50 mg kg^{-1} i.v.) produced a significant level of damage in the rat jejunum after 15 min, ($P < 0.05$ compared to saline control) which was characterized macroscopically as a diffuse hyperaemia (damage score, 1.3 ± 0.2, $n = 15$). L-NMMA (10–50 mg kg^{-1}, i.v.) administered prior to LPS, dose-dependently enhanced the jejunal damage. The higher dose of L-NMMA produced extensive damage, with haemorrhage into the jejunal lumen. The enantiomer, D-NMMA (50 mg kg^{-1}) had no significant effect on LPS-induced jejunal damage (Figure 1). In control experiments, treatment with L-NMMA (50 mg kg^{-1}) alone did not produce jejunal damage ($n = 4$). Administration of L-arginine (150 and 300 mg kg^{-1}, i.v.) after L-NMMA, dose-dependently

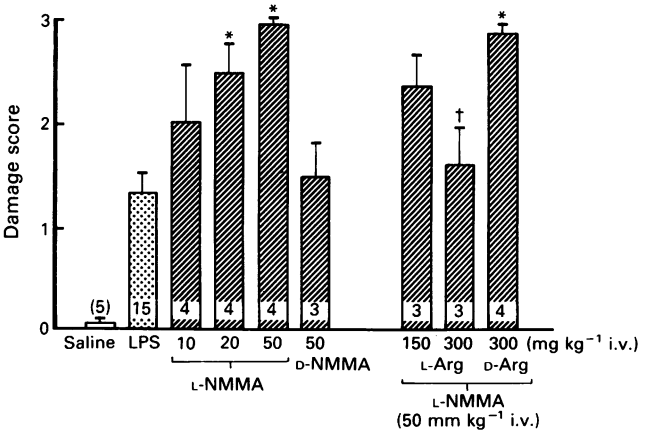

Figure 1 Enhancement of *E. coli* lipopolysaccharide (LPS; 50 mg kg^{-1}, i.v.)-induced macroscopic jejunal damage in the rat by pretreatment with N^{G}-monomethyl-L-arginine. (L-NMMA, 10–50 mg kg^{-1}, i.v.) and the effect of L-arginine (150 and 300 mg kg^{-1}, i.v.) or D-arginine (300 mg kg^{-1}, i.v.). Jejunal damage was scored macroscopically (0–3 scale) in a randomized manner in segments of tissue taken 15 min after LPS. Results are the mean and vertical bars indicate s.e.mean of n (number in column) rats per experimental group. Statistically significant difference from LPS control, is shown as * $P < 0.05$ and the effect of L-arginine as † $P < 0.05$.

inhibited the enhancement of LPS-induced jejunal damage. Thus, L-arginine (300 mg kg^{-1}) completely reversed the damage to the level produced by LPS alone, whereas D-arginine (300 mg kg^{-1}) had no significant effect (Figure 1). L-Arginine (300 mg kg^{-1}) alone had no significant effect on the jejunal damage produced by LPS alone ($n = 4$).

Histological damage

LPS produced moderate vasocongestion in the jejunal villi (score 0.9 ± 0.1, $n = 4$) as shown in Figure 2. However, as described for the macroscopic damage, pretreatment with L-NMMA (50 mg kg^{-1}) enhanced the jejunal vasocongestion produced by LPS and also induced distinct haemorrhage (Figure 2). Furthermore, the enhancement of histological damage was reduced (80 ± 24% inhibition, $n = 6$, $P < 0.05$) by L-arginine (300 mg kg^{-1}, i.v.) but not by D-arginine (Table 1).

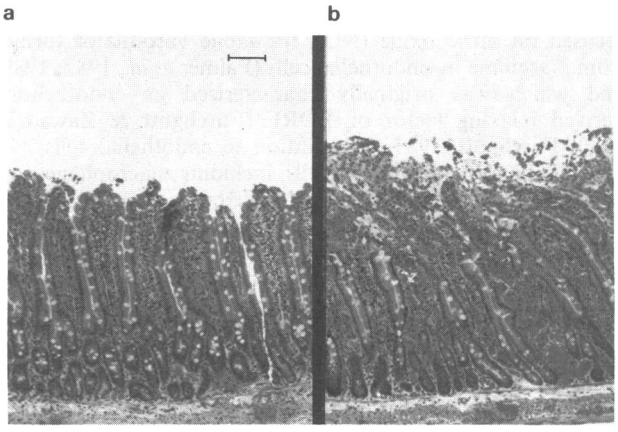

Figure 2 Section of jejunum from rat treated with *E. coli* lipopolysaccharide (LPS: 50 mg kg^{-1}, i.v.) (a) alone or (b) following pretreatment with N^{G}-monomethyl-L-arginine (L-NMMA, 50 mg kg^{-1}, i.v.). The vasocongestion following LPS is limited to the upper portion of the jejunal villi. In animals pretreated with L-NMMA, the vasocongestion and engorgement extent throughout the entire depth of the mucosa. In addition there is haemorrhage and loss of cells from the tips of the villi. (Haematoxylin and eosin).

Table 1 Enhancement of *E. coli* lipopolysaccharide (LPS)-induced jejunal damage by N^G-monomethyl-L-arginine (L-NMMA) and prevention by L-arginine

Treatment ($mg\,kg^{-1}$ i.v.)	(*n*)	*Histological damage*	*% LPS control*
LPS (50)	(4)	0.9 ± 0.1	100 ± 11
LPS + L-NMMA (50)	(6)	2.7 ± 0.2**	300 ± 22**
LPS + L-NMMA + L-arginine (300)	(6)	1.3 ± 0.4	144 ± 44
LPS + L-NMMA + D-arginine (300)	(7)	2.6 ± 0.3**	289 ± 33**

Histological jejunal damage after 15 min was assessed in sections (4 μm) stained with haematoxylin and eosin in a randomised blinded manner by a scoring system of 0 = normal; 1 = focal regions of vasocongestion; 2 = extensive vasocongestion of subepithelial vessels and congestion of deeper mucosa; 3 = extensive vasocongestion of the entire depth of the mucosa and mucosal haemorrhage. Results are given as mean ± s.e.mean of (*n*) experiments, where statistical significant difference from control using Mann-Whitney U-test is shown as ** $P < 0.01$.

Plasma leakage

LPS alone produced a significant plasma leakage into the jejunum (net leakage, $286 \pm 44\,\mu l\,g^{-1}$ of tissue, $n = 10$, $P < 0.001$) that was enhanced dose-dependently by pretreatment with L-NMMA (10–50 $mg\,kg^{-1}$) as shown in Figure 3. At the highest dose, L-NMMA (50 $mg\,kg^{-1}$) enhanced net plasma leakage by $70 \pm 4\%$ ($n = 13$, $P < 0.01$), whilst D-NMMA (50 $mg\,kg^{-1}$) had no effect. L-Arginine (300 $mg\,kg^{-1}$), inhibited the enhanced plasma leakage ($85 \pm 15\%$ inhibition, $n = 5$, $P < 0.05$) produced by L-NMMA (50 $mg\,kg^{-1}$, i.v.), whereas D-arginine (300 $mg\,kg^{-1}$, i.v.) was without effect (Figure 3).

LPS also induced plasma leakage in the stomach, duodenum and ileum (Figure 4) and this was also significantly ($P < 0.05$) enhanced by pretreatment with L-NMMA (50 $mg\,kg^{-1}$). There was, however, no significant plasma leakage in the colon after LPS alone, or following pretreatment with L-NMMA (Figure 4).

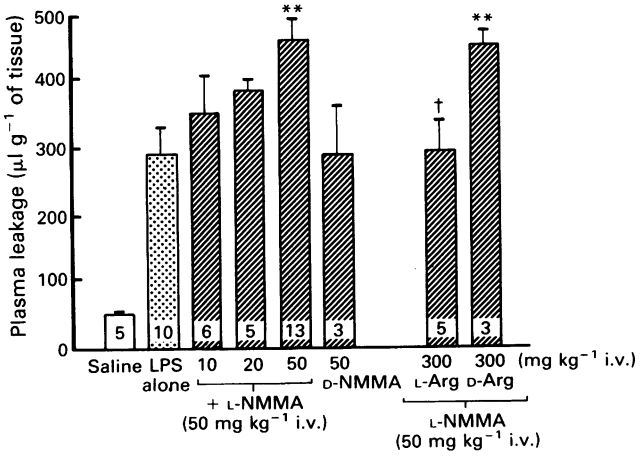

Figure 3 Enhancement of *E. coli* lipopolysaccharide (LPS; 50 $mg\,kg^{-1}$, i.v.) induced increases in intestinal plasma leakage by pretreatment with N^G-monomethyl-L-arginine (L-NMMA, 10–50 $mg\,kg^{-1}$, i.v.) and the effect of L-arginine (L-Arg, 300 $mg\,kg^{-1}$, i.v.) or D-arginine (D-Arg, 300 $mg\,kg^{-1}$ i.v.). The leakage of [^{125}I]-labelled human serum albumen after 15 min administered i.v. 30–40 min prior to LPS was measured in segments of rat jejunum. Results are the mean, and vertical bars indicate s.e.mean of *n* (number in column) rats per group. Statistically significant difference from LPS control is shown as ** $P < 0.01$ and the effect of L-arginine as † $P < 0.05$.

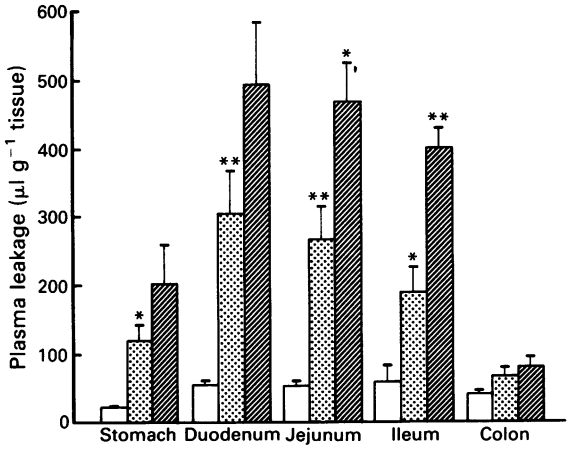

Figure 4 Increases in plasma leakage induced by *E. coli* lipopolysaccharide (LPS, 50 $mg\,kg^{-1}$, i.v.) in different regions of the gastrointestinal tract and enhancement by N^G-monomethyl-L-arginine (L-NMMA, 50 $mg\,kg^{-1}$, i.v.). The plasma leakage of [^{125}I]-labelled human serum albumen, administered 30–40 min before either saline (open columns) LPS (stippled columns) or LPS and L-NMMA (hatched columns) was measured in segments of gastro-intestinal tissue after 15 min. Results are the mean and vertical bars indicate s.e.mean of 4 rats per group. Statistically significant differences between either saline control vs LPS or between LPS vs LPS and L-NMMA are shown as * $P < 0.05$, ** $P < 0.01$.

Systemic arterial blood pressure

The initial resting mean systemic arterial blood pressure (BP) of 127 ± 4 mmHg was reduced to 90 ± 8 mmHg ($n = 11$, $P < 0.05$), 15 min after LPS. L-NMMA (10–50 mg kg^{-1}, i.v.) produced a dose-dependent increase in BP (of 22 ± 1 mmHg; 28 ± 3 mmHg and 31 ± 2 mmHg, at 10, 20 and 50 $mg\,kg^{-1}$ respectively, $P < 0.001$, $n = 4$), that persisted for the duration of the experiment (30 min). The systemic vasopressor effect of L-NMMA (50 $mg\,kg^{-1}$) did not, however, prevent the decrease in BP induced by LPS, which fell to a level (95 ± 5 mmHg, $n = 15$), not significantly different from that induced by LPS alone. When L-arginine (300 $mg\,kg^{-1}$) was administered 5 min after L-NMMA, BP returned to resting levels within 5 min, while D-arginine (300 $mg\,kg^{-1}$) did not reverse the pressor effects of L-NMMA ($n = 4$). D-NMMA (50 $mg\,kg^{-1}$) had no significant effect on BP ($n = 4$).

Haematological analysis

The mean haematological parameters for control blood samples were HCT, $48 \pm 1\%$; RBC, $6.8 \pm 2 \times 10^6\,mm^{-3}$ and WBC, $5.8 \pm 0.4 \times 10^3\,mm^{-3}$. LPS induced a small increase in HCT ($7 \pm 1\%$ increase above control, $n = 6$, $P < 0.05$) and RBC count ($12 \pm 1\%$ increase, $n = 6$; $P < 0.01$) after 15 min (Figure 5). Pretreatment with L-NMMA (50 $mg\,kg^{-1}$) markedly potentiated the increase in HCT and RBC produced by LPS (to $30 \pm 3\%$ and $21 \pm 5\%$ increase above control HCT and RBC respectively, ($n = 8$, $P < 0.01$). Despite the haemoconcentration induced by LPS alone and following pretreatment with L-NMMA, there was no significant change in WBC count. There was, however, a reduction in the WBC/RBC ratio of $11 \pm 1\%$ ($n = 6$) after LPS and of $18 \pm 2\%$ ($n = 8$) after L-NMMA and LPS, suggesting a relative loss of leucocytes from the circulation. The haematological parameters were not affected by L-NMMA (50 $mg\,kg^{-1}$) or L-arginine (300 $mg\,kg^{-1}$) alone ($n = 4$).

Effect of phenylephrine

Intravenous infusion of phenylephrine ($10\,\mu g\,kg^{-1}\,min^{-1}$) produced a sustained increase in BP of 32 ± 7 mmHg ($n = 3$), comparable to that induced by L-NMMA (50 $mg\,kg^{-1}$).

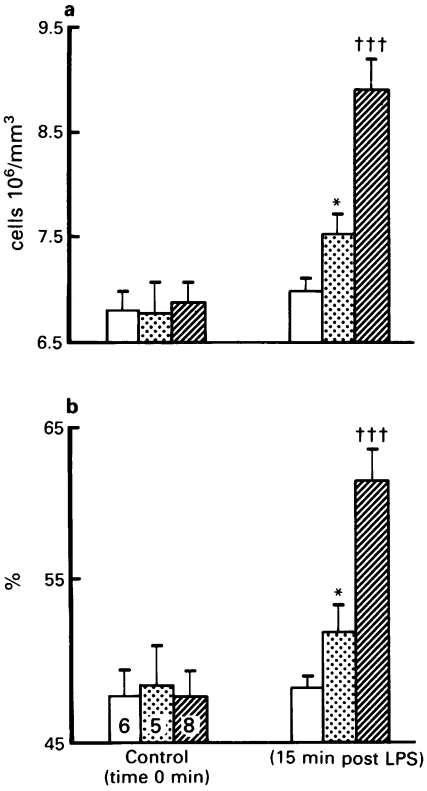

Figure 5 Enhancement of *E. coli* lipopolysaccharide (50 mg kg^{-1}, i.v.)-induced increases in (a) red blood cell count (RBC) and (b) haematocrit (HCT) by pretreatment (15 min) with N^G-monomethyl-L-arginine (L-NMMA, 50 mg kg^{-1}, i.v.). RBC and HCT were measured in blood samples collected from the carotid artery before (time 0) and 15 min after saline (open columns), LPS (stippled columns) or L-NMMA and LPS (hatched columns). Results are the mean, vertical bars indicate s.e.mean of 5–8 rats per group. Statistically significant difference from saline control is shown as * $P < 0.05$ and enhancement above LPS control as ††† $P < 0.001$.

However, concurrent administration of phenylephrine did not significantly affect the jejunal damage (score, 0.8 ± 0.6, $n = 3$) or net plasma leakage (215 ± 70 μl g^{-1} tissue, $n = 3$) induced by LPS.

Discussion

The present study demonstrates that the arginine analogue, L-NMMA, a specific inhibitor of NO synthesis (Palmer *et al.*, 1988), enhances LPS-induced intestinal damage that occurs after 15 min and that the enhancement is prevented by L-arginine, the physiological precursor of NO synthesis. These findings, therefore, indicate that the synthesis of NO from L-arginine has an important role in maintaining the integrity of the intestinal mucosa following acute challenge with LPS.

The enhancement by L-NMMA of the acute macroscopic and histological jejunal damage and the plasma leakage induced by LPS was enantiomer specific, since D-NMMA, which does not inhibit NO biosynthesis (Palmer *et al.*, 1988), had no effect, while the effects of L-NMMA were not reversed by D-arginine. The nature of the jejunal damage, which involved vasocongestion and distinct haemorrhage, implicates vascular injury as an important primary event. In addition to these local intestinal effects, L-NMMA also enhanced the increase in systemic erythrocyte count and haematocrit produced by LPS, suggesting changes in vascular permeability.

Although in the present study, L-NMMA did not attenuate the acute fall in blood pressure induced by this dose of LPS, its systemic vasopressor effects may have confounded any distinct inhibitory actions. Thus, the contribution of endogenous NO release in the complex cardiovascular changes associated with endotoxin shock is not clear. These systemic vasopressor actions of L-NMMA do not seem to be the mechanism of enhanced intestinal damage, since phenylephrine, infused at a dose sufficient to produce a similar vasopressor response as L-NMMA, did not enhance LPS-induced intestinal damage. It is possible however, that L-NMMA and phenylephrine may exert differential local responses in vascular beds, and the effect of L-NMMA on the jejunum may thus be a consequence of changes in regional microvascular blood flow. The inhibition of NO synthesis by L-NMMA may, by removing this endogenous vasodilator, indirectly produce local vasoconstriction and decrease intestinal vascular perfusion. Indeed, L-NMMA substantially decreases vascular conductance in the mesenteric vascular bed of conscious rats (Compton *et al.*, 1989; Gardiner, 1990). In addition, L-NMMA in comparable doses to those used in the present study, has been shown to decrease rat gastric mucosal blood flow (Pique *et al.*, 1989) and also to lead to mucosal damage when the release of other local vasodilator mediators was concurrently inhibited (Whittle *et al.*, 1990).

In previous studies, inhibitors of the potent vasoconstrictor TXA_2 attenuated the intestinal damage produced by LPS (Boughton-Smith *et al.*, 1989). Therefore, the extent of intestinal damage may depend on the local balance within the intestinal microcirculation of damaging vasoconstrictors and protective vasodilators. Indeed, in animal models of endotoxic shock, infusion of the potent vasodilator, prostacyclin, exerted protective effects (Lefer *et al.*, 1989; Krausz *et al.*, 1981; Smith *et al.*, 1985; Ditter *et al.*, 1988), which were attributed to vasodilatation, as well as to inhibition of platelet aggregation and suppression of thromboxane formation. Since NO is also a potent vasodilator that inhibits platelet aggregation, and in addition can inhibit platelet adhesion to the vascular endothelium (Radomski *et al.*, 1987a,b) it may, like prostacyclin, have a local protective role under these conditions. The simultaneous activation of adenylate cyclase by prostacyclin and of guanylate cyclase by NO, as has been demonstrated in platelets (Radomski *et al.*, 1986c), may produce a potentiating interaction on different cells affecting the vasculature, and thereby act synergistically to maintain vascular integrity. The effects of NO-releasing nitrovasodilators, alone or in combination with prostacyclin, in models of vascular damage such as that following endotoxic shock therefore warrant investigation.

The enhancement of acute intestinal damage by L-NMMA resulted in haemorrhage, and therefore the observed increases in intestinal plasma leakage ([^{125}I]-HSA) represent, at least in part, overt vascular damage. The marked enhancement by L-NMMA of LPS-induced haemoconcentration, suggests that a significant part of the plasma leakage is also due to a profound increase in vascular permeability. The mechanism by which the removal of NO by L-NMMA may reduce blood flow to the jejunum but paradoxically increase plasma leakage is not clear, but may reflect the substantial extent to which vascular integrity is compromised by LPS.

Several studies have shown that LPS can stimulate the synthesis of NO. Treatment *in vivo* with *E. coli* LPS increases the amount of NO_3^-, an oxidative product of NO, in the urine of rats (Wagner *et al.*, 1983) and in the blood of mice (Stuehr & Marletta, 1985). Furthermore, in a study *in vitro*, LPS stimulates the formation of NO-like activity by vascular endothelium (Salvemini *et al.*, 1989) and increases NO, NO_2^- and NO_3^- production by mouse macrophages, (Stuehr & Marletta, 1985; Marletta *et al.*, 1988). In addition, the synthesis of NO by rat neutrophils can be stimulated by the bacterial peptide fMet-Leu-Phe (FMLP) and leukotriene B_4 (McCall *et al.*, 1989). In the present study, therefore, the enhancement of acute intestinal damage by L-NMMA could be due to both inhibition of the basal synthesis of NO and also prevention of increases in NO synthesis by leukocytes or endothelial cells in response to LPS stimulation. The failure of L-arginine to reverse the intestinal damage produced by LPS alone suggests that under these conditions, there is sufficient endogenous substrate for NO synthesis to be maximally stimulated.

The mechanisms by which LPS increases vascular permeability, vascular damage and haemorrhage in the jejunum may also involve the generation of reactive oxygen molecules. Endotoxin can prime phagocytic leukocytes to release oxygen radicals (Pabst & Johnston, 1980; Weiss & LoBuglio, 1982) and can stimulate the formation of oxygen radicals indirectly by releasing secondary mediators, such as PAF, or by activation of the complement system which additionally stimulate neutrophils (Sacks *et al.*, 1978). There is considerable evidence that oxygen radicals derived from activated neutrophils can damage endothelial cells and produce increases in vascular permeability (Fantone & Ward, 1982, for review). In addition, the small intestine is also particularly sensitive to ischaemia-reperfusion injury, which has been shown in a cat model to be neutrophil- and oxygen radical-dependent (Granger *et al.*, 1981; Hernandez *et al.*, 1987). This may explain why in the present study, the plasma leakage induced by LPS and its enhancement by L-NMMA was greatest in the small intestine, with no effect on the colon. Therefore, the mechanism of intestinal injury by LPS may involve reactive radicals formed by either activated leukocytes, or possibly, by endothelial cells. Studies *in vitro* have shown that NO can interact with the superoxide radical (O_2^-) to produce a loss in activity of both moieties (Palmer *et al.*, 1987; McCall *et al.*, 1989). Furthermore, similar biological effects of stimulated neutrophils on platelet aggregation can be achieved by either scavenging O_2^- with superoxide dismutase or by the addition of L-arginine, which increases the level of NO formation (McCall *et al.*, 1989). Thus endogenous NO released from endothelial cells or activated phagocytic leukocytes may serve to reduce the acute microvascular damage produced by LPS by scavenging the O_2^- moiety.

The apparent loss of leukocytes from peripheral blood observed following LPS and its enhancement by L-NMMA may be due to neutrophil aggregates becoming lodged in the intestinal microcirculation. The formation of such aggregates has been previously described following PAF-induced gastrointestinal damage (Wallace & Whittle 1986) and may be an important underlying mechanism in the vascular damage produced by PAF and LPS. Neutrophil aggregation can be inhibited by NO (McCall *et al.*, 1988) and it is feasible that NO may also prevent neutrophil adhesion to the endothelium, as has previously been shown for the adhesion of platelets (Radomski *et al.*, 1987b). Such effects may therefore be additional mechanisms by which NO could limit neutrophil-dependent damage to the vascular endothelial cells.

The present study with L-NMMA suggests that endogenous NO has an important acute protective role in the intestinal microvasculature against blood-borne toxins and tissue-destructive mediators. The role of NO in other inflammatory conditions in which there are increases in vascular permeability involving either neutrophils, oxygen radicals or vasoactive mediators, thus requires investigation.

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The effects of drugs on Sephadex-induced eosinophilia and lung hyper-responsiveness in the rat

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1 Rats given an intravenous injection of Sephadex particles (0.5 mg of G200 in 1 ml of saline) on days 0, 2 and 5 had a blood eosinophilia which was maximal on day 7.

2 On day 7, broncho-alveolar lavage (BAL) fluids taken from the rats contained an increased number of eosinophils and fewer mononuclear cells but there was no change in the small number of neutrophils. In addition the rats were hyper-sensitive to the increase in resistance to artificial respiration produced by 5-hydroxytryptamine (5-HT), given intravenously, with a shift to the left of the log dose-response curve. Lung parenchymal strips, taken from the rats on days 6, 7 and 8, were hyper-reactive to 5-HT with an increase in slope of the log dose-response curve.

3 Compounds with a wide variety of activities were evaluated for their effects on the blood eosinophilia on day 7 when given before each injection of Sephadex. The eosinophilia was reduced by glucocorticosteroids, β-adrenoceptor agonists, aminophylline, dapsone and phenidone.

4 Dexamethasone, isoprenaline, dapsone and phenidone at doses that reduced the blood eosinophilia also reduced the changes in number of leucocytes in the BAL fluids and the hyper-responsiveness to 5-HT *in vivo* and *in vitro*, except that the effects of dapsone on the hyper-sensitivity to 5-HT *in vivo* did not reach significance. Aminophylline was the least effective of the drugs at reducing the blood eosinophilia and its effects on the other changes did not reach significance. Sodium cromoglycate reduced the BAL eosinophilia but had no effect on the other changes produced by Sephadex.

5 The correlation coefficients between blood eosinophil numbers and reactivity to 5-HT *in vitro* and sensitivity *in vivo* were $r = 0.76$, ($n = 88$; $P < 0.001$) and $r = 0.53$, ($n = 61$; $P < 0.001$) respectively.

6 Doses of dexamethasone, isoprenaline, dapsone and phenidone that reduced the blood eosinophilia when given before each injection of Sephadex were inactive when given up to 8 h after the Sephadex.

7 These data show an association between blood eosinophilia and hyper-responsiveness of the lung. The blood eosinophilia in the rats was triggered within the first few hours of injecting the Sephadex and drugs have been identified which inhibit this trigger.

Introduction

The need for a new treatment for asthma is highlighted by the current increase in prevalence, severity and mortality of the disease in many developed countries (Mitchell, 1985; Friday & Fireman, 1988). It is now recognised that chronic asthma involves an inflammatory response in the lung and that treatments should be directed to reducing this (Barnes, 1989). The inflammatory response is characterized by the presence of the eosinophil which is thought to contribute to the pathology of the disease (Wardlaw & Kay, 1987; Gleich *et al.*, 1988). The peripheral blood eosinophil count in asthma correlates with the severity of the disease and there is a direct relationship between the ability of glucocorticosteroids to reduce blood eosinophil counts in asthma and clinical benefit whether given orally (Horn *et al.*, 1975) or by inhalation (Harris, 1980). Asthmatics have an exaggerated response to a wide variety of stimuli that can produce an increase in resistance to airflow in the lung and the responsiveness of asthmatics to inhaled histamine was found to correlate with the blood eosinophil count (Taylor & Lukza, 1987). The evidence suggests therefore that a compound that reduced eosinophilia in asthmatics would be of clinical benefit.

We have produced a blood eosinophilia in rats by the intravenous injection of Sephadex particles (Laycock *et al.*, 1986). We have used this model as a screen to detect compounds which reduce eosinophilia. The blood eosinophilia was accompanied by an increase in number of eosinophils and a fall in number of mononuclear cells in the broncho-alveolar lavage (BAL) fluids of the rats and, in addition, the lungs of the rats were hyper-responsive to the spasmogenic effects of 5-HT *in vivo* and *in vitro* (Spicer *et al.*, 1989). We have investigated the effects of reducing the blood eosinophilia with drugs on these other changes.

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Methods

Administration of Sephadex and drugs

Sephadex G200, particle size 40 to 120 μm, when fully swollen in water, was suspended in sterile, isotonic saline at 0.5 mg ml^{-1} and stored at 4°C for 48 h; 1 ml of the suspension was injected intravenously into the hind foot vein of Charles Rivers Sprague Dawley rats, 250–350 g, on days 0, 2 and 5. Rats in a control group received saline. Drugs were given before each injection of Sephadex and, in most experiments, with a contact time expected to give maximum activity at the time of the administration of the Sephadex. Six rats, in a control group, were given Sephadex without a drug each time that drugs were evaluated for their effects on leucocyte numbers in the blood or broncho-alveolar lavage fluids.

The drugs were given parenterally as freshly made neutral solutions in isotonic saline or orally as solutions or suspensions in 0.5% methylcellulose at 0.2 ml for every 100 g of body weight.

Broncho-alveolar lavage

Rats were anaesthetized by the intraperitoneal injection of Sagatal at 0.1 ml 100 g^{-1} of body weight. The trachea were cannulated and 1.5 ml of isotonic saline, containing 6 units ml^{-1} of heparin, was injected into the airways from a syringe connected to the cannula. The liquid was gently

sucked back into the syringe and transferred to the centrifuge tube. This was carried out 4 times. The combined washings, (4 to 5 ml), were centrifuged at 150 g for 5 min, the supernatant removed and the pellet resuspended in 500 μl of saline. Samples of 20 μl were used to determine leucocyte counts. The number of cells are quoted as millions per ml of the suspension in the 500 μl of saline.

Total and differential leucocyte counts

Samples of blood (20 μl) taken from the tail vein of the rats, or resuspended BAL fluid, were added to 10 ml of Isoton II, and within 30 min, Zaponin (3 drops) was added, to lyse the erythrocytes, 5 min before the determination of total cell counts with a Coulter Counter Model DN. Differential leucocyte counts were carried out by fixing and staining a blood smear on a microscope slide with May-Grunwald and Giesma stains. Smears of BAL fluid were stained with a Wright's and Giesma stain (Speirs & Dreisback, 1956). A minimum of 400 cells was counted on each slide. Blood and BAL fluids were collected between 09 h 00 min and 11 h 00 min.

Preparation of lung strips

Blood samples, for leucocyte counts, were taken from the tail vein of rats which were then stunned and bled and the heart and lungs removed and placed in a modified Tyrode solution at room temperature, containing ($g l^{-1}$): NaCl 8.0, $NaHCO_3$ 1.0, glucose 1.0, NaH_2PO_4 0.032, $MgCl_2$ 0.2, KCl 0.04 and $CaCl_2$ 0.05. The initial 2 to 3 mm of the left lobe was removed and then two consecutive strips were cut at right angles to the bronchus 3 to 4 mm wide. The lung strip was suspended in 4 ml organ baths which contained modified Tyrode solution at 37°C through which was bubbled a mixture of 95% O_2 and 5% CO_2. The response of the tissue was recorded with a UFI isometric transducer and a Kipp and Zonen 2 channel pen recorder. A tension of 1 g was applied to the tissue and the tissue was allowed to stabilize for 1 h. During this time the bathing fluid was changed by upward displacement at 15 min intervals and the tension restored after each wash.

Cumulative dose-response curves of the parenchymal lung strips to 5-hydroxytryptamine

5-Hydroxytryptamine (5-HT), as its creatinine sulphate salt, was dissolved in isotonic saline at a concentration of 800 $\mu g\,ml^{-1}$ of the free base. The solution was then diluted by five-fold serial dilutions seven times to 10.24 $ng\,ml^{-1}$. Volumes of 0.1 ml of each concentration of 5-HT, starting with the most dilute, was added in turn to the organ bath at 3 min intervals, without washing out, to produce a cumulative dose-response curve. The area under the log dose curve was calculated over the concentration range of 5-HT from 1.4×10^{-9} to 1.2×10^{-4} M against the increase in tension over 1 g. Three to four animals were given Sephadex and carrier fluid without drug on each occasion that a drug was evaluated. The results obtained on both lung strips from each animal were used to compare the effects of treatments. Some of the lung strips were weighed after the dose-response to 5-HT was obtained. The strips were placed on blotting paper to remove excess solution before weighing.

Increase in resistance to artificial respiration produced by 5-HT

Resistance to respiratory airflow was measured in anaesthetised artificially respired rats by the overflow method of Konzett & Rossler (1940). Rats were anaesthetized by the intraperitoneal injection of a 25% urethane solution in saline (0.6 ml $100\,g^{-1}$ of body weight). The trachea was cannulated and the animal was artificially respired with a Palmer Ideal respiratory pump set to exceed the normal lung capacity at 90 strokes min^{-1}. The overflow volume was measured with Ugo Basile monitor No. 7020 with a Devices DC 3461 amplifier connected to a Devices MX212 recorder. The carotid artery was cannulated for recording blood pressure with a Bell and Howell type 4-422-0001 physiological pressure transducer connected via a Devices 3552 amplifier to the Devices recorder. The jugular vein was cannulated for intravenous dosing. The level of anaesthesia was maintained to suppress spontaneous respiration, by the intraperitoneal injection of additional urethane when required.

The 100% resistance to respiratory airflow was taken as the overflow volume obtained by momentarily clamping the air supply to the trachea and the zero value was the maximum overflow volume during the normal respiratory cycle. After allowing the animal to stabilize for 30 min, doses of 5-HT were given intravenously in 0.1 ml of saline at 5 min intervals, starting with a dose of 1.5 $\mu g\,kg^{-1}$, as the free base, and increasing by two fold serial amounts until the resistance to respiratory airflow was in excess of 80% of maximum, or a dose of 96 $\mu g\,kg^{-1}$ of 5-HT was reached. The doses of 5-HT in excess of threshold produced a rapid increase in overflow volume which returned to the baseline more slowly and was ended by momentarily clamping the overflow tube if it had not returned to baseline after 3 min. To confirm that the sensitivity of the rat to 5-HT had not changed during the experiment, the dosing with 5-HT was repeated. For each rat, the second dose-response curve was not significantly different from the first. Both sets of results were used to plot two dose-response curves for each rat. Rats were tested in groups and treatments were randomised within the groups, each containing at least one positive and one negative control rat, given either Sephadex or saline, respectively, without drugs. The log dose-response curves for each rat were plotted and the ED_{30} values were estimated.

Hyper-sensitivity and hyper-reactivity

We have used the term hyper-sensitivity to mean a parallel shift to the left of the log dose-response curve, with hyper-reactivity being used to describe an increase in the steepness of the slope, as suggested by Orehek *et al.* (1977), with hyper-responsiveness being used as a general term.

Correlation between number of eosinophils in the blood and the responsiveness to 5-HT

The numbers of eosinophils in the blood of rats was determined from differential leucocyte counts made from blood samples taken from the tail veins of the rats immediately before taking lung strips or anaesthetising the rats for measurement of resistance to artificial respiration. Lung strips were taken on days 6, 7 or 8 and measurements of resistance to artificial respiration were made on day 7. The number of eosinophils in the blood was compared with the area under the log dose-response curve for the lung strips *in vitro* or with the $\log_{10}$ of the ED_{30} value *in vivo*. The mean of the two determinations made for each rat was used. Data from all the rats in the treatment groups shown on Table 3 were used except that data for rats given indomethacin were not used for the correlations between blood eosinophilia and sensitivity *in vivo* since indomethacin appeared to potentiate the sensitivity to 5-HT.

Drugs and chemicals

Sodium cromoglycate was a gift from Fisons. Phenidone (1-phenyl-3-pyrazolidone) was obtained from Sigma. Dexamethasone sodium phosphate solutions were prepared from a Decadron solution, 4 $mg\,ml^{-1}$, purchased from Merck Sharpe and Dohme. Urethane, Giemsa, May Grunwald and Wrights stains were from BDH, heparin sodium (mucous), 1000 u ml^{-1} from Weddel, Isoton 11 and Zaponin from Coulter Electronics, Sagatal (pentobarbitone sodium 60 $mg\,ml^{-1}$) from May and Baker, and Sephadex G200 from Pharmacia. Other compounds were obtained from commercial suppliers.

Statistical analyses

The variability of the results for blood and BAL leucocyte numbers was examined using residual plots following an analysis of variance. Analyses on a logarithmic scale were found to be more appropriate. For BAL neutrophils, analysis was performed on log 10 ($x + 0.005$) because of recorded zero counts. Results for Sephadex-treated control animals varied significantly over experiments and therefore treatments were assessed relative to control means within experiments. For each treatment the difference between its mean value and that for its control was calculated and a weighted mean difference was derived over experiments to allow for unequal replication. The weights used were the reciprocals of the variances of the differences, using a pooled error term from all treatment and control groups. Differences were derived on a logarithmic scale with 95% confidence intervals, which when back transformed represented ratios between geometric means. Additionally P values (2 tailed) were determined for these comparisons. Results were quoted as geometric means with 95% confidence intervals or as arithmetic means with s.e.mean.

Results for areas under the 5-HT dose-response curve on lung strips were analysed in a similar way. Control mean values were found to be consistent over experiments and treatment comparisons were made against a pooled control mean.

The responses to 5-HT *in vivo* were analysed in terms of $\log_{10} ED_{30}$ values comparing each treatment group with the Sephadex-treated controls by Student's t test (unpaired, two-tailed). The mean values and s.e.mean were used to derive geometric means with corresponding 95% confidence intervals.

Correlation coefficients were computed by least square analysis. The significance of the differences between other results was assessed by unpaired Student's t test (two-tailed).

Results

Effects of drugs on Sephadex-induced blood eosinophilia

Rats given Sephadex intravenously on days 0, 2 and 5 had an increase in number of eosinophils in the blood which reached a maximum on days 7 to 8 (Figure 1). There was no change in number of other blood leucocytes at the times measured. Compounds, with a variety of pharmacological activities were tested for their ability to reduce the blood eosinophilia, on day 7, when given before each injection of Sephadex. Most were inactive (Table 1) but the eosinophilia was reduced by the adrenoceptor agonists, isoprenaline, salbutamol, and adrenaline and by aminophylline, glucocorticosteroids, as exemplified by dexamethasone and triamcinolone, and by phenidone and dapsone (Figure 2).

The drugs had no effect on numbers of other leucocytes in the blood except for dapsone and the glucocorticosteroids. Dapsone at $100\,mg\,kg^{-1}$ given orally, 30 min before each injection of Sephadex, increased the number of mononuclear cells (from $9.7 \pm 0.67 \times 10^6\,ml^{-1}$, in control animals given Sephadex alone, to $12.7 \pm 0.9 \times 10^6\,ml^{-1}$, $P < 0.001$, $n = 43$) and neutrophils (from $2.8 \pm 0.27 \times 10^6\,ml^{-1}$ to $3.7 \pm 0.22 \times 10^6\,ml^{-1}$, $P < 0.001$). Dexamethasone at $0.1\,mg\,kg^{-1}$ reduced the number of mononuclear cells (from $11.0 \pm 0.75 \times 10^6\,ml^{-1}$ to $7.5 \pm 0.44 \times 10^6\,ml^{-1}$, $P < 0.001$, $n = 37$), arithmetic mean values ± s.e.mean, but had no significant effect on numbers of neutrophils. The effects produced by triamcinolone at $4\,mg\,kg^{-1}$, p.o., given 4 h before the Sephadex were similar to those produced by dexamethasone.

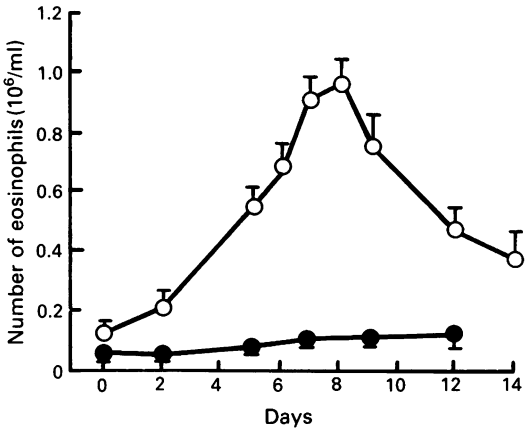

Figure 1 The number of eosinophils in the blood of rats given an intravenous injection of Sephadex, G200, 0.5 mg in 1 ml of saline (○), or saline (●), on days 0, 2 and 5. Blood was collected before the injections on the days when they were given. The values are arithmetic means of $n = 12$ or more for Sephadex-treated rats and 6 or more for rats given saline; s.e.mean shown by vertical bars.

Effects of drugs on changes in the BAL fluids produced by Sephadex

Most of the cells in the BAL fluids of negative control rats, given saline instead of Sephadex, were mononuclear cells, less than 5% of the cells were neutrophils and no eosinophils were detected. Eosinophils were found, on day 7, in the BAL fluids of rats given Sephadex, at the time of the peak in blood eosinophil numbers. At this time there was also a fall in number of mononuclear cells but there was no change in the number of neutrophils. Drugs which reduced the blood eosinophilia were tested for their effects on the changes in the BAL fluids. Doses of dexamethasone, isoprenaline, aminophylline, dapsone and phenidone which reduced the blood eosinophilia when given before each injection of Sephadex also reduced the increase in number of eosinophils in the BAL fluids, although the reduction produced with aminophylline did not reach significance. These drugs also reduced the fall in number of mononuclear cells in the BAL fluid but again this did not reach significance with aminophylline. Sodium cromoglycate, which had no effect on the blood eosinophilia, produced some reduction of the eosinophilia in the BAL fluids but did not prevent the fall in number of mononuclear cells.

Table 1 Compounds that had no effect on the increase in number of eosinophils in the blood of rats given Sephadex

Compound	*Dose* ($mg\,kg^{-1}$)	*Route*	*Time* (h)	*Compound*	*Dose* ($mg\,kg^{-1}$)	*Route*	*Time* (h)
Aspirin	300	p.o.	1	Noradrenaline	0.5	s.c.	0.17
Atropine	10	s.c.	0.5	Papaverine	10	s.c.	0.5
Chloroquine	100	p.o.	1	Phentolamine	2.5	s.c.	0.5
Cyproheptadine	4	s.c.	1	Phenoxybenzamine	2	s.c.	0.5
Sodium cromoglycate	100	s.c.	0.17	Phenylephrine	4	s.c.	0.5
Indomethacin	5	p.o.	1	Propranolol	5	s.c.	0.25
Ketotifen	20	p.o.	0.25	Quinidine	50	p.o.	0.5
Mepacrine	100	p.o.	1	Quinine	100	p.o.	0.5
Mepyramine	20	s.c.	0.5	Verapamil	50	p.o.	0.25

Rats were given Sephadex, G200 0.5 mg in 1 ml of saline, intravenously on days 0, 2 and 5. The numbers of eosinophils in the blood were counted on day 7. Compounds were given at the stated dose, route and time before each injection of Sephadex.

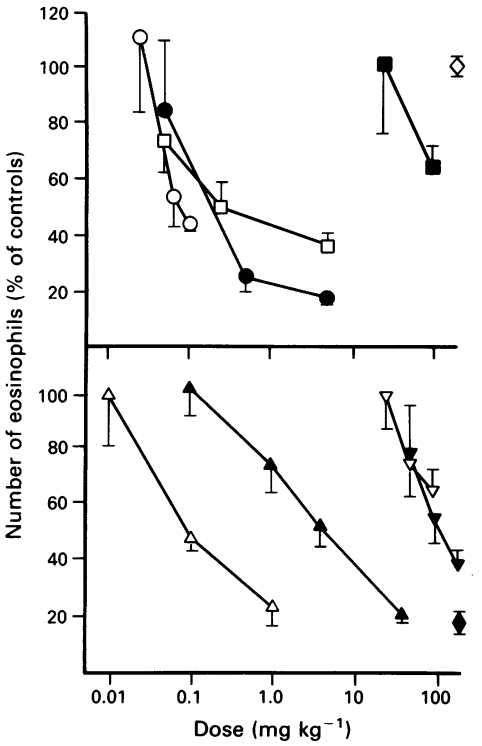

Figure 2 The effect of drugs on the number of eosinophils in the blood of rats on day 7 after the i.v. injection of Sephadex, G200, 0.5 mg in 1 ml of saline on days 0, 2 and 5. The drugs were given by the stated route and time before each injection of Sephadex: isoprenaline (○), salbutamol (●) and adrenaline (□) s.c. 10 min; aminophylline (■) p.o. 30 min; dexamethasone (△) and triamcinolone (▲) p.o. 4 h; phenidone (▽) and dapsone (▼) p.o. 30 min. The values are given as a percentage of the mean values for similar numbers of rats given Sephadex but no drug in the same experiments. For typical groups of control rats given Sephadex but no drug (◇) or saline only (◆) the number of eosinophils in the blood, as 10^6 ml^{-1} were 0.86 ± 0.05, $n = 53$, and 0.14 ± 0.01, $n = 56$ as arithmetic means ± s.e.mean. The drugs had no effect on the number of other leucocytes in the blood except for dapsone and the glucocorticosteroids (see text). Points represent arithmetic means and the vertical lines s.e.mean, $n = 6$ to 43.

Indomethacin had no effect on any of the changes in leucocyte numbers. The number of neutrophils remained low for all the treatments. Aminophylline and isoprenaline significantly reduced the number of neutrophils but the numbers in the Sephadex-treated controls were low and not different from the saline controls, so that this reduction is unlikely to be of importance and is probably due to the inaccuracy that results from counting cells in such low numbers (Table 2).

Effects of drugs on lung hyper-responsiveness

Rats given Sephadex had increased sensitivity to the increase in resistance to artificial respiration produced by the intravenous injection of 5-HT as shown by a shift to the left of the 5-HT log dose-response curve as compared to that for control rats given saline (Figure 3a). *In vitro* parenchymal lung strips taken from Sephadex treated rats were hyper-reactive to 5-HT in that they responded over the same dose range of 5-HT as did strips from control rats but the slope of the log dose-response curve was steeper (Figure 3b). The results can be expressed as mg tension per mg of tissue weight rather than as mg tension. When expressed in either way the differences in the response of lung strips obtained from untreated to Sephadex-treated rats were similar. The area under the log dose-response curve for tissue from untreated rats, as a percentage of that for tissue from Sephadex-treated rats, was $27 \pm 2\%$ as mg tension and $26 \pm 4\%$ as mg tension per mg of tissue ($n = 22$ and 15 respectively, as arithmetic means ± s.e.mean).

The effects on the hyper-responsiveness of the lungs to 5-HT of drugs that reduced blood eosinophilia were studied. Dexamethasone, isoprenaline, aminophylline, dapsone and phenidone were given to the rats before each injection of Sephadex at doses at which they reduced the blood eosinophilia. The drugs reduced the hyper-reactivity to 5-HT *in vitro* and hyper-sensitivity *in vivo* (Figure 3, Table 3), with the exception that the apparent reduction in hyper-sensitivity *in vivo* produced by aminophylline and dapsone did not reach significance and aminophylline had little effect on hyper-reactivity *in vitro*. Sodium cromoglycate and indomethacin had no effect on any of the parameters.

Blood eosinophil numbers for each rat were compared with either the reactivity of lung parenchymal strips to 5-HT or with the sensitivity to 5-HT *in vivo*. There was good corre-

Table 2 The effects of drugs on the changes in number of leucocytes in the broncho-alveolar lavage (BAL) fluids of rats produced by Sephadex

		Number of leucocytes					
		Mononuclear cells		*Neutrophils*		*Eosinophils*	
Treatment	n	× 10^6/ml	% of controls	× 10^6/ml	% of controls	× 10^6/ml	% of controls
Controls							
Sephadex, no drug	43	1.22	100	0.05	100	0.54	100
Saline, no drug	12	2.48	198 (148 to 264)***	0.03	45 (20 to 103)	0.00	0
Dexamethasone (0.1 mg kg^{-1}, p.o., 4 h)	12	1.96	133 (100 to 178)*	0.12	64 (28 to 145)	0.07	12 (7 to 21)***
Isoprenaline (0.1 mg kg^{-1}, s.c., 10 min)	6	2.03	152 (101 to 229)*	0.01	22 (6 to 70)*	0.09	17 (8 to 37)***
Aminophylline (100 mg kg^{-1}, p.o., 30 min)	6	1.82	137 (91 to 206)	0.01	25 (7 to 80)*	0.28	57 (27 to 120)
Dapsone (100 mg kg^{-1}, p.o., 30 min)	30	1.67	137 (114 to 166)***	0.08	80 (47 to 136)	0.06	11 (8 to 15)***
Phenidone (100 mg kg^{-1}, p.o., 30 min)	17	1.58	131 (103 to 166)*	0.02	84 (42 to 167)	0.18	44 (28 to 68)***
Sodium cromoglycate (100 mg kg^{-1}m s.c., 10 min)	10	0.92	83 (60 to 114)	0.05	95 (38 to 237)	0.31	48 (27 to 87)*
Indomethacin (5 mg kg^{-1}, p.o., 1 h)	6	1.55	117 (77 to 176)	0.01	32 (10 to 101)	0.61	123 (58 to 259)

Drugs were given at the dose route and time stated before each injection of Sephadex, 0.5 mg in saline, i.v., on days 0, 2 and 5. BAL washings were collected on day 7, cells were separated by centrifugation and suspended in 0.5 ml of saline. The values as 10^6/ml represent the number in this suspension and are geometric means. For each drug treatment, leucocyte numbers were compared with the values for a similar number of control rats given Sephadex, on the same occasion, to give the % of control values together with 95% confidence intervals in parentheses. Comparisons were by analysis of variance on a logarithmic scale, as described in the test, P values are quoted for these comparisons. The control values given are for typical controls given Sephadex or saline. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

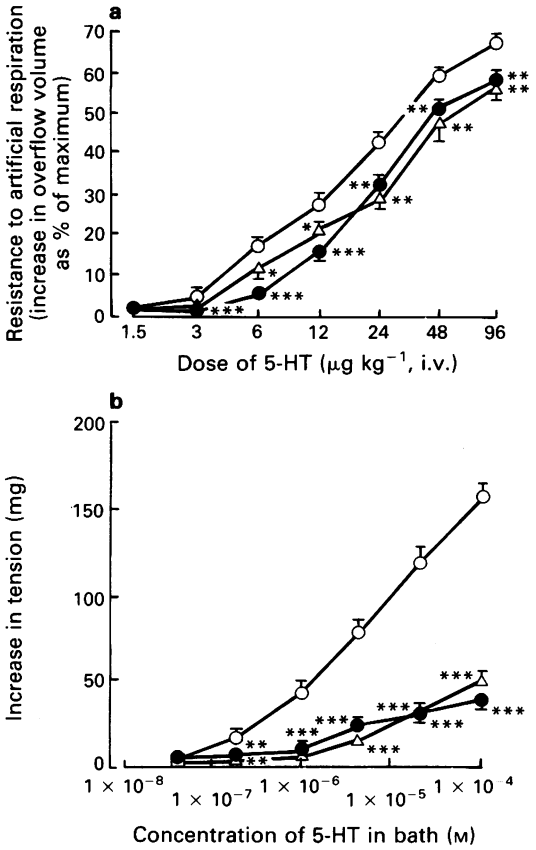

Figure 3 Effect of dexamethasone on the increase in lung responsiveness to 5-hydroxytryptamine (5-HT) produced in rats given Sephadex (a) *in vivo* and (b) on lung parenchymal strips *in vitro*. Rats were given Sephadex G200, 0.5 mg in 1 ml of saline (○) or saline alone (●), i.v., on days 0, 2 and 5. Other rats were given dexamethasone orally at 0.1 mg kg^{-1}, 4 h before each dose of Sephadex (△). Measurements *in vivo* were made on day 7 and lung strips were taken on days 6, 7 and 8. Numbers of rats for *in vivo* and *in vitro* determinations respectively were, for rats given: Sephadex, 15, and 22; saline, 20 and 21; dexamethasone and Sephadex, 6 and 7. Points represent arithmetic means and vertical lines s.e.mean. Student's *t* test (2-tailed) was used to assess the significance of the difference from rats given Sephadex: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

lation between blood eosinophil numbers and reactivity *in vitro*, as measured by the area under the log dose-response curve $r = 0.76$ ($n = 80$; $P < 0.001$). The correlation was less between blood eosinophil numbers and sensitivity *in vivo*, as measured by the $\log_{10}$ of the ED_{30} value, $r = -0.53$ ($n = 61$; $P < 0.001$). This was also less than the value reported previously when only Sephadex- and saline-treated animals were compared (Spicer *et al.*, 1989). In this study when the values in these control rats were compared the correlation was increased, $r = -0.66$ ($n = 25$; $P < 0.001$).

The drugs could have a direct effect on the responsiveness of the lungs of rats to 5-HT. Therefore dexamethasone, isoprenaline, aminophylline, dapsone and phenidone were given to groups of 6 rats at the same dose and dosage regimen as outlined in Table 3 except that saline was given to the rats instead of Sephadex. A control group of 6 rats was given saline only. When the rats given the drugs were compared with this control group the log dose-response curves for the effects of 5-HT given intravenously and for its effects on lung parenchymal strips were not different (data not shown). Thus the drugs by this dosage regimen, did not antagonize the effects of 5-HT.

Effects of drugs on the blood eosinophilia when given after each dose of Sephadex

Drugs which reduced the eosinophilia when given before the Sephadex showed a reduced activity when given after Sephadex. Of the drugs tested, dexamethasone could be given for the longest period of time after the Sephadex and still be active but it produced no significant inhibition when given at 8 h (Table 4).

Discussion

Effect of drugs on eosinophilia

The blood eosinophilia in rats given Sephadex was reduced by glucocorticosteroids, β-adrenoceptor agonists, aminophylline, dapsone and phenidone. It is remarkable that the first three of these are used for the treatment of asthma and dapsone has been shown to have a steroid sparing effect in asthmatics (Berlow *et al.*, 1990).

At the time of the peak in the blood eosinophilia, BAL fluids taken from the rats contained an increased number of eosinophils, a reduced number of mononuclear cells but there was no change in the small number of neutrophils. The glucocorticosteroid, dexamethasone, the β-adrenoceptor agonists, isoprenaline, dapsone, and phenidone reduced these changes at the same doses at which they reduced the blood eosinophilia. Aminophylline appeared to have similar effects but these did not reach significance. Aminophylline was the least effective of these drugs at reducing the blood eosinophilia. Sodium cromoglycate, whilst it had no effect on the blood eosinophilia or the fall in number of BAL mononuclear cells, did reduce the BAL eosinophilia.

Others have studied the effects of drugs on a BAL eosinophilia in the guinea-pig produced by the inhalation of PAF or antigen. Similar findings, to ours were that the eosinophilia was reduced by dexamethasone, aminophylline and sodium cromoglycate (Sanjar *et al.*, 1990a,b). However in these studies, in contrast to our findings in the rat, ketotifen was effective and no activity was demonstrated for salbutamol. Others found that while antigen induced BAL eosinophilia in the guinea-pig was reduced by methylprednisolone, no activity was demonstrated for ketotifen (Havill *et al.*, 1990). The finding of activity for a drug in one study but not in another could be due to differences in dosage regimens. For example; in the two studies in which salbutamol failed to reduce the BAL eosinophilia in the guinea-pig it was given over a period of days from an implanted minipump so that tolerance may have developed.

Sodium cromoglycate has been reported to reduce the number of eosinophils in the BAL fluids in asthmatics (Diaz *et al.*, 1984) and the ability of β-adrenoceptor agonists, aminophylline and glucocorticosteroids to produce an eosinopenic effect in man has been known for many years (Ohman *et al.*, 1972). In addition, asthmatics can show tolerance to the eosinopenic effects of β-adrenoceptor agonists (Reed *et al.*, 1970).

Eosinophilia and lung hyper-responsiveness

Hyper-responsiveness of the lungs, to spasmogens, has been produced in animals by a variety of techniques, most of which involve producing inflammation in the lung. In many of the studies the hyper-responsiveness was associated with a cellular infiltration, often of neutrophils (Smith, 1989). However, it is only in the dog that there is strong evidence that the neutrophil is the cause of the hyper-responsiveness (O'Byrne *et al.*, 1984). In other species such as the rabbit (Coyle *et al.*, 1988), guinea-pig (Silbaugh *et al.*, 1987) and rat (Pauwels *et al.*, 1986), the airway responsiveness could remit whilst a neutrophilia was still present in the lung. An eosinophilia in the lung has been associated with a lung hyper-responsiveness in sheep (Abraham, 1987), monkey (Grundel *et al.*, 1990) and rabbit (Coyle *et al.*, 1988). In the guinea-pig, inhaled antigen produced an eosinophilia in the BAL fluids and an *in vivo* hyper-reactivity to inhaled spasmogens. However, there was no direct relationship between the two since the hyper-reactivity could be reduced by drugs which had no effect on the BAL

Table 3 The effects of drugs on the blood eosinophilia and lung hyper-responsiveness to 5-hydroxytryptamine (5-HT) *in vitro* and *in vivo* produced in rats given Sephadex

Treatment	Number of eosinophils % of value in Sephadex-treated controls	Response to 5-HT *in vitro* AUC	Response to 5-HT *in vivo* EC_{30} ($\mu g\,kg^{-1}$ i.v.)
Controls			
Sephadex, no drug	100	100	12.3 (9.8 to 15.5)
Saline, no drug	14 (11 to 18)***	27 (21 to 34)***	26.1 (22.0 to 30.9)***
Dexamethasone ($0.1\,mg\,kg^{-1}$, p.o., 4 h)	47 (38 to 59)***	35 (25 to 49)***	22.8 (15.8 to 32.8)**
Isoprenaline ($0.1\,mg\,kg^{-1}$, s.c., 10 min)	36 (25 to 52)***	53 (39 to 72)***	22.5 (16.7 to 30.4)**
Aminophylline ($100\,mg\,kg^{-1}$, p.o., 30 min)	62 (39 to 99)*	80 (56 to 114)	15.5 (11.6 to 20.8)
Dapsone ($100\,mg\,kg^{-1}$, p.o., 30 min)	50 (40 to 62)***	45 (32 to 62)***	14.6 (10.6 to 20.2)
Phenidone ($100\,mg\,kg^{-1}$, p.o., 30 min)	66 (52 to 83)***	65 (48 to 89)**	19.8 (16.0 to 24.4)**
Sodium cromoglycate ($100\,mg\,kg^{-1}$, s.c., 10 min)	87 (63 to 118)	108 (76 to 154)	12.6 (10.3 to 15.3)
Indomethacin ($5.0\,mg\,kg^{-1}$, p.o., 1 h)	98 (62 to 156)	108 (77 to 154)	8.4 (6.5 to 10.7)

Drugs were given at the stated dose route and time before each injection of Sephadex, 0.5 mg in 1 ml of saline, i.v., on days 0, 2 and 5. Blood was taken and sensitivity to 5-HT *in vivo* was determined on day 7. Lung strips were taken on days 6, 7 or 8. For each drug-treatment the eosinophil numbers were compared with the values for a similar number of control rats given Sephadex on the same occasion. The means for the area under the log dose-response curves (AUC) were compared with the pooled control values for rats given Sephadex. Comparisons were made by an analysis of variance on a logarithmic scale as described in the text. *P* values are quoted for these comparisons. The EC_{30} values are the i.v. doses increasing the overflow volume to 30% of the maximum and are geometric means. The $\log_{10}$ of the EC_{30} values were compared by unpaired Student's *t* test, two tailed, with the values in Sephadex-treated rats. Number of rats, for each drug treatment, were 12 to 43 for blood eosinophils 6 to 7 for AUC and for EC_{30} values, with two determinations for each rat, numbers for Sephadex-treated controls were 24 for AUC and 15 for EC_{30} values. 95% confidence intervals are given in parentheses. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

eosinophilia and conversely the eosinophilia could be reduced by drugs which had no effect on the hyper-reactivity. In one study ketotifen, PAF receptor antagonists and prednisolone reduced the hyper-reactivity but only prednisolone reduced the eosinophilia (Havill *et al.*, 1990). These results were made somewhat confusing by the finding in another study that ketotifen had the converse effect in that it reduced the eosinophilia but not the hyper-reactivity and whilst dexamethasone reduced the eosinophilia it had no effect on the hyper-reactivity (Sanjar *et al.*, 1990b).

In our study in the rat we found a close association between a blood eosinophila and the reactivity of lung strips to 5-HT. Lung strips, taken from the rats given Sephadex at the time of the peak in blood eosinophilia, were hyper-reactive to 5-HT with an increase in the slope of the log dose-response curve. Drugs that reduced the eosinophilia also reduced the hyper-reactivity to 5-HT except that the effects of aminophylline on the hyper-reactivity did not reach significance. Rats given Sephadex also had an hyper-sensitivity to the increase in resistance to artificial respiration produced by the intravenous injection of 5-HT with a shift to the left of the log dose-response curve. The effects of reducing the eosinophilia with drugs on the hyper-sensitivity were less clear cut. Whilst dexamethasone, isoprenaline and phenidone reduced the hyper-sensitivity, the effects of dapsone and aminophylline did not reach significance, at doses at which they reduced the blood eosinophilia. The correlation between blood eosinophil numbers and lung hyper-responsiveness was greater *in vitro* than *in vivo*. This could be due to the variability of the measurements *in vivo* and the correlation was better when results in non-drug treated control rats were used. The nature of the smooth muscle in the lung showing the hyper-responsiveness to 5-HT is not known and the extent of the involvement of respiratory or vascular tissue is being investigated.

Table 4 Effects of drugs on the blood eosinophilia when given after each dose of Sephadex

Drugs	Time (h)	Eosinophils (% of controls)
Dexamethasone	1	34.3 ± 6.3
	4	60.8 ± 7.8
	8	81.2 ± 11.6
Isoprenaline	8	105.0 ± 19.0
Dapsone	1	54.6 ± 7.3
	4	82.0 ± 14.2
Phenidone	0.5	63.7 ± 10.7
	1	83.1 ± 15.3

The drugs were given at the same dose and route as shown on Table 3, but at the time stated after each injection of Sephadex on days 0, 2 and 5. Blood was taken on day 7. The numbers of eosinophils are given as a percentage of the mean value in control rats tested at the same time and given Sephadex but no drug; 6 rats were used for each test and control except for isoprenaline when 4 rats were used. The values are expressed as arithmetic mean ± s.e.mean.

In the rats given Sephadex there was a marked correlation between the number of blood eosinophils and the reactivity of the lungs, to 5-HT, *in vitro* which suggests, but does not prove, a causal relationship. The eosinophil can secrete a variety of cytotoxic and spasmogenic materials which are potential mediators of the hyper-reactivity, such as basic proteins, peroxidase, leukotriene C_4, PAF and 15-lipoxygenase products (Wardlaw & Kay, 1987). Tracheal smooth muscle of guinea-pigs and dogs treated with major basic protein from eosinophils was hyper-reactive to spasmogens (Flavåhan *et al.*, 1988; Brofman *et al.*, 1989).

If the eosinophil is causal of the lung hyper-responsiveness it may be the number of activated eosinophils rather than the total numbers that are important. Eosinophils taken from the peritoneal cavities of rats given three injections of Sephadex were activated when compared with eosinophils from rats given saline, or a single injection of Sephadex, as shown by enhanced cytotoxicity in a variety of assays (Cook *et al.*, 1987). It was assumed that the eosinophils would be activated in the BAL fluids of guinea-pigs given antigen by inhalation, since they had crossed several body compartments (Sanjar *et al.*,

1990b). However, this applies also to the eosinophils in the peritoneal cavity of the rat and since it is possible to detect difference in levels of activation in peritoneal eosinophils the same could apply to eosinophils in the lung.

The lung hyper-responsiveness of the rats given Sephadex has similarities with that shown by asthmatics. Asthmatics have a mixed hypersensitivity and hyper-reactivity to the acute bronchoconstrictor effects of inhaled histamine with a shift to the left and an increase in the slope of the log dose-response curve (Snashall, 1987) and, in two recent studies, lung tissues from asthmatics was found to be hyper-reactive to spasmogens (De Jongste *et al.*, 1987; Bai, 1990).

Mechanism of drug action

The mechanism by which the drugs reduce the eosinophilia is not known. It is possible that some compounds are active because they produce endogenous glucocorticosteroid release. However dexamethasone reduced the eosinophilia at doses at which it reduced the number of mononuclear cells in the blood and this was not found with the other active compounds. Phenidone is an inhibitor of the cyclo-oxygenase and lipoxygenase pathways of arachidonic acid metabolism (Blackwell & Flower, 1978). Inhibitors of the cyclo-oxygenase pathway such as indomethacin and aspirin had no effect on the eosinophilia suggesting that inhibition of lipoxygenase might be a relevant activity. The other active compounds have been also shown to be capable of inhibiting the release of lipoxygenase products: β-adrenoceptor agonists and theophylline (Orange & Austen, 1971), glucocorticosteroids (Blackwell *et al.*, 1980), and dapsone (Bonney *et al.*, 1983). The possible involvement of lipoxygenase products in the triggering of the eosinophilia warrants further study.

Mechanism of eosinophilia

Sephadex particles when injected intravenously into rats, embolised the lung vasculature to form a localised inflammatory reaction or granuloma (Walls & Beeson, 1972). The number of granuloma in the lung was not reduced by isoprenaline, dexamethasone or dapsone when given before each injection of Sephadex at doses at which they inhibited the blood eosinophilia and therefore they were not active merely because they prevented the embolisation of the vasculature. The mature granuloma contained mainly mononuclear cells but for the first few hours the predominant cell was the neutrophil (Cook *et al.*, 1989). Drugs which reduced the eosinophilia, when given before the Sephadex, were inactive when given 8 h afterwards. The changes produced by the Sephadex during the first few hours after being injected must therefore trigger the eosinophilia. These changes may be those taking place in the early stages of the formation of the granuloma involving interactions between leucocytes, particularly neutrophils, and vascular endothelial cells. In the lungs a large number of vascular endothelial cells are close to the external environment and 60 to 75% of the neutrophils in the blood are marginated in the lung vasculature (Worthen *et al.*, 1987), and are therefore suitably placed for this type of interaction. It was reported in 1953, that lung tissue from guinea-pigs, which had been previously given an intraperitoneal injection of antigen-antibody complexes, when placed into the peritoneal cavity of normal guinea-pigs produced an eosinophilia, suggesting that the lungs could be a site of formation of eosinopoietic factors (Samter *et al.*, 1953).

In conclusion these studies provide data showing an association between eosinophilia and hyper-responsiveness of the lung. The eosinophilia in the rats was triggered within the first few hours of injecting the Sephadex and drugs have been identified which inhibit this trigger. The mechanism by which the eosinophilia is produced warrants further study.

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Effects of carbachol and (−)-N^6-phenylisopropyladenosine on myocardial inositol phosphate content and force of contraction

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1 The effects of carbachol and the A_1-adenosine receptor agonist (−)-N^6-phenylisopropyladenosine (PIA) on force of contraction and inositol lipid metabolism were studied in electrically driven left auricles and papillary muscles isolated from guinea-pig hearts. Both carbachol and PIA (0.01–10 μM) had concentration-dependent negative inotropic effects in auricles. In papillary muscles PIA had no inotropic effect. Carbachol also had no inotropic effect at low concentrations (0.01–1 μM) but at 10–100 μM it exerted a slight positive inotropic effect.

2 In auricles and papillary muscles both carbachol and PIA concentration-dependently increased inositol trisphosphate (IP_3; significant at 1 μM). Accordingly phosphatidylinositol bisphosphate (PIP_2), the precursor of IP_3, was reduced. All effects of carbachol and PIA were antagonized by atropine (10 μM) and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX; 20 μM) respectively, indicating receptor-mediated effects.

3 In auricles the negative inotropic effects of carbachol and PIA preceded the increase in IP_3.

4 In papillary muscles the increase in IP_3 preceded the slight positive inotropic effect of carbachol, indicating that the M-cholinoceptor-mediated increase in IP_3 and force of contraction may be related. However, PIA showed a comparable increase in IP_3 but no inotropic effect, indicating a dissociation between those parameters.

5 In conclusion, in previous studies a close relation between increases in IP_3 and force of contraction has been shown after α_1-adrenoceptor stimulation. The present study with carbachol supports this view. However, the present data for PIA could not show such a close relationship, questioning the role of IP_3 as an endogenous regulator of force of contraction.

Introduction

The M-cholinoceptor agonist carbachol and the A_1-adenosine receptor agonist (−)-N^6-phenylisopropyladenosine (PIA) have different effects on myocardial contractility in different parts of the heart. In auricles both agents exert pronounced negative inotropic effects. In papillary muscles carbachol and PIA have slight positive and no inotropic effects at high concentrations respectively. But in ventricular tissue both PIA and carbachol reduce force of contraction in the presence of agents that increase adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels such as isoprenaline (Böhm *et al.*, 1984; 1985; Löffelholz & Pappano, 1985; Brückner *et al.*, 1985; Linden *et al.*, 1985; Endoh, 1987). Furthermore, cholinoceptor agonists reportedly cause an enhanced incorporation of [^{32}P]-phosphate into phosphatidylinositol in the heart (Quist, 1982; Brown & Brown, 1983). By use of [^{3}H]-inositol, an increased inositol phosphate formation after stimulation with carbachol has been demonstrated in preparations of chick, rat and guinea-pig hearts (Brown *et al.*, 1985; Brown & Jones, 1986; Scholz, 1989). The initial step in the inositol lipid metabolism is a phospholipase C-mediated hydrolysis of phosphatidylinositol bisphosphate (PIP_2) resulting in the generation of the two presumed second messengers diacylglycerol (DG) and inositol trisphosphate (IP_3; Berridge & Irvine, 1984; 1989). DG activates a protein kinase C (Nishizuka, 1986) while IP_3 releases calcium from intracellular stores in many tissues (for review see Berridge & Irvine, 1984; 1989; Scholz, 1989). It is still a matter of debate whether or not IP_3 releases calcium from cardiac sarcoplasmic reticulum (Hirata *et al.*, 1984; Movsesian *et al.*, 1985), although there is evidence that IP_3 is indeed an intracellular calcium mobilizing agent in cardiac muscle (Nosek *et al.*, 1986; Fabiato, 1986; Kentish *et al.*, 1990). The existence of inositol lipid metabolism has also been shown in the human heart (Kohl *et al.*, 1989).

The close resemblance of the cardiac effects of M-cholinoceptor and A_1-adenosine receptor agonists (for review see Endoh, 1987) led us to compare the concentration-dependent and time-dependent effects of carbachol and PIA on different products of inositol lipid metabolism and on force of contraction in auricles and papillary muscles from guinea-pigs. The aim of the study was twofold. Firstly, since in previous studies a close relation between increase in IP_3 and force of contraction has been shown after α_1-adrenoceptor stimulation (Poggioli *et al.*, 1986; Schmitz *et al.*, 1987a) the present study investigates this phenomenon by a comparative study of the effects of carbachol and PIA. Secondly, since it has been shown in auricles that pertussis toxin treatment converted the negative inotropic effect of carbachol into a positive inotropic effect (Tajima *et al.*, 1987) the effects of carbachol and PIA on inositol phosphates were also studied in auricles.

Some of these results were presented at the 29th Spring Meeting of the German Society of Pharmacology and Toxicology (Scholz *et al.*, 1988a).

Methods

Force of contraction

The experiments were performed on electrically driven (frequency 1 Hz, duration 5 ms, intensity 20% greater than threshold) left auricles and papillary muscles isolated from guinea-pigs (body weight 200–250 g). The animals were killed by a blow on the neck and bled from the carotid arteries. The preparations were attached to a bipolar platinum stimulating electrode and suspended individually in 10 ml glass tissue chambers for recording contractions as described previously (Scholz *et al.*, 1988b). All animals were pretreated with reserpine (5 mg kg^{-1} i.p. 18 h before they were killed) to

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prevent interference from endogenous catecholamines. The bathing solution was a modified Tyrode solution containing (mM) NaCl 119.8, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.05, NaH_2PO_4 0.42, $NaHCO_3$ 22.6, Na_2EDTA 0.05, ascorbic acid 0.28, glucose 5.0. It was gassed continuously with 95% O_2 plus 5% CO_2 and maintained at 35°C with a pH of 7.4. The force of contraction was measured with an inductive force transducer (W. Fleck, Mainz, FRG). Each muscle was stretched to the length at which force of contraction was maximal. The resting force (approximately 10 mN in the auricles and 5 mN in the papillary muscles) was kept constant throughout the experiment. After mechanical stabilization the substances were added.

Determination of inositol lipid products

Electrically driven left auricles and papillary muscles were labelled for 6 h with 20 μCi ml^{-1} of [^{3}H]-inositol in 10 ml bathing solution (composition see above), gassed with 95% O_2 plus 5% CO_2. Then they were washed for 10 min in [^{3}H]-inositol-free bathing solution and preincubated for 30 min with adenosine deaminase (1 μg ml^{-1}; only in the experiments with PIA to exclude interference from endogenous adenosine; Böhm *et al.*, 1985) and for 10 min with lithium chloride which was present throughout the remainder of the experiments (10 mM; to facilitate the measurement of phosphoinositide products; Scholz *et al.*, 1988b). Thereafter, the muscles were incubated in bathing solution containing carbachol or PIA (plus adenosine deaminase). At the end of each experiment the muscles were frozen in liquid nitrogen, homogenized with a microdismembrator (Braun, Melsungen, FRG), followed by the addition of 1 ml of chloroform/methanol/ hydrochloric acid (100:200:2) and extracted with water (310 μl) and chloroform (310 μl). The inositol phosphates (inositol phosphate, IP_1; inositol bisphosphate, IP_2; inositol trisphosphate, IP_3) were eluted from Dowex 1X8 anion exchange columns (formate form) according to the method of Berridge *et al.* (1983) as described previously (Schmitz *et al.*, 1987b; Scholz *et al.*, 1988b). The phospholipids (phosphatidylinositol, PI; phosphatidylinositol phosphate, PIP; phosphatidylinositol bisphosphate, PIP_2) were washed and dried under a stream of nitrogen. Thereafter they were separated on h.p.t.l.c. silica-gel plates (impregnated with potassium oxalate) running in one dimension with chloroform/methanol/acetone/acetic acid/ water (40:13:15:12:7). Thereafter the silica-gel plates were placed into a vessel with iodine vapour and identified by co-chromatographed standards. The radioactively labelled products were counted in a liquid scintillation counter.

Drugs

Substances used were carbamoylcholine chloride (Sigma, St. Louis, U.S.A.), (−)-N^6-phenylisopropyladenosine (Boehringer, Mannheim, FRG), atropine sulphate (Merck, Darmstadt, FRG), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, a gift from Dr M.J. Lohse, Heidelberg, F.R.G.), LiCl (Merck, Darmstadt, FRG), phosphoinositides (Sigma, St. Louis, U.S.A.), myo-[2-^{3}H]-inositol (20 Ci mmol^{-1}, Amersham, Braunschweig, FRG), AG 1X8 anion exchange resin (formate form; Bio-Rad Laboratories, München, FRG), h.p.t.l.c.-silica-gel plates 60 (Merck, Darmstadt, FRG), Ready-Value scintillation cocktail (Beckmann, München, FRG). All other chemicals were of analytical or best grade commercially available. All substances were freshly dissolved in prewarmed and pregassed bathing solution. Deionized and twice distilled water was used throughout.

Statistics

The values presented are means ± s.e.mean. Statistical significance was estimated with Student's *t* test for unpaired observations. A *P* value of less than 0.05 was considered significant.

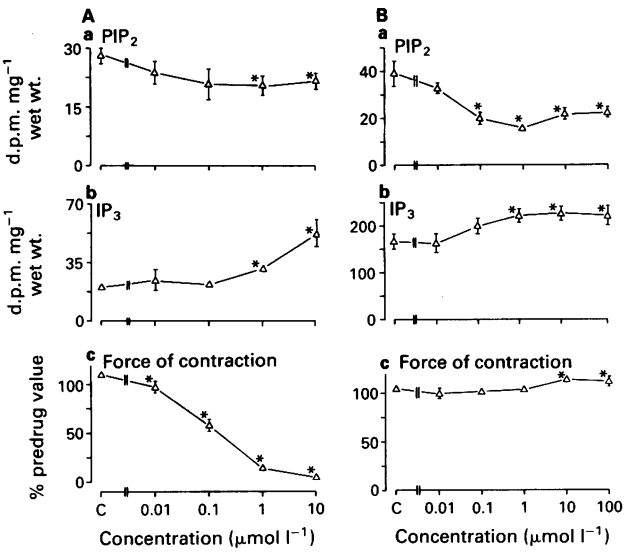

Figure 1 Effects of carbachol in guinea-pig auricles (A) and papillary muscles (B). Shown are concentration-response curves for the effects of carbachol on inositol lipid products (a,b) or force of contraction (c) of guinea-pig isolated electrically driven heart muscle preparations in the presence of lithium chloride (10 mM). Ordinates: phosphatidylinositol bisphosphate (PIP_2; a), inositol trisphosphate (IP_3; b) in d.p.m. mg^{-1} wet weight and force of contraction as a percentage of predrug value (c). Abscissae: Concentration of carbachol in μM. The pre-carbachol value of force of contraction was 2.9 ± 0.3 mN (n = 25; A(c)) and 1.3 ± 0.1 mN (n = 32; B(c)). The incubation time was 5 min for each drug concentration. C = control. n = 4–6 for (A) and 5–7 for (B). * $P < 0.05$ vs control.

Results

Concentration-dependent effects of carbachol

Auricles Figure 1A(a,b) shows concentration-response curves for the carbachol-induced effects on inositol lipid metabolism in guinea-pig left auricles. Accumulation of IP_3 or degradation of PIP_2 began at 1 μM carbachol. At 10 μM carbachol, the highest concentration investigated, the effects apparently did not reach a maximum. The concentration-dependence of the effect of carbachol on force of contraction is shown in Figure 1A(c). The negative inotropic effect was significant at 0.01 μM of carbachol. At 10 μM carbachol the myocardial force decreased to about 4.9% of the predrug value. All effects of carbachol (10 μM) on inositol lipid metabolism and force of contraction were blocked by the M-cholinoceptor-antagonist atropine (10 μM; Table 1A). PI remained unchanged under all conditions.

Papillary muscles Figure 1B shows the results obtained in papillary muscles. Carbachol had no inotropic effect at low concentrations (0.01–1 μM; Figure 1B(c)) but at 10 to 100 μM it exerted a slight positive inotropic effect, up to 115% of control. Carbachol concentration-dependently increased IP_3 (significant at 1 μM). Accordingly PIP_2 was reduced (Figure 1B(a,b)). These effects were also blocked by atropine (10 μM; Table 1B).

Time-dependent effects of carbachol

Auricles The time course of the inositol lipid metabolism in the absence and presence of carbachol (10 μM) is shown in Figure 2A(a,b). Carbachol increased IP_3 to about 226% of control at 5 min. PIP_2 decreased within 5 min to about 73% of control. Figure 2A(c) shows the time course of force of contraction. Carbachol exerted a strong negative inotropic effect which could already be detected at 10 s, reached maximum at 1 min and remained nearly constant thereafter. Thus, in auricles the negative inotropic effect of carbachol preceded the increase in IP_3.

Table 1 Effects of carbachol (CCh, 10 μM) and carbachol in the presence of atropine (Atr, 10 μM) on inositol lipid products (d.p.m. mg^{-1} wet weight) or force of contraction (in % of predrug value) of electrically driven left auricles (A) or papillary muscles (B) in the presence of lithium chloride (10 mM)

	Control	*CCh*	*CCh + Atr*
(A) *Guinea-pig left auricles* (n = 6)			
IP_1	101 ± 11	152 ± 9*	102 ± 11
IP_2	30 ± 3	97 ± 9*	28 ± 1
IP_3	20 ± 2	47 ± 8*	19 ± 2
PI	1125 ± 142	1110 ± 104	1171 ± 192
PIP	46 ± 4	29 ± 3*	44 ± 4
PIP_2	31 ± 3	22 ± 2*	31 ± 3
Force	109 ± 1.6	4.9 ± 2.7*	104 ± 6.1
(B) *Guinea-pig papillary muscles* (n = 6)			
IP_1	262 ± 38	376 ± 37*	284 ± 34
IP_2	195 ± 26	288 ± 25*	181 ± 28
IP_3	139 ± 13	199 ± 19*	150 ± 15
PI	4998 ± 680	4332 ± 392	4175 ± 230
PIP	494 ± 74	280 ± 51*	492 ± 46
PIP_2	434 ± 47	221 ± 60*	393 ± 62
Force	105 ± 1.5	115 ± 1.4*	102 ± 0.2

All 6 inositol lipid products were measured in each muscle. The products determined were inositol phosphate (IP_1), inositol bisphosphate (IP_2), inositol trisphosphate (IP_3), phosphatidylinositol (PI), phosphatidyl inositol phosphate (PIP) and phosphatidylinositol bisphosphate (PIP_2). The pre-carbachol value of force of contraction was 2.8 ± 0.5 mN in auricles and 1.5 ± 0.3 mN in papillary muscles respectively. * Denotes significant differences versus control ($P < 0.05$). The incubation time was 5 min. n = number of preparations.

Papillary muscles The slight positive inotropic effect of carbachol (10 μM; Figure 2B(c)) was significant at 3 min amounting to about 115% of control. The increase in IP_3 was significant at 2 min, reached a maximum at 10 min and was accompanied by a decrease of PIP_2 (Figure 2B(a,b)). Thus, in papillary muscles the increase in IP_3 preceded the increase in force of contraction induced by carbachol.

Concentration-dependent effects of (−)-N^6-phenylisopropyladenosine

Auricles Figure 3A(a,b) shows concentration-response curves for the PIA-induced effects on inositol lipid metabolism in guinea-pig left auricles. Accumulation of IP_3 or degradation of PIP_2 began at 0.1–1 μM PIA. The concentration-dependence of the effect of PIA on force of contraction is shown in Figure 3A(c). The negative inotropic effect was significant at 0.01 μM PIA. At 10 μM PIA the force of contraction decreased to about 5% of the predrug value. All effects of PIA (10 μM) on inositol lipid metabolism and force of contraction were blocked by the A_1-adenosine receptor-antagonist DPCPX (20 μM; Table 2A). DPCPX was used, because it is a potent and selective A_1-adenosine receptor antagonist (700 fold A_1-selectivity; Lohse *et al.*, 1987; Leyen *et al.*, 1989). PI remained unchanged under all conditions.

Papillary muscles In Figure 3B the same experiments are shown for the papillary muscle. PIA had no inotropic effect (0.01–100 μM; Figure 3B(c)) but PIA concentration-dependently increased IP_3 (significant at 1 μM) and PIP_2 was reduced (Figure 3B(a,b)). Again these effects were all blocked by DPCPX (20 μM; Table 2B).

Time-dependent effects of (−)-N^6-phenylisopropyladenosine

Auricles The time course of the inositol lipid metabolism in the absence and presence of PIA (10 μM) is shown in Figure 4A(a,b). PIA increased IP_3 to about 171% of control, significant at 5 min. PIP_2 was decreased within 5 min to about 67% of control. Figure 4A(c) shows the time course of force of contraction. PIA exerted strong negative inotropic effects which could already be detected at 10 s, and remained nearly constant from 1–15 min. Thus, in auricles the negative inotropic effect of PIA preceded the increase in IP_3.

Papillary muscles PIA (10 μM; Figure 4B) had no inotropic effect. In contrast, the increase in IP_3 was significant at 2 min, reached a maximum thereafter and was accompanied by a decrease of PIP_2. Thus, in papillary muscles the increase in

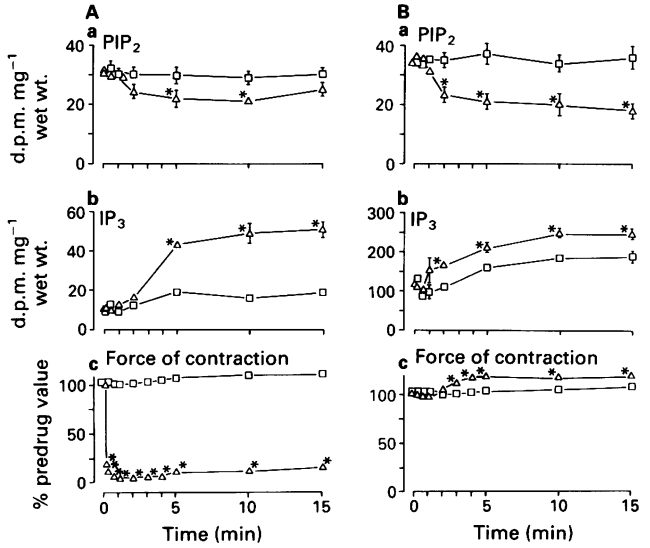

Figure 2 Effects of carbachol in guinea-pig auricles (A) and papillary muscles (B). Shown are time courses of inositol lipid products (a,b) or force of contraction (c) of guinea-pig isolated electrically driven heart muscle preparations in the absence (□) and presence (△) of carbachol (10 μM). All experiments were performed in the presence of lithium chloride (10 mM). Ordinates: phosphatidylinositol bisphosphate (PIP_2; a) inositol trisphosphate (IP_3; b) in d.p.m. mg^{-1} wet weight and force of contraction as percentage of predrug value (c). Abscissae: time of incubation with carbachol in min. The value of force of contraction at zero time was 3.9 ± 1.2 mN (n = 6; A(c)) and 1.6 ± 0.6 mN (n = 5; B(c)). n = 4–6 for (A) carbachol and n = 6 for (A) control; n = 5–7 for (B) carbachol and n = 6 for (B) control. * $P < 0.05$ vs control.

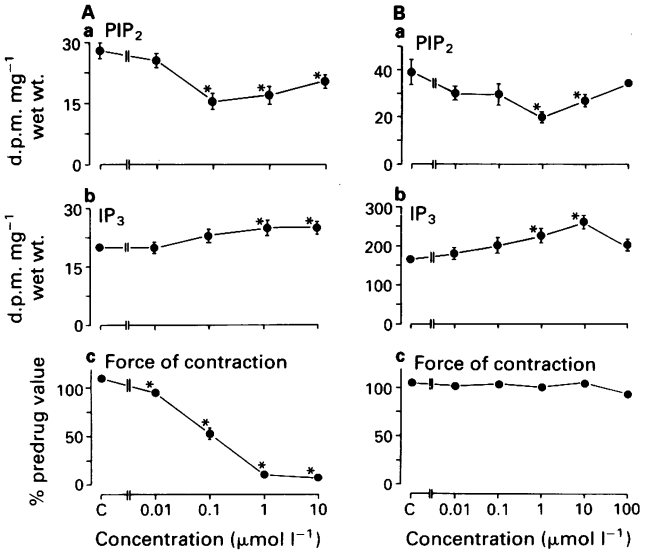

Figure 3 Effects of (−)-N^6-phenylisopropyladenosine (PIA) in guinea-pig auricles (A) and papillary muscles (B). Shown are concentration-response curves for the effects of PIA on inositol lipid products (a,b) or force of contraction (c) of guinea-pig isolated electrically driven heart muscle preparations in the presence of lithium chloride (10 mM). Ordinates: phosphatidylinositol bisphosphate (PIP_2; a) inositol trisphosphate (IP_3; b) in d.p.m. mg^{-1} wet weight and force of contraction as percentage of predrug value (c). Abscissae: Concentration of PIA in μM. The pre-PIA value of force of contraction was 3.4 ± 0.3 mN (n = 25; A(c)) and 1.4 ± 0.1 mN (n = 30; B(c)). The incubation time was 5 min for each drug concentration. C = control. n = 5–7 for (A) and (B). * $P < 0.05$ vs control.

Table 2 Effects of (−)-N^6-phenylisopropyladenosine (PIA, 10 μM) and PIA in the presence of 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 20 μM) on inositol lipid products (d.p.m. mg^{-1} wet weight) or force of contraction (in % of predrug value) of electrically driven left auricles (A) or papillary muscles (B) in the presence of lithium chloride (10 mM)

	Control	*PIA*	*PIA + DPCPX*
(A) *Guinea-pig left auricles* (n = 5)			
IP_1	94 ± 8	139 ± 9*	102 ± 9
IP_2	30 ± 2	51 ± 3*	28 ± 2
IP_3	20 ± 2	46 ± 2*	20 ± 2
PI	1044 ± 64	980 ± 107	1010 ± 60
PIP	51 ± 2	28 ± 1*	51 ± 3
PIP_2	29 ± 2	20 ± 2*	27 ± 1
Force	109 ± 1.6	8.5 ± 1.3*	99 ± 3.2
(B) *Guinea-pig papillary muscles* (n = 6)			
IP_1	311 ± 24	392 ± 26*	314 ± 15
IP_2	196 ± 17	278 ± 14*	209 ± 18
IP_3	175 ± 14	251 ± 21*	166 ± 14
PI	3936 ± 438	3907 ± 602	3792 ± 627
PIP	472 ± 45	336 ± 30*	461 ± 55
PIP_2	398 ± 25	253 ± 20*	377 ± 28
Force	105 ± 1.5	104 ± 1.9	104 ± 1.3

All 6 inositol lipid products were measured in each muscle. The products determined were inositol phosphate (IP_1), inositol bisphosphate (IP_2), inositol trisphosphate (IP_3), phosphatidylinositol (PI), phosphatidylinositol phosphate (PIP) and phosphatidylinositol bisphosphate (PIP_2). The pre-PIA value of force of contraction was 2.9 ± 0.5 mN in auricles and 1.4 ± 0.2 mN in papillary muscles respectively. * Denotes significant differences versus control ($P < 0.05$). The incubation time was 5 min. n = number of preparations.

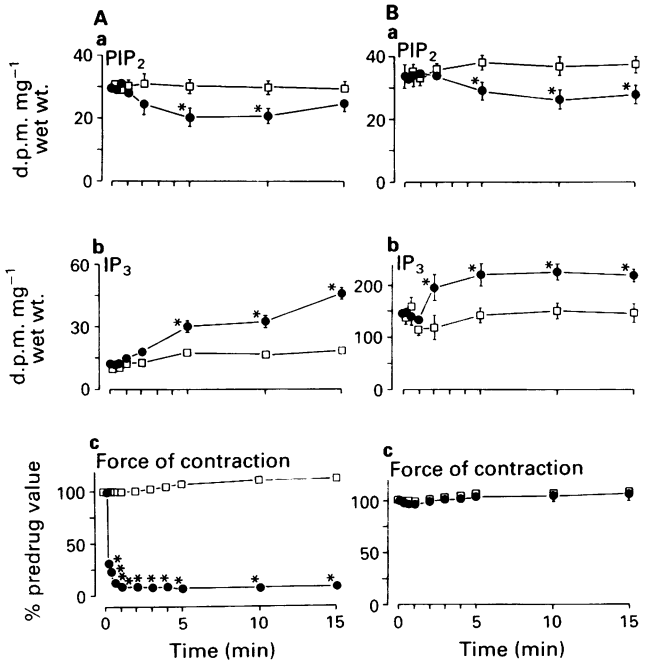

Figure 4 Effects of (−)-N^6-phenylisopropyladenosine (PIA) in guinea-pig auricles (A) and papillary muscles (B). Shown are time courses of inositol lipid products (a,b) or force of contraction (c) of guinea-pig isolated electrically driven heart muscle preparations in the absence (□) and presence (●) of PIA (10 μM). All experiments were performed in the presence of lithium chloride (10 mM). Ordinates: phosphatidylinositol bisphosphate (PIP_2; a) inositol trisphosphate (IP_3; b) in d.p.m. mg^{-1} wet weight and force of contraction as percentage of predrug value (c). Abscissae: time of incubation with PIA in min. The value of force of contraction at zero time was 3.7 ± 0.3 mN (n = 5; A(c)) and 1.7 ± 0.2 mN (n = 6; B(c)). n = 7–9 for (A) PIA and n = 6–9 for (A) control; n = 6–8 for (B) PIA and n = 8 for (B) control. * $P < 0.05$ vs control.

IP_3 resembles that induced by carbachol, while there is no inotropic effect.

Discussion

The present study shows that carbachol and PIA had similar effects on inositol lipid metabolism and on force of contraction in the mammalian heart. However, a close relation between increase in IP_3 and force of contraction could not be demonstrated. Carbachol or PIA decreased PIP_2 and PIP and increased the content of IP_3 and its congeners IP_2 and IP_1. All effects were blocked by the M-cholinoceptor antagonist atropine or the A_1-adenosine receptor antagonist DPCPX, indicating receptor-mediated effects. For clarity the effects of carbachol and PIA will be discussed separately.

Carbachol

In auricles (Figure 1A) there is an apparent dissociation between increase in IP_3, which supposedly is a second messenger for positive inotropic effects (Renard & Poggioli, 1987; Scholz *et al.*, 1988b), and force of contraction because carbachol had a negative inotropic effect. In addition, the negative inotropic effect was significant at lower concentrations than the increase in IP_3 and preceded the increase in IP_3. Thus, the increase in IP_3 after stimulation with carbachol is unlikely to be responsible for the negative inotropic effect. This is not surprising because the negative inotropic effect in auricles is due to an activation of atrial potassium channels through a guanine nucleotide binding protein (G-protein; Pfaffinger *et al.*, 1985; Böhm *et al.*, 1986). Carbachol increases potassium conductance, hyperpolarizes the membrane, decreases action potential duration and thereby reduces influx of calcium, leading to the negative inotropic effect.

In auricles the increase in potassium conductance conceivably overrides a possible IP_3-induced positive inotropic effect. Recently, it could be demonstrated (Tajima *et al.*, 1987; Kohl *et al.*, 1990) that pertussis toxin treatment converted the negative inotropic effect of carbachol (starting at 10 μM) in auricles into a positive inotropic effect which was still accompanied by an increase in inositol phosphates, indicating that 2 different G-proteins are involved: a pertussis toxin-sensitive G-protein which regulates potassium conductance at low concentrations of agonists and a different pertussis toxin-insensitive as yet unidentified G-protein which couples the M-cholinoceptor to the inositol-lipid-metabolism in the heart at high concentrations of agonists. Alternatively two different subtypes of M-cholinoceptors may be involved. The pertussis toxin-sensitive effect of carbachol on potassium conductance prevails over the pertussis toxin-insensitive effects on inositol lipid metabolism. Hence a negative inotropic effect of carbachol is normally observed despite an increase in IP_3. A positive inotropic effect of carbachol possibly due to the IP_3 increase can only be observed after elimination of the effect on potassium conductance with pertussis toxin. A pertussis toxin-insensitive G-protein has also been shown for the α_1-adrenoceptor-mediated effects on inositol lipid metabolism in the heart (Schmitz *et al.*, 1987b) and in other tissues (Cockroft, 1987; Rosenthal & Schultz, 1988). All effects of carbachol were blocked by atropine, indicating that the effects on force of contraction and inositol lipid metabolism are mediated via M-cholinoceptors.

In papillary muscles carbachol alone induced a slight positive inotropic effect and an increase in IP_3 content (Figure 1B). The increase in force developed slowly being first significant at 3 min whereas the increase in IP_3 was significant at 2 min (Figure 2B). It is evident that the increase in IP_3 preceded the increase in force, fulfilling a prerequisite for a second messenger role of IP_3. The concentration- and time-dependent effects of carbachol on force of contraction and IP_3 are compatible with an IP_3-mediated positive inotropic effect.

(−)-N^6-phenylisopropyladenosine

In auricles the effects of PIA on force of contraction and inositol lipid metabolism were similar to the effects of carbachol. The negative inotropic effect after stimulation with PIA was also faster (Figure 4A) and occurred at lower concentrations (Figure 3A) than did the increase in IP_3 content. This leads to similar conclusions. In brief, a possible IP_3-induced positive inotropic effect could be overriden by an increase in potassium conductance, leading to the negative inotropic effect. Moreover, DPCPX antagonized all effects of PIA, indicating an A_1-adenosine receptor-mediated effect (Leyen *et al.*, 1989).

In papillary muscles PIA had no inotropic effect but increased inositol phosphates and decreased phospholipids (Figure 3B). Thus, concentration- and time-dependent effects (Figure 4B) of PIA on inositol lipid products are comparable with the results observed with carbachol. However, the reason for the lack of effect of PIA on force of contraction is unclear. Firstly, it could be due to unspecific effects of the adenosine analogue, because the parent compound adenosine, like carbachol, has a slight positive inotropic effect at high concentrations (100 μM; Brückner *et al.*, 1985; Legsseyer *et al.*, 1988). Secondly, it could indicate a dissociation between increase in IP_3 and increase in force of contraction. Thirdly, compartmentation of IP_3, as has been shown for cyclic AMP in cardiomyocytes (Buxton & Brunton, 1983) cannot be excluded. In contrast to the present study, no stimulation of the inositol lipid metabolism in atrial and ventricular myocytes was found after stimulation with PIA (Leung *et al.*, 1986). However, a similar increase in IP_3 was found with adenosine in rat papillary muscles (Legssyer *et al.*, 1988).

In summary, atrial and ventricular M-cholinoceptors and adenosine A_1-receptors are both coupled to inositol lipid metabolism. In atrial tissues there is an apparent dissociation between increase in IP_3 and force of contraction. However, a possible IP_3 induced positive inotropic effect of carbachol can only be observed after elimination of the effect on potassium conductance. In ventricular tissues the positive inotropic and IP_3 increasing effects of carbachol revealed similar time- and concentration-dependencies and hence might be closely related. In contrast, PIA failed to cause an increase in force of contraction. Thus, the present data could not show a close relationship between increase in IP_3 and an increase in force of contraction as has been shown for α_1-adrenoceptor-stimulation.

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Effects of the cyclo-oxygenase inhibitor, fenbufen, on clenbuterol-induced hypertrophy of cardiac and skeletal muscle of rats

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1 When rats were fed with clenbuterol for 7 days skeletal muscle mass increased by 21% in the tonic soleus and phasic plantaris muscles and a 16% hypertrophy of the heart was also induced. Fenbufen, fed to rats for the same period, blocked the hypertrophy of the heart but not that of the skeletal muscles.

2 When feeding of fenbufen commenced 3 days before the administration of clenbuterol, plasma prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was reduced by 79%; there was again no effect of fenbufen on clenbuterol-induced increases in the RNA or protein content of plantaris, nor in the increased area of fast or slow twitch fibres in the soleus. In the heart the clenbuterol-induced increases in the RNA (+21%) and protein content (+20%) were totally inhibited.

3 The effects of clenbuterol on heart muscle appear to be mediated by a cyclo-oxygenase metabolite of arachidonic acid whilst the effects on skeletal muscle are not.

Introduction

The mode of action of the β-adrenoceptor agonist, clenbuterol, in promoting muscle protein deposition remains unknown. The suggestion that muscle hypertrophy occurred principally as a result of a decrease in protein degradation (Reeds *et al.*, 1986) contrasts with the observation of a large increase in protein synthesis rates in denervated muscle (Maltin *et al.*, 1987). Even in the innervated muscle, increases in protein synthesis rates after 3 days have been observed (Maltin *et al.*, 1989), suggesting that a transient rise in synthesis is at least partly responsible for the hypertrophy in phasic muscles.

The β-adrenoceptor antagonist, propranolol, was shown to block the effects of clenbuterol on skeletal muscle in the study of MacLennan & Edwards (1989) whereas in other studies, β-adrenoceptor antagonists blocked effects of clenbuterol on the heart whilst having no effect on skeletal muscle anabolism (Reeds *et al.*, 1988). This latter observation has led to the suggestion that the skeletal muscle-directed effect of clenbuterol is separate from its β-mediated effects. Thus the possibility that the ability of clenbuterol to promote muscle hypertrophy was indirect and mediated by other hormones or growth factors has been considered. Interactions between β-adrenoceptor agonists and insulin binding have been reported in muscle (Webster *et al.*, 1986) but McElligott *et al.* (1987) demonstrated that clenbuterol was effective in diabetic rats, suggesting that the response was not insulin-mediated. The ability of clenbuterol to cause muscle hypertrophy in Snell dwarf mice (Pell *et al.*, 1987) suggests that its action is independent of prolactin, GH and thyroid hormones in which these animals are deficient.

Non-steroidal anti-inflammatory drugs (NSAIDs), such as indomethacin and meclofenamate, which reduce prostaglandin release by inhibition of cyclo-oxygenase, block increases in protein synthesis induced by stretch (Smith *et al.*, 1983) and by insulin *in vitro* (Reeds & Palmer, 1983) and *in vivo* (Reeds *et al.*, 1985). These observations have implicated cyclo-oxygenase metabolites of arachidonic acid, specifically prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Smith *et al.*, 1983) in the control of protein synthesis. Another NSAID, fenbufen (a pro-drug, which is metabolized in the liver to 4-biphenyl acetic acid) has also been shown to affect rates of protein synthesis in rat muscle (McMillan *et al.*, 1987). The present work was undertaken to investigate the effects of inhibition of prostaglandin synthesis with fenbufen on clenbuterol-induced muscle hypertrophy.

Methods

Male hooded Lister rats of the Rowett strain were weaned at 19 days and treated prior to experiments as described previously (Maltin *et al.*, 1986). During the experiments the rats were housed individually and fed on PW3 diet (Pullar & Webster, 1977).

Clenbuterol was incorporated into the diet at a concentration of $2\,mg\,kg^{-1}$ diet, a concentration at which a significant hypertrophy of skeletal muscle occurred without any adverse effect on food consumption (Reeds *et al.*, 1986). Fenbufen (Lederle Laboratories, Gosport, Hants, U.K.) was fed at a concentration in the diet of $1200\,mg\,kg^{-1}$; this was the maximum dose which could be fed to rats without causing gastric ulceration whilst inducing a near maximal inhibition of prostaglandin release (McMillan, 1987).

In Experiment 1, the diets were fed for 7 days, after which the animals (4 groups of 6) were killed and tissues removed, frozen in liquid N_2 and stored at −20°C until analysed.

In Experiment 2, feeding of fenbufen started 3 days before the addition of clenbuterol to the diet; thus the rats were fed fenbufen for a total of 10 days and clenbuterol for 7 days. Ten rats were subjected to each dietary treatment; on the final day of the experiment 6 rats from each group were injected via a lateral tail vein with 150 μmol L-phenylalanine plus 75 μCi L-[2,6-^{3}H]phenylalanine per 100 g body weight (Garlick *et al.*, 1980). Precisely 10 min later the animals were killed and tissues were dissected and treated either for measurement of total protein and RNA and rates of protein synthesis (Reeds *et al.*, 1986), or for fibre area and frequency (Maltin *et al.*, 1986). The remaining 4 rats/group were not injected with phenylalanine. These rats were killed by decapitation, blood was collected from the neck and muscles removed, frozen in liquid N_2 and stored at −70°C for $PGF_{2\alpha}$ assay.

$PGF_{2\alpha}$ was measured in serum and muscle after extraction of 0.5 ml serum or homogenization of plantaris muscles in ethyl acetate: isopropanol: 0.2 M HCl (3 : 3 : 1, v/v/v) followed by the addition of 2 ml ethyl acetate and 3 ml H_2O. After mixing, 3 ml of the organic layer was evaporated to dryness,

Table 1 Effects of fenbufen on clenbuterol-induced tissue hypertrophy

Treatment	*Control*	*Clenbuterol*	*Fenbufen*	*Clenbuterol + fenbufen*
Weight				
Whole body (g)	111 ± 3	113 ± 2	108 ± 2	106 ± 1
Heart (mg)	467 ± 10	544 ± 13***	439 ± 12	489 ± 11##
Soleus (mg)	53.3 ± 2.2	64.3 ± 2.9*	52.3 ± 2.1	62.7 ± 1.4**
Plantaris (mg)	82.0 ± 2.5	99.2 ± 2.7**	81.2 ± 2.1	92.7 ± 1.8**

Rats were fed on clenbuterol (2 mg kg^{-1}, diet) and/or fenbufen (1200 mg kg^{-1}, diet) for 7 days. Values are means ± s.e.mean (n = 6).
By Student's t test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ v control; ## $P < 0.01$ v clenbuterol alone.

resuspended and analysed by radioimmunoassay (Dade [^{3}H]-$PGF_{2\alpha}$ RIA kit; Steranti, St. Albans, Herts).

One way analysis of variance was used to assess the data; the significance of differences between groups was determined by Student's t test.

L-[2,6-^{3}H]phenylalanine was purchased from Amersham International (Amersham, Bucks, U.K.). All other reagents were from Sigma, or B.D.H. (both Poole, Dorset).

Results

In the first experiment feeding of clenbuterol and fenbufen was started simultaneously and continued for 7 days. There were no significant effects of any treatment on the body weight of the rats (Table 1). Gut and liver weights were also unaffected (data not shown). All 4 of the muscles examined (gastrocnemius, soleus, plantaris and extensor digitorum longus) showed significant hypertrophy in response to clenbuterol with no apparent effect of fenbufen on the hypertrophy; data for two of these muscles are presented in Table 1. The heart hypertrophied by 16% in response to clenbuterol alone and fenbufen appeared partially to reverse this effect: in the presence of fenbufen the clenbuterol-induced hypertrophy of the heart was significantly ($P < 0.01$) less than with clenbuterol alone. This effect of fenbufen was not apparent when the small and non-significant differences in final body weight were taken into account. Heart weight/100 g body weight was increased from 423 ± 5 mg (control) to 481 ± 6 mg, $P < 0.001$ (clenbuterol alone). In the presence of fenbufen the increase was from 410 ± 7 (fenbufen alone) to 464 ± 8 mg per 100 g body wt., $P < 0.001$ (fenbufen + clenbuterol).

The second experiment therefore examined the effect of pre-feeding the rats on fenbufen for 3 days to induce a reduction in prostaglandin synthesis before clenbuterol administration. Serum $PGF_{2\alpha}$ was reduced by 79% after fenbufen had been fed to the rats for 10 days but was not affected by clenbuterol; muscle $PGF_{2\alpha}$ was reduced by 50% (plantaris) (Table 2). RNA and protein accretion in both plantaris (Table 3) and soleus muscle (data not shown) were stimulated by clenbuterol; this response was unaffected by fenbufen. Clenbuterol also signifi-

Table 2 Effects of fenbufen and clenbuterol on prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) levels in serum and plantaris muscle

	$PGF_{2\alpha}$ concentration	
	pg per 50 µl serum	pg per muscle
Control	890 ± 108	118 ± 12
Clenbuterol	890 ± 78	108 ± 26
Fenbufen	190 ± 12**	64 ± 19
Clenbuterol + fenbufen	280 ± 96##	62 ± 21

Fenbufen (1200 mg kg^{-1}) was fed for a total of 10 days; clenbuterol (2 mg kg^{-1}) was fed for 7 days. Values are means ± s.e.mean (n = 4).
By Student's t test: ** $P < 0.01$ v control; ## $P < 0.01$ v clenbuterol alone.

Table 3 Effects of fenbufen and clenbuterol on the RNA and protein content and fractional rate of protein synthesis (k_s) of plantaris and cardiac muscle

	Total RNA (µg)	*Total protein* (mg)	*k_s* (%/day)
Plantaris			
Control	151 ± 3	16.6 ± 0.2	16.4 ± 0.8
Clenbuterol	202 ± 4***	20.0 ± 0.5***	17.1 ± 0.7
Fenbufen	150 ± 3	16.3 ± 0.4	15.3 ± 0.3
Clenbuterol + fenbufen	200 ± 14**	21.6 ± 0.8***	15.1 ± 0.6
Heart			
Control	1548 ± 71	59.2 ± 1.6	17.9 ± 0.3
Clenbuterol	1872 ± 69**	71.3 ± 1.7**	18.6 ± 0.6
Fenbufen	1476 ± 49	54.7 ± 1.1	16.6 ± 0.5
Clenbuterol + fenbufen	1426 ± 57##	52.1 ± 4.0##	17.0 ± 0.6

Fenbufen (1200 mg kg^{-1}) was fed for a total of 10 days; clenbuterol (2 mg kg^{-1}) was fed for 7 days. Values are means ± s.e.mean (n = 6).
By Student's t test: ** $P < 0.01$; *** $P < 0.001$ v control; ## $P < 0.01$ v clenbuterol alone.

Table 4 Effect of fenbufen on clenbuterol-induced increase in area and frequency of fast oxidative glycolytic (FOG) and slow oxidative (SO) fibres in soleus muscle

	Mean area (sq µm)		*Mean frequency* (%)		*Mean area* (%)	
Fibre type	FOG	SO	FOG	SO	FOG	SO
Control	973 ± 31	1226 ± 212	43.0 ± 0.8	57.0 ± 0.8	37.5 ± 0.7	62.5 ± 0.7
Clenbuterol	1425 ± 69***	1470 ± 77*	47.4 ± 1.6*	52.6 ± 1.6*	46.7 ± 1.6	53.3 ± 1.7
Fenbufen	983 ± 23	1212 ± 30	45.3 ± 1.3	54.7 ± 1.3	40.2 ± 1.4	59.8 ± 1.4
Clenbuterol + fenbufen	1427 ± 155*	1431 ± 144	43.5 ± 1.7	56.5 ± 1.7	43.3 ± 1.3	56.7 ± 1.3

Fenbufen (1200 mg kg^{-1}) was fed for a total of 10 days; clenbuterol (2 mg kg^{-1}) was fed for 7 days. Values are means ± s.e.mean for 6 observations except in the clenbuterol + fenbufen group where n = 5.
By Student's t test: * $P < 0.05$; $P < 0.001$ v control.

cantly increased the mean area of both fast-twitch oxidative glycolytic (FOG) and slow-twitch oxidative (SO) fibres in the soleus (Table 4) and this response too was unaffected by fenbufen. However, the mean fibre frequency, which was significantly changed by clenbuterol alone (a 10% increase in the frequency of FOG fibres) was apparently inhibited by fenbufen. In the heart the significant hypertrophy (increases of 21% in total RNA and 20% in total protein) was totally inhibited by fenbufen (Table 3).

Discussion

The mechanism of action of clenbuterol in increasing muscle mass remains poorly understood and has been claimed to result from changes in both rates of protein degradation and synthesis. A recent study has shown significant effects on protein synthesis, particularly in denervated phasic muscles (Maltin *et al.*, 1989). This increase in the rate of protein synthesis in normal, innervated muscle was transient which has led to the conclusion (Reeds *et al.*, 1986) that in the rat the effect of clenbuterol is mainly on protein degradation. The present study was undertaken to investigate the possibility that prostaglandin metabolism was involved in the clenbuterol-induced hypertrophy of muscle. Prostaglandins have been implicated in the control of both protein synthesis and protein degradation. One prostaglandin, PGE_2, has been shown to stimulate protein degradation (Rodemann & Goldberg, 1982) and is believed to be involved in some pathological states involving muscle wasting where a large increase in rates of protein degradation has been shown to be inhibited by NSAIDs such as indomethacin and naproxen (Ruff & Secrist, 1984; Tian & Baracos, 1989).

$PGF_{2\alpha}$ was shown to stimulate protein synthesis in rat (Rodemann & Goldberg, 1982) and rabbit muscle (Smith *et al.*, 1983) *in vitro*. The non-steroidal anti-inflammatory drug, indomethacin, which inhibits the action of cyclo-oxygenase and thus reduces the metabolism of arachidonic acid to prostanoids, was shown to inhibit the acute effects of insulin *in vivo* (Reeds *et al.*, 1985) and *in vitro* (Reeds & Palmer, 1983). Fenbufen, a pro-drug which is an active inhibitor of cyclo-oxygenase only after metabolism to 4-biphenyl acetic acid in the liver, is preferred in prolonged treatments since the gastric ulceration caused by feeding indomethacin and aspirin is prevented. Fenbufen inhibited prostaglandin release by 80% and reduced rates of protein synthesis in normal muscle and in muscle undergoing hypertrophy in response to tenotomy of a synergist (McMillan *et al.*, 1987). However, the ability of the muscle to hypertrophy in response to tenotomy was unimpaired in that experiment, suggesting that protein degradation had also been reduced. The results presented here are similar in that $PGF_{2\alpha}$ was reduced by fenbufen in plantaris muscle and in serum. However, the reduction in the fractional rate of protein synthesis in heart and plantaris muscle of the rats fed fenbufen was in no case statistically significant in the present study. Together with the ability of plantaris muscle to respond to clenbuterol in the presence of fenbufen, the data suggest that the response of skeletal muscle to the β-adrenoceptor agonist is not prostaglandin-mediated. They also provide further, albeit indirect evidence that the effect of clenbuterol is not insulin-mediated, since NSAIDs block both insulin action (Reeds & Palmer, 1983) and insulin secretion (Jepson & Millward, 1989).

Although the hypertrophy of the skeletal muscles was unaffected by fenbufen it is interesting to note that the effects of clenbuterol on fibre type were inhibited (Table 4). The clenbuterol-induced change in fibre frequency, consistent with previous data (Maltin *et al.*, 1986) and resulting in a significant shift (+10%) towards more fast-twitch oxidative glycolytic fibres, was not observed when clenbuterol and fenbufen were fed in combination. Thus it appears that the clenbuterol-induced change in fibre frequency can be separated from fibre hypertrophy by an agent which inhibits cyclo-oxygenase. The patho-physiological basis for this change in fibre frequency is not clear. Clenbuterol may impair the normal developmental fibre type conversion (Zeman *et al.*, 1988); alternatively the drug may interact directly or indirectly to alter muscle contractile and metabolic properties (Maltin *et al.*, 1986). Thus it may be speculated that since the clenbuterol-induced change in fibre frequency is sensitive to fenbufen, metabolites of cyclo-oxygenase may be involved in the determination of fibre frequency.

Noticeably different from the clenbuterol-induced hypertrophy of skeletal muscle in the presence of fenbufen was the effect on cardiac muscle, in which the hypertrophy induced by clenbuterol was completely abolished by the NSAID. This contrasts with work in the perfused rat heart where both insulin and pressure overload stimulated protein synthesis and 4-biphenylacetic acid (the active metabolite of fenbufen) failed to inhibit the effect of either stimulus (Smith & Sugden, 1987). Thus previous published data suggest that cardiac muscle is less likely to be sensitive to prostaglandins than skeletal muscle and the work presented here surprisingly suggests that the opposite is true in the case of clenbuterol-induced hypertrophy.

However, the results of the present study with fenbufen are remarkably similar to those obtained by Reeds *et al.* (1988). In that study a complete inhibition of the clenbuterol-induced hypertrophy of the heart was achieved with the β-adrenoceptor antagonists, propranolol and atenolol, which, like fenbufen had no effect on skeletal muscle hypertrophy. It has been proposed that the effects on the heart result from the increased blood flow, tachycardia or stretch of the heart muscle, that these effects are β-adrenoceptor-mediated and that the action of clenbuterol on skeletal muscle is separate from the β-adrenoceptor-mediated fat loss and cardiac hypertrophy (Reeds *et al.*, 1988; Maltin *et al.*, 1989). These proposals are not disputed in the present study. Clearly, increased stretch and work load are likely to be involved in the effects on the heart and less likely to be involved in the hypertrophy of the skeletal muscles. Although no direct effect of prostaglandins in the action of β-agonists has been proposed previously, the involvement of prostaglandins in secondary, stretch-related effects of increased blood flow is perhaps not unexpected.

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Differential effects of chronic lorazepam and alprazolam on benzodiazepine binding and $GABA_A$-receptor function

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1 Chronic benzodiazepine administration has been associated with tolerance and with downregulation of γ-aminobutyric $acid_A$ ($GABA_A$)-receptor binding and function. However, effects of individual benzodiazepines on brain regions have varied.

2 To compare the effects of chronic lorazepam and alprazolam, we have administered these drugs to mice for 1 and 7 days ($2\,mg\,kg^{-1}\,day^{-1}$) and determined benzodiazepine receptor binding *in vivo* with and without administration of CL 218,872, $25\,mg\,kg^{-1}$ i.p., and GABA-dependent chloride uptake in 3 brain regions at these time points.

3 Benzodiazepine binding was decreased in the cortex and hippocampus at day 7 compared to day 1 of lorazepam, with an increase in CL 218,872-resistant (Type 2) sites in both regions. Maximal GABA-dependent chloride uptake was also decreased in the cortex and hippocampus at day 7.

4 Binding was decreased only in the cortex after 7 days of alprazolam, with no significant change in Type 2 binding. Maximal GABA-dependent chloride uptake was also decreased only in the cortex.

5 These data suggest that the effects of chronic benzodiazepine administration on the $GABA_A$-receptor may be both region-specific and receptor subtype-specific.

Introduction

In clinical use, chronic benzodiazepine administration is associated with the development of tolerance to anticonvulsant and hypnotic effects (e.g., Greenblatt & Shader, 1978). Tolerance has also been observed in a number of animal models with a variety of benzodiazepines, including 'classical' benzodiazepines and the newer triazolobenzodiazepines (Garratt *et al.*, 1989). In previous studies, we demonstrated the development of tolerance during chronic administration of the classical benzodiazepine lorazepam (Miller *et al.*, 1988a) and the triazolobenzodiazepine alprazolam (Miller *et al.*, 1989c). In both cases, tolerance was associated temporally with benzodiazepine receptor downregulation and decreased $GABA_A$-receptor function. Similar results have been obtained by other investigators for flurazepam (Tietz *et al.*, 1986) and diazepam (Marley & Gallager, 1989).

However, our results indicated that receptor downregulation produced by alprazolam and lorazepam had differing regional specificity. Receptor alterations induced by lorazepam occurred in the cortex, hypothalamus, and hippocampus, whereas those associated with alprazolam occurred in the cortex and hypothalamus only. A possible mechanism for this discrepancy is differential effects of the two drugs on benzodiazepine receptor subtypes (Sieghart, 1989), although binding studies do not indicate a substantial difference in this regard (Haefely *et al.*, 1985). We did not assess regional specificity for γ-aminobutyric acid (GABA)-dependent chloride uptake, although other investigators have demonstrated effects in the cortex but not the cerebellum after chronic diazepam (Marley & Gallager, 1989).

To assess possible region-specific effects of lorazepam and alprazolam during chronic administration, we evaluated benzodiazepine binding *in vivo* and GABA-dependent chloride uptake in several brain regions both before (day 1) and after (day 7) the development of tolerance to both compounds. In addition, we evaluated the relative proportion of benzodiazepine subtype binding by use of the subtype-specific ligand CL 218,872 (Sato & Neale, 1989).

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Methods

Male CD1 mice, 6–8 weeks of age, were obtained from Charles River Laboratories (Wilmington, MA), given food and water *ad libitum*, and maintained on a 12 h light/dark cycle.

Lorazepam and alprazolam ($2\,mg\,kg^{-1}\,day^{-1}$) were dissolved in PEG 400 and administered by subcutaneously implanted osmotic pumps as previously described (Miller *et al.*, 1988a). Day 1 was chosen as a point before the development of tolerance and receptor alterations, and day 7 as a point associated with tolerance and receptor changes (Miller *et al.*, 1988a). Benzodiazepine receptor subtypes were distinguished by use of CL 218,872, which appears to bind preferentially to Type 1 sites (Sieghart, 1989). CL 218,872 was dissolved in ethanol and diluted with saline to a final ethanol concentration of 0.1%. Vehicle contained 0.1% ethanol diluted with saline.

Benzodiazepine binding *in vivo* was performed as previously described (Miller *et al.*, 1988a). Briefly, mice were injected i.v. with 3 μCi [^{3}H]-Ro15-1788. After 20 min, animals were killed and brains rapidly removed and dissected on ice. After the brain regions had been weighed they were dissolved in Protosol (40°C for 24 h) and then counted by scintillation spectrometry. For subtype specific binding mice were injected with CL 218,872, $25\,mg\,kg^{-1}$ i.p., 30 min before the radioligand.

GABA-dependent chloride uptake was performed as previously described (Miller *et al.*, 1988a). Briefly, cortical synaptoneurosomes were prepared and resuspended in assay buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, pH 7.4). After incubation for 10 min at 30°C, 100 μl of membrane suspension mixed with 100 μl of a solution containing muscimol (1–50 μM) and $^{36}Cl^-$, $0.2\,\mu Ci\,ml^{-1}$ assay buffer. After 6 s the incubation was terminated by addition of 0.5 ml cold assay buffer containing 6 μM picrotoxin and filtration on Whatman GF/C filters by a Brandel M24 apparatus. Filters were washed twice with cold buffer and counted by scintillation spectrometry.

[^{3}H]-Ro15-1788 (flumazenil, spec. act. 80 Ci mmol^{-1}) and $^{36}Cl^-$ (spec. act. 25 Ci mg^{-1}) were obtained from New England Nuclear (Boston, MA). Muscimol was obtained from Sigma and polyethylene glycol 400 (PEG 400) from J.T. Baker (St. Louis, MO). Osmotic pumps were obtained from Alza (Palo Alto, CA). Alprazolam was a gift from Upjohn (Kalamazoo, MI), lorazepam from Wyeth (Philadelphia, PA) and CL 218,872 from Dr Joseph Moerschbaecher.

Data were analysed by Student's t test, the Mann-Whitney test, or analysis of variance with Dunnett's test.

Results

Benzodiazepine receptor binding *in vivo* was significantly decreased in cortex and hippocampus at day 7 compared to day 1 of lorazepam (cortex, day 1: 1609 ± 191 fmol g^{-1}; day 7: 1182 ± 150 fmol g^{-1}; $n = 6$–9; $P < 0.05$; hippocampus, day 1: 2509 ± 254 fmol g^{-1}; day 7: 1718 ± 145 fmol g^{-1}; mean ± s.e., $n = 6$–9, $P < 0.05$; Figure 1). These results are similar to those previously obtained (Miller *et al.*, 1988a), except that decreased binding in the hypothalamus did not reach significance ($P < 0.10$). Differences in the cerebellum were not significant (day 1: 668 ± 18 fmol g^{-1}, day 7: 277 ± 27 fmol g^{-1}; $n = 6$–9; $P > 0.15$). In contrast, Type 2 benzodiazepine receptor binding (binding remaining after administration of 25 mg kg^{-1} CL 218,872) was increased in the cortex and hippocampus at day 7 compared to day 1. Type 2 binding was increased both in absolute terms (cortex, day 1: 254 ± 14 fmol g^{-1}; day 7, 405 ± 45 fmol g^{-1}; $P < 0.05$; hippocampus, day 1: 554 ± 23 fmol g^{-1}; day 7: 754 ± 91 fmol g^{-1}; $P < 0.05$) and even more dramatically as a percentage of total specific binding (Figure 1 inset). Changes in Type 2 binding in the cerebellum (day 1: 241 ± 18 fmol g^{-1}; day 7: 277 ± 27 fmol g^{-1}; $P > 0.15$) and other regions were not significant.

For alprazolam, benzodiazepine receptor binding *in vivo* was significantly decreased in the cortex at day 7 compared to day 1 (day 1: 1918 ± 127 fmol g^{-1}; day 7: 1595 ± 127 fmol g^{-1}; $n = 7$–9; $P < 0.05$; Figure 2). No alterations in other brain regions were observed (hippocampus, day 1: 2522 ± 214 fmol g^{-1}; day 7: 2436 ± 145 fmol g^{-1}; $n = 7$–9; $P > 0.40$; cerebellum, day 1: 914 ± 82 fmol g^{-1}; day 7: 818 ± 77 fmol g^{-1}; $n = 7$–9, $P > 0.30$). Unlike in previous studies (Miller *et al.*, 1989c), alterations in the hypothalamus did not achieve significance (day 1: 2141 ± 214 fmol g^{-1}; day 7: 1809 ± 214 fmol g^{-1}; $n = 7$–9; $P < 0.15$). Type 2 benzodiazepine receptor binding was slightly but not significantly increased in the cortex at day 7 compared to day 1, either as specific binding (day 1, 295 ± 20 fmol g^{-1}; day 7, 332 ± 25 fmol g^{-1}; $P < 0.15$) or as a percentage of total specific binding (Figure 2 inset). Changes in Type 2 binding in the hippocampus (day 1: 527 ± 36 fmol g^{-1}; day 7: 618 ± 41 fmol g^{-1}; $P > 0.10$), cerebellum (day 1: 223 ± 14 fmol g^{-1}; day 7: 241 ± 18 fmol g^{-1}; $P > 0.20$) and other brain regions were not significant.

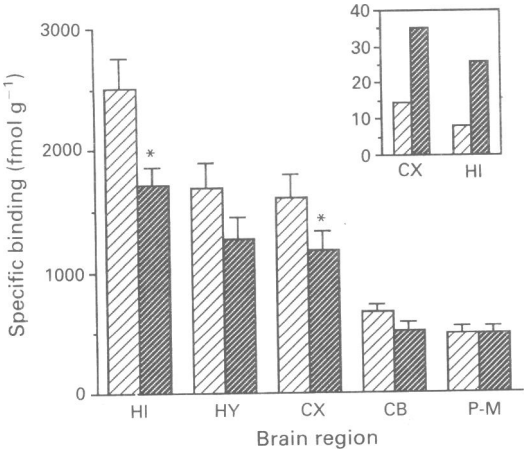

Figure 1 Effects of chronic lorazepam on benzodiazepine binding *in vivo*. Binding was determined by specific uptake of [^{3}H]-Ro15-1788. Results are mean and vertical bars show s.e.mean, $n = 7$–10. CB = cerebellum, CX = cortex, HI = hippocampus, HY = hypothalamus, and P-M = pons-medulla. *$P < 0.05$ vs. day 1. Inset: effects of chronic lorazepam on Type 2 benzodiazepine binding. Binding was determined as above after administration of CL 218,872, 25 mg kg^{-1} i.p. Results are means expressed as a percentage of total binding, $n = 6$–9. Results in cortex and hippocampus for lorazepam are significant ($P < 0.05$). (▨) Day 1; (▨) day 7.

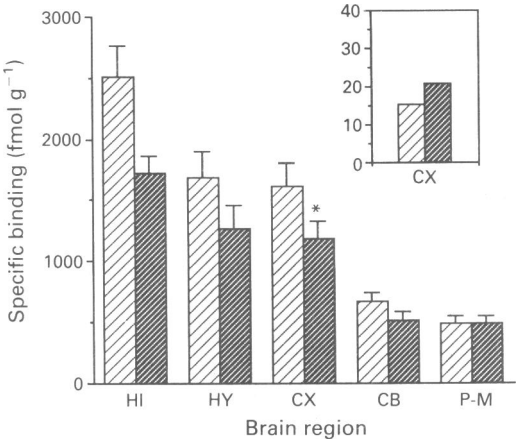

Figure 2 Effects of chronic alprazolam on benzodiazepine binding *in vivo*. Binding was determined by specific uptake of [^{3}H]-Ro15-1788. Results are mean and vertical bars show s.e.mean, $n = 7$–10. CB = cerebellum, CX = cortex, HI = hippocampus, HY = hypothalamus, and P-M = pons-medulla. *$P < 0.05$ vs. day 1. Inset: effects of chronic alprazolam on Type 2 benzodiazepine binding. Binding was determined as above after administration of CL 218,872, 25 mg kg^{-1} i.p. Results are means expressed as a percentage of total binding, $n = 6$–9. Results in the cortex are not significant. (▨) Day 1; (▨) day 7.

GABA-dependent chloride uptake in the cortex was decreased at day 7 compared to day 1, as previously found (Miller *et al.*, 1988a; Figure 3). Maximal chloride uptake was decreased, but the EC_{50} for muscimol was not altered (day 1: 3.2 μM; day 7; 3.8 μM). A similar decrement in maximal uptake without a change in the EC_{50} was observed in the hippocampus (EC_{50}: day 1, 4.2 μM; day 7, 3.4 μM), but no changes in either maximal uptake or EC_{50} for muscimol were observed in the cerebellum.

For alprazolam, maximal GABA-dependent chloride uptake was decreased at day 7 compared to day 1 (Figure 4) with no change in the EC_{50} (day 1: 3.9 μM; day 7: 3.1 μM), as previously demonstrated (Miller *et al.*, 1989c). A small, non significant decrease in uptake was observed in the hippocampus, and no changes in the EC_{50} for the maximal uptake of muscimol were observed in the cerebellum.

Discussion

These data corroborate previous studies indicating down-regulation of $GABA_A$-receptor binding and function after chronic lorazepam and alprazolam administration (Miller *et al.*, 1988a; 1989c). For both lorazepam and alprazolam, decreases in benzodiazepine binding and chloride uptake were observed in the cortex at day 7 compared to day 1. Binding was decreased in the hippocampus after lorazepam but not alprazolam, as previously found. For both drugs decreases in binding in the hypothalamus did not achieve significance, in contrast to previous findings (Miller *et al.*, 1988a; 1989c).

The present study extends our previous data in two respects. First regional alterations observed in

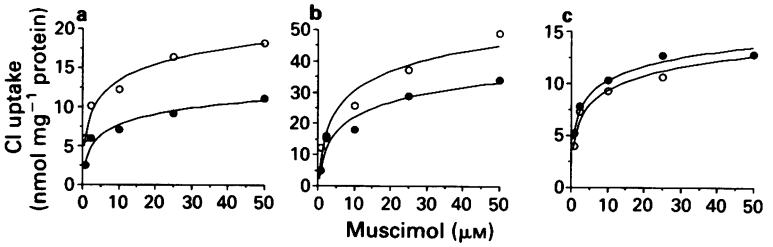

Figure 3 Effects of chronic lorazepam on $GABA_A$-receptor function in (a) cortex, (b) hippocampus and (c) cerebellum. Chloride uptake was determined in the presence (1–50 μM) and absence of muscimol. Maximal uptake was decreased at day 7 (●) compared to day 1 (○) for lorazepam in the cortex and hippocampus ($P < 0.05$). Results are means of 3–5 determinations at each point.

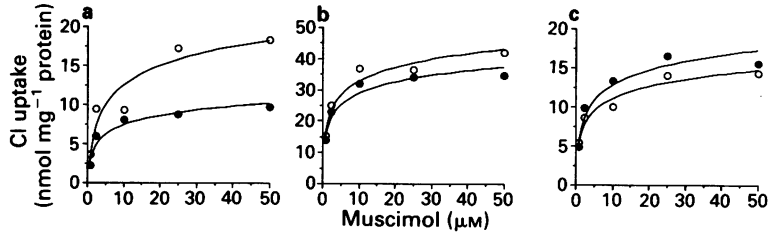

Figure 4 Effects of chronic alprazolam on $GABA_A$-receptor function in (a) cortex, (b) hippocampus and (c) cerebellum. Chloride uptake was determined in the presence (1–50 μM) and absence of muscimol. Maximal uptake was decreased at day 7 (●) compared to day 1 (○) for alprazolam in the cortex ($P < 0.05$). Results are means of 3–5 determinations at each point.

$GABA_A$-receptor function were analogous to those previously observed in binding studies. That is, lorazepam effects on chloride uptake were observed in cortex and hippocampus but not cerebellum, and alprazolam in cortex alone. Alterations in cortex but not cerebellum are similar to results obtained for diazepam by other investigators (Marley & Gallager, 1989). In addition, the association of changes in benzodiazepine binding and GABA-dependent chloride uptake observed in this study has been observed in most (Lopez *et al.*, 1990a,b; Miller *et al.*, 1989a,b), but not all (Lopez *et al.*, 1989), previous studies.

Second, alterations in binding after chronic lorazepam appear to have a greater effect on Type 2 benzodiazepine receptors compared to Type 1 sites. Not only did the binding in the cortex and hippocampus resistant to CL 218,872 (Type 2) increase after 7 days of lorazepam, but the percentage of Type 2 sites increased substantially. A non-significant increase in Type 2 binding was observed with alprazolam. These data suggest that chronic benzodiazepine treatment may preferentially affect one subclass of benzodiazepine receptors, despite the use of a ligand (lorazepam) which does not appear to bind differentially at the two sites. An alternative explanation, although it remains speculative, is that chronic lorazepam administration leads to altered receptor structure so that the ligand used, Ro15-1788, bound preferentially to one site.

For both binding and functional studies, results of chronic (7 days) treatment were compared to short-term treatment (1 day) as in previous studies. Short-term treatment rather than vehicle is the appropriate comparison for chronic treatment, since the presence of a benzodiazepine in the tissue would be expected to alter the results of both binding and functional assays. We have previously demonstrated that brain concentrations achieved by implanted pumps are unchanged at days 1 and 7 for both lorazepam and alprazolam (Miller *et al.*, 1988a; 1989c), indicating that the comparison of these time points is appropriate.

Substantial neurochemical evidence (e.g., Sato & Neale, 1989; Sieghart, 1989), and recently molecular biological evidence (e.g., Shivers *et al.*, 1989; Olsen & Tobin, 1990), support the existence of multiple benzodiazepine receptors. A number of benzodiazepine, β-carboline, and non-benzodiazepine ligands have been demonstrated to distinguish between receptor subtypes. The identification of multiple variants of the α, β and γ subunit mRNAs, together with *in situ* hybridization studies demonstrating region-specific localization of several subunits (Shivers *et al.*, 1989), strongly support the existence of receptor subtypes with different structural and perhaps functional characteristics. Recent transfection studies confirm differences in benzodiazepine binding related to the α_1 versus α_3 subunits (Pritchett *et al.*, 1989). Thus, it is likely that several $GABA_A$-receptors exist with differing benzodiazepine binding characteristics and these subtypes are likely to have differing regional specificity.

Several mechanisms might account for the alteration in the percentage of Type 1 and Type 2 sites in cortex associated with chronic lorazepam. It is possible that lorazepam might mediate interconversion of the two receptor states, either at the genome or post-translationally. Alternatively, the lack of alteration in subtype binding in cerebellum, where Type 1 sites predominate, may indicate a specific effect of chronic lorazepam on Type 2 sites. Finally, chronic lorazepam might alter receptors such that the radioligand used, Ro15-1788, binds preferentially to one site.

Our results suggest region-specific effects for chronic lorazepam and alprazolam on binding and function at the $GABA_A$-receptor. It is unlikely that these effects are related to dose or drug concentration, since the same doses were used and the regimen used maintains similar chronic drug concentrations in brain. However, it remains possible that different concentrations of individual benzodiazepines are required to alter receptors in different regions. Our results over a broad dose range for lorazepam ($1–10\,mg\,kg^{-1}\,day^{-1}$) argue against this hypothesis (Miller *et al.*, 1988b). That is, results are similar with different lorazepam doses but are distinct from alprazolam at each dose evaluated. Rather, it appears more likely that chronic lorazepam downregulates benzodiazepine sites in the cortex and hippocampus, with a preference for Type 2 sites. In contrast, alprazolam downregulates receptors in the cortex only, without a significant effect on either receptor subtype. Effects on $GABA_A$-receptor function exhibit similar regional specificity. It is possible that these differential effects are due to the binding characteristics of lorazepam and alprazolam. It is also possible that either compound might effect another neurotransmitter system, with indirect effects on the $GABA_A$ complex. Finally, the two drugs might differentially affect the $GABA_A$-receptor subunit gene expression. Additional studies examining the effects of these compounds on the $GABA_A$-receptor gene regulation may shed light on this hypothesis.

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Myocardial uptake of lignocaine: pharmacokinetics and pharmacodynamics in the isolated perfused heart of the rabbit

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1 The uptake kinetics and pharmacodynamics of lignocaine were studied in the isolated perfused heart of the rabbit.

2 Six hearts were perfused with increasing concentrations of lignocaine in a modified Krebs-Henseleit buffer. The effluent concentration together with the increase in QRS duration were measured during lignocaine infusion and during 20 min after cessation of lignocaine infusion.

3 Lignocaine disposition and elimination were best described by a two-compartment open model. Terminal half-life was 11.0 ± 2.9 min. The unidirectional transfer was slower from central to peripheral compartment than from peripheral to central compartment ($T_{1/2,12}$ = 42.6 ± 10.5 min whereas $T_{1/2,21}$ = 10.7 ± 2.8 min). The myocardium/perfusate concentration-ratio was 4.7 ± 0.4.

4 The pharmacodynamic effect was best described in the central compartment by using the Hill equation. Calculated maximum QRS duration (E_{max}) was 77 ± 8 ms. E_{max} was also directly measured in four additional rabbits by infusing ten times the dose of lignocaine used in the main experiment: the value of E_{max} measured in these conditions was 92.5 ± 9.6 ms, i.e. a QRS widening of 150%. The steady-state perfusate concentration producing half the effect (C_{50}) was 15.7 ± 7.6 $\mu g\,ml^{-1}$.

6 In conclusion, the specific lignocaine binding leading to increase in QRS duration appeared to be more closely related to the vascular stream than non specific binding leading to a deeper accumulation process.

Introduction

Lignocaine is a widely used class Ib antiarrhythmic agent. It is presumed to exert almost no effect over QRS duration (Goodman & Gilman, 1985). Numerous experiments have studied lignocaine myocardial uptake as a function of regional coronary blood flow and/or as a function of myocardial ischaemia (Zito *et al.*, 1981; Patterson *et al.*, 1982; Horowitz *et al.*, 1986). Nevertheless, myocardial uptake of lignocaine remains to be quantified in term of kinetics and dynamics as: (i) the myocardial tissue uptake kinetics of various drugs seems to be directly related to their lipophilicity expressed as the octanol/water partition coefficient (Lüllmann *et al.*, 1979), and (ii) various drugs exhibit hysteresis when comparisons are made between pharmacokinetics and pharmacodynamics (Galeazzi *et al.*, 1976; Sheiner *et al.*, 1979; Keefe & Kates, 1981). This hysteresis between pharmacokinetic and pharmacodynamic data may indicate that the site of effect is located far from the sampling site, i.e. usually the central compartment. This lack of correlation between tissue uptake and effect has been related to different locations between the sites of accumulation and the sites of action: while the myocardial tissue uptake kinetics of various drugs seem to be directly related to their lipophilicity, the effect dynamics are probably determined by the receptor site location (Lüllmann *et al.*, 1979; Pang & Sperelakis, 1983). Thus, the aim of the present study was to quantify the relationship between the pharmacokinetics of lignocaine myocardial uptake and the increase in QRS duration induced by lignocaine. For this purpose, we have chosen an isolated single pass perfused rabbit heart model.

We have measured lignocaine uptake by and release from the heart in conjunction with its effects on the QRS duration. Both the physico-chemical properties of the molecule (octanol/water partition coefficient = 79 (molality), pKa = 7.57) and the clinical experience led us to presume that lignocaine myocardial uptake and release is rapid. We, thus, used increasing infusion concentrations in order to obtain multiple pseudo steady-state concentrations of lignocaine.

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Methods

Heart preparation

Six male New Zealand rabbits weighing 2215 ± 650 g, (mean ± s.d.), (range 1350–3200 g) were anaesthetized with urethane, 2.5 $g\,kg^{-1}$ i.p. A tracheostomy was performed and the animals were ventilated with a Braun Melsugen (874070) apparatus. The chest was opened via a midline incision and the heart was removed and mounted quickly on the perfusion apparatus: the aorta was cannulated and perfused retrogradely.

In order to determine the maximum attainable increase in QRS duration, four additional rabbits were perfused with ten times the dose of lignocaine used in the main experiment.

Perfusion procedure

We used a modification of the procedure described by Gillis & Kates, 1986. The heart was perfused with a modified Krebs-Henseleit buffer in a single pass design. A mixture of 95% O_2 and 5% CO_2 was bubbled through the perfusate. The perfusate flowing through the coronary arteries was maintained at a constant inflow of 20 $ml\,min^{-1}$ with a pump (Watson-Marlow). The drug was delivered into the affluent perfusate just before the aorta via an automatic syringe. Teflon tubing was used for the pump and syringe connections to avoid non specific adsorption. The temperature and pressure were measured just before the aorta. The temperature was 38.2 ± 0.5°C and the pressure was 25 ± 4 mmHg. The pulmonary artery was cannulated for collection of the effluent perfusate. The coronary effluent flow, measured from the pulmonary artery, was 19.5 ± 0.7 $ml\,min^{-1}$. The hearts were paced atrially with a bipolar electrode, positioned in the region of the sinus node at a cycle length of 300 ms, by use of a Hugo Sachs type 17071 A1 stimulator.

The buffer composition consisted of (millimolar): NaCl 106, KCl 4.0, $MgSO_4$, $7H_2O$ 1.1, KH_2PO_4 0.9, $NaHCO_3$ 40.0, dextrose 5.5, pyruvic acid 2.0 and $CaCl_2$ 1.8. No heparin was

injected during the whole procedure. Lignocaine hydrochloride was infused via the automatic syringe at five increasing amount rates: 0.10 μM min^{-1} (30 μg min^{-1}) during the first 8 min; 0.21 μM min^{-1} (60 μg min^{-1}) from 8 to 16 min; 0.52 μM min^{-1} (150 μg min^{-1}) from 16 to 24 min; 1.04 μM min^{-1} (300 μg min^{-1}) from 24 to 32 min and 2.08 μM min^{-1} (600 μg min^{-1}) from 32 to 42 min. The last infusion period was maintained longer than the preceding ones in order to approach a steady-state. The rates corresponded to concentrations of 5, 10, 26, 52, 104 nM ml^{-1} (1.5, 3, 7.5, 15 and 30 μg ml^{-1}) in buffer.

Measurements

Four wire ECG electrodes were positioned on the epicardial surface of the heart, one on the right atria, one on the left atria, one on the apex and one on the free wall of the right ventricle. Two leads of the ECG were monitored continuously on a Honeywell CM 130 electrocardioscope and recordings were made with a Beckman Dynograph chart recorder at a paper speed of 250 mm s^{-1}. The QRS duration of three consecutive beats were measured manually, and averaged. The heart was perfused for 20–25 min to allow stabilization of the ECG. After obtaining a stable baseline ECG, the infusion of lignocaine was started. Lignocaine concentration was measured in the effluent before lignocaine infusion and 2, 3, 4, 6, 8, 9, 10, 11, 12, 14, 16, 17, 18, 19, 20, 22, 24, 25, 26, 27, 28, 30, 32, 33, 34, 35, 36, 38, 40 and 42 min after starting the infusion. Lignocaine infusion was stopped after 42 min. Lignocaine was measured in the effluent 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 18, 23, 28, 33 and 38 min after cessation of lignocaine infusion. ECGs were recorded before lignocaine infusion and then at each minute until cessation of lignocaine infusion. After cessation of lignocaine infusion, ECGs were recorded at the same time periods as the effluent was sampled.

Lignocaine assay

Lignocaine was measured by a gas chromatographic assay (Coyle & Denson, 1986). Briefly, after a single extraction in toluene using etidocaine as the internal standard, 3 μl of the extract were injected in a Varian 3400 gas chromatograph equipped with a 3 m × 2 mm i.d. glass column fitted with 3% OV-11 on 100–120 mesh Chromosorb W (AW/DMCS). The detection was made with a nitrogen specific detector.

Kinetic-dynamic analysis

Rationale for kinetic and dynamic analysis The outflow concentration-time data were fitted to both a one and a two compartment model (Figure 1). The discrimination between these two models was made by using both visual inspection and the Akai criterion (Yamaoka *et al.*, 1978). The data were always best fitted by using the two compartment model.

The following abbreviations are used: C_0 concentration in the afferent perfusate; C concentration in the efferent perfusate; t time; T time of infusion. While infusion is continuing, T = t and varies with time. When infusion ceases, T becomes a constant corresponding to the duration of infusion. k_{ij} rate constant from compartment i to compartment j; λ_1 hybrid rate constant (initial phase); λ_z hybrid rate constant (terminal phase); $T_{1/2\,ij}$ unidirectional half-life from compartment i to compartment j; $T_{1/2}$ terminal half-life; K myocardium/perfusate concentration-ratio at steady-state; E observed QRS duration; E_0 resting QRS duration; E_{max} theoretical maximum QRS duration; A_{50} amount of drug in the central compartment producing half the maximum increase in QRS duration at steady-state; C_{50} afferent perfusate concentration producing half the maximum increase in QRS duration at steady-state; A_1 amount of drug in the central compartment; A_2 amount of drug in the peripheral compartment.

Similarly, we compared a linear proportional model (Gillis & Kates, 1986) and the simplest Hill model for the effect-time data fitting (increase in QRS duration). For that purpose we perfused four additional rabbit hearts with ten times the highest concentration used in the main experiment, i.e. 21 μM min^{-1} in order to determine approximately the maximum QRS widening.

The dynamic data (increase in QRS duration) were comparatively fitted to the central or peripheral compartment concentrations or to a special effect compartment concentration (Sheiner *et al.*, 1979), by using the Akaike criterion (Yamaoka *et al.*, 1978), whereas the comparison between the linear proportional model and the Hill model used the F ratio test (Boxenbaum *et al.*, 1974).

Pharmacokinetics A two-compartment open model, with elimination from the central compartment, was used to describe the concentration-time data measured in the effluent perfusate (Figure 1).

By remembering that the rate of appearance of drug in the outflow perfusate (Q × C) is directly related to the amount of drug in the central compartment:

$$Q \times C = k_{10} \times A_1$$

one obtains the following equation describing the concentration in the effluent (Gibaldi & Perrier 1982, p. 64):

$$C = C_0(R'_1(e^{-\lambda_1(t-T)} - e^{-\lambda_1 t}) + R'_z(e^{-\lambda_z(t-T)} - e^{-\lambda_z t})),$$

with

$$R'_1 = \frac{\lambda_z(k_{21} - \lambda_1)}{k_{21}(\lambda_z - \lambda_1)} \quad \text{and} \quad R'_z = \frac{\lambda_1(k_{21} - \lambda_z)}{k_{21}(\lambda_1 - \lambda_z)},$$

i.e. $$R'_z = 1 - R'_1$$

Since the concentration in the central compartment is not known, we cannot estimate any volume.

The following parameters also were derived: The terminal half-life ($T_{1/2}$) = 0.693/λ_z, the ratio of drug mass between the peripheral and the central compartments at steady-state expressed by the ratio of the intercompartmental rate constants, k_{12}/k_{21} and the myocardium tissue/afferent perfusate concentration ratio at steady-state (K).

Pharmacodynamics The QRS duration was fitted according to the simplest model of the Hill equation (Gibaldi & Perrier, 1982; p. 222 and 233) with respect to the amount of drug in the central compartment (assuming no time-dependent change in volumes):

$$E = E_0 + \frac{(E_{max} - E_0) \times A_1}{(A_{50} + A_1)}$$

where E is the observed QRS duration, E_0 is the resting QRS duration, E_{max} is the theoretical maximum QRS duration, A_{50} is the amount of drug in the central compartment producing half of the maximum increase in QRS duration. C_{50}, the affer-

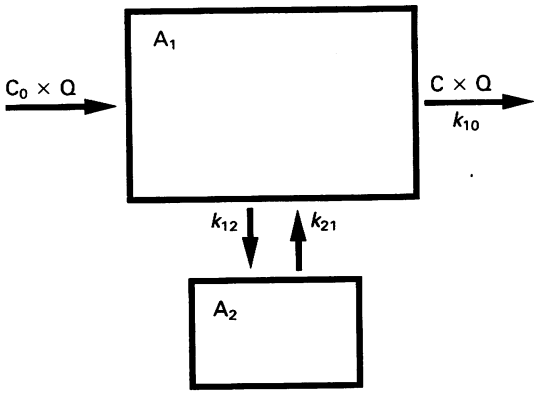

Figure 1 Open two compartment model used for kinetics and dynamics. Abbreviations are defined in text.

ent perfusate steady-state concentration producing half of the maximum increase in QRS duration also was calculated.

The two sets of data were independently fitted using a non-linear regression programme (PCNONLIN). Estimates of the kinetic parameters were used for the dynamic analysis. A constant coefficient of variation of the lignocaine assay was assumed so that the data were iteratively reweighted using the inverse of the concentration squared. A constant variance was assumed for the QRS duration measurement and the effect data were iteratively reweighted using the inverse of the measured QRS duration.

All the results are expressed as the mean ± s.d.

Results

Myocardial uptake kinetics (Table 1 and Figure 2)

The assumptions of immediate mixing and linear pharmacokinetics provided excellent fitting. Both visual inspection and Akaï's criterion showed that the data were best adjusted to the two compartment model rather than to a one compartment model (Figure 2). The asymptotic correlation coefficient was greater than 0.985 for all observations.

The exchange kinetics were rapid as it was presumed from the physicochemical properties of the molecule. The elimination rate constant k_{10} was 0.642 ± 0.091 min^{-1} and the terminal (elimination from the heart) half-life ($T_{1/2}$) was 11.0 ± 2.9 min. Despite a marked two-compartment profile, most of the drug remained in the central compartment: the unidirectional transfer was much slower from central to peripheral compartment than from peripheral to central compartment ($T_{1/2,12}$ = 42.6 ± 10.5 min, whereas $T_{1/2,21}$ = 10.7 ± 2.8 min). In other words, at steady state, only one fifth of the drug actually in the heart was distributed in the peripheral compartment (k_{12}/k_{21} = 0.25 ± 0.04).

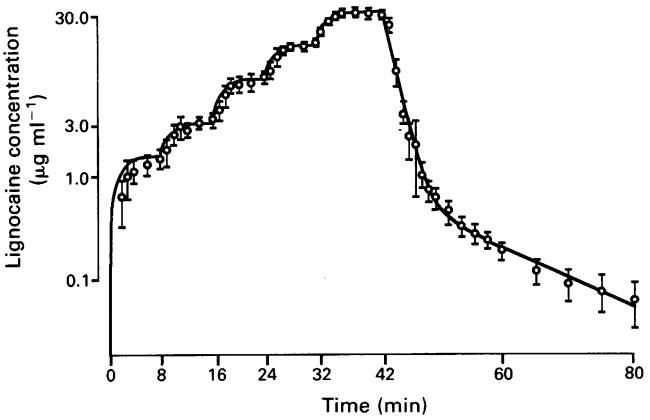

Figure 2 Concentration-time data observed in the effluent perfusate in the six rabbits (mean values are shown with s.d. indicated by vertical bars). The solid line represents the computer simulation obtained with the average estimates of the parameters.

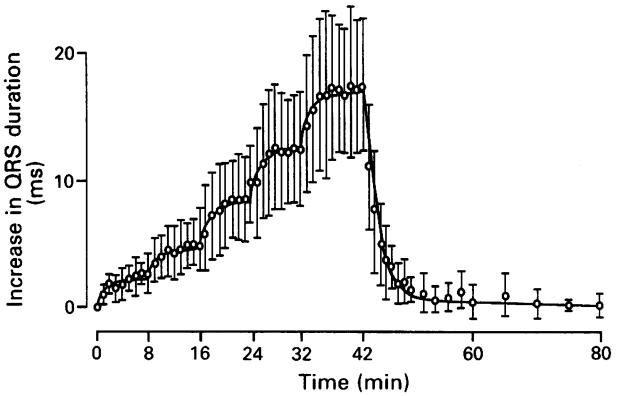

Figure 3 Increase in QRS duration ($E_{max} - E_0$) observed in the six rabbits (mean values are shown with s.d. indicated by vertical bars). The solid line represents the computer simulation obtained with the average estimates of the parameters.

Pharmacodynamics (Table 1 and Figure 3)

After 20–25 min equilibration time, basal heart rate and QRS duration remained stable. When paced at 300 ms, the rabbit hearts exhibited a resting QRS duration (E_0) of 36 ± 3 ms. All rabbit hearts receiving 21 $\mu M\,min^{-1}$ of lignocaine stopped beating within 45 s after the beginning of infusion after a short period of bradycardia (the stimulation voltage was not raised in an attempt to overcome the asystole). The asystole period was brief and all the hearts totally recovered within 5 min after cessation of lignocaine infusion. These hearts receiving 21 $\mu M\,min^{-1}$ of lignocaine exhibited a measured maximum QRS duration of 92.5 ± 9.5 ms (150 ± 20% increase in QRS duration).

The QRS duration increased rapidly with lignocaine infusion, even at the lower doses. Nevertheless, the atrial stimulation was efficient for all the experiments throughout the study. The effector site responsible for the increase in QRS duration appeared to be located in the central rather than in any other compartment (peripheral or special 'effect' compartment) (Figure 3). The adequacy of fitting was excellent with respect to the imprecision of the measure of the QRS duration: the asymptotic correlation coefficient was higher than 0.96 for all observations.

Discussion

In the present *in vitro* experiment, myocardial uptake kinetics of lignocaine appeared likely to be described by a two compartment model. This fact may be related to the studies by Lüllmann *et al.* (1979). Large differences in the rate and degree of binding to guinea-pig atria between drugs have actually been attributed by these authors to differences in lipophilicity. They reported that the myocardium is made up of four different successive compartments: (1) the extracellular fluid representing about one third of the volume of the muscle, (2) the phospholipid layer, (3) the intracellular water and (4) the

Table 1 Measured and estimated parameters from the six rabbit hearts

Rabbit	k_{12} (min^{-1})	k_{21} (min^{-1})	λ_1 (min^{-1})	λ_z (min^{-1})	E_0 (ms)	E_{max} (ms)	C_{50} ($\mu g\,ml^{-1}$)	K
1	0.0130	0.0480	0.5726	0.0468	40	64	14.5	5.4
2	0.0187	0.0555	0.7476	0.0540	36	73	12.4	4.3
3	0.0131	0.0628	0.6589	0.0614	33	87	29.7	4.7
4	0.0153	0.0637	0.5416	0.0617	36	81	13.7	4.3
5	0.0268	0.1146	0.7878	0.1101	33	82	7.2	4.7
6	0.0170	0.0732	0.6596	0.0710	38	75	17	5.1
Mean	0.0173	0.0696	0.6613	0.0675	36	77	15.7	4.7
s.d.	0.0052	0.0232	0.0956	0.0224	3	8	7.6	0.4

intracellular components. In the case of lignocaine, two compartments can be clearly differentiated. The first (central) compartment is the most important with 80% of the drug distributed in it at steady-state. This distribution profile prevents the heart from accumulating lignocaine in a deeper compartment and thus, may explain in part that the effect of lignocaine rapidly disappears upon infusion discontinuation. The steady-state concentration-ratio of lignocaine between myocardium and perfusate was $K = 4.7 \pm 0.4$. This value is in accordance with a partition of 2.8 between myocardium and arterial whole blood in the anaesthetized sheep (Nancarrow *et al.*, 1987) and a myocardium/blood ratio of 5.4 reported in anaesthetized rabbits (Halpern *et al.*, 1984). In the present study performed on rabbit isolated hearts, no protein and/or blood cells were added to the perfusate. Thus, K may not adequately reflect the *in vivo* steady-state myocardium/blood concentration-ratio. In fact, only the free fraction of a drug is presumed available for equilibration between the different compartments of the body (Tozer, 1981). Since lignocaine is about 60–80% bound in serum as well as in whole blood (McNamara *et al.*, 1981; Coyle & Denson, 1984; Nancarrow *et al.*, 1987), the *in vivo* steady-state myocardium/perfusate concentration-ratio may be three to four times lower than that observed in the present study.

The pharmacodynamic data were best fitted by the Hill (E_{max}) model rather than the linear proportional model (F test). We observed a 150% maximum increase in QRS duration in the four rabbits in which ten times the dose of lignocaine was infused. This value may be interpreted with care since the hearts stopped beating rapidly, which may have led to an underestimation of E_{max}. Albeit lower when estimated than when directly measured (77 ± 8 vs. 92.5 ± 9.5 ms), E_{max} was of the same order of magnitude in the two groups. Thus, lignocaine may induce an increase in QRS duration even at relatively low perfusate concentrations (Figure 3). Although lignocaine uptake kinetics were best described by using a two-compartment model, the effect dynamics (increase in QRS duration) appeared to occur in the central rather than in the peripheral compartment. A lack of correlation between non-specific (distribution) and specific (effect) binding may explain this difference: a similar lack of correlation between myocardial uptake kinetics of several calcium channel blocking agents and their onset and duration of effect has been described (Pang & Sperelakis, 1983). The commonly accepted explanation for these discrepancies is a dissociation between specific and non-specific binding of the molecule. C_{50}, the steady-state perfusate concentration producing half the effect, was $15.7 \pm 7.6\,\mu g\,ml^{-1}$. This value is about three to four times the effective clinical serum concentration. However, this C_{50} concentration in the afferent perfusate may not adequately reflect the *in vivo* concentrations needed to produce a similar effect, since the perfusate was free of proteins and blood cells and C_{50} may be underestimated by the same proportion as the myocardium/perfusate concentration-ratio may have been overestimated.

In summary, myocardial uptake of lignocaine was best described by a two-compartment model, suggesting that lignocaine distribution occurs both in the extracellular and in the intracellular space, in the rabbit isolated heart. The transient residual effect of lignocaine observed upon discontinuation may be explained both by a central compartment prevalent for the disposition kinetics, and by the fact that the measured effect (increase in QRS duration) was related to the central compartment concentration with no time-lag. The lack of specificity in lignocaine myocardial binding seems to prevent the pharmacodynamic effect from being directly related to the uptake kinetics.

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Sensitivity of hippocampal neurones to kainic acid, and antagonism by kynurenate

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1 The sensitivity to kainic acid of neurones in the CA1 and CA3 regions of rat hippocampal slices has been examined by microiontophoresis and by superfusion methods.

2 When the iontophoretic currents needed to produce comparable plateaux of firing were compared, neurones in the pyramidal cell layer of the CA3 region were approximately 5 times more sensitive than cells in the CA1 region. No difference was noted in sensitivity to N-methyl-D-aspartate (NMDA) or quisqualate.

3 When kainate was superfused at known concentrations, the threshold for eliciting excitation in CA1 was 2.1 μM. The threshold concentration in CA3 was 0.24 μM.

4 Two weeks after the stereotaxic intrahippocampal injection of colchicine, the granule cells of the dentate gyrus and thus the mossy fibre projections to CA3 were destroyed. In slices prepared from animals thus treated the threshold concentration of kainate for eliciting excitation had risen to 1.64 μM.

5 Kainate was less effective in promoting the development of epileptiform bursts of neuronal firing in colchicine-treated slices than in controls.

6 Kynurenic acid antagonized the excitation of CA1 neurones elicited by kainate, NMDA or quisqualate. In the CA3 region kynurenate antagonized selectively responses to microiontophoretic NMDA, with little effect on responses to kainate or quisqualate.

7 In slices taken from colchicine-treated rats kynurenate was able to block responses to kainate in the CA3 area in parallel with responses to NMDA.

8 Taken together the results suggest that the excitatory responses to kainate in the CA3 region may be partly due to a presynaptic action on mossy fibre terminals to release endogenous amino acids. The differential action of kynurenate in normal and lesioned slices may, therefore, indicate that the postsynaptic kainate receptors are sensitive to antagonism by this compound whereas the presynaptic receptors are resistant to kynurenate.

Introduction

High affinity binding sites for kainic acid are located in many regions of the central nervous system but occur with highest density in the CA3 region of the hippocampus (Foster *et al.*, 1981; Unnerstall & Wamsley, 1983; Greenamyre *et al.*, 1985; Monaghan *et al.*, 1985). This location corresponds to a high sensitivity of neurones to locally applied kainic acid (de Montigny & Tardif, 1981; Robinson & Deadwyler, 1981). De Montigny & Tardif (1981) demonstrated that pyramidal neurones in the CA3 region were approximately 80 fold more sensitive to iontophoretically applied kainate than pyramidal neurones in the CA1 region.

However, the kainate binding sites in CA3 appear to be localized, at least partly, on the presynaptic terminals of mossy fibre afferents arriving from dentate gyrus granule cells, since destruction of this pathway causes a profound loss in the number of binding sites (Represa *et al.*, 1987). This change is accompanied by a reduced excitatory potency of iontophoretically applied kainate (de Montigny *et al.*, 1987). It is possible therefore that the greater sensitivity of CA3 neurones to kainate is partly the result of its acting presynaptically to promote the release of endogenous compounds, which contribute to the excitatory response. Consistent with this idea is the demonstration that kainic acid can promote the release of both glutamate and aspartate from nerve terminals (Collins *et al.*, 1983; Ferkany & Coyle, 1983; Potashner & Gerard, 1983; Connick & Stone, 1988). In the present study we have therefore compared the potency of kainic acid on CA3 pyramidal cells in the normal hippocampal slice and slices prepared from animals pretreated with colchicine to remove the mossy fibre projection.

Kynurenic acid was described as a selective amino acid antagonist in the cerebral cortex by Perkins & Stone (1982) and has since been used widely to block the three major classes of excitatory amino acid receptors (N-methyl-D-aspartate, NMDA, kainate and quisqualate) in many regions of the nervous system and a variety of synaptic pathways (reviewed in Stone *et al.*, 1989). However, on some neurones in the CNS kynurenic acid will distinguish, to a variable extent, between NMDA receptors and those for kainate and quisqualate, usually producing a greater degree of blockade of the former (Ganong *et al.*, 1983; Herrling, 1985; Elmslie & Yoshikami, 1985). As a second objective in the present study therefore we have attempted to examine the effects of kynurenic acid in both CA1 and CA3 regions, in order to determine whether the removal of the mossy fibre projection changes the sensitivity of amino acids to blockade by kynurenate.

Methods

Slices were prepared at a thickness of 500 μm from male Wistar rats killed by stunning and cervical dislocation. The slices were preincubated in an artificial CSF medium (composition in mM: NaCl 115, KCl 5, KH_2PO_4 1.5, $NaHCO_3$ 25, $CaCl_2$ 2.5, $MgSO_4$ 1.2, glucose 10) saturated with 5% CO_2 in O_2. The slices were maintained in this medium at room temperature (21–24°C) for at least one hour before experimentation. Individual slices were then transferred onto a nylon net submerged in a 0.5 ml perfusion chamber, where they were superfused with medium of the same composition at a rate of approximately 3 ml min^{-1} and a temperature of 30°C.

In order to obtain quantitative estimates of the sensitivity of neurones to kainate, two methods were used. Firstly the compound was applied by microiontophoresis (Stone, 1985) in a regular sequence with quisqualate and NMDA, so as to

produce a plateau of cell firing rate of around 30 Hz. The ejecting currents of each agonist required to produce these plateaux were then taken as an indication of the relative sensitivity of the tested neurones. Compounds were applied from 5 or 7 barrelled micropipettes (Clarke Electromedical) broken back to an overall tip diameter of about 2 μm. The barrels were filled with a selection of the following solutions in water: kainate 5 mM, pH 7; N-methyl-D-aspartate (NMDA) 20 mM, pH 7; quisqualate 5 mM, pH 7.5. One barrel contained 3 M NaCl for current balancing and ejection was performed by use of a regular time cycle, so as to prevent changes in the amount of compound released for a given current.

In order to obtain more accurate estimates of the absolute sensitivity of neurones to kainate, the agonist was applied directly into the superfusing medium at known concentrations while single cell firing was recorded by a microelectrode filled with 1 M NaCl. This arrangement also served to detect epileptiform bursts of localised groups of cells which sometimes developed following the initial excitation of units by kainate. In all such superfusion experiments 2 min kainate applications were made at least 15 min apart, so as to minimize any tendency to overdepolarization, desensitization or excitotoxicity. Since virtually all the cells tested in this way were initially silent, it was possible to quantify the degree of excitation by counting the total number of spikes produced during the response using a digital counter (Neurolog).

For the subsequent examination of the antagonism of amino acid excitation by kynurenate, a comparison was made of the sensitivity to several agonists applied by microiontophoresis. The details of solutions used are noted above. Recordings of cell firing rate were made through a separate single electrode filled with 1 M NaCl and glued alongside the multibarrel assembly (Stone, 1985). The position of the recording electrode was adjusted so that the tip protruded 40–50 μm beyond the multibarrelled iontophoretic assembly.

Action potentials were amplified, discriminated and counted to produce instantaneous ratemeter recordings and histogram records of firing rate. In order to minimize the recognised variability which can exist in the ejecting characteristics of different iontophoretic electrodes, cell sensitivity was always compared with the same electrode in the CA1 and CA3 areas on any one slice.

Mossy fibre lesions

Small amounts of colchicine can be used to destroy selectively the mossy fibre projection from the granule cells to CA3 pyramidal cells (Goldschmidt & Steward, 1980; Represa *et al.*, 1987; de Montigny *et al.*, 1987). Rats were anaesthetized with pentobarbitone, 60 mg kg^{-1} i.p., and a small burr hole made in the skull to allow the stereotaxic insertion of a microsyringe into the hippocampal formation. Colchicine 2 μmol was injected into the hippocampus in 0.25 μl of saline at coordinates AP 3 mm (caudal to bregma), lateral 2.8 mm, vertical 3.0 mm according to the atlas of Pellegrino *et al.* (1981). The scalp was sutured and the animal allowed to recover for two weeks, at which time the animal was killed for the preparation of brain slices. One slice from each animal was preserved in 10% formaldehyde in saline for subsequent histology to confirm the extent of lesioning.

Quantitative data are presented as mean ± s.e.mean. Statistical comparisons were made by use of Student's two-tailed t test for superfusion data (between slices) and paired t test for iontophoretic data (in which comparisons were made between subregions of each slice).

Results

The sensitivity of CA1 and CA3 pyramidal cells was compared initially by use of microiontophoresis. The application of the three agonists kainate, quisqualate and NMDA by microiontophoresis readily produced excitation of all cells

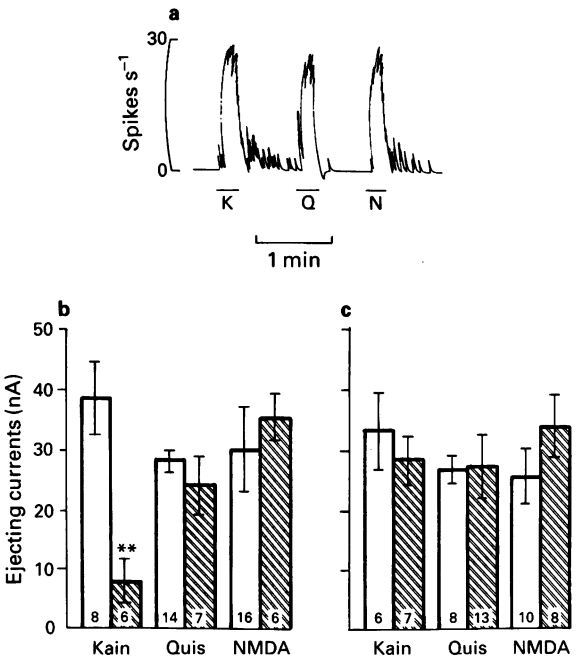

Figure 1 (a) Ratemeter records of the responses of a neurone in the CA1 pyramidal layer to the iontophoretic application of kainate (Kain) with a current of 32 nA, quisqualate (Quis) with a current of 22 nA and N-methyl-D-aspartate (NMDA) with a current of 36 nA. The currents were adjusted to produce plateaux of firing rate of approximately 30 Hz. (b and c) Show the ejecting currents required in control (b) and colchicine-lesioned (c) rats to elicit plateau firing of cells in the CA1 (open columns) or CA3 (hatched columns) regions of hippocampal slices. Each column shows the mean current (n = number in column) and bars represent s.e.mean. ** $P < 0.05$.

tested in both regions of the hippocampus. Figure 1 illustrates the type of responses obtained by adjusting the ejecting currents so as to induce a plateau of firing rate at about 30 Hz, and also summarizes the iontophoretic currents required to elicit these plateaux. It is clear that the currents required for NMDA and quisqualate are similar in CA1 and CA3 pyramidal cells, but the sensitivity to kainate is approximately 5 times greater in the CA3 region. Indeed the difference in sensitivity to kainate was the only significant difference noted between the two regions.

Superfusion of the slices with medium containing kainate at various concentrations in the range 0.01 to 10 μM caused concentration-related increases of the firing frequency of both spontaneously active and initially silent units. In the CA1 region excitation was produced by perfusion with kainate at threshold concentrations (the smallest concentration eliciting an observable increase of firing rate) ranging from 0.8 to 3.5 μM (mean 2.1 ± 0.41 μM, $n = 6$). However, in the CA3 region a threshold excitation was elicited by concentrations in the range 0.05–0.40 μM (mean 0.24 ± 0.06, $n = 6$) (Figure 2).

Above these threshold levels the dose-response relationships for kainate superfusion were similar on both populations of pyramidal cells and appeared to be parallel (Figure 3). However, no attempt was made to seek maximum responses, because of the problems of achieving this with a potent compound which can cause overdepolarization or excitotoxicity on repeated applications of high concentrations.

Microiontophoresis on colchicine-lesioned slices

Destruction of the mossy fibre projection from the dentate gyrus to CA3 pyramidal cells was essentially complete when viewed by light microscopy in most of the animals treated with colchicine. The histologically observable damage in a typical lesioned hippocampus is illustrated in Figure 4. In slices taken from such animals the threshold range for eliciting CA3 cell excitation by kainate superfusion was 0.35–2.5 μM.

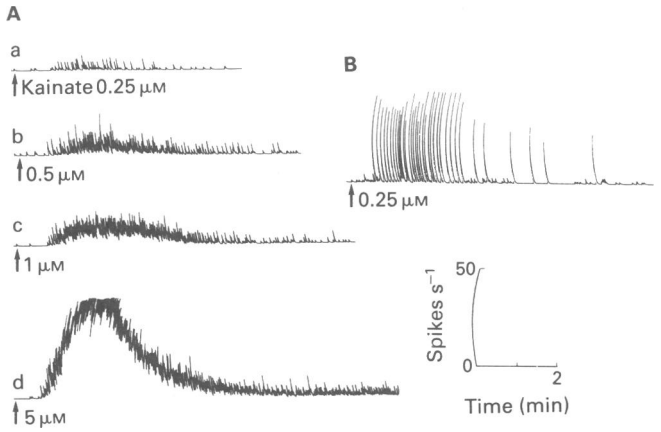

Figure 2 (A, a–d) Ratemeter records of the activity of a CA3 neurone in response to superfusion with kainate; (a) threshold of 0.25 μM, (b) 0.5 μM, (c) 1 μM, (d) 5 μM. (B) Record showing epileptiform bursts of activity of a cell in the CA3 region of a different slice, induced by kainate (0.25 μM) in the absence of any overt change of single cell firing. The scales shown apply to all records.

The mean value of $1.64 \pm 0.35\,\mu M$ ($n = 6$) was significantly greater than that obtained from intact slices ($P < 0.05$).

Kainate-induced epileptiform activity

Not all slices from which an excitatory response to kainate had been recorded went on to develop epileptiform bursts of cell firing. Equally, some cells which did not show an immediate increase of firing rate in response to kainate were involved in epileptiform bursts of activity, which arose from a few seconds to several minutes after the kainate superfusion. An example of such a cell is illustrated in Figure 2. The concentrations of kainate eliciting such bursts therefore did not exactly parallel the concentrations causing excitation. On 8 control slices bursts occurred only following kainate applications at a mean concentration of $1.19 \pm 0.16\,\mu M$. In colchicine-treated slices this value rose significantly to $6.25 \pm 1.25\,\mu M$ ($n = 4$; $P < 0.05$).

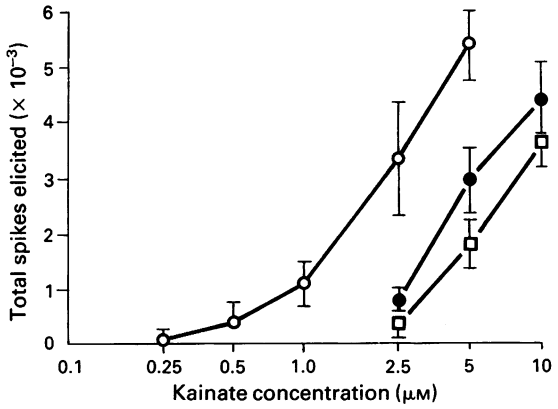

Figure 3 Partial dose-response curves for the excitation caused by kainate of neurones in the CA1 region (□), neurones in the CA3 region of control rats (●) and neurones in the CA3 region of colchicine lesioned animals (○). The ordinate scale indicates the total spike count during the response to 2 min superfusion of kainate. Points indicate the mean and vertical bars show s.e.mean for 4–6 slices. * Significantly different from control slices at $P < 0.05$.

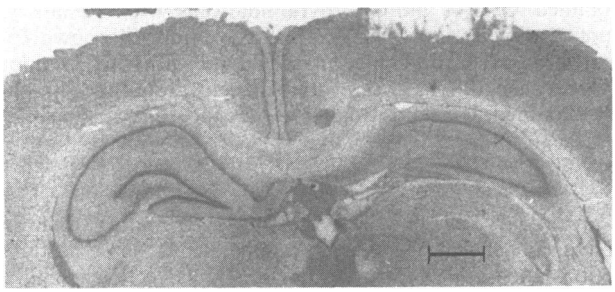

Figure 4 Photomicrograph of a section through the hippocampus of a rat treated with colchicine (2.5 μmol) 14 days previously. In comparison with the untreated side, the lesioned hippocampus is seen to lack the granule cell layer of the dentate region, which normally projects to the CA3 layer of pyramidal cells. The scale bar represents 1 mm.

Antagonism by kynurenic acid

Figure 5 illustrates the responses of a neurone in the CA1 region excited by each of the three excitants tested. Superfusion with kynurenic acid at a concentration of 200 μM decreased sensitivity to all three agents. On some cells NMDA responses were antagonized to a relatively greater extent than kainate and quisqualate, but no concentration of kynurenic acid was found which would produce any reduction of NMDA responses without affecting the other two agonists to some extent. At the concentration illustrated (200 μM) all three agonists could be fully antagonized on all the neurones tested. At the lower concentration of 100 μM responses to all three

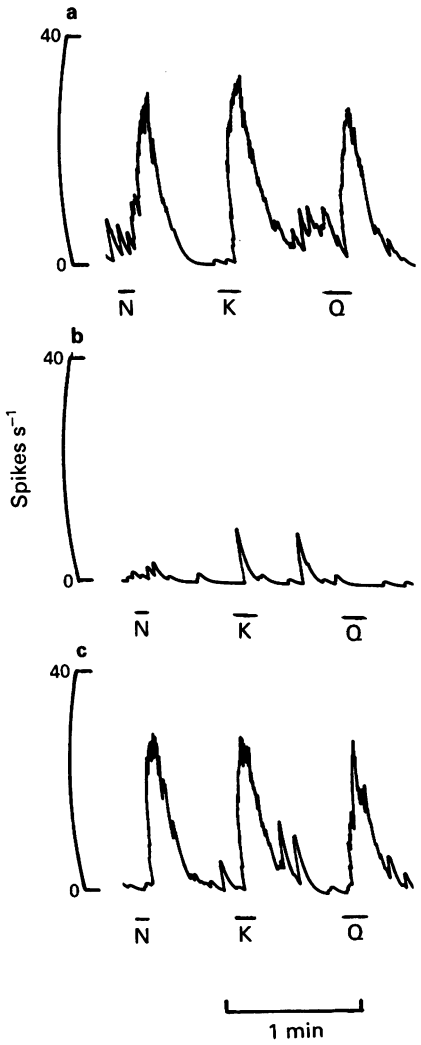

Figure 5 Records of the firing rate of a neurone in the CA1 pyramidal cell region in response to the iontophoretic application of N-methyl-D-aspartate (N) 24 nA, kainate (K) 40 nA and quisqualate (Q) 28 nA. (a) Shows control responses, (b) the non-selective blockade of all three agonists by kynurenic acid 200 μM, and (c) illustrates recovery 6 min later.

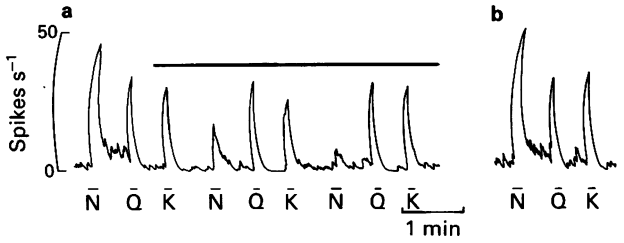

Figure 6 (a) Records of the firing rate of a neurone in the CA3 region of a normal hippocampus in response to the iontophoretic application of N-methyl-D-aspartate (NMDA, N) 35 nA, quisqualate (Q) 18 nA, and kainate (K) 35 nA. Superfusion of the slice with kynurenic acid 200 μM blocked selectively the responses to NMDA. (b) Shows recovery 5 min later.

agents were reduced partially – kainate by 52 ± 11% (n = 10), quisqualate by 40 ± 4% (n = 12) and NMDA by 58 ± 10% (n = 12).

Figure 6 illustrates the responses of a neurone in the CA3 region. In this case kynurenic acid at a concentration of 200 μM produced a total blockade of the responses to NMDA without affecting sensitivity to kainate (92 ± 16% of control, n = 9) or quisqualate (98 ± 11%, n = 10). Even at a concentration of 1 mM kynurenic acid had no significant effect on the kainate or quisqualate responses.

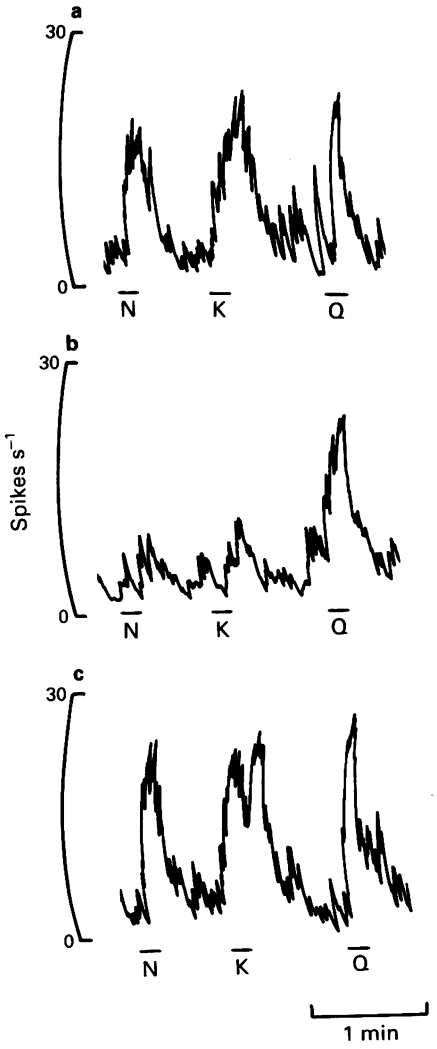

Figure 7 (a) Records of the firing rate of a neurone in the CA3 region of a hippocampus taken from a colchicine-treated rat. Excitation is seen in response to the iontophoretic application of N-methyl-D-aspartate (NMDA, N) 30 nA, kainate (K) 30 nA and quisqualate (Q) 20 nA. (b) Superfusion with kynurenic acid 200 μM antagonised the responses to NMDA and kainate while having little effect on sensitivity to quisqualate. (c) Shows recovery 5 min after the addition of kynurenate.

Colchicine-lesioned slices

The sensitivity of a CA3 neurone in a colchicine lesioned hippocampus is illustrated in Figure 7. In this case it is now clear that even at a concentration of 200 μM kynurenic acid was able to reduce sensitivity to kainate as well as NMDA. At lower concentrations of 150 μM or 100 μM the antagonism of both compounds was usually partial, NMDA responses being reduced to 47 ± 8% (n = 6) and 79 ± 10% (n = 8), respectively. However, no clear differences of sensitivity between NMDA and kainate were apparent, since kainate responses were also now reduced to 39 ± 11% (n = 6) and 72 ± 5% (n = 8), respectively. Quisqualate responses on the other hand were reduced by about 25% on 3 of the cells tested at 200 μM kynurenate, but remained unchanged by kynurenate even at 1 mM on 6 others (overall reduction of 8 ± 12%, n = 9).

Discussion

Kainic acid binding sites are widely distributed throughout the CNS. Their occurrence in organisms as primitive as *Hydra* (London *et al.*, 1980) suggests a fundamental role in cellular function. Of the many studies which have examined kainate receptor distribution in the mammalian brain, most are agreed that these sites are localized to synaptic regions (Foster *et al.*, 1981) and that by far the greatest density of binding is restricted to the stratum lucidum in the CA3 area of the hippocampus (Foster *et al.*, 1981; Unnerstall & Wamsley, 1983; Greenamyre *et al.*, 1985; Monaghan *et al.*, 1985).

Kainate sensitivity

This localization may account for the greater sensitivity of CA3 cells to the excitatory and burst inducing effects of superfused kainate observed in the present study. However, de Montigny *et al.* (1987) have shown previously that, using microiontophoretic applications, the CA3 neurones were approximately 80 fold more sensitive to kainate excitation than cells in the CA1 region, while no differences were noted in the sensitivity to acetylcholine or glutamate. This was not the case in the present work, where a difference of sensitivity of around 5 fold was seen with microiontophoretic applications of agonists.

The explanation for this difference is not clear, but the variability of iontophoretic ejection characteristics is notorious (Stone, 1985). In addition, the use of a separate recording electrode in the present work, protruding 40–50 μm beyond the iontophoretic barrels, may have resulted in a different cellular distribution of the ejected compounds relative to the recording tip. A comparable difference of sensitivity was obtained for CA1 and CA3 pyramidal cells when kainate was applied directly into the superfusing medium at known concentrations. The difference in sensitivity with this technique was between 10 fold at threshold concentrations and 4 fold at the maximum concentrations tested (Figure 2).

Presynaptic kainate receptors

A great deal of evidence suggests that the excitatory, toxic and convulsant effects of kainate are mediated at least partly by actions on presynaptic terminals. Thus colchicine deafferentation of the CA3 pyramidal cells greatly reduces the toxic effects of kainate on those cells (Okazaki & Nadler, 1988). The ability of kainate to induce epileptiform bursting in hippocampal slices (Westbrook & Lothman, 1983; Fisher & Alger, 1984; Neuman *et al.*, 1988) is similarly diminished after colchicine lesions (Okazaki *et al.*, 1988). In addition, kainate

has been shown directly to elicit a release of endogenous amino acids *in vivo* (Young *et al.*, 1988), from olfactory cortex (Collins *et al.*, 1983) and hippocampal synaptic terminals (Ferkany & Coyle, 1983; Connick & Stone, 1988) and from astroglia *in vitro* (Lehmann & Hansson, 1988), and a release of radiolabelled D-aspartate from cerebral cortex, striatum and cerebellum (Potashner & Gerard, 1983; Gallo *et al.*, 1989). On this basis it has been concluded that the kainate binding which results from mossy fibre lesions probably reflects the loss of receptors on the presynaptic terminals. The deafferentation of CA3 pyramidal cells may also induce postsynaptic changes which could contribute to the change in kainate sensitivity.

After mossy fibre lesions de Montigny *et al.* (1987) found CA3 cells to be only 2.6 times more sensitive to kainate than CA1 cells, a 30 fold decrease in CA3 cell sensitivity. In the present study a significant diminution in sensitivity of only 5 fold was obtained when tested by microiontophoresis. This may be another reflection of kainate gaining access to different regions of the cell surface in the present work because of the electrode arrangement noted above.

It is interesting to note that Sawada's group has claimed that two distinct types of CA3 cell response can be observed with kainate, a fast response obtained with kainate application into the stratum oriens or stratum radiatum, and a slower response obtainable only in the stratum lucidum (Sawada & Yamamoto, 1984). It is possible therefore that these qualitatively different responses may involve the mossy fibre terminals to different degrees, and that their relative contributions to the excitatory responses seen by de Montigny *et al.* (1987) and the present author may account for our quantitative differences. However, it must be stated that no obvious or consistent variations comparable to those described by Sawada and Yamamoto (1984) were observed in the present work in the latency or duration of kainate responses in stratum pyramidale, probably because the durations of application were far longer than the 200 ms pulses used in the intracellular recording experiments (Sawada & Yamamoto, 1984).

Kynurenic acid

Undoubtedly the dominant new finding of the present work was the different degree of antagonism by kynurenic acid of kainate-induced excitation of CA3 cells in control and colchicine lesioned slices. The non-selective nature of the antagonism of excitatory amino acids by kynurenate was noted in the original study of this activity (Perkins & Stone, 1982), and a similar non-specificity has since been found in most regions of the neuraxis (Stone & Burton, 1988). There are several examples where kynurenate is a more effective antagonist of NMDA than of kainate or quisqualate (Ganong *et al.*, 1983; Herrling, 1985; Elmslie & Yoshikami, 1985), a finding which probably owes much to the ability of kynurenate to displace glycine from the strychnine-sensitive glycine site on the NMDA receptor complex (Birch *et al.*, 1988; Mayer *et al.*, 1988). The differentiation between NMDA and other receptors in these studies was only relative, and increasing the dose of kynurenate was usually able to block other amino acid agonists. This may be a consequence of the ability of kynurenate to interact directly with glutamate binding sites (Danysz *et al.*, 1989).

In most such areas the major excitant activity of all three primary amino acid agonists is thought to involve postsynaptic receptors. The main exception is the CA3 region where, as illustrated in Figure 6, kynurenate has a relatively selective ability to antagonize NMDA responses, even at high (mM) concentrations in the superfusing medium. This represents a marked contrast with CA1 neurones where no such selectivity is apparent.

In view of the likely involvement of presynaptic receptors in the control responses to kainate in CA3, the mossy fibre projection was therefore removed in order to reassess the selectivity of kynurenate antagonism on a relatively pure population of CA3 postsynaptic receptors. The results show that under these circumstances the selectivity towards NMDA is lost, responses to both NMDA and kainate being susceptible to blockade at similar doses of the antagonist.

It was notable that responses to quisqualate remained largely unaffected by kynurenate, an observation made also by Ganong & Cotman (1986). This may indicate some subtle variations in the properties of the quisqualate receptor in different regions of the CNS, but will require careful quantification. Preliminary experiments in which quisqualate was administered directly into the superfusion medium show that quisqualate responses are more variable and less reproducible with this method than are responses to kainate. This makes quantification and further analysis difficult; it may be more appropriate to approach this problem by use of d.c. field potential responses of a population of CA3 and CA1 neurones. Alternatively the quisqualate resistance may be a reflection of some non-specificity in the action of quisqualate at non-amino acid receptors. It would be of interest to repeat some of this work using the more selective quisqualate analogue DL-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). However, it should be emphasized that kainate has been found to be more sensitive to kynurenate antagonism than quisqualate in other regions of CNS, such as spinal cord (Evans *et al.*, 1987) and cerebellar granule cells in culture (Gallo *et al.*, 1987).

The present results suggest that the presynaptic receptors for kainate, participating in kainate responses in control but not lesioned slices, are relatively resistant to the blocking action of kynurenate. The postjunctional receptors, studied in isolation after mossy fibre removal, appear to behave as do postjunctional sites in most other regions of CNS including the CA1 region. This implies that the presynaptic and postsynaptic receptors for kainate can be discriminated pharmacologically, a proposal voiced previously by Sawada *et al.* (1988). Kynurenic acid may be a useful tool in this regard, able to antagonize the postsynaptic but not the presynaptic kainate receptors.

This conclusion makes it likely that the presynaptic kainate receptors release a substance which acts on kynurenate-insensitive receptors to cause excitation and raises questions concerning the identity of that substance. Since the endogenous amino acids glutamate and aspartate have not been tested in this study it is quite possible that these could be released and act on kynurenate resistant sites. Glutamate responses have been retained in previous studies after blockade of NMDA and non-NMDA receptor populations in olfactory cortex (Surtees & Collins, 1985; see Stone & Burton, 1988). Precedent also exists in the brainstem, where kynurenate blocks synaptic transmission and responses to kainate, AMPA and NMDA but not the effects of glutamate (Leone & Gordon, 1989). Alternatively other endogenous compounds such as cholecystokinin which, together with its receptors, is known to be highly localized to the CA3 region (Innis *et al.*, 1979; Vanderhaegen *et al.*, 1980; Zarbin *et al.*, 1983) and causes a powerful depolarization of pyramidal cells (Dodd & Kelly, 1981) could be released.

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Effects of repeated infusions of substance P and vasoactive intestinal peptide on the weights of salivary glands subjected to atrophying influences in rats

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1 The long-term influence of substance P (SP) and vasoactive intestinal peptide (VIP) on rat salivary gland weight was investigated after parasympathetic denervation or on feeding soft food.

2 The parotid gland lost about one-third of its weight within 4–5 days following parasympathetic postganglionic denervation or change in dietary regimen, from pellets to liquid diet, thought to reduce nerve reflex activity.

3 Daily i.v. infusions with SP or VIP diminished or largely prevented the fall in parotid gland weight, whereas infusions with pentagastrin, bethanechol and saline had no effect. The infusions were preceded by administration of α- and β-adrenoceptor antagonists; these antagonists were also given to the control animals.

4 The effect of SP and VIP on the parotid gland weight appeared to be related to cell size rather than to cell number, as judged by measurements of RNA and DNA.

5 Observations on the two other major salivary glands underlined the fact that different gland types in the same animal behave differently. Parasympathetic preganglionic denervation (decentralization) lowered the weights of the sublingual and submandibular glands, whereas liquid diet only reduced the weight of the sublingual gland. SP and VIP did not affect the weights of the submandibular glands, but VIP prevented the slight fall in sublingual gland weight induced by liquid diet.

6 The present results suggest a trophic role in rats for SP and VIP on parotid glands and for VIP on sublingual glands. Such an influence may be exerted naturally as a result of their release from nerves containing these peptides around acini.

Introduction

In rats, the parotid gland loses 30–40% of its weight after parasympathetic denervation or diminished nerve reflex activation induced by dietary change, from pellets to liquid diet (Schneyer & Hall, 1966). Surprisingly, the weight of this gland does not decrease in response to prolonged treatment with antimuscarinic agents; in fact, it increases somewhat and this is not dependent on an intact sympathetic innervation (Ekström, 1974). In this species, stimulation of the parasympathetic innervation evokes a flow of parotid saliva in the presence of atropine and adrenoceptor antagonists (Ekström *et al.*, 1983a). Nerves occur in the rat parotid gland that contain substance P (SP, Sharkey & Templeton, 1984; Ayer-Le Lievre & Seiger, 1985), of the tachykinin family, and vasoactive intestinal peptide (VIP, Wharton *et al.*, 1979; Uddman *et al.*, 1980) and are found in fibres surrounding the acini. SP- and VIP-containing nerve fibres reach the parotid gland via the parasympathetic auriculo-temporal nerve, and SP and VIP are thought to be transmitters involved in the 'atropine-resistant', parasympathetic nerve-evoked secretion of parotid saliva (see Ekström, 1987; Ekström *et al.*, 1989a). In the present study we have considered the possibility that SP and VIP exert long-term influences on salivary gland size. Therefore, they were administered over a period of time in order to assess their efficacy in preventing the expected fall in parotid gland weight following parasympathetic denervation or reduced nerve reflex activity. For comparisons, some observations were made on the sublingual and submandibular glands as well as on the pancreatic gland.

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Methods

Altogether 145 adult, female Sprague-Dawley rats were used. Preliminary surgery was performed in animals anaesthetized by inhalation of diethyl ether. In order to denervate the parotid gland parasympathetically, the auriculo-temporal nerve was cut (Ekström, 1974; Alm & Ekström, 1976); in some experiments the chorda-tympani nerve (carrying preganglionic parasympathetic nerve fibres, Houssay *et al.*, 1962) was also cut where it crosses over the auriculo-temporal nerve, to cause parasympathetic decentralization of the sublingual and submandibular glands. These denervation and decentralization procedures were performed unilaterally. An intravenous polyethylene catheter for chronic use was inserted into the femoral vein and the tip was placed in the inferior caval vein. The other end of the catheter was inserted subcutaneously to an interscapular position and fitted with a modified Venflon wing (Viggo AB, Helsingborg, Sweden), which was sutured to the muscle fascia and allowed to protrude through the skin. This end of the catheter was sealed with a rubber membrane, through which repeated needle insertions could be made to gain access to the circulation. At the end of each experiment the venous position of the catheter and its function were checked. Food and water were given *ad libitum*. Liquid diet was prepared daily by mixing two parts of water with one part of a powder made from a commercial pelleted diet (Astra-Ewos, Södertälje, Sweden). Rats in the denervation/decentralization study and those serving as controls in the liquid diet study were maintained on this pelleted diet. The drug treatment started 12 h after denervation/decentralization (SP, 2 μg kg^{-1} min^{-1}; VIP, 5 μg kg^{-1} min^{-1}; and bethanechol, 20 μg kg^{-1} min^{-1}) or change to liquid diet (SP, 5 μg kg^{-1} min^{-1}; VIP, 5 μg kg^{-1} min^{-1}; and pentagastrin

$10\,\mu g\,kg^{-1}\,min^{-1}$). These drugs (or sterile saline) were infused twice daily for 1 h over a period of five days. During the infusions the animals were awake and restrained to Bollman cages, to which they had been accustomed. The animals were given the α-adrenoceptor blocking agent dihydroergotamine ($0.5\,mg\,kg^{-1}$ i.p.) and the β-adrenoceptor blocking agent propranolol ($0.5\,mg\,kg^{-1}$ i.p.) 10 min before the i.v. infusion began. The control rats in the denervation/decentralization study and those given a pelleted diet in the liquid diet study were kept in Bollman cages and subjected to the adrenoceptor antagonists following the same protocol as mentioned above. The doses of adrenoceptor antagonists used were sufficiently large to prevent a possible sympathetic influence on the gland cells in connection with the infusions.

Twelve hours after the last infusion the animals were killed by diethyl ether and both parotid glands (and in some experiments the submandibular and sublingual glands as well as the pancreatic gland) were removed, weighed, and either heated to 110°C for 48 h (dry weights, denervation/decentralization study) or frozen and stored at −70°C for determination of gland protein, RNA and DNA (liquid diet study). In the latter study the mean value of left and right salivary glands of each animal was used for statistical calculations. RNA and DNA were extracted according to the procedure of Schmidt & Thannhauser (1945), modified by Schneider (1946) and then, measured as described by Mejbaum (1939) and Burton (1956), respectively. The standards used were RNA from baker's yeast (Sigma, Chemical Co, Mo, St Louis, U.S.A.) and DNA from calf thymus (Sigma). Protein was measured by the method of Lowry *et al.* (1951), the standard being bovine serum albumin. In preliminary experiments on non-denervated animals (kept on pellets) mean arterial blood pressure was monitored continuously (pressure transducer) via a catheter placed into a femoral (anaesthetized animals) or tail artery (awake animals). To measure flow of parotid saliva in anaesthetized rats (chloralose, $100\,mg\,kg^{-1}$ i.v.), the parotid duct was cannulated and drops of saliva falling from the cannula were photoelectrically recorded and collected over 10 min periods in tubes and weighed. Drugs were infused either via a femoral vein or a tail vein (awake animals); the animals were pretreated with α- and β-adrenoceptor blockers as mentioned above.

Drugs

Adrenaline tartrate (ACO, Solna, Sweden), carbamyl β-methylcholine chloride (Bethanechol, Sigma), dihydroergotamine methansulphonate (Sandoz, Basel, Switzerland), pentagastrin (ICI Pharmaceuticals, Macclesfield, U.K.), propranolol hydrochloride (ICI), SP (Sigma), and VIP (kindly supplied by Professor V. Mutt, Karolinska Institutet, Stockholm, Sweden).

Group matching and statistics

In the groups to be compared, the rats were age- and weight-matched. At the end of the experimental period no differences between the body-weights of the rats in each group were found (see legends to figures). Student's *t* test for unpaired data was used, and the Bonferroni method was applied to make multiple comparisons (Wallenstein *et al.*, 1980). *P* values <0.05 were considered to be significant. Values given are means ± s.e.mean.

Results

Mean arterial blood pressure and salivary secretion

With respect to blood pressure, the pattern was the same in anaesthetized and non-anaesthetized animals. After an initial drop in pressure at the start of the infusion the bethanechol- and SP-treated animals regained their pre-infusion levels of blood pressure, while in the VIP-treated rats the recovery was not complete; the pressure was about 100 mmHg during the major part of the infusion period (Figure 1, awake animals). Total amount of parotid saliva secreted over 60 min in response to SP ($2\,\mu g\,kg^{-1}\,min^{-1}$) was $700 \pm 84\,\mu l$ ($n = 3$ glands and rats) and to bethanechol ($20\,\mu g\,kg^{-1}\,min^{-1}$) $616 \pm 91\,\mu l$ ($n = 3$). During infusion of SP the salivary flow rate declined, while it was steady during the bethanechol infusion. There was a small flow of viscous saliva in response to VIP ($5\,\mu g\,kg^{-1}\,min^{-1}$, $n = 3$). However, the cannula was repeatedly plugged which made measurements of the volume secreted difficult.

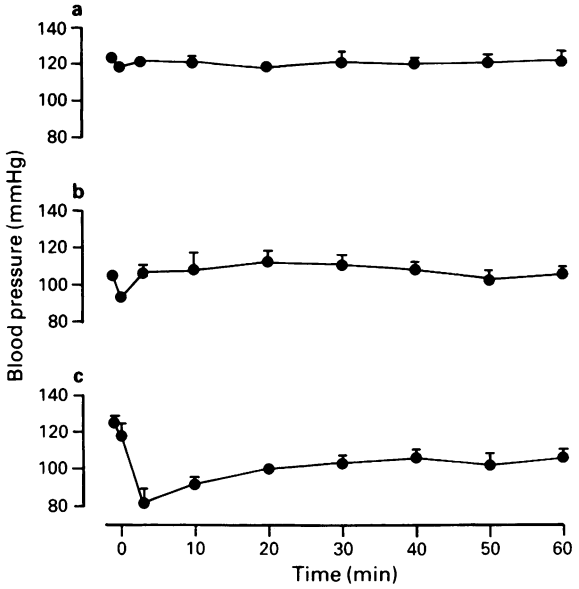

Figure 1 Mean arterial blood pressure monitored in (9) awake rats infused i.v. with either (a) bethanechol ($20\,\mu g\,kg^{-1}\,min^{-1}$, $n = 3$), (b) substance P ($2\,\mu g\,kg^{-1}\,min^{-1}$, $n = 3$) or (c) vasoactive intestinal peptide ($5\,\mu g\,kg^{-1}\,min^{-1}$, $n = 3$) over a period of 60 min. The animals were pretreated with α- and β-adrenoceptor blocking agents.

Control experiments with adrenoceptor antagonists

Control experiments in 10 rats after adrenoceptor blockade showed adrenaline ($10\,\mu g\,kg^{-1}$ i.v.) to be ineffective in evoking secretion of saliva from the parotid and submandibular glands and the response to stimulation of the sympathetic innervation (20 Hz in bursts of 1 s every 10 s, see Anderson *et al.*, 1988) to be reduced by 80%. Three hours after the injection of the antagonists a certain recovery in the secretory response had occurred. However, the responses were far from pre-injection levels. It should also be noted that much larger doses of adrenoceptor antagonists than presently used are ineffective in lowering salivary gland weights in rats (Brenner & Stanton, 1970).

Parasympathetic denervation or decentralization

Parotid glands Figure 2a shows the gradual fall in the weight of the parotid gland after denervation. The expected fall in weight caused by denervation was diminished or largely prevented by infusions of SP and VIP. The weights of denervated glands of rats treated with these peptides were significantly heavier than those of denervated glands of untreated controls (+27%, $P < 0.05$, and +32%, $P < 0.01$, respectively, Figure 3a,b). After VIP treatment the weights of denervated glands were not significantly different from those of the intact contralateral glands from the control animals. After bethanechol, the weights of denervated glands were higher than those from

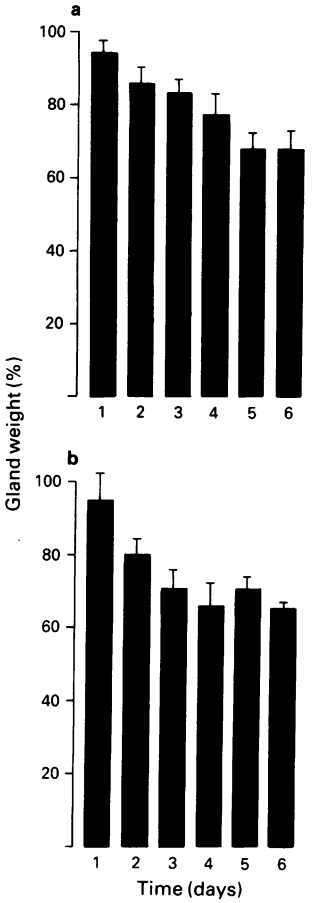

Figure 2 Effects on parotid gland (wet) weights over time following (a) parasympathetic denervation and (b) change in dietary regimen, from pellets to liquid diet. (a) At each time of observation following the unilateral denervation three rats were used. The weight of the denervated gland was expressed as a percentage of the weight of the contralateral gland (the contralateral glands did not show any compensatory hypertrophy with time; weight of contralateral glands 166 ± 5 mg, $n = 18$). (b) At each time of observation following the change to liquid diet three rats were used. The gland weights (mean of left and right glands) were expressed as percentages of mean gland weight of the same control group of six rats given pelleted diet (143 ± 5 mg). Values are means and bars show s.e.mean.

controls (+11%, Figure 3c), but the difference did not achieve statistical significance. Also the weights of contralateral glands of bethanechol- and VIP-treated rats tended to be higher than those of corresponding glands of controls (+11% and +18%, respectively), but again the differences were not statistically significant.

Sublingual and submandibular glands The dry weights of decentralized sublingual and submandibular glands of control rats after 5 days of saline infusion were 35% less (5.1 ± 0.2 mg versus 7.9 ± 0.2 mg, $n = 11$, $P < 0.001$) and 23% less (40.4 ± 1.9 mg versus 52.2 ± 2.0 mg, $n = 13$, $P < 0.001$), than those of intact contralateral glands. Neither in the SP-treated (nine observations for each type of gland) nor in the VIP-treated animals (four observations) were the decentralized glands heavier than the corresponding decentralized glands of untreated animals.

Liquid diet

Parotid glands The fall in gland weight following the change from pellets to liquid diet is shown in Figure 2b. The fall in weight was not diminished by saline (−42%, $P < 0.001$) (Figure 4a) but was less after SP and VIP. The SP and VIP-treated glands were significantly heavier than those of saline-treated animals (SP +32%, VIP +45% $P < 0.001$). In fact

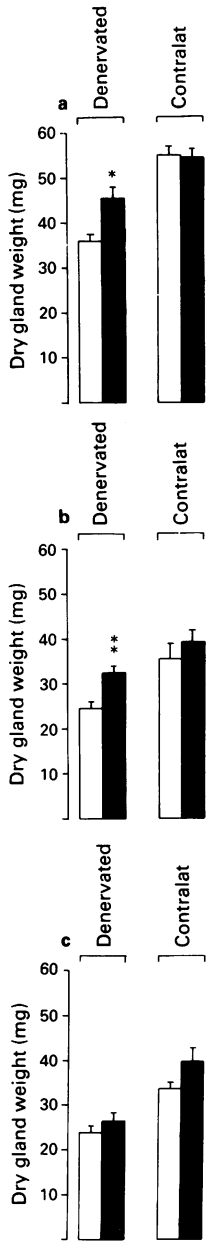

Figure 3 Effects on the weights of denervated and contralateral parotid glands of i.v. infusions (one hour twice daily for 5 days) of (a) substance P (SP, $2\,\mu g\,kg^{-1}\,min^{-1}$, body weights of treated 274 ± 8 g, $n = 9$, and untreated rats 276 ± 8 g, $n = 9$), (b) vasoactive intestinal peptide (VIP, $5\,\mu g\,kg^{-1}\,min^{-1}$, body weights of treated 215 ± 7 g, $n = 6$, and untreated rats 222 ± 8 g, $n = 6$) and (c) bethanechol ($20\,\mu g\,kg^{-1}\,min^{-1}$, body weights of treated 219 ± 8 g, $n = 6$, and untreated rats 217 ± 4 g, $n = 6$). Before each infusion period the animals were given α- and β-adrenoceptor blocking agents; the untreated, control rats were subjected to the same protocol. The weights of the glands of the drug-treated animals (solid columns) were compared with those of corresponding glands of untreated, control animals (open columns). Values are means and bars show s.e.mean. $^*P < 0.05$, $^{**}P < 0.01$.

after VIP treatment the gland weight did not differ significantly from that of the animals on the pelleted diet. In this study the dose of substance P used was larger than that in the denervation/decentralization study. However, this increase in dose did not prevent a reduction in gland weight. The gland weight of pentagastrin-treated rats was not different from that of saline-treated ones. Bethanechol was not tested in this part of the study. Total gland protein in saline-treated rats on a liquid diet was reduced by as much as 52% ($P < 0.001$), whereas the reduction after SP- and VIP-treatment was less

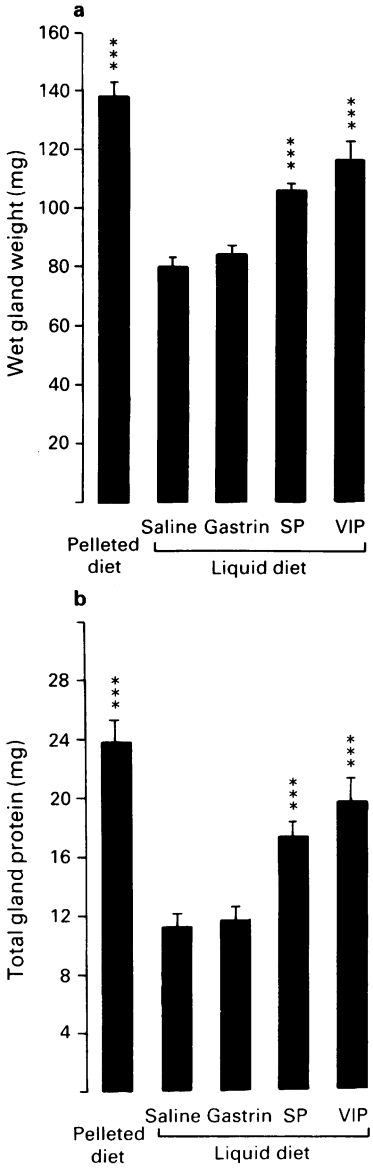

Figure 4 Effects on (a) weight and (b) total protein of parotid glands of rats maintained on liquid diet of i.v. infusions (one hour twice daily for 5 days) of saline (bodyweight 219 ± 5 g, $n = 6$), pentagastrin (Gastrin, 10 $\mu g\,kg^{-1}\,min^{-1}$, bodyweight 220 ± 7 g, $n = 6$), substance P (SP, 5 $\mu g\,kg^{-1}\,min^{-1}$, bodyweight 215 ± 3 g, $n = 6$) and vasoactive intestinal peptide (VIP, 5 $\mu g\,kg^{-1}\,min^{-1}$, bodyweight 218 ± 10 g, $n = 6$). For comparison, the glands of a group of rats kept on pelleted diet are included (bodyweight 225 ± 2 g, $n = 9$). Before each infusion period the animals were given α- and β-adrenoceptor blocking agents; the animals given pellets underwent the same treatment. Values are means and bars show s.e.mean. Differences between the glands of saline-treated rats and those of each of the other four groups of rats were analysed. $P < 0.001$.

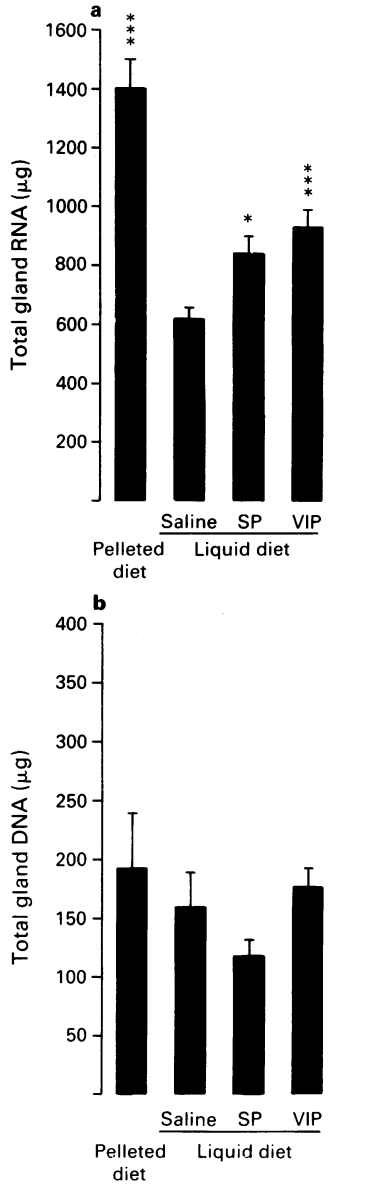

Figure 5 Effects on total amounts of (a) RNA and (b) DNA in parotid glands of rats maintained on liquid diet of i.v. infusions of saline, substance P (SP) and vasoactive intestinal peptide (VIP). For further details, see legend of Figure 4. * $P < 0.05$, *** $P < 0.001$.

(Figure 4b). Thus, total gland protein was significantly higher in SP- and VIP-treated rats than in the saline-treated ones (SP +53%, VIP +73%, $P < 0.001$). The value for the VIP-treated animals was not significantly different from that for the animals on the pelleted diet. In pentagastrin-treated rats, total gland protein was the same as in the saline-treated animals. The reduction in the total amount of RNA was 56% ($P < 0.001$) in the glands of saline-treated animals (Figure 5a). The amount of RNA was significantly larger in SP- and VIP-treated rats than in saline-treated ones (SP +35%, $P < 0.05$, VIP +50%, $P < 0.001$). The total amount of DNA in the glands of the saline-treated rats was not significantly different from that in the glands of rats maintained on pellets (Figure 5b). Neither were there any statistically significant differences between gland DNA content of saline-treated animals and that of SP- and VIP-treated rats, although the DNA content after SP treatment was notably low. The nucleic acids were not analysed in the glands of the pentagastrin-treated rats.

Sublingual and submandibular glands Sublingual glands of rats kept on a liquid diet and infused with saline (5.9 ± 0.2 mg dry weight, $n = 6$) weighed significantly less (−17%) than glands of rats given pellets (7.1 ± 0.3 mg, $n = 9$, $P < 0.05$). However, the glands of VIP-treated rats (7.4 ± 0.4 mg, $n = 4$) were significantly heavier than those of saline-treated rats (+25%, $P < 0.01$). The sublingual gland weights of SP- (6.3 ± 0.6 mg, $n = 5$) and pentagastrin- (6.6 ± 0.4 mg, $n = 6$) treated rats were not significantly different from those of saline-treated animals.

The weight of the submandibular gland, which was not affected by the liquid diet, did not change in response to the various infusions.

Pancreas The pancreatic gland (dry) weights of rats given pellets (287 ± 11 mg, $n = 4$) and liquid diet combined with infusions of either saline (265 ± 20 mg, $n = 6$) or VIP (242 ± 19 mg, $n = 4$) were about the same, while those of rats

treated with pentagastrin (346 ± 26 mg, $n = 6$) were significantly greater ($P < 0.05$, compared with saline-treated rats).

Discussion

The fall in weight on the parotid gland as a result of parasympathetic denervation or liquid diet was diminished or largely prevented by treatment with SP or VIP. Obviously, the effect of the two neuropeptides was not due to an increase in glandular water content, as judged by dry weights and total amounts of gland protein and RNA. Treatment with the parasympathetic drug bethanechol and pentagastrin had no significant effect on the parotid gland weight. However, the bethanechol treatment tended to reduce the magnitude of the weight loss of the denervated gland and to cause the contralateral gland to gain in weight. That pentagastrin, in the dose used, had trophic potential was shown in the pancreas, since the pancreatic gland weight increased as previously found (Mayston & Barrowman, 1971).

The neuropeptides were administered in the presence of α- and β-adrenoceptor antagonists. This was to exclude the possibility that a peptide-induced release of noradrenaline from sympathetic nerve terminals, or of catecholamines from the adrenal medulla (Malhotra & Wakade, 1987), caused the glandular effects observed in response to SP and VIP. A β-adrenoceptor-mediated hypertrophy of salivary glands in rats is a well-known phenomenon (see e.g. Schneyer, 1962; Brenner & Stanton, 1970). In the denervated glands a release of acetylcholine or other substances from parasympathetic nerve terminals elicited by SP and VIP can also be excluded as a cause of the effect of these peptides on glandular weight. Furthermore, the finding that SP and VIP affected significantly the denervated gland, but not the contralateral gland in the same animal underlines the fact that a parasympathetic background activity was not a prerequisite for the phenomenon to occur. Thus, it seems unlikely that the effect of SP and VIP observed in the rats given the liquid diet was due to an indirect mechanism, depending on the presence of parasympathetic nerve fibres in the glands. Previously, intraperitoneal injections of the nonmammalian tachykinin physalaemin (over a period of 15 days) have been shown to cause an increase in parotid and submandibular gland weights of rats, whereas eledoisin, another nonmammalian tachykinin, did not cause any increase in gland weight (Bertaccini *et al.*, 1966; Cantalamessa *et al.*, 1975); in these studies pretreatment with adrenoceptor antagonists was not undertaken.

The effect of SP and VIP on parotid gland weight was not solely a reflection of their capacity to increase the flow of saliva, since the dose of VIP used produced much less secretion of saliva (but a very protein-rich one, Ekström *et al.*, 1983b) than bethanechol, and the dose of SP used produced about the same amount of saliva as bethanechol. The same conclusion can be drawn by comparing the effects of the β-adrenoceptor agonist isoprenaline, which causes a very large increase in parotid gland weight but only a small, protein-rich, salivary flow, with those of pilocarpine, which causes none or only a slight increase in gland weight but a copious flow of saliva (Schneyer, 1969; Ekström, 1977). The enlargement of the rat parotid gland occurring in response to β-adrenoceptor stimulation is associated with increases in adenosine 3′:5′-cyclic monophosphate (cyclic AMP, Butcher & Putney, 1980). In this gland, VIP is thought to act via cyclic AMP as intracellular messenger (Dehaye *et al.*, 1985), but in contrast to β-adrenoceptor stimulation the VIP activation of the gland cells also involves calcium (Christophe *et al.*, 1989); parasympathomimetics and SP are also thought to act via the calcium pathway (Putney, 1986). Evidently, there must be complex interactions which maintain the normal gland size since, for example, agonists thought to use the same intracellular pathway differ in their effectiveness in causing trophic responses. Furthermore, different gland types within the same animal behave differently, as illustrated in the present study.

Following a liquid diet, Schneyer (1970) obtained a reduced amount of RNA, but unchanged amount of DNA, in the parotid gland, indicating that the weight loss was probably attributable to a decrease in the size of individual cells and not to a decrease in the number of cells. Similar findings were obtained in the present study. The fact that RNA, but not DNA, was found to be higher in the glands of SP- and VIP-treated animals than in the saline-treated animals, favours the idea that these peptides affected cell size rather than cell number. Notably, with respect to DNA, there was in fact a tendency for a decrease to occur in response to treatment with substance P.

There was no statistically significant increase in the weights of the contralateral parotid glands (supplied with an intact innervation) in response to the various drug infusions. Increased sensitivity to chemical stimuli, such as SP and VIP, of the secretory cells of the parotid glands subjected to parasympathetic denervation (Ekström, 1980; Ekström & Wahlestedt, 1982; Ekström *et al.*, 1983b) or liquid diet (Ekström & Templeton, 1977) may have created favourable conditions for revealing trophic effects.

The sublingual, but not the submandibular, glands showed a slight fall in weight in response to liquid diet, as previously observed (Wells & Peronace, 1967). In the present study, VIP, but not SP, prevented the reduction in sublingual gland weight. The weights of the atrophied, parasympathetically-decentralized sublingual and submandibular glands, which do also develop supersensitivity to SP and VIP (for references, see above), were not affected by the treatment with these neuropeptides. A comparison of the different behaviour of the three major salivary glands to the various agents, shows that the parotid glands are particularly susceptible to treatment with SP and VIP. This may be combined with the observation that prolonged treatment with antimuscarinic agents reduces the weights of the sublingual and submandibular glands of the rat (Ohlin & Perec, 1966; Ekström, 1974), but not the weight of the parotid gland which increases slightly (Ekström, 1974).

Polyamines are known to be coupled to protein synthesis and cell growth (Tabor & Tabor, 1984). Interestingly, in rat parotid and sublingual glands the polyamine formation increases in response to infusions (for 3 h in the anaesthetized rat) with VIP, in particular, but also to SP (Ekström *et al.*, 1989b). Further, in these glands polyamine formation is influenced by non-adrenergic non-cholinergic transmitters in response to stimulation of the parasympathetic innervation (Ekström *et al.*, 1989c). The present results seem to suggest a trophic role for SP and VIP: loss of SP and VIP bombardment on secretory cells after parasympathetic denervation and liquid diet may contribute to the parotid gland atrophy. After parasympathetic denervation SP and VIP disappear from this gland (Ekström *et al.*, 1984) and these peptides are also reduced in the gland by liquid diet (Månsson *et al.*, 1990). Interestingly, *in vitro* studies on mammalian cell cultures suggest that SP and VIP are growth-promoting factors (Dalsgaard *et al.*, 1989).

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Effects of cyclic AMP-affecting agents on contractile reactivity of isolated mesenteric and renal resistance arteries of the rat

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1 Effects of adenosine 3':5'-cyclic monophosphate (cyclic AMP)-affecting agents were compared in mesenteric and renal resistance arteries that had been isolated from 20 week old Wistar-Kyoto rats, chemically sympathectomized, stretched to their optimal diameter for mechanical performance and made to contract in response to 30 mM potassium.

2 In mesenteric resistance arteries, isoprenaline, dopamine, NaF, forskolin, isobutyl-methylxanthine, milrinone and dibutyryl-cyclic AMP induced relaxation. Clonidine induced further increases in tension that could be reduced by pertussis toxin and prazosin but not by yohimbine. Clonidine also reduced relaxant responses to isoprenaline.

3 In renal resistance arteries, isoprenaline and dopamine failed to induce relaxation. Compared to mesenteric resistance arteries, renal vessels were less sensitive to the relaxant effect of NaF, forskolin and isobutyl-methylxanthine. Relaxant responses to dibutyryl-cyclic AMP did not differ between the two resistance arteries.

4 Indirect evidence thus suggests that in mesenteric resistance arteries, adenylate cyclase is susceptible to pharmacological activation and inhibition and is functionally coupled to relaxation. The refractory nature of renal resistance arteries to the relaxant effects of isoprenaline and dopamine could be due primarily to absence of appropriate receptors and to a relatively low activity of adenylate cyclase.

Introduction

Selective renal vasodilatation could be beneficial for the therapy of hypertension (Struyker-Boudier, 1980; Ackerman *et al.*, 1983) and congestive heart failure (Renne, 1986; Dzau, 1987). This can be achieved by the use of prodrugs (Drieman *et al.*, 1990) and theoretically also by a direct action on vasodilator systems that are selectively present in renal blood vessels. The pharmacological properties of the renal vasculature differ both quantitatively and qualitatively from those in other organs. It is well known for instance that atrial natriuretic peptides and dopamine display in some species selectivity for the renal vasculature (Goldberg, 1984; Hintze *et al.*, 1985; De Mey *et al.*, 1987) and that there is a relative lack of postjunctional α_2-adrenoceptors in renal blood vessels (Pettinger *et al.*, 1987; Wolff *et al.*, 1987; Edwards & Trizna, 1988). In addition, renal arteries, unlike arteries from other organs, fail to relax in response to β-adrenoceptor agonists (Bomzon, 1983; Boonen *et al.*, 1990). As for most aspects of vascular heterogeneity, the mechanisms that underly this latter regional difference are unclear.

β-Adrenoceptor agonists dilate blood vessels through activation of adenylate cyclase which leads to an increased sarcoplasmic concentration of adenosine 3':5'-cyclic monophosphate (cyclic AMP) and subsequent activation of cyclic AMP-dependent protein kinases (Kukovetz *et al.*, 1981; Krall *et al.*, 1983). β-Adrenoceptor-induced relaxation of vascular smooth muscle is reduced following prolonged exposure to agonist, ageing and hypertension (Triner *et al.*, 1975; Cohen & Berkowitz, 1976; Fleisch, 1980; Harden, 1983; Tsujimoto *et al.*, 1986). These changes have been attributed to alterations at the level of the β-adrenoceptors, the guanosine triphosphate (GTP)-binding regulatory proteins that link the receptors to the cyclase, the adenylate cyclase itself or to changes of cyclic AMP-dependent kinases (Bhalla *et al.*, 1976; Tsujimoto *et al.*, 1986; Asano *et al.*, 1988a,b).

To localize the steps within the receptor-cyclase-kinase chain that could be responsible for regional differences of β-adrenoceptor responses of vascular smooth muscle, we recorded effects of cyclic AMP-affecting agents on the contractile reactivity of isolated mesenteric and renal blood vessels. The experiments were performed in vessels that were small enough to contribute to the regulation of mesenteric and renal vascular resistance *in vivo*. Parts of this study were presented at the 1989 summer meeting of the American Society for Pharmacology and Experimental Therapeutics (Heesen *et al.*, 1989).

Methods

Experiments were performed on resistance arteries isolated from 20-week-old male Wistar-Kyoto rats (local inbred, Rijksuniversiteit Limburg, Maastricht). The animals were killed by cervical dislocation and exsanguination. Fourth- to fifth-order side branches of the superior mesenteric artery and interlobar renal arteries were isolated and chemically sympathectomized with 6-hydroxydopamine (Aprigliano & Hermsmeyer, 1976). Two stainless-steel wires (diameter 40 μm) were inserted in the lumen of the resistance arterial segments, which were then mounted horizontally in an organ chamber (volume 10 ml) between an isometric force transducer (Kistler Morse DSC6, Seattle WA, U.S.A.) and a displacement device (Mitatoyo, Tokyo, Japan) (Mulvany & Halpern, 1977; De Mey *et al.*, 1987). The organ chamber was filled with Krebs-Ringer bicarbonate solution (KRB, composition in mM: NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$, 25.0 and glucose 11) which was maintained at 37°C and continuously aerated with 95% O_2 and 5% CO_2. Before experimentation the resistance arterial segments were stretched to their individual optimal lumen diameter for mechanical performance (De Mey *et al.*, 1987). Their diameter was therefore initially set at 65 μm and increased by 20 μm increments at 10 min intervals. Intermittently, the preparations were exposed to activating solution (K-KRB, KRB in which all NaCl was replaced by an equimolar amount of KCl). This procedure was continued until maximal contractile responses to potassium were obtained. Subsequent experimentation was performed at this optimal diameter. In all experiments a mesenteric and a renal resistance-sized arterial segment from the same animal were mounted in the same organ chamber and were studied in parallel.

To study relaxant responses to pharmacological agents, the resistance arterial segments were first made to contract in

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response to 30 mM potassium by replacing the fluid in the organ chamber with a prewarmed and oxygenated solution containing four parts KRB and one part K-KRB. Changes with time of the response to this contractile stimulus were recorded in each individual experiment and were taken into account when evaluating the effects of agents added on top of the contraction induced by 30 mM potassium. The pharmacological agents that were used included (−)-isoprenaline sulphate, (±)-dopamine hydrochloride, prostaglandin E_2, forskolin, dibutyryl-cyclic adenosine monophosphate, 3-isobutyl-1-methylxanthine, 6-hydroxydopamine hydrochloride, prazosin hydrochloride, and clonidine hydrochloride which were obtained from Sigma Chemicals (Saint Louis, MO, U.S.A.). Pertussis toxin was obtained from Janssen Chimica (Beerse, Belgium). The stable analogue of prostacyclin, iloprost and the inhibitor of phosphodiesterase III, milrinone were kind gifts from Shering AG and Sterling Winthrop, respectively.

Contractile responses were expressed as increases in wall tension (increases in force/2 × length of arterial segment). Effects of pharmacological agents were expressed as percentage change from the pre-existing contractile tension. Data are shown as means ± s.e.mean. Statistical significance of differences was evaluated by Student's t test for paired or unpaired observations or by analysis of variance followed by Bonferroni's t test, where applicable (Wallenstein *et al.*, 1980). $P < 0.05$ was taken to denote statistical significance.

Results

Sympathectomized resistance arteries that had been isolated from rat mesenteries and kidneys had comparable optimal diameters (Table 1). Maximal contractile responses to 125 mM potassium were significantly larger in mesenteric than renal preparations (Table 1). Potassium (30 mM) induced responses that reached 70 to 80% of the maximal response to depolarization in both the mesenteric and the renal resistance arteries (Table 1). In neither mesenteric nor renal preparations was the amplitude of the contractile response to 30 mM potassium significantly affected by the presence of either 1 μM prazosin alone or of both 1 μM prazosin and 1 μM propranolol (data not shown). Exposure of mesenteric and renal resistance arteries to pertussis toxin (1 μg ml^{-1}) for 90 min did not affect their resting wall tension. Following pretreatment with the toxin, responses to 125 mM potassium were reduced in mesenteric resistance arteries and increased in renal vessels (Table 1). The relative amplitude of responses to 30 mM potassium was reduced in mesenteric preparations and not affected in renal resistance arteries that had been exposed to pertussis toxin (Table 1).

In the presence of 1 μM prazosin, mesenteric resistance arteries that had been made to contract with 30 μM potassium responded to isoprenaline with concentration-dependent relaxations. Renal resistance arteries failed to respond to isoprenaline (Figure 1 and Table 2). Similarly, mesenteric but not renal resistance arteries, relaxed in response to dopamine when they had been made to contract with 30 mM potassium in the presence of both prazosin (1 μM) and propranolol (1 μM) (Table 2). Similar observations were obtained with the D_1-receptor agonist, fenoldopam (0.1 to 100 μM, $n = 4$, not shown).

Prostaglandin E_2 and the stable analogue of prostacyclin, iloprost, which in a variety of systems have been observed to stimulate the production of cyclic AMP (Gryglewski *et al.*, 1988), did not induce relaxation in either type of resistance artery. Prostaglandin E_2 rather increased contractile responses to 30 mM potassium in both the mesenteric and renal preparations (Table 2). The maximal effect and sensitivity for prostaglandin E_2 did not differ between the two types of resistance artery (Table 2). Iloprost, on the other hand, increased contractile responses in the renal resistance arteries but did not affect mesenteric resistance arteries (Table 2).

NaF, which in combination with trace amounts of aluminium ions directly activates GTP-binding regulatory proteins (Sternweis & Gilman, 1982), caused biphasic effects in precontracted resistance arteries (Figure 2). Low concentrations of NaF induced relaxation, while concentrations of NaF above 10 mM induced transient further increases in tension. The relaxant effect induced by 3 mM NaF was significantly larger in mesenteric (67 ± 4%, $n = 6$) than renal (42 ± 8%, $n = 6$) resistance arteries. Forskolin, a diterpene that directly activates the catalytic subunit of adenylate cyclase (Seamon & Daly, 1981), caused concentration-dependent relaxations in both precontracted mesenteric and renal resistance arteries (Figure 3). The mesenteric preparations were significantly more sensitive to forskolin than the renal vessels (Table 2). Forskolin fully relaxed mesenteric and renal resistance arteries that had been contracted with 30 mM potassium (Table 2).

In contrast to receptor-mediated and direct activators of adenylate cyclase, dibutyryl-cyclic AMP similarly relaxed precontracted mesenteric and renal resistance arteries (Figure 4). Neither the sensitivity for, nor the maximal effect of the degradation-resistant analogue of cyclic AMP, differed significantly between both types of resistance arteries (Figure 4).

We considered whether in addition to the presence of appropriate receptor sites and of adenylate cyclase, differences in the activity of phosphodiesterases and in receptor-mediated inhibition of adenylate cyclase could also contribute to regional differences in responsiveness to cyclic AMP affecting agents. Isobutyl-methylxanthine and milrinone, a non-selective phosphodiesterase inhibitor and an inhibitor of phosphodiesterase III, respectively (Weishaar *et al.*, 1986), induced

Table 1 Effects of pertussis toxin (PTX) on contractile responses to potassium in isolated mesenteric and renal resistance arteries[a]

Resistance artery	*Mesenteric*	*Renal*
Optimal diameter, $n = 24$ (μm)	265 ± 10	295 ± 11
Before PTX, $n = 24$		
125 mM K^+ (mN mm^{-1})	2.5 ± 0.3	1.7 ± 0.3*
30 mM K^+ (/K125)	0.701 ± 0.063	0.774 ± 0.045
After PTX, $n = 6$		
125 mM K^+ (mN mm^{-1})	1.5 ± 0.6†	2.6 ± 0.6
30 mM K^+ (/K125)	0.465 ± 0.065†	0.793 ± 0.059*

[a] Data are shown as means ± s.e.mean for isolated sympathectomized resistance arteries, before and after 90 min exposure to PTX 1 μg ml^{-1}. Contractile responses to 125 mM potassium were expressed as increases in wall tension; those to 30 mM potassium as a fraction of the response to 125 mM potassium. * and †: The difference between mesenteric preparations and between observations before and after exposure to PTX is statistically significant.

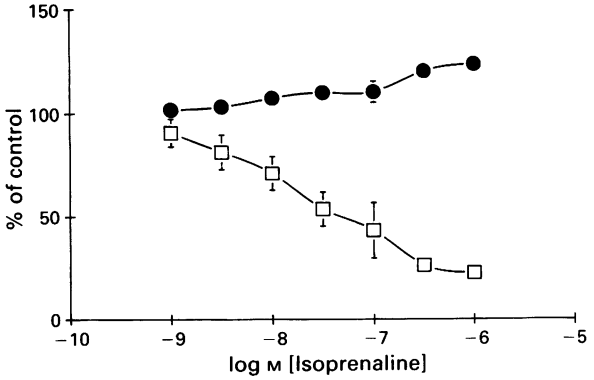

Figure 1 Effects of isoprenaline in isolated mesenteric (□) and renal (●) resistance arteries that had been contracted with 30 mM potassium in the presence of 1 μM prazosin. The data are expressed as a percentage of the response to potassium in the absence of agonist (for absolute value see Table 1) and are shown as means ($n = 24$) with s.e.mean indicated by vertical bars.

Table 2 Effects of pharmacological agents in precontracted resistance arteries[a]

	Maximal effect (% change)		*Sensitivity* (−log M ED_{50})	
Resistance artery	*Mesenteric*	*Renal*	*Mesenteric*	*Renal*
Control				
Isoprenaline[b] (24)	−84 ± 1	+11 ± 2*	7.5 ± 0.2	<5*
Forskolin (12)	−97 ± 1	−100 ± 0	6.5 ± 0.2	5.8 ± 0.2*
Dopamine[b,c] (6)	−48 ± 3	−0 ± 6*	6.2 ± 0.4	<5*
Prostaglandin E_2 (6)	+128 ± 79	+106 ± 16	7.2 ± 0.2	6.9 ± 0.3
Iloprost (6)	+9 ± 3	+99 ± 36*	<5	6.7 ± 4*
Isobutyl-methylxanthine (6)	−92 ± 2	−90 ± 5	6.1 ± 0.1	5.5 ± 0.2*
Milrinone (6)	−89 ± 3	−92 ± 4	5.2 ± 0.3	5.3 ± 0.3
In presence of 10 μM clonidine				
Isoprenaline[b] (6)	−42 ± 11†	−1 ± 8*	7.7 ± 0.2	<5*
Forskolin (6)	−98 ± 1	−100 ± 0	6.5 ± 0.3	5.3 ± 0.2*†

[a] Data are shown as means ± s.e.mean for isolated sympathectomized resistance arteries that had been made to contract with 30 mM potassium. A negative effect indicates relaxation; a positive effect a further increase in tension. For agents that hardly induced an effect the change induced by 10^{-5} M was taken as the maximal effect. The number of observations is given in parentheses.
[b] In the presence of 1 μM prazosin.
[c] In the presence of 1 μM propranolol.
* and †: The difference between mesenteric preparations and between observations in the absence and presence of clonidine is statistically significant.

relaxation in both mesenteric and renal resistance arteries. Mesenteric resistance arteries were more sensitive to isobutyl-methylxanthine than renal preparations (Table 2). Milrinone, on the other hand, had a similar effect on both types of resistance arteries (Table 2).

Up to 10 μM clonidine failed to induce contraction in resting mesenteric or renal resistance arteries (Table 3). In vessels that had been made to contract with 30 mM potassium, the α_2-adrenoceptor agonist induced further increases in tension (Figure 5). The sensitivity for clonidine and its maximal potentiating effect did not differ significantly between mesenteric and renal preparations (Table 3). Following pretreatment of mesenteric and renal resistance vessels with pertussis toxin

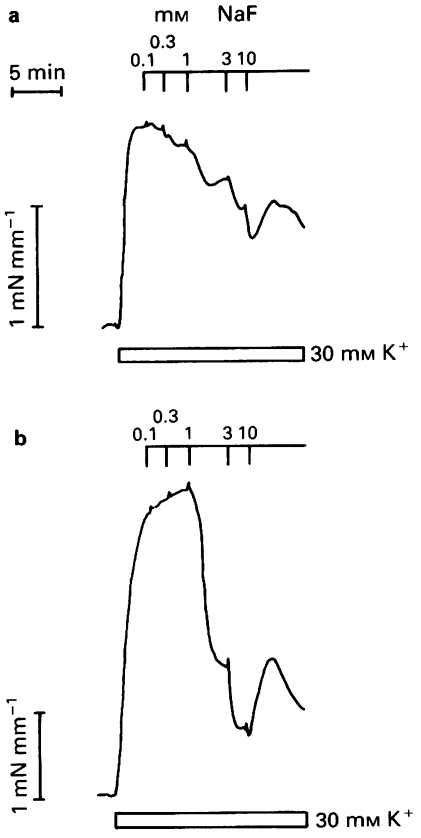

Figure 2 Tracings of isometric wall tension versus time obtained in an isolated renal (a) and mesenteric artery (b) of the same rat. The tracings illustrate the effect of increasing concentrations of sodium fluoride (NaF) on contractile responses to 30 mM potassium (presence indicated by open bar). The figure is representative of observations in six rats.

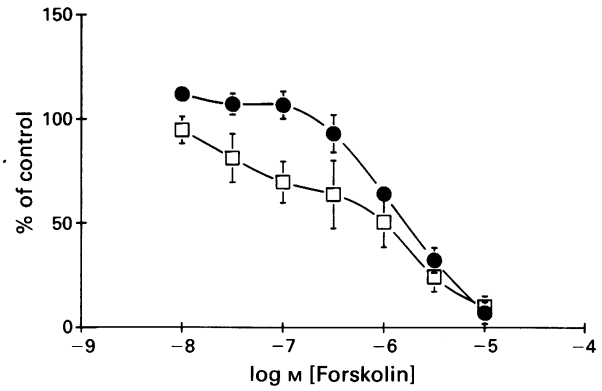

Figure 3 Effects of forskolin in isolated mesenteric (□) and renal (●) resistance arteries that had been contracted with 30 mM potassium. The data are expressed as a percentage of the response to potassium in the absence of pharmacological agents and are shown as means (n = 12) with s.e.mean indicated by vertical bars.

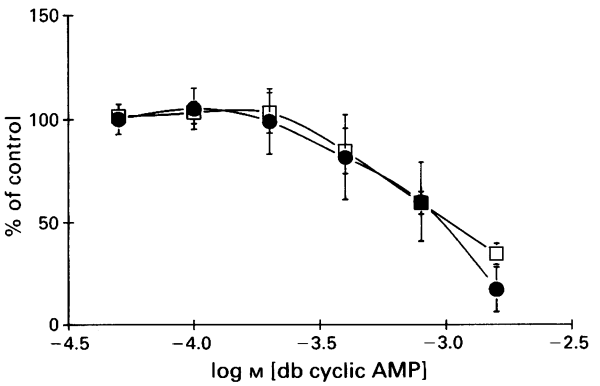

Figure 4 Effects of dibutyryl cyclic AMP (db cyclic AMP) in isolated mesenteric (□) and renal (●) resistance arteries that had been contracted with 30 mM potassium. Data are expressed as a percentage of the active wall tension in the absence of the cyclic AMP-analogue and are shown as means (n = 6) with s.e.mean indicated by vertical bars.

Table 3 Effects of clonidine in mesenteric and renal resistance arteries[a]

Resistance artery	Maximal effect		Sensitivity (−log M ED_{50})	
	Mesenteric	Renal	Mesenteric	Renal
Resting (mN mm^{-1})	0.0	0.0	<5	<5
K^+ 30 mM (% change)				
Control	+104 ± 31	+51 ± 9	6.7 ± 0.5	6.9 ± 0.2
Prazosin 1 μM	+34 ± 3	+36 ± 4	<5†	<5†
Yohimbine 1 μM	+90 ± 8	+65 ± 7	6.6 ± 0.4	6.7 ± 0.3
PTX 1 μg ml^{-1}	+110 ± 52	+57 ± 13	5.5 ± 3†	5.2 ± 3†

[a] Data are shown as means ± s.e.mean (n = 6). When the maximal effect was apparently not reached at 10^{-5} M, the effect of this concentration is indicated. Prazosin and yomhibine were applied 30 min before clonidine; pertussis toxin (PTX) was applied during a 90 min pretreatment.
†: The difference from control observations is statistically significant.

1 μg ml^{-1}, concentration-response curves for the potentiating effect of clonidine on contractile responses to 30 mM potassium, were shifted to higher concentrations (Table 3). Also prazosin (1 μM), but not yohimbine (1 μM) rendered resistance arteries less sensitive to the potentiating effect of clonidine (Table 3). In the presence of prazosin (1 μM), clonidine (10 μM) reduced the amplitude of relaxant responses to isoprenaline in mesenteric resistance arteries (Table 2). In renal resistance vessels, clonidine did not modify the absence of responses to isoprenaline but reduced the sensitivity to forskolin (Table 2).

Discussion

In contrast to isolated mesenteric resistance arteries, renal resistance arteries failed to relax in response to isoprenaline. Indirect observations suggest that this is primarily due to absence of β-adrenoceptors on renal resistance arterial smooth muscle cells or inappropriate coupling of these receptors to adenylate cyclase.

Effects of β-adrenoceptor agonists in various cell types including arterial smooth muscle cells, are mediated by cyclic AMP (Kukovetz *et al.*, 1981; Krall *et al.*, 1983). The binding of agonist to its receptor is transduced by stimulatory GTP-binding regulatory proteins (G_s proteins) to activation of the catalytic subunit of adenylate cyclase (Gilman, 1987). The enzyme catalyzes conversion of ATP into cyclic AMP. This cyclic nucleotide in turn activates certain protein kinases. In arterial smooth muscle this leads to relaxation. The cyclic AMP that is formed by adenylate cyclase can be degraded by phosphodiesterases, several types of which are present in arterial smooth muscle (Weishaar *et al.*, 1986). In several cell types, adenylate cyclase can not only be activated by β-adrenoceptor agonists, but also by dopamine, prostaglandin E_2 and prostacyclin (Goldberg, 1984; Gilman, 1987; Grylewski *et al.*, 1988). Other pharmacological agents can inhibit adenylate cyclase. This has been documented for the α_2-adrenoceptor agonist, clonidine (Pettinger *et al.*, 1987), the effect of which is mediated by pertussis toxin-sensitive GTP-binding regulatory proteins (G_i proteins; Limbird, 1988).

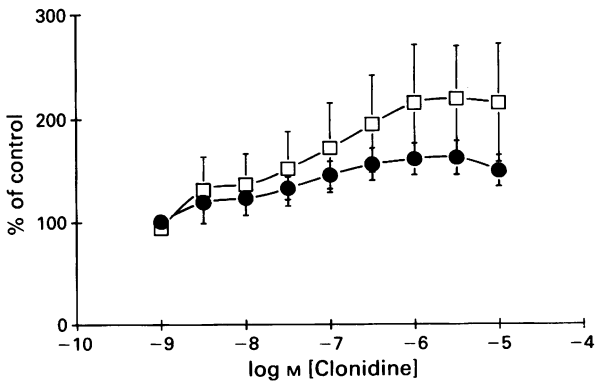

Figure 5 Effects of clonidine in isolated mesenteric (□) and renal (●) resistance arteries that had been contracted with 30 mM potassium. Data are expressed as a percentage of the active wall tension in the absence of α-adrenoceptor agonist and are shown as means (n = 6) with s.e.mean indicated by vertical bars.

Whether these pathways and components are all operative in smooth muscle of arteries that are small enough to regulate total peripheral resistance and local blood flow, is unknown. Ideally this should be demonstrated by receptor-binding and other biochemical assays. Some of these have previously been performed in resistance arterial preparations (see Kwan *et al.*, 1987). They require pooling of large numbers of resistance arteries. This can be done for certain vascular beds such as the mesentery, but is virtually excluded for renal resistance arteries that are laborious to isolate. Haemodynamic data (for review see Bomzon, 1983) as well as preliminary observations in isolated resistance arteries (Boonen *et al.*, 1990) indicate, however, that the susceptibility of the resistance part of the circulation to cyclic AMP affecting agents is not uniform and could differ between vascular beds. We therefore used an indirect pharmacological approach to document the responsiveness of isolated mesenteric and renal resistance arteries to cyclic AMP affecting agents and to evaluate the mechanisms that could be involved in the regional differences. Since observations in large arteries following long term exposure to agonist, hypertension and ageing, indicated that every step in the receptor-cyclase-kinase chain could be subject to modulation (see Triner *et al.*, 1975; Cohen & Berkowitz, 1976; Bhalla *et al.*, 1978; Fleisch, 1980; Harden, 1983; Tsujimoto *et al.*, 1986; Asano *et al.*, 1988a,b), we evaluated effects of potential receptor-mediated and direct activators of adenylate cyclase as well as those of a degradation-resistant cyclic AMP-analogue. Taking into account that interventions such as depolarizing solution, isoprenaline, clonidine, and dopamine can affect arterial function not only through a direct action on smooth muscle but also by interferences with adrenergic neurotransmission (Vanhoutte *et al.*, 1981), the experiments were performed in resistance arteries that had been acutely sympathectomized.

To judge from responses to pharmacological agents, all components of the pathway that was outlined above, seem to be present in mesenteric resistance arteries. These vessels relaxed in response to isoprenaline and dopamine, potential receptor-mediated activators of adenylate cyclase. Relaxant responses to isobutyl-methylxanthine and milrinone, also directly activate GTP-binding regulatory proteins (Sternweis & Gilman, 1982), the catalytic subunit of adenylate cyclase (Seamon & Daly, 1981) and cyclic AMP-dependent protein kinases, further strengthen the view that adenylate cyclase and protein kinase A are ultimately coupled to relaxation of the resistance arterial smooth muscle. As judged from relaxant responses to isobutyl-methylxanthine and milrinone, also phosphodiesterases are present in mesenteric resistance arteries and sufficient cyclic AMP would be generated under unstimulated conditions to cause relaxation when the phosphodiesterases are inhibited. Since in the presence of an

α_1-adrenoceptor antagonist, clonidine reduced relaxant responses to isoprenaline, α_2-adrenoceptor inhibition of adenylate cyclase (Pettinger *et al.*, 1987; Limbird, 1988) could also be operative in mesenteric resistance arterial smooth muscle. This is also suggested by the biphasic nature of relaxing responses to NaF, which can activate both G_s- and G_i-proteins (Sternweis & Gilman, 1982).

The mesenteric resistance arteries are thus equipped with a pharmacologically modulatable adenylate cyclase system that can interfere with contractile reactivity. Observations with clonidine and pertussis toxin deviate, however, from the classical picture. The α_2-adrenoceptor agonist on its own did not contract mesenteric resistance arteries. This indicates that in mesenteric as in renal resistance arteries (Edwards & Trizna, 1988), postjunctional α_2-adrenoceptors are not directly coupled to a contractile mechanism. The potentiating effect was reduced by pertussis toxin, the bacterial toxin that irreversibly ADP-ribosylates GTP-binding proteins involved in receptor-mediated inhibition of adenylate cyclase (Limbird, 1988). In mesenteric resistance arteries, as in a variety of other systems (Limbird, 1988; Pettinger *et al.*, 1987) α_2-adrenoceptor activation would thus inhibit adenylate cyclase. However, the potentiating effect of clonidine was also inhibited by prazosin and not affected by yohimbine. Clonidine thus displayed α_1-adrenoceptor agonist properties. That clonidine lacked a direct contractile effect but increased responses to potassium in a prazosin-sensitive way indicates that the intrinsic activity of the partial α_1-adrenoceptor agonism of clonidine can be increased by preconstriction or depolarization. Interference of pertussis toxin with the potentiating effect of clonidine thus can be due to both inactivation of G-proteins that are negatively coupled to adenylate cyclase and to the existence of pertussis toxin-sensitive components within excitation-contraction coupling during α_1-adrenoceptor activation (Nichols *et al.*, 1989).

In addition to interfering with effects of clonidine, pretreatment with pertussis toxin decreased contractile responses to potassium in mesenteric resistance arteries and increased those in renal preparations. The mechanisms underlying these effects are at present unclear since the direct contractile effect of depolarization on smooth muscle is supposed to be due to an effect on sarcolemmal calcium channels without the involvement of G-proteins or enzymes. One possibility is that pertussis toxin interferes with endogenously released neuropeptides which (i) are abundantly present in perivascular nerves that resist chemical sympathectomy, (ii) can be released by depolarization and which (iii) can modulate contractile responses to exogenous stimuli (see Wharton & Gulbenkian, 1987; Kawasaki *et al.*, 1988). This however remains to be established.

Unlike mesenteric preparations, renal resistance arteries failed to relax in response to isoprenaline or dopamine. They relaxed, albeit to a lesser extent, in response to NaF and forskolin. Dibutyryl-cyclic AMP, on the other hand, affected mesenteric and renal resistance arteries similarly. These indirect pharmacological observations suggest that in renal resistance arteries, β-adrenoceptors and dopamine binding sites are absent or not coupled to adenylate cyclase, which in turn is present to a lesser extent than in mesenteric resistance arterial smooth muscle.

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Rabbit brain contains an endogenous inhibitor of endothelium-dependent relaxation

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1 Supernatants prepared from the rabbit brain, lung and liver caused an endothelium-dependent and volume-related contraction of the phenylephrine-pretreated rabbit aorta and inhibited relaxation due to acetylcholine (ACh).

2 Perfusion *in situ* of the rabbit lung or liver with Krebs solution substantially reduced or removed the endothelium-dependent inhibitor. Spectrophotometric analysis revealed the presence of substantial amounts of haemoglobin (1.8–2.1 μM) in these organ supernatants.

3 Supernatants prepared from the Krebs-perfused rabbit brain retained the ability to contract the phenylephrine-pretreated rabbit aorta and to inhibit relaxation due to ACh and substance P (SP). Rabbit brain supernatant did not reduce the vasodilator effect of sodum nitroprusside (NP) or nitric oxide (NO).

4 Rabbit brain supernatant contained low ($<0.35\,\mu$M) concentrations of haemoglobin.

5 The inhibitory effect of rabbit brain supernatant was reversed by L-arginine (500 μM) but not D-arginine (500 μM).

6 The inhibitor of endothelium-dependent vasodilatation present in rabbit brain was not removed by dialysis (24 h, 4°C) but was partially precipitated by ammonium sulphate (30% w/v).

7 Rabbit brain contains an endogenous inhibitor of vascular NO biosynthesis. The identity of this inhibitor is not known although it seems likely to be a large peptide or protein.

Introduction

Nitric oxide (NO) accounts for the vasodilator effect of endothelium-derived relaxing factor (EDRF) and is released from isolated blood vessels and cultured vascular endothelial cells challenged with a range of drugs including acetylcholine (ACh) and substance P (SP) (Moncada *et al.*, 1989). Recently, NO biosynthesis has been described in rat cerebellar slices stimulated with N-methyl-D-aspartate (Garthwaite *et al.*, 1989). An enzyme which converts L-arginine to NO has also been identified in the rat brain and may be inhibited by L-N^G-monomethyl arginine (L-NMMA) (Knowles *et al.*, 1989). We have recently reported that a second guanidino-substituted L-arginine derivative. L-N^G-nitro arginine (L-NOARG), inhibits NO formation by the rabbit aorta and mouse anococcygeus preparations (Gibson *et al.*, 1990; Moore *et al.*, 1990). We show here the existence in rabbit brain homogenates of an endogenous inhibitor of endothelium-dependent vasodilatation which parallels the effect of both L-NMMA and L-NOARG on the rabbit isolated aorta preparation.

Methods

Rabbits (male, New Zealand White, 2.5–3.5 kg) were killed by an overdose of pentobarbitone (60 mg kg^{-1}, i.v.). Brain, lung and liver were removed and placed on ice. In some experiments, residual blood trapped at the moment of death was removed by perfusion (5 min, 7 ml min^{-1}) of organs *in situ* with warmed (37°C), oxygenated (95% O_2:5% CO_2) Krebs solution (composition, mM: NaCl 118.1, KCl 4.7, $MgSO_4$ 1.0, KH_2PO_4 1.0, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, glucose 11.1) via cannulae inserted into the carotid, pulmonary or splanchnic arteries. All organs were weighed, homogenized in 50 mM Tris-HCl buffer (pH 8.0) in an Ultra-Turrax homogenizer (1:4 w/v) and centrifuged (1000 *g*, 10 min) at room temperature. Supernatant was removed and either (i) assayed immediately for ability to inhibit endothelium-dependent relaxation of the rabbit aorta, (ii) dialysed (24 h, 4°C) against 20 volumes of Krebs solution prior to assay of both the dialysate and residual supernatant or (iii) mixed with ammonium sulphate (30% w/v) to prepare a protein fraction which was collected by centrifugation (1000 *g*, 10 min) and resuspended in Krebs solution (1:1 w/v) prior to assay.

In order to determine the effect of organ fractions on endothelium-dependent vasodilatation, rabbit aortae were removed and mounted as intact rings in 15 ml organ baths containing warmed (37°C), oxygenated (5% CO_2: 95% O_2) Krebs solution to which indomethacin (7 μM) was added to inhibit vascular prostacyclin biosynthesis. In some experiments, aortic rings were rubbed on the intimal surface to remove endothelial cells. Aliquots of brain, lung and liver supernatant (5–100 μl), ammonium sulphate-precipitated brain protein fraction (10–100 μl), brain dialysate (1.0 ml) or dialysed brain supernatant (100 μl) were added to rabbit aortic rings which had been precontracted with phenylephrine (0.75 μM) to produce 80% of the maximum response. After a 12 min preincubation period the effect on relaxation of the aorta due to cumulative addition of ACh (0.1–1.0 μM), SP (0.0001–0.1 μM) or sodium nitroprusside (NP, 0.1–1.0 μM) was determined as described previously (Moore *et al.*, 1990). The effect of rabbit brain supernatant/protein fraction on the response of the aorta to bolus administration of NO (0.16–1.6 μM) was also determined. Reversal of the inhibitory effect of rabbit brain, lung and liver supernatant and rabbit brain protein fraction was assessed following preincubation (6 min) of aortic rings with either L- or D-arginine (500 μM).

Haemoglobin concentration of aliquots (20 μl) of organ supernatants fraction was estimated spectrophotometrically following conversion to cyanmethaemoglobin with a commercially available kit (Sigma Ltd.). Ferrous haemoglobin was prepared by reaction of bovine (type II) haemoglobin with sodium dithionite and dialysis overnight (4°C) against 100 volumes of distilled water as described by Martin *et al.* (1985). Dialysis membrane was obtained from Sigma. NO (BDH Ltd.) solution was prepared and bath concentrations calculated as described previously (Bhardwaj & Moore, 1989). Other drugs were purchased from Sigma and dissolved in 0.9% w/v saline with the exception of indomethacin which was dissolved in 0.5% Na_2CO_3.

Results show mean ± s.e.mean. Statistical analysis of differences between groups was determined by unpaired Student's *t*

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test. A probability (P) value of 0.05 or less was taken to indicate statistical significance.

Results

Effect of organ supernatants on the rabbit aorta

Aliquots (100 μl) of non-perfused rabbit brain, lung and liver supernatant produced a volume-related additional contraction (14.6 $\pm$ 4.5%, $n = 8$; 12.5 $\pm$ 1.6%, $n = 5$; 11.8 $\pm$ 2.5%, $n = 5$) of the phenylephrine-pretreated rabbit aorta (= 100%). Contractions persisted for approximately 5 min and were not observed in endothelium-denuded preparations. Supernatant prepared from the Krebs-perfused rabbit brain also caused a similar contraction of the rabbit aorta under these circumstances (8.7 $\pm$ 1.9%, $n = 14$) whilst supernatants prepared from perfused lung were significantly ($P < 0.05$) less active (2.7 $\pm$ 0.4%, $n = 5$). Supernatants prepared from the perfused rabbit liver did not contract the phenylephrine-pretreated aorta (data from 5 separate experiments).

Aliquots of non-perfused rabbit brain, lung and liver supernatant additionally inhibited aortic relaxation due to ACh. Again, following perfusion of each organ *in situ* with Krebs solution, only supernatant prepared from the rabbit brain retained significant ability to inhibit endothelium-dependent relaxation in response to ACh (Figure 1). These results indicate that both the observed contractile and inhibitory activity of rabbit lung and liver (but not brain) was due to a substance(s) present in trapped blood.

Since rabbit brain supernatant appeared to contain an inhibitor of endothelium-dependent vasodilatation unlike that observed in other organs, we decided to investigate the nature of this inhibitor in more detail. Rabbit brain supernatant inhibited not only ACh but also the relaxant effect of SP on the rabbit aorta. For example, 100 μl rabbit brain supernatant inhibited the relaxant effect of SP (0.0011 μM) by approximately 77% (16.2 $\pm$ 8.4% of the maximum response cf. 71.2 $\pm$ 5.3% in the absence of supernatant, $n = 6$, $P < 0.01$). In contrast, brain supernatant did not affect responses due to cumulative addition of NP (e.g. 0.5 μM; 64.6 $\pm$ 5.4% in the presence and 55.8 $\pm$ 4.2% in the absence of 100 μl supernatant, $n = 5$, $P > 0.5$) or bolus addition of NO (e.g. 0.32 μM; 1.71 $\pm$ 0.13 g relaxation in the presence and 1.74 $\pm$ 0.16 g in the absence of 100 μl supernatant). Of interest was the finding that L-arginine (500 μM) but not D-arginine (500 μM) partially reversed the inhibitory effect of rabbit brain supernatant on ACh-induced relaxation of the rabbit aorta without reversing the inhibition due to rabbit lung or liver supernatant (Table 1).

Effect of dialysed rabbit brain supernatant and resuspended brain proteins on response of the rabbit aorta to acetylcholine

Preliminary attempts have been made to characterize the rabbit brain inhibitor. Dialysis of rabbit brain supernatant did not significantly influence its ability to inhibit ACh-mediated relaxation of the rabbit aorta. Accordingly, no inhibitory activity was detected in the Krebs dialysate (Figure 2). However, aliquots of the resuspended brain protein fraction prepared by mixing brain supernatant with ammonium sulphate did cause a volume-related inhibition of the effect of ACh on the rabbit aorta (Figure 3). The protein fraction retained approximately 70% of the inhibitory effect of the crude brain supernatant and, like the brain supernatant, was partially reversed by L- but not D-arginine (500 μM) (Table 1).

Measurement of haemoglobin in organ supernatants: effect of ferrous haemoglobin on the rabbit aorta

Since ferrous haemoglobin is known to inhibit the response of the rabbit isolated aorta to ACh it was considered impor-

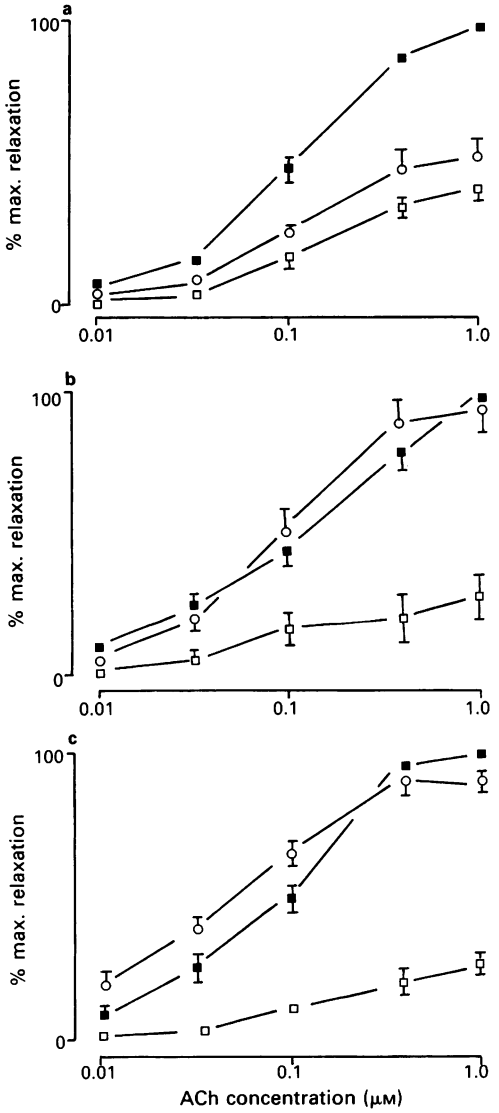

Figure 1 Relaxant effect of acetylcholine (ACh) in rabbit isolate aorta in the absence (■) and presence of 100 μl supernatant prepare from homogenized perfused (○) and non-perfused (□) rabbit brai (a), liver (b) and lung (c). Results indicate mean of $n = 5$–16; s.e.mea shown by vertical bars.

Table 1 Effect of L- and D-arginine on the effect of rabbit brain, lung and liver supernatant on acetylcholine (ACh)-induced relaxation of the rabbit aorta

	% *maximum response to ACh* (1 μM)
Control (no inhibitor)	81.6 $\pm$ 2.9
+ Brain supernatant	42.2 $\pm$ 2.8*
+ Brain + L-arginine	76.4 $\pm$ 7.9
+ Brain + D-arginine	45.5 $\pm$ 3.2*
+ Protein	56.5 $\pm$ 4.9*
+ Protein + L-arginine	73.5 $\pm$ 5.8
+ Protein + D-arginine	54.4 $\pm$ 3.2*
+ Liver supernatant	36.4 $\pm$ 4.4*
+ Liver + L-arginine	39.3 $\pm$ 5.1*
+ Lung supernatant	17.3 $\pm$ 8.2*
+ Lung + L-arginine	22.1 $\pm$ 6.6*

Reversal by L-arginine (500 μM) of the inhibitory effect of ACh (0.33 μM) on the rabbit isolated aorta by crude rabbit brain supernatant (100 μl) and protein fraction (100 μl) produced therefrom by ammonium sulphate (30%) precipitation. D-Arginine (500 μM) did not reverse the inhibitory effect of rabbit brain supernatant or protein fraction. L-Arginine did not reverse inhibition due to addition of either crude rabbit liver or lung supernatant (both 100 μl). Results indicate mean $\pm$ s.e.mean, $n = 10$–50. * $P < 0.05$.

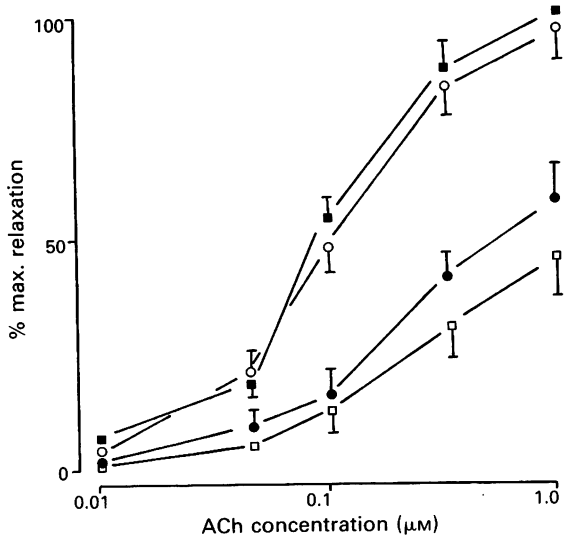

Figure 2 Relaxant effect of acetylcholine (ACh) in rabbit isolated aorta in the absence (■) and presence of 100 μl undialysed (□) or dialysed (24 h, ●) rabbit brain supernatant or 1 ml of the corresponding dialysate (○). Results indicate mean of $n = 5$; s.e.mean shown by vertical bars.

tant to estimate haemoglobin concentration in the supernatant and protein fraction prepared from perfused and non-perfused rabbit brain. Spectrophotometric analysis of total haemoglobin revealed low concentrations in brain supernatants. By calculation, the maximum concentration of haemoglobin possible in the organ bath following addition of 100 μl non-perfused and perfused rabbit brain supernatant was therefore 0.28 ± 0.1 μM and 0.11 ± 0.05 μM (both $n = 7$) respectively. Higher concentrations of haemoglobin were detected in supernatants prepared from non-perfused rabbit lung (2.1 ± 0.2 μM, $n = 5$) and liver (1.8 ± 0.17 μM, $n = 3$). In contrast, haemoglobin was undetectable in supernatants prepared from perfused rabbit lung and liver and in the resuspended brain protein fraction. In separate experiments, ferrous haemoglobin (0.5 μM) failed to influence the relaxant effect of ACh on the rabbit aorta. Increasing the bath concentration of ferrous haemoglobin to 10 μM completely prevented relaxation due to ACh without influencing the response to a

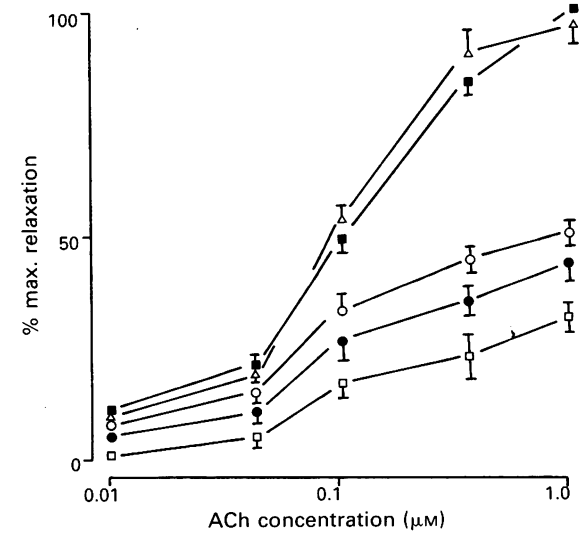

Figure 3 Relaxant effect of acetylcholine (ACh) in rabbit isolated aorta in the absence (■) and presence of 100 μl rabbit brain supernatant (□) or protein fraction (100 μl, ●; 75 μl, ○; 25 μl, △). Results indicate mean of $n = 8$; s.e.mean shown by vertical bars.

submaximally effective (ED_{80}) concentration (0.8 μM) of NP. The effect of 5 μM haemoglobin, which produced approximately 60% inhibition of responses to ACh, was not reversed by preincubation of aortic rings with L-arginine (500 μM).

Discussion

These data suggest the existence in several rabbit organs of substance(s) which inhibit endothelium-dependent blood vessel relaxation. Supernatants prepared from rabbit lung and liver contracted the phenylephrine-pretreated rabbit aorta and additionally inhibited relaxation due to ACh. Both of these effects are mimicked by drugs which either (a) generate superoxide anions to destroy endothelium-derived NO (e.g. phenidone) or (b) bind endothelium-derived NO (e.g. haemoglobin) or (c) inhibit NO biosynthesis from L-arginine (e.g. L-NMMA and L-NOARG).

Several findings suggest that the inhibitor found in rabbit lung and liver belongs in group (a) and/or (b). For example, perfusion of these two organs *in situ* with Krebs solution either substantially reduced or abolished the ability of supernatants to contract the aorta and inhibit the effect of ACh. Furthermore, L-arginine failed to reverse the inhibitory effect of either supernatant. Finally, measurement of haemoglobin in lung and liver supernatant revealed concentrations which would be expected to inhibit ACh-induced aortic relaxation. Thus, it seems likely that the inhibitor present in rabbit lung and liver is primarily haemoglobin which is trapped in the pulmonary or hepatic vascular beds at the time of death.

Rabbit brain supernatant also inhibited relaxation of the aorta in response to endothelium-dependent (ACh, SP) without influencing responses to endothelium-independent vasodilators (NP, NO). In contrast to the lung and liver, perfusion of the rabbit brain *in situ* with Krebs solution failed to affect the ability of supernatants prepared therefrom to inhibit ACh-mediated relaxation of the rabbit aorta. Thus, it seems unlikely that the brain inhibitor occurs in trapped blood. Furthermore, spectrophotometric measurement of total haemoglobin in perfused and non-perfused rabbit brain supernatants revealed concentrations which were at or only just above the limit of detection of the assay. Even in the unlikely event that all of the haemoglobin present in brain supernatant is in the ferrous state the calculated maximum concentration of haemoglobin achieved in the organ bath following addition of the largest volume of brain supernatant (100 μl) is <0.30 μM. Authentic ferrous haemoglobin at this concentration did not contract the phenylephrine-pretreated rabbit aorta or inhibit the response to ACh. In addition, the inhibitory effect of rabbit brain supernatant was reversed by L- but not D-arginine. Thus, rabbit brain supernatant mimics the effect of L-NMMA and L-NOARG (but not haemoglobin) to produce an L-arginine-reversible inhibition of endothelium-dependent relaxation in this preparation (Moore *et al.*, 1990). Consequently, these results effectively rule out the possibility that haemoglobin accounts for the action of brain supernatant and, furthermore, implies that rabbit brain contains an endogenous inhibitor of NO biosynthesis with a similar mechanism of action to both L-NMMA and L-NOARG.

The identity of the endogenous inhibitor is not known although some preliminary biochemical characterization has been attempted. For example, the brain inhibitor is at least partially precipitated by ammonium sulphate treatment suggesting that it may be either a protein, peptide or amino acid. However, the inhibitor is not susceptible to dialysis with a commercially available membrane which excludes proteins having a molecular weight in excess of 12,000. For this reason it seems likely that a relatively large peptide or protein is responsible for the inhibition of aortic NO biosynthesis observed. In this context, it may be of interest that L-NMMA has been detected in the protein hydrolysate of both bovine (Nakajima *et al.*, 1971) and human (Kakimoto *et al.*, 1975)

brain. Whether such an L-NMMA-containing protein exists in rabbit brain is not known.

The present experiments suggest a novel mechanism for the biochemical control of NO biosynthesis within the brain. Whether this inhibitor occurs in neurones or some other type of cell (e.g. glial cells) is not known. The precise identity of the inhibitor and the part which it plays (if any) in the, as yet, unknown biological functions of NO in the brain requires further investigation.

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The antinociceptive activity of paracetamol in zymosan-induced peritonitis in mice: the role of prostacyclin and reactive oxygen species

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1 Oral administration of high doses of paracetamol (600 mg kg^{-1} or more) resulted in inhibition of the writhing and reduced the levels of prostacyclin (PGI_2, measured as 6-keto-$PGF_{1\alpha}$) induced by intraperitoneal administration of zymosan in mice. The high oral doses of paracetamol required were accompanied by behavioural toxicity which may have contributed to the inhibition of writhing.

2 The number of writhes per mouse and the proportion of mice writhing at least once correlated significantly with the levels of 6-keto-$PGF_{1\alpha}$. However, inhibition of writhing by paracetamol occurred at higher levels of 6-keto-$PGF_{1\alpha}$ than was previously observed with acidic non-steroidal anti-inflammatory agents.

3 When injected i.p., PGI_2, carbacyclin and iloprost (agonists at the PGI_2 receptor) induced writhing. Intraperitoneal injection of PGI_2 reversed the inhibition of writhing induced by indomethacin (1 mg kg^{-1}, p.o.) but not that induced by oral administration of paracetamol.

4 Paracetamol at 800 mg kg^{-1}, p.o., inhibited carbacyclin-induced writhing but indomethacin at 1 mg kg^{-1} p.o. did not. Paracetamol administered i.p. at 100 mg kg^{-1} reduced the peritoneal levels of 6-keto-$PGF_{1\alpha}$ and inhibited zymosan-induced but not carbacyclin-induced writhing and did not produce behavioural toxicity.

5 The *in vitro* potency of paracetamol as a prostaglandin synthesis inhibitor is known to be reduced by the presence of lipid peroxides. However, no lipid peroxides, measured as thiobarbituric acid reactive material, were detected in the peritoneal lavage fluid of zymosan-injected mice.

6 Intraperitoneal administration of a mixture of superoxide dismutase and catalase reduced detectable superoxide anion by 98% without inhibiting the writhing response to zymosan or the antinociceptive potency of paracetamol.

7 The data are consistent with the suggestion that inhibition of PGI_2 synthesis in the peritoneal cavity by paracetamol is responsible for only a part of its antinociceptive activity in this test. However, extremely high oral doses of paracetamol were required which produced behavioural toxicity which clearly contributed to the inhibition of writhing. The low potency of paracetamol in this model cannot be attributed to the generation of lipid peroxides via the oxidative burst.

Introduction

Prostaglandins have been shown to be important mediators of inflammatory pain (Moncada *et al.*, 1978; Ferreira, 1981; Brune & Lanz, 1984). Non-steroidal, acidic, anti-inflammatory drugs (NSAIDs e.g. aspirin, indomethacin, ibuprofen) exert their analgesic effects in inflammatory pain largely by inhibiting the synthesis of prostaglandins in the inflammatory lesion (Vane, 1971; Nakamura & Shimizu, 1981; Nakamura *et al.* 1983; Doherty *et al.*, 1987; Doherty, 1987). N-(4-hydroxyphenyl) acetamide (acetaminophen, paracetamol) also inhibits inflammatory pain but, despite extensive study, its mechanism of action remains obscure. For example, the effect of paracetamol on prostaglandin synthesis in the most widely used cell-free system (ram seminal vesicle microsomes) is highly dependent on experimental conditions: in the presence of high levels of lipid peroxides which inactivate the enzyme, paracetamol enhances prostaglandin synthesis; in the presence of low levels of lipid peroxides, paracetamol inhibits prostaglandin synthesis (Lands *et al.*, 1976; Robak *et al.*, 1978; Hanel & Lands, 1982; Lands & Hanel, 1982; Hertz & Cloarec, 1984). These results make it difficult to predict the *in vivo* effects of paracetamol on prostaglandin synthesis in a particular *in vivo* situation. Few whole animal studies on the effect of paracetamol on prostaglandin synthesis at the site of an inflammatory reaction have been reported (Flower *et al.*, 1972).

In contrast to the above, paracetamol was shown to be a relatively potent inhibitor of cell-free prostaglandin synthetase from neural tissue (Flower & Vane, 1972) and to inhibit the increased synthesis of prostaglandins which occurs in the central nervous system (CNS) of animals with fever (Feldberg & Gupta, 1973; Feldberg & Milton, 1978). These observations, plus evidence that increased levels of prostaglandins in the CNS play a role in inflammatory pain (Okuyama & Aihara, 1985; Taiwo & Levine, 1986; Horiguchi *et al.*, 1986; Okuyama & Aihara, 1986) have led to the suggestion that paracetamol exerts its analgesic activity largely or exclusively by inhibiting CNS prostaglandin synthesis, rather than prostaglandin synthesis in inflamed peripheral tissues (Flower & Vane, 1972; Tolman *et al.*, 1983; Hertz & Cloarec, 1984). There is also evidence that inhibition of CNS prostaglandin synthesis may contribute to the analgesic activity of NSAIDs under some circumstances (Ferreira *et al.*, 1978; Bhattacharaya & Das, 1984; reviewed in Doherty, 1987).

In the present report, the effect of paracetamol on the levels of prostaglandin in an inflammatory lesion (zymosan-induced peritonitis in mice) has been examined and related to the nociceptive response (writhing) that also occurs in these animals. The role of superoxide radicals, generated by the zymosan-stimulated peritoneal macrophages and a potential source of lipid peroxides, in determining the activity of paracetamol was also examined. A preliminary account of this work was presented at the 4th International Conference of the Inflammation Research Association (October 23–27, 1988, White Haven, PA, U.S.A.).

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Methods

Zymosan-induced peritonitis

The methods used have been comprehensively described previously (Doherty *et al.*, 1985; 1987). Briefly, inflammation (oedema and leukocyte accumulation) and a nociceptive response (writhing) were induced by the intraperitoneal injection of zymosan (1 mg/mouse in 0.5 ml saline) into male CD1 mice (Charles River Breeding Labs, Wilmington, MA, U.S.A.). The number of writhes was counted over a 15 min period, 5–20 min after injection of zymosan, after which the mice were killed and the peritoneal cavity lavaged with saline. The levels of 6-keto prostaglandin $PGF_{1\alpha}$ (6-keto $PGF_{1\alpha}$, a measure of prostaglandin I_2 (PGI_2) synthesis) and prostaglandin E_2 (PGE_2) were measured with commercially available radioimmunoassay kits (New England Nuclear, Boston, MA, U.S.A.). Prostaglandins were also determined by gas chromatograph/mass spectrometry (GC/MS), as described previously (Doherty *et al.*, 1987).

Determination of lipid peroxides

The levels of thiobarbituric acid reactive material, indicative of lipid peroxides, in the peritoneal lavage fluids were determined by the method described by Uchiyama & Mihara (1978). Malonyldialdehyde was used to construct the calibration curve.

Chemiluminescence measurements

Zymosan-induced lucigenin-dependent chemiluminescence by resident peritoneal macrophages was measured by a method based on those described by Allen (1986). For *in vitro* chemiluminescence studies, resident peritoneal macrophages were obtained from CD-1 mice by lavage with 8 ml of RPMI 1640 buffer containing 25 mM HEPES and sodium heparin 136 mg l^{-1}. The cells were centrifuged (400 *g* for 15 min) and resuspended in RPMI buffer. Cells pooled from 25 mice (final concentration of 1×10^6 ml^{-1}) were suspended in RPMI buffer containing 50 μM lucigenin (Sigma Chemical Co., St. Louis, Mo, U.S.A.) and varying concentrations of inhibitors or enzymes. Aliquots of cells (1 ml) were placed in a Picolite chemiluminometer (Los Alamos Diagnostics, Los Alamos, NM, U.S.A.) and allowed to incubate in the dark for 30 min. The cells were then stimulated with unopsonized zymosan (0.4 mg ml^{-1}, final concentration) and the chemiluminescence measured over 4 h. The inhibitory effects of the treatments of the macrophage were determined by comparing the integrals of the chemiluminescence curves.

To assess the *in vivo* effect of treatments, mice were injected i.p. with 0.5 ml of saline or saline containing superoxide dismutase plus catalase. After 20 min, each animal was lavaged with 5 ml of Hanks' balanced salt solution and the cells obtained counted and chemiluminescence measured as above in aliquots from individual mice. Results were corrected for differences in cell number and expressed as counts per 1×10^6 cells. This procedure avoids washing the cells free of the enzymes administered *in vivo* but results in at least a 5 fold dilution of the compounds or enzymes present in the peritoneal cavity at the time of collection.

Drug administration

For oral administration, compounds were prepared as suspensions in water containing 2 drops of Tween 80 (Fisher Scientific, Fairlawn, NJ, U.S.A.) per 10 ml and were homogenized in a teflon-in-glass homogenizer to reduce particle size. The dose volume used was 10 ml kg^{-1}. For subcutaneous administration, compounds were prepared in saline. For intraperitoneal injection, PGI_2 sodium salt was dissolved in ice cold pH 10 carbonate buffer immediately before use, in order to minimize spontaneous breakdown (Cho & Allen, 1978). The PGI_2 analogues, carbacyclin and iloprost, were prepared similarly.

Indomethacin was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.; paracetamol from S.B. Penick & Co., Lyndhurst, NJ, U.S.A.; PGI_2 (sodium salt) from Chemical Dynamics Corp., South Plainfield, NJ, U.S.A.; superoxide dismatase (SOD) from DDI Pharmaceuticals, Inc., Mountain View, CA, U.S.A.; catalase from Sigma. Carbacyclin and iloprost were gifts from Upjohn (Kalamazoo, MI, U.S.A.) and Schering AG (Berlin), respectively.

Animals were allocated to treatments randomly. Treatment groups were compared with controls by analysis of variance followed by Dunnett's test (Steel & Torrie, 1960), or, when homogeneity of variance problems made analysis of variance inappropriate, by multiple *t* tests with Bonferoni's adjustment (Neter & Wasserman, 1974). For comparison of proportions (e.g. % of mice writhing), Fisher's exact test was used and for assessment of the significance of the linear relationship between parameters, least squares regression was performed (Steel & Torrie, 1960).

Results

Paracetamol administered orally proved to be a very weak inhibitor of zymosan-induced writhing: significant inhibition required doses of 600 mg kg^{-1}, p.o., or above (Table 1). The doses which inhibited writhing also produced behavioural changes (decreased spontaneous motor activity, piloerection). Despite this behavioural toxicity, inhibition of writhing was obtained at non-lethal doses: over a 7-day observation period, no deaths occurred in groups of 5 mice given paracetamol at doses up to 800 mg kg^{-1}, p.o., but 3 out of 5 died after 1600 mg kg^{-1}, p.o.

High doses of paracetamol also reduced the levels of 6-keto-$PGF_{1\alpha}$ in the peritoneal lavage fluid (Table 1). Pooling data from several experiments showed that as the levels of 6-keto-$PGF_{1\alpha}$ were reduced, the number of writhes per mouse (Figure 1a) and the proportion of mice which showed a writhing response (regardless of the number of writhes) (Figure 1b) decreased. Although there was a significant ($P = 0.0006$) relationship between levels of 6-keto-$PGF_{1\alpha}$ and the number of writhes, the correlation was poor ($r = 0.4638$). However,

Table 1 Effect of oral administration of paracetamol (PA) on zymosan-induced writhing and levels of 6-keto-$PGF_{1\alpha}$ and PGE_2

PA (mg kg^{-1}, p.o.)	*No. of writhes*	*Responders/ Total*	*6-keto* $PGF_{1\alpha}$ (ng/mouse)	PGE_2 (ng/mouse)
0	8.6 ± 2.2	8/10	79.2 ± 10.8	0.96 ± 0.08
200	4.3 ± 1.6	6/6	58.4 ± 10.6	0.79 ± 0.17
400	4.0 ± 1.7	5/6	41.4 ± 4.8*	0.41 ± 0.08*
600	0*	0/6*	27.7 ± 3.5*	0.41 ± 0.09*

Values are mean ± s.e.mean.
Paracetamol or vehicle (water) was administered 1 h before intraperitoneal administration of zymosan (1 mg in 0.5 ml). The number of writhes per mouse was counted over the 5–20 min period following zymosan injection. The mean number of writhes per mouse (including those which failed to writhe) and the number of mice which writhed at least once (responders) are shown. Twenty minutes after zymosan injection the mice were killed and the total amount of 6-keto-$PGF_{1\alpha}$ and PGE_2 in the peritoneal lavage fluid was determined by RIA.
* $P < 0.05$ compared with vehicle-treated control.

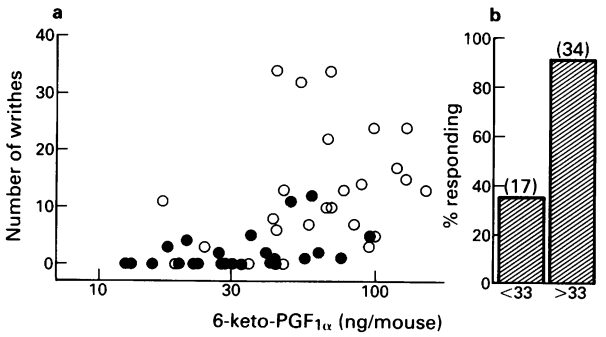

Figure 1 6-Keto-$PGF_{1\alpha}$ levels and the writhing response to zymosan. (a) The total number of writhes (counted over the period 5–20 min after zymosan administration) and the levels of 6-keto-$PGF_{1\alpha}$ (ng/mouse) are shown for each mouse: (○) vehicle-treated; (●) paracetamol-treated (100–800 mg kg^{-1}, p.o.) (b) The proportion of mice writhing at least once, grouped according to whether peritoneal levels of 6-keto-$PGF_{1\alpha}$ were above or below 33 ng/mouse. The numbers at the top of the columns indicate the number of mice within that range of 6-keto-$PGF_{1\alpha}$ levels.

even the highest doses of paracetamol used did not lower levels of 6-keto-$PGF_{1\alpha}$ to the extent achieved with acidic non-steroidal anti-inflammatory agents (Doherty *et al.*, 1987). The levels of 6-keto-$PGF_{1\alpha}$ below which the percentage of mice writhing at least once was reduced (below 33 ng/mouse compared to above 33 ng/mouse, $P = 0.00006$, Fisher's exact test) was higher than was found in previous studies with acidic non-steroidal anti-inflammatory agents (10 ng/mouse, Doherty *et al.*, 1987). In fact, even the highest doses of paracetamol did not reduce 6-keto-$PGF_{1\alpha}$ levels below 10 ng/mouse. By use of GC/MS, the inhibition of 6-keto-$PGF_{1\alpha}$ synthesis was confirmed and no increases in the very low levels of PGE_2, $PGF_{2\alpha}$ or thromboxane B_2 (TxB_2) were detected (data not shown). By use of a sensitive radioimmunoassay, the levels of PGE_2 were found to fall in parallel with the levels of 6-keto-$PGF_{1\alpha}$ (Table 1).

In several experiments (not shown), paracetamol at 100 mg kg^{-1} administered orally had no effect on writhing or prostaglandin levels. When paracetamol was administered locally (intraperitoneally, mixed with the zymosan), 100 mg kg^{-1} was the lowest dose which consistently inhibited writhing (not shown and Table 2a). This dose also reduced levels of 6-keto-$PGF_{1\alpha}$ (Table 2a) without exhibiting behavioural toxicity. No increase in LDH levels in the lavage fluid was detected (data not shown), indicating the absence of a direct cytotoxic effect on peritoneal cells under these conditions.

In previous studies, it was found that intraperitoneal administration of PGI_2 was able to induce writhing and to reverse completely the inhibition of writhing produced by indomethacin and other acidic prostaglandin synthesis inhibitors (Doherty *et al.*, 1987). These data have been confirmed for indomethacin in the present series of experiments (Table 3) and two PGI_2 analogues, carbacyclin and iloprost, have been shown to induce writhing when injected intraperitoneally (Table 4). Paracetamol (800 mg kg^{-1}, s.c.) was found to block partially the PGI_2 reversal of inhibition of writhing produced by indomethacin (Table 3). Different routes of administration were used for paracetamol and indomethacin to avoid possible interference of one compound on the absorption of the other. In addition, PGI_2 produced only partial reversal of the ability of paracetamol (800 mg kg^{-1}, p.o.) to inhibit writhing (Table 3).

Paracetamol administered orally at 800 mg kg^{-1} did block carbacyclin-induced writhing while indomethacin at 1 mg kg^{-1} p.o. did not (Table 2c). In contrast, paracetamol administered i.p. at 100 mg kg^{-1} did not block carbacyclin-induced writhing (Table 2c).

No increase in thiobarbituric acid reactive material was detectable in lavage fluids collected immediately, 10 to 20 min after injection of zymosan, as compared to saline injected controls (not shown). The lower limit of detection in this assay is 400 ng of malonyldialdehyde equivalents/mouse. In other experiments, the zymosan was administered in a saline solution of malonyldialdehyde (400 μM) and mice were killed and lavaged either immediately or 20 min after injection. Analysis of the malonyldialdehyde levels in the lavage fluid showed 23% was recovered immediately after injection and 6% 20 min after injection (not shown).

When macrophages were removed from the peritoneal cavity of untreated mice and then stimulated with zymosan *in vitro*, a burst of lucigenin-dependent chemiluminescence was generated which peaked at about 30 min and disappeared over a 4 h period, as reported by others (Allen, 1986). The area under the curve for the 0–4 h period was used to quantify the response in subsequent studies. When superoxide dismutase (SOD) was present in the *in vitro* incubation medium, this response was inhibited by 95% (not shown). Intraperitoneal administration of SOD plus catalase (0.5 ml of saline containing 2 mg ml^{-1} each of SOD and catalase), produced a marked reduction of *ex vivo* zymosan induced chemiluminescence measured 20 min after i.p. administration (Table 5) indicating that effective levels of the enzymes remained in the peritoneal cavity over this period of time. This dose of SOD plus catalase did not induce writhing, did not inhibit zymosan induced

Table 2 Effect of paracetamol (PA) and indomethacin on zymosan-induced writhing and 6-keto-$PGF_{1\alpha}$ levels and carbacyclin-induced writhing

Treatment	*No. of writhes* (% inhibition)	*Responders/ Total*	*6-keto-$PGF_{1\alpha}$* (ng/mouse) (% inhibition)
(a) *Zymosan-induced writhing*			
Vehicle	16.3 ± 3.0	14/14	63.8 ± 8.8
PA 100 mg kg^{-1}, i.p.	0.7 ± 0.5(96*)	2/7	23.7 ± 4.3(63*)
(b) *Carbacyclin-induced writhing*			
Vehicle	6.0 ± 1.8	10/10	—
PA 100 mg kg^{-1}, i.p.	5.3 ± 1.4(12)	7/10	—
(c) *Carbacyclin-induced writhing*			
Vehicle	4.1 ± 0.7	9/10	—
PA 800 mg kg^{-1}, p.o.	0.2 ± 0.2 (95*)	1/10	—
Indomethacin 1 mg kg^{-1}, p.o.	6.1 ± 1.5 (−49)	8/10	—

Values are mean ± s.e.mean.

* $P < 0.05$ cf. vehicle control. Percentage inhibition shown in parentheses. In (a) and (b), the zymosan (1 mg) and PA, or carbacyclin (2 μg) and PA, were administered i.p., simultaneously. In (c), the PA and indomethacin were administered one hour prior to i.p. injection of carbacyclin (2 μg).

Table 3 Effect of paracetamol (PA) on (a,b,c) the ability of PGI_2 to reverse the inhibition of zymosan-induced writhing produced by indomethacin; (d) zymosan-induced writhing in the presence and absence of PGI_2

PA (mg kg^{-1})	*Indomethacin* (mg kg^{-1})	*PGI_2* (i.p.)	*No. of writhes*	*Responders/ Total*
(a)				
— (Control)	—	–	3.0 ± 1.5	5/7
—	1 s.c.	–	0.3 ± 0.2*	2/7
—	1 s.c.	+	4.6 ± 1.0	6/7
800 p.o.	1 s.c.	–	0.1 ± 0.1*	1/7
800 p.o.	1 s.c.	+	0.9 ± 0.7	2/7
(b)				
— (Control)	—	–	3.3 ± 1.6	7/10
—	1 p.o.	–	0 ± 0*	0/10
—	1 p.o.	+	3.6 ± 1.4	7/10
800 s.c.	—	–	0.1 ± 0.1*	1/10
800 s.c.	1 p.o.	+	0.5 ± 0.2*	4/10
(c)				
— (Control)	1 p.o.	+	4.8 ± 2.1	7/10
800 s.c.	1 p.o.	+	1.3 ± 0.6*	5/10
(d)				
— (Control)	—	–	6.4 ± 1.1	10/10
800 p.o.	—	–	0 ± 0*	0/10
800 p.o.	—	+	1.2 ± 1.0*	2/10

Values are mean ± s.e.mean.
Zymosan was administered i.p. at 1 mg/mouse in 0.5 ml saline to all mice. PGI_2 (2 μg/mouse, i.p.) was administered 10 min after the zymosan.
* $P < 0.05$ cf. control.
(a) PA and indomethacin were administered 1 h before zymosan.
(b)(c) PA and indomethacin were administered 0.5 h and 1 h before zymosan, respectively.
(d) PA was administered 1 h before zymosan.

Table 4 Induction of writhing by intraperitoneal administration of two prostacyclin analogues, carbacyclin and iloprost

		Expt. 1		*Expt. 2*	
Compound	*Dose* (μg/mouse)	*No. of Writhes*	*Responders/ Total*	*No. of Writhes*	*Responders/ Total*
Vehicle	—	0.3 ± 0.2	3/10	0 ± 0	0/10
Iloprost	0.02	0.3 ± 0.2	2/10	0 ± 0	0/10
Iloprost	0.22	0.8 ± 1.3*	6/10	4.5 ± 1.8*	8/10
Iloprost	2	4.9 ± 0.8*	10/10	6.6 ± 1.9*	10/10
Carbacyclin	0.02	0 ± 0	0/10	—	
Carbacyclin	0.21	0.1 ± 1.1	1/10	—	
Carbacyclin	2	4.7 ± 1.1*	9/10	—	

* $P < 0.05$ cf. vehicle-treated group.
Groups of 10 mice were injected intraperitoneally with 0.5 ml of carbonate buffer (pH 10), or buffer plus PGI_2 analogue. The numbers of writhes over the subsequent 30 min were counted.

Table 5 Effect of *in vivo* treatment of mouse peritoneal macrophages on *in vitro* zymosan-induced superoxide anion generation

Treatment	*Dose*	*Superoxide generation* (Mean counts[1] (× 10^4))	*% inhibition*
Vehicle	—	46.6 ± 12.2 (10)	—
Superoxide dismutase + Catalase	4 mg ml^{-1} + 4 mg ml^{-1}	0.9* ± 0.4 (7)	98.1

Values are mean ± s.e.mean.
Groups of mice were injected intraperitoneally with 0.5 ml saline containing the indicated concentrations of enzymes. After 20 min peritoneal macrophages were collected from individual mice without washing, stimulated with zymosan (0.4 mg ml^{-1}) and superoxide generation measured by chemiluminescence. Number in parentheses = *n*.
* $P < 0.05$ cf. vehicle control.
[1] Total chemiluminescence counts.

writhing and did not alter the anti-writhing potency of paracetamol (not shown).

Discussion

It is immediately apparent that paracetamol, although effective, has very low potency in inhibiting zymosan-induced writhing. The ED_{50} was between 400 and 600 mg kg^{-1}, p.o. while therapeutic doses in man are 5–15 mg kg^{-1}, p.o. (Flower *et al.*, 1985). In contrast, the ED_{50} for indomethacin in this test (0.3 mg kg^{-1}, p.o., unpublished observations) is within the range of therapeutic doses used in man (0.3–1.5 mg kg^{-1}, p.o.) (Flower *et al.*, 1985). The high doses required in this test also produced behavioural side effects which may have indirectly interfered with the animals ability to writhe, in addition to exerting a specific antinociceptive action. Since the doses required to inhibit zymosan-induced writhing were higher than those reported for paracetamol in most animal models of inflammatory pain (Vinegar *et al.*, 1976; Winter *et al.*, 1979; Pruss *et al.*, 1980; Foote *et al.*, 1988), it seems possible that

there is some aspect of this model that makes it particularly unresponsive to paracetamol.

The doses of paracetamol which inhibited writhing also reduced the synthesis of PGI_2, as indicated by the reduced levels of immunoreactive 6-keto-$PGF_{1\alpha}$. GC/MS analysis confirmed the RIA data and indicated that arachidonic acid, the precursor of PGI_2, had not been shunted into the other cyclo-oxygenase products examined. This indicates that paracetamol inhibited cyclo-oxygenase itself rather than one of the endoperoxide isomerases. As with the NSAIDs (Doherty *et al.*, 1987), there was a significant correlation between the levels of 6-keto-$PGF_{1\alpha}$ and both the proportion of mice that writhed at least once, and with the number of writhes per mouse. However, the level of 6-keto-$PGF_{1\alpha}$ below which the proportion of responders decreased was higher than that noted previously for the NSAIDs. In fact, even the highest doses of paracetamol used did not lower the levels of 6-keto-$PGF_{1\alpha}$ to the extent found to be associated with inhibition of writhing by acidic non-steroidal anti-inflammatory agents. This difference in threshold could be due to the ability of the behavioural toxicity of paracetamol to suppress writhing, independently of 6-keto-$PGF_{1\alpha}$ levels.

As has been reported previously (Doherty *et al.*, 1987), PGI_2, injected i.p., can both induce writhing and reverse the ability of indomethacin to inhibit zymosan-induced writhing. Carbacyclin (as already reported by Smith *et al.*, 1985) and iloprost, two stable agonists at the PGI_2 receptor, also induced writhing when injected i.p., supporting the suggestion that PGI_2 is involved in inflammatory pain in the mouse peritoneal cavity.

Several other lines of evidence support the suggestion that oral doses of paracetamol inhibit zymosan-induced writhing by a mechanism in addition to lowering PGI_2 levels: (1) Oral administration of paracetamol, in contrast to the NSAID tested previously (Doherty *et al.*, 1987), partially blocked the ability of PGI_2 to reverse inhibition of zymosan-induced writhing produced by indomethacin. (2) Intraperitoneal administration of PGI_2 failed to reverse the ability of paracetamol to inhibit zymosan-induced writhing. (3) Oral administration of paracetamol ($800\,mg\,kg^{-1}$), but not indomethacin ($1\,mg\,kg^{-1}$, p.o.), inhibited carbacyclin-induced writhing.

It is, however, noteworthy that $100\,mg\,kg^{-1}$ paracetamol administered i.p. inhibited writhing and reduced 6-keto-$PGF_{1\alpha}$ levels without producing behavioural toxicity. This activity cannot be attributed to antagonism at the prostacyclin receptor since the same dose of paracetamol administered i.p. failed to block writhing induced by i.p. injection of carbacyclin. Therefore, local inhibition of PGI_2 synthesis in the peritoneal cavity by paracetamol can clearly inhibit writhing without involving behavioural toxic effects.

Since the *in vitro* potency of paracetamol, as an inhibitor of prostaglandin synthesis, is regulated by levels of peroxides in the reaction medium (Lands *et al.*, 1976; Robak *et al.*, 1978; Lands & Hanel, 1982; Hertz & Cloarec, 1984), attempts were made to measure levels of lipid peroxides in the peritoneal cavity. However, a standard assay method failed to detect lipid peroxides, possibly because of rapid elimination as was demonstrated here and has been discussed by Salaris & Babbs (1988). An alternative approach was, therefore, investigated. Zymosan activates the oxidative burst in leukocytes and the superoxide anion generated can be converted to highly reactive species which may generate lipid peroxides (reviewed in Chow, 1988; Marshall & Lands, 1986). It therefore seems reasonable to suppose that the oxidative burst could be a major source of lipid peroxides in zymosan-induced peritonitis and attempts were made to regulate this phenomenon.

One means of regulating the consequences of the oxidative burst is to eliminate the superoxide anion generated. This was achieved by local *in vivo* administration of a mixture of SOD and catalase. However, despite 98% reduction in detectable superoxide, no inhibition of writhing or modification of the potency of paracetamol was seen. Therefore, there is no evidence to implicate the oxidative burst in determining the antinociceptive potency of paracetamol in this model.

Overall, the data presented here are consistent with the suggestion that inhibition by paracetamol of PGI_2 synthesis in the peritoneal cavity is responsible for only a part of its antinociceptive activity in this test. However, the extremely high oral doses required produce behavioural toxicity which clearly contributes to the inhibition of writhing. The large difference in potency between the mouse and man suggests that in man, paracetamol may have additional mechanisms of action, not represented in this mouse model. One possible mechanism is inhibition of CNS prostaglandin synthesis triggered by release of interleukin-1 or other cytokines from the inflammatory lesion, as previously suggested (Doherty, 1987). In the acute model used here (20 min duration) there is insufficient time for synthesis and release of cytokines, consistent with the absence of a febrile response (data not shown). It seems unlikely that the low potency of paracetamol in this model is due to high levels of lipid peroxide generated from the oxidative burst, although other sources of lipid peroxides may be present. In particular, paracetamol itself has been shown to generate lipid peroxides when it is metabolized (Van De Straat *et al.*, 1988) and these could be a factor at the high doses used here.

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Non-adrenergic, non-cholinergic contractions in the electrically field stimulated guinea-pig trachea

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1 The effects of drugs and altering stimulus parameters on neurogenic responses to electrical field stimulation (EFS) have been investigated in distal and proximal portions of the guinea-pig trachea.

2 In the presence of indomethacin (3 μM) and propranolol (1 μM) two contractile phases were evident in both the proximal and distal trachea. The first rapid phase was abolished by atropine (0.1 μM), whereas the prolonged, second phase was abolished by capsaicin (10 μM) pretreatment. Tetrodotoxin (3 μM) abolished the initial response and greatly inhibited the second phase. In proximal trachea this second phase was evident only in 9 of 22 preparations. The addition of the peptidase inhibitor thiorphan (10 μM) however, caused a second phase to be seen in all the proximal tissues examined.

3 The two phases of the contractions to EFS were differentially sensitive to the pulse duration applied. The initial, cholinergic contractions were evident at lower pulse durations than were the prolonged capsaicin-sensitive contractions, with the first phase being approximately 10 fold more sensitive than the second phase.

4 The magnitude of the capsaicin-sensitive contraction to EFS was significantly greater in the distal trachea than in the proximal trachea. This difference prevailed in the presence of thiorphan, an inhibitor of neutral endopeptidase. In contrast, concentration-response curves to capsaicin were similar in segments of proximal and distal trachea.

5 The non-adrenergic non-cholinergic (NANC) relaxant responses were studied in tissues in which excitatory neurogenic responses were pharmacologically abolished by capsaicin and atropine treatment. The NANC relaxant responses in the proximal trachea were evident at lower pulse frequencies and were of greater magnitude compared with NANC relaxant responses in the distal trachea.

6 These results indicate that, by pharmacologically manipulating the trachea and by selecting optimum stimulation parameters, a NANC contractile response to EFS can be seen throughout the length of the guinea-pig trachea. This NANC response is most likely to be due to the release of tachykinins from capsaicin-sensitive sensory fibres. It is suggested that NANC relaxant responses mask NANC contractile responses especially in the proximal trachea where NANC relaxant responses predominate.

Introduction

The contractile response of guinea-pig tracheal smooth muscle to electrical field stimulation (EFS) can be abolished by muscarinic receptor antagonists and thus is considered to be cholinergic in nature (Carlyle, 1964; Coburn & Tomita, 1973; Coleman & Levy, 1974). In contrast to the trachea, EFS of the guinea-pig bronchus results in contractions that are both cholinergic and noncholinergic in nature (Szolcsanyi & Bartho, 1982; Lundberg *et al.*, 1983; Karlsson *et al.*, 1984). The noncholinergic component is thought to be secondary to release of neurokinins such as substance P (SP) from intrinsic sensory C-type fibres (see Karlsson, 1986). Although a noncholinergic contractile innervation to the trachea has been alluded to in the literature (Andersson & Grundstrom, 1983), to our knowledge it has not been systematically evaluated.

Since the guinea-pig trachea is known to receive strong relaxant innervation, the lack of a noncholinergic contractile response to EFS may be due to the functional antagonism afforded by the relaxant innervation. In the present study, experiments were designed to test this hypothesis. Under specified experimental conditions we found that EFS evokes noncholinergic contractions in both the proximal and distal trachea and that noncholinergic contractions may be functionally antagonized by the nonadrenergic relaxant innervation. It is suggested that the functional antagonism of the contractile response to EFS by NANC relaxant innervation is more prevalent in the proximal than distal trachea.

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Methods

General

Male, Dunkin-Hartley guinea-pigs (400–650 g: Charles River), were stunned, exsanguinated and the trachea removed. The trachea was placed in a modified Krebs solution (composition mM: NaCl 118, $CaCl_2$ 1.9, KH_2PO_4 1.0, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, glucose 11.1). Following removal of fat and connective tissue, the trachea was dissected into two sections each 1 cm long. One section was closest to the larynx and was designated as proximal, whereas the other section was closest to the bronchus and was designated as the distal section. The two sections were opened longitudinally, and transverse strips consisting of two adjacent cartilage rings were prepared and suspended between platinum ring electrodes in 10 ml organ baths containing Krebs solution maintained at 37°C and gassed with 5% CO_2 in O_2. Preparations were connected to force-displacement transducers for measurement of isometric tension responses. Before each experiment the tissues were equilibrated for 60 min at an initial resting tension of 1.5 g, and washed with fresh solution every 15 min. Indomethacin (3 μM) and propranolol (1 μM) were present in the tissue baths except where stated. In one group of experiments transverse strips of the main right bronchus were also similarly prepared.

Field stimulation studies

Rectangular pulses were delivered to the electrodes from a Grass S48 stimulator the output of which was passed through a Med-Lab Stimu-Splitter (Med-Lab Instruments, Fort

Collins, Colorado, U.S.A.) for signal amplification. In order to study the contractile innervation, frequency-response curves were generated by applying stimuli (20 V, 1 ms, 1–16 Hz) for 15 s. Experiments on the effects of pulse duration were carried out by varying the pulse duration from 0.01 to 1 ms at a frequency of 2 Hz. In preliminary studies we found 20 V, when delivered via the Stimu-Splitter, to be supramaximal for stimulation of cholinergic and noncholinergic nerve endings in the tissue.

Control frequency-response curves were obtained in each preparation before the addition of atropine (0.1 μM) or tetrodotoxin (3 μM), 15 min prior to the construction of a second curve. A second frequency-response curve obtained in the absence of any drug showed that the responses were reproducible. When capsaicin (10 μM) was used, a second curve was not obtained until the tension of the tissues had returned to baseline about 90 min later. When the effect of spantide (10 μM) was studied, control responses to field stimulation (2 Hz, 20 V, 1 ms for 15 s) and to exogenously added substance P (SP) (0.1 μM) were obtained before the addition of spantide. After contractions to spantide had diminished, 10 μM spantide was added again before responses to field stimulation and to SP were obtained. At the end of each experiment a maximum contractile response was obtained by the addition of 1 mM carbachol.

NANC relaxant responses were studied in tissues pretreated with capsaicin (10 μM at the start of the experiment), in order to abolish the noncholinergic contractile response. After 90 min the tissues were treated with atropine (1 μM) and contracted with histamine (10 μM). Two sets of experiments were then carried out. In the first, frequency-response curves (20 V, 1 ms, 0.1–50 Hz for 15 s) were obtained in both proximal and distal preparations. In the second set, the effects of increasing pulse duration (0.01–1 ms, 2 Hz, 20 V for 15 s) were examined in proximal preparations. At the end of each experiment the preparations were maximally relaxed with 30 μM sodium nitroprusside.

Drugs

Atropine H_2SO_4, (±)-propranolol HCl, tetrodotoxin (TTX), indomethacin, carbamylcholine chloride (carbachol), histamine PO_4, sodium nitroprusside (SNP), (±)-thiorphan and capsaicin were obtained from the Sigma Chemical Co., Missouri, U.S.A. [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]-substance P (spantide) was obtained from Bachem, California, U.S.A. (kindly provided by Dr William Karbon, Nova Pharmaceuticals, Baltimore, Maryland, U.S.A.). Atropine, propranolol, TTX, carbachol, histamine, SNP, thiorphan and spantide were each dissolved in 0.9% w/v NaCl solution. Indomethacin and capsaicin were dissolved in absolute ethanol at stock concentrations of 10 mM with subsequent dilutions in buffer.

Results

As previously shown (Coburn & Tomita, 1973), EFS (4 Hz, 20 V, 0.2 ms for 15 s) of the proximal or distal trachea in the absence of pharmacological inhibitors resulted in rapid contractions followed by long-lasting relaxations. The contractile component was abolished by atropine (1 μM) (data not shown). Indomethacin (3 μM) substantially reduced the intrinsic tone of the trachea, thereby masking the relaxation component to EFS. Thus, we re-examined the effect of EFS in the presence of indomethacin (3 μM) and propranolol (1 μM).

Distal trachea

All preparations (n = 37), when stimulated at 2 Hz (1 ms, 20 V for 15 s), exhibited a biphasic contractile response with an initial phase which faded rapidly and a second phase which was greatly prolonged after cessation of stimulation (Figure 1c). At its peak, the magnitude of the second phase of the contraction averaged 21 ± 1% of the maximum response of the tissue which could be obtained by the addition of 1 mM carbachol to the organ bath. This corresponded to 61 ± 4% of the first-phase of the contraction.

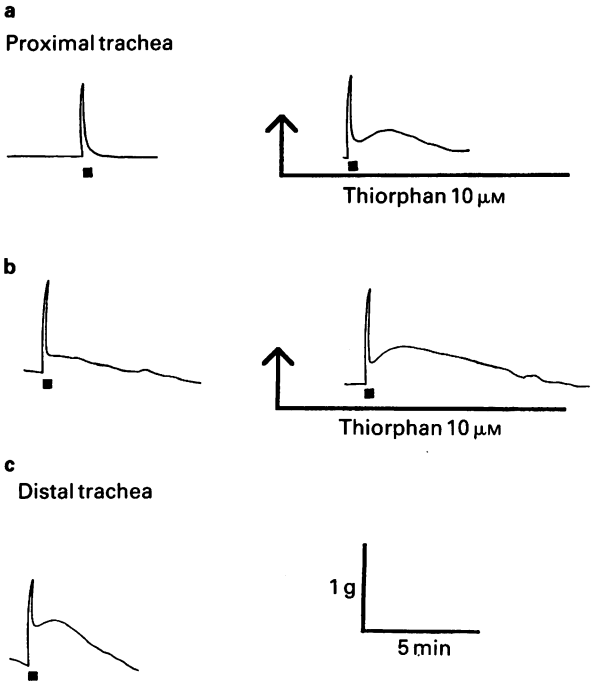

Figure 1 Guinea-pig trachea treated with indomethacin (3 μM) and propranolol (1 μM): representative tracings of contractile responses to electrical field stimulation (EFS) at 2 Hz, 1 ms, 20 V for 15 s. (a) Effect of thiorphan on a segment of proximal trachea which initially exhibited a monophasic contractile response to EFS. (b) Effect of thiorphan on a segment of proximal trachea which initially exhibited a biphasic response to EFS. (c) Typical biphasic response of a segment of distal trachea to EFS. The resting tension of the tissues was about 0.25 g.

Pulse duration The effects of pulse duration on the two phases was examined by stimulating preparations at 20 V, 2 Hz for 15 s and varying the pulse duration from 0.01 to 1 ms. The first phase was evident at the lowest pulse duration which could be delivered by the stimulator (0.01 ms) and had already reached a near maximal response at 0.04 ms, the pulse duration at which the second phase became evident (Figure 2). From Figure 2 it can be seen that the second phase is approximately 10 fold less sensitive to pulse duration than the first phase as measured by the pulse duration required to elicit a half maximal response.

Tetrodotoxin Tetrodotoxin (3 μM) was added to the bath 15 min prior to the second frequency-response curve. TTX abolished the initial phase of the response and profoundly reduced the second phase indicating the neurogenic nature of the responses (Figure 3).

Atropine The addition of atropine (0.1 μM), 15 min prior to the second frequency curve abolished the initial phase and reduced the second phase by about 25% (Figure 3). In the presence of atropine, responses to electrical stimulation were not seen until 8–10 s after the start of stimulation period.

Capsaicin desensitization The addition of capsaicin (10 μM) to the bath produced a large increase in tension equivalent to 66 ± 4% (n = 6) of the maximal contraction produced by 1 mM carbachol. When tension returned to the baseline value about 90 min later a second frequency curve was constructed. Upon stimulation the tissue responded with an initial contractile response which was reduced by about 20% when compared to control, whereas no second contractile phase was evident (Figure 3).

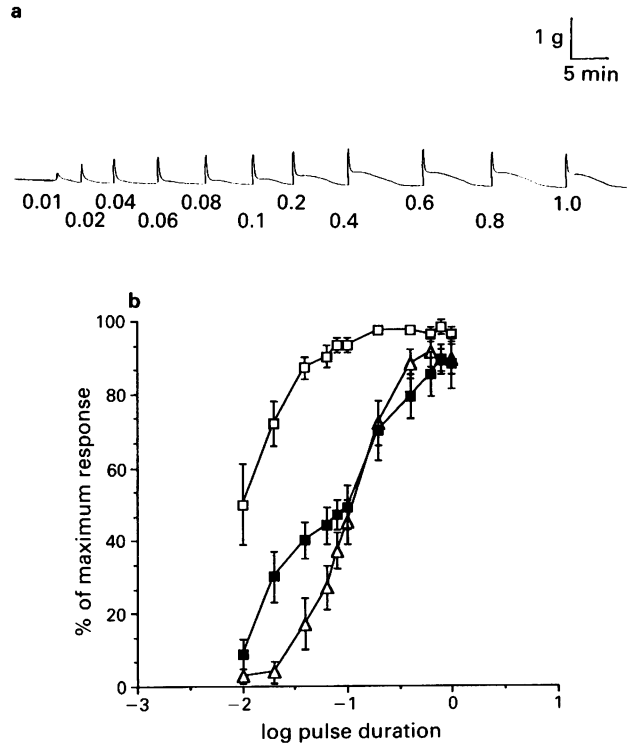

Figure 2 (a) Guinea-pig distal trachea treated with indomethacin (3 μM) and propranolol (1 μM): representative tracing of responses of the tissue to electrical field stimulation at 2 Hz, 20 V, 15 s and varying pulse duration from 0.01–1.0 ms. The resting tension of the tissue was about 0.25 g. (b) Effect of pulse duration on responses to electrical field stimulation in guinea-pig trachea treated with indomethacin (3 μM) and propranolol (1 μM). (□) First contractile phase in distal trachea; (△) second contractile phase in distal trachea; (■) NANC inhibitory phase in proximal trachea pretreated with capsaicin (10 μM), atropine (1 μM) and contracted with histamine (10 μM). Each point is the mean of at least six observations; s.e.mean shown by vertical bars.

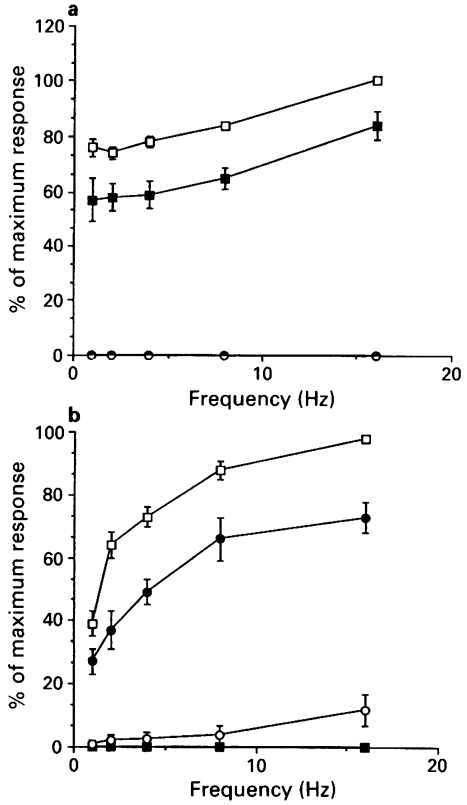

Figure 3 Guinea-pig distal trachea treated with indomethacin (3 μM) and propranolol (1 μM): (a) Frequency-response curve for the first contractile phase of the response to electrical field stimulation (20 V, 1 ms for 15 s). (□) Controls, $n = 19$; (■) in the presence of capsaicin (10 μM), responses to stimulation greater than 1 Hz are significantly different from controls ($P < 0.05$, paired t test), $n = 6$; (●) in the presence of atropine (0.1 μM), $n = 7$; (○) in the presence of tetrodotoxin (3 μM), $n = 6$. (b) Frequency-response curve for the second contractile phase of the response to electrical field stimulation (20 V, 1 ms for 15 s). (□) Controls, $n = 19$: (■) in the presence of capsaicin (10 μM), $n = 6$; (●) in the presence of atropine (0.1 μM), all responses to stimulation greater are significantly different from controls ($P < 0.05$, paired t test), $n = 7$; (○) in the presence of tetrodotoxin (3 μM), $n = 6$. Vertical lines indicate s.e.mean.

Spantide Upon addition to the bath, spantide (10 μM) produced large contractions which averaged 76 ± 4% of the maximum response to carbachol. This contraction was long lasting with tension not returning to baseline values for over 60 min. When tension had returned to baseline the further addition of 10 μM spantide was without effect. Subsequent field stimulation (2 Hz, 1 ms, 20 V for 15 s) of the tissues, in the presence of spantide, produced both cholinergic and NANC contractions which were unchanged from responses prior to the addition of spantide. Spantide also had no effect on contractions to 0.1 μM SP (Table 1). The effect of 10 μM spantide was also studied on the field-stimulated right bronchus. As in the trachea, spantide had no significant effect on responses to field stimulation or to exogenously added SP (Table 1).

Proximal trachea

In 9 of 22 preparations, EFS of the proximal trachea (2 Hz, 1 ms, 20 V for 15 s) caused a biphasic contractile response as observed in the distal trachea (Figure 1b). The magnitude of the second-phase averaged 11 ± 2% of the maximum response to carbachol, corresponding to 29 ± 4% of the first-phase. In the remaining preparations only the rapid first-phase of the contractions were observed. Both phases were abolished by TTX (3 μM) and, as in the distal trachea, the second phase was abolished by capsaicin pretreatment (data not shown).

Differences in proximal vs. distal preparations

In tissues stimulated at 2 Hz, 1 ms, 20 V for 15 s, the cholinergic first phase showed no differences between proximal and distal preparations. Responses were 31 ± 2 and 33 ± 3% respectively of the maximum response to carbachol. The magnitude of the second capsaicin-sensitive phase however, was much greater in the distal trachea than in the proximal trachea (see above).

Table 1 Summary of the effect of spantide on contractile responses to electrical field stimulation (EFS) and substance P in the guinea-pig trachea and bronchus

	Control	*Spantide* (10 μM)	
Trachea			
Cholinergic contraction to EFS	26 ± 3	29 ± 2	$n = 6$
NANC contraction to EFS	16 ± 4	17 ± 3	$n = 6$
Substance P (0.1 μM)	28 ± 6	26 ± 10	$n = 4$
Bronchus			
Cholinergic contraction to EFS	32 ± 2	32 ± 4	$n = 4$
NANC contraction to EFS	20 ± 3	18 ± 3	$n = 4$
Substance P (0.1 μM)	12 ± 4	11 ± 3	$n = 4$

Guinea-pig distal trachea and right main bronchus treated with indomethacin (3 μM) and propranolol (1 μM): effect of spantide on cholinergic and NANC contractions elicited by field stimulation (2 Hz, 20 V, 1 ms for 15 s) and on contractions elicited by exogenously added substance P (0.1 μM). Values are mean ± s.e.mean expressed as the % of maximum response to carbachol (1 mM).

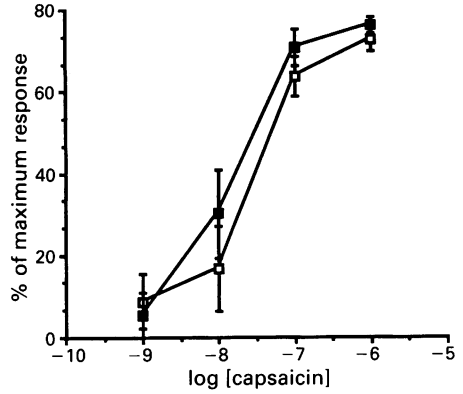

Figure 4 Guinea-pig trachea treated with indomethacin (3 μM) and propranolol (1 μM): concentration-response for contraction induced by capsaicin. (□) Proximal trachea; (■) distal trachea. Each point is the mean of 6 observations; vertical bars show s.e.mean.

Three hypotheses that might explain the marked differences between the second-phase contractions to EFS in the proximal and distal trachea were addressed experimentally: (1) there may be differential innervation by capsaicin-sensitive fibres between the two regions of the trachea; (2) there may be a greater degree of breakdown of the sensory transmitter in the proximal region of the trachea; or (3) there may be differential innervation by NANC relaxant fibres capable of functionally antagonizing the second-phase contractile response along the trachea.

In order to test the first hypothesis the contractile response to cumulative exposure to capsaicin, in the presence of indomethacin (3 μM) and propranolol (1 μM), was compared in proximal vs. distal portions of the trachea. No differences in either the sensitivity or maximum responses were observed between the two tissues (Figure 4). The negative logarithm of the EC_{50} (M) of capsaicin (mean ± s.e.mean) was 6.3 ± 0.2 and 6.6 ± 0.2 and the maximum contractions to capsaicin were 73 ± 2% and 76 ± 2% of the maximum response to carbachol (1 mM) in six proximal and six distal tracheal strips, respectively.

The second potential explanation was examined by quantifying the second-phase contraction to EFS in the presence of the neutral endopeptidase inhibitor thiorphan. Thiorphan (10 μM), added 10 min prior to the next stimulation, was then studied in 13 proximal preparations, 6 of which showed no evidence of a second contractile response. Subsequently, all of the tissues exhibited a biphasic contractile response (Figure 1). In the six tissues which had previously exhibited a second phase, thiorphan slightly increased the magnitude (1.9 ± 0.3 fold) and greatly increased the duration (3.7 ± 1.4 fold) of this phase compared with control responses. In the presence of thiorphan the second phase was 14 ± 3% of the maximum response to carbachol. Thus, even in the presence of thiorphan, the second phase in the proximal trachea is less than the second phase in the distal trachea without thiorphan, as in the absence of thiorphan the second phase in the distal trachea averaged 21 ± 1% of the maximum response to carbachol. Thiorphan had no significant effect on the magnitude of the first-phase of the contractile response to EFS.

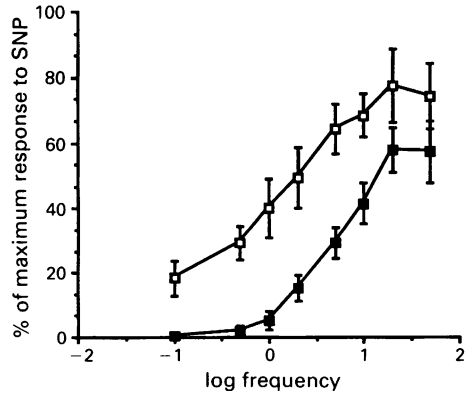

Figure 5 Guinea-pig trachea pretreated with capsaicin (10 μM), atropine (1 μM), indomethacin (3 μM), propranolol (1 μM) and contracted with histamine (10 μM): frequency-response curve for the NANC relaxant response induced by electrical field stimulation (20 V, 1 ms for 15 s); (□) proximal trachea; (■) distal trachea. Responses in the distal trachea to stimulation at 10 Hz or less are significantly different from responses in the proximal trachea ($P < 0.01$, unpaired t test). Each point is the mean of 6 observations. Vertical bars show s.e.mean.

To test the third hypothesis, the NANC relaxant response was evaluated in the proximal vs. distal trachea. These experiments were carried out on tissues in which the β-adrenoceptor response, as well as both phases of the contractile response were pharmacologically abolished. In capsaicin (10 μM) pretreated tissues, in the presence of atropine (1 μM), propranolol (1 μM) and indomethacin (3 μM), both proximal and distal preparations responded to histamine (10 μM) with contractions of equal magnitude. The frequency-response curves (20 V, 1 ms, 0.1–50 Hz for 15 s) obtained under these conditions show that NANC relaxant responses in the proximal portion of the trachea are evident at lower frequencies and that maximal responses are greater when compared to responses in the distal portion (Figure 5). Thus at 2 Hz the response in the proximal portion was 49 ± 9% ($n = 6$) of the maximal relaxation to SNP, whereas in the distal portion it was only 15 ± 4% ($n = 6$). It is interesting to note that the pulse duration effect curve for the NANC relaxant response was more akin to that for the capsaicin-sensitive second phase contractile response than for the first-phase cholinergic response (Figure 2b).

Discussion

That the proximal and distal portions of the trachea in the presence of indomethacin and propranolol respond to electrical field stimulation (EFS) with an initial atropine-sensitive contraction is in accordance with the literature (Coburn & Tomita, 1973; Coleman & Levy, 1974). We also observed, however, that a second NANC contractile response can be uncovered in both portions if experimental parameters are optimal. Our results suggest that optimal conditions include a preparation with little or no intrinsic tone and with endopeptidase activity inhibited. In the present study this was accomplished by treating the tissues with indomethacin and thiorphan, respectively. We also found it necessary to stimulate tissue with electrical current of sufficient intensity. We found that using supramaximal voltage, pulse duration of above 0.6 ms were required to evoke maximal noncholinergic neuronal responses in the trachea.

It is not surprising that, as for bronchus, a NANC contractile response to EFS exists in the trachea as evidence for sensory innervation in the guinea-pig trachea has been reported in the literature: capsaicin produces a contraction in the trachea (Molnar *et al.*, 1969), SP-like immunoreactivity is seen throughout the tracheobronchial tree (Lundberg *et al.*, 1984) and nicotine produces an atropine insensitive contraction (Kizawa & Takayanagi, 1987).

Our results show that there is regional variation in NANC responses to EFS in the trachea in that, in the distal portion, a second contractile component can always be seen using the stimulation parameters employed, whereas only about 40% of proximal portions show a second response. It should be noted however, that NANC contractile innervation exists throughout the trachea as the second phase could be unmasked by thiorphan in those preparations of the proximal trachea which previously had shown no second contraction to EFS. There are three possible explanations to explain this regional variability: (1) although a NANC contractile innervation is present

throughout the trachea, it appears to be graded being more prominent in the distal region of the trachea. This is unlikely given our present observation that capsaicin has similar effects in both regions and also that it has been reported that SP-like immunoreactivity is similar in both regions (Lundberg *et al.*, 1984) and that levels of the peptide are not different between the two regions (Ghatei *et al.*, 1982); (2) the level of endopeptidase activity which breaks down the peptide(s) responsible for the second phase may be higher in the proximal than in the distal region of the trachea. This is unlikely to be the case as even in the presence of thiorphan, an endopeptidase inhibitor the second phase in the proximal trachea was not as great as that in the distal trachea. (3) NANC relaxant innervation, which masks the second phase, is more predominant in the proximal region than in the distal region of the trachea. Until this NANC relaxant response can be selectively antagonised, this cannot be directly tested. Nevertheless our results favour this latter hypothesis. Evidence to support this comes from the frequency-response curves which show that the NANC relaxant response is both larger and is seen at lower frequencies in the proximal trachea compared with the distal trachea.

This study attempted to identify the transmitter responsible for the NANC excitatory response by using the putative SP antagonist, spantide (Jacoby *et al.*, 1986; Kamikawa & Shimo, 1989). We were unable to show an inhibition by spantide of the capsaicin-sensitive contractile response to EFS or of the contractions evoked by 0.1 μM SP in both the distal trachea and the right main-stem bronchus. Others have cautioned that the effects of the SP antagonists currently available may be due to non-specific actions (Karlsson *et al.*, 1984; Konishi & Otsuka, 1985), thus more selective antagonists will be required before the transmitter(s) responsible can be positively identified. It is likely however, that the innervation is a continuum of the capsaicin-sensitive sensory innervation of the bronchus (Lundberg & Saria, 1982; Lundberg *et al.*, 1983; Karlsson *et al.*, 1984). This is supported by evidence from this present study. Firstly capsaicin, which releases tachykinins from and then ultimately degenerates sensory C fibres, completely inhibited the NANC contractile response to EFS. Secondly responses were either enhanced or unmasked by the neutral endopeptidase inhibitor thiorphan which has been shown to potentiate both electrically- and capsaicin-induced contraction in the guinea-pig bronchi (Djokic *et al.*, 1989, Undem *et al.*, 1990). Thirdly, responses were favoured by the use of high pulse durations which are needed to stimulate capsaicin-sensitive fibres (Undem *et al.*, 1990).

NANC-cholinergic interactions

We found that approximately 25% of the second-phase (capsaicin-sensitive phase) contraction to EFS was inhibited by atropine. This is consistent with our previous findings that atropine significantly inhibited the second-phase contraction elicited by vagus nerve stimulation in the guinea-pig isolated bronchus (Undem *et al.*, 1990). This is unlikely to be due simply to an overlap of the first phase with the second phase as the first phase in absence of a second phase shows a very rapid decay on cessation of stimulation with a decay half-life of about 5 s whereas the second phase reaches a maximum 1–2 min after the stimulation period and is maintained over 5–10 min. Two other possibilities exist. Firstly acetylcholine by an action on muscarinic receptors may enhance the effects of the tachykinin involved in the second phase. Secondly C-fibre activation may itself cause the release of acetylcholine. This is supported by observations that substance P contractions in rat (Joos *et al.*, 1986), rabbit (Tanaka & Grunstein, 1984) and ferret (Sekizawa *et al.*, 1987) airways are mediated partially through the release of acetylcholine. Regardless of the mechanism, this observation suggests that the responses to stimulation of parasympathetic cholinergic fibres and capsaicin-sensitive sensory fibres in guinea-pig central airways are not totally independent of one another.

Capsaicin was also shown to inhibit the rapid first phase of the response. This may be due to the observation that tachykinins released from C-fibres enhance cholinergic transmission (Sekizawa *et al.*, 1987; Hall *et al.*, 1989). Another possibility is that the rapid first phase response is due to the addition of the cholinergic response and the response to C-fibre stimulation as the first phase reached a maximum at the end of the stimulation (15 s) and that in the presence of atropine a second phase was evident 8–10 s after the start of the stimulation period. Capsaicin may also inhibit the first phase by non-selectively damaging the parasympathetic neurones. However, we have previously reported that in tissues where the second contractile phase is minimal or absent, capsaicin has no effect on vagally induced cholinergic contractions in the guinea-pig bronchus suggesting that this is not the case (Undem *et al.*, 1990).

The guinea-pig trachea is frequently employed as a model for the study of NANC relaxant innervation of the airways. In this study we provide evidence that smooth muscle in both the proximal and distal portions of the guinea-pig trachea receive capsaicin-sensitive contractile innervation. This non-cholinergic contractile innervation, which may go unobserved under conditions where intrinsic tone is present, may nevertheless influence other neuronal responses in the trachea. Based on our findings, therefore, it would seem prudent to abolish pharmacologically this capsaicin-sensitive contractile innervation before performing experiments aimed at specifically evaluating the relaxant innervation of the trachea.

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Different profiles of desensitization dynamics in guinea-pig jejunal longitudinal smooth muscle after stimulation with histamine and methacholine

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1 In the present study we investigated desensitization phenomena of guinea-pig jejunal longitudinal smooth muscle responses after stimulation with 100 μM histamine or methacholine, using a superfusion method.

2 Histamine H_1-receptor-mediated contractions appear to be rapidly reduced after application of 100 μM histamine. Muscarinic responses were not affected following desensitization with 100 μM histamine, indicating a homologous desensitization.

3 Initial contractions to 0.3 μM histamine were reduced by 90%, recovered quickly, but did not reach control levels within 1 h. Desensitization of histamine responses could be separated into two phases; a rapid, but transient, desensitization and a more sustained desensitization. As a consequence of this sustained effect the pD_2 for histamine shifted from 6.7 $\pm$ 0.1 (control) to 6.1 $\pm$ 0.1 (desensitized).

4 Desensitization with 100 μM methacholine caused a heterologous desensitization, reflected by the development of a refractory period, in which neither histamine nor methacholine was able to elicit a contraction. After a few minutes responses to both agents recovered to control levels.

5 During the refractory period after methacholine desensitization, muscle strips were still responsive to 40 mM KCl but did not contract in response to 10 mM caffeine, suggesting that the heterologous desensitization is caused by a modification of an intracellular Ca^{2+}-store, which is used by both histamine and methacholine.

6 The recovery of the responses after methacholine desensitization was not dependent on extracellular Ca^{2+}, suggesting that the recovery is not dependent on refilling of the intracellular Ca^{2+} store with extracellular Ca^{2+}.

7 The protein kinase C activator, phorbol-12,13-dibutyrate, concentration-dependently inhibited histamine- and methacholine-induced contractions. Protein kinase C seems therefore not to be implicated in the observed homologous H_1-receptor desensitization.

8 These data suggest that different forms of desensitization can be distinguished in this model, each with a different time course and dependent on the applied stimulus.

Introduction

Prolonged exposure of biological systems to high concentrations of stimulating agents often leads to the development of a negative feedback response (Hollenberg, 1985a). Such a desensitization occurs both *in vivo* and *in vitro*. Levels of various neurotransmitters and hormones are regulated by several (patho)physiological conditions and might be elevated for a long period of time. For example, in allergic conditions histamine is released from mast cells (Ring *et al.*, 1985) and is responsible for some of the clinical relevant symptoms of allergy after interaction with the H_1-receptor. It has been shown that histamine responses can be modulated *in vivo* (Manning *et al.*, 1987) and *in vitro*. Histamine-induced desensitization of the H_1-receptor has been documented in various isolated cell systems (Lewis Baenzinger *et al.*, 1981; Brown *et al.*, 1986; Nakahata & Harden, 1987; McDonough *et al.*, 1988) and smooth muscle preparations (Anderson *et al.*, 1979; Kenakin & Cook, 1979; Bielkiewicz & Cook, 1984; Hishinuma & Uchida, 1988).

Recently Hishinuma & Uchida (1988) studied the short-term desensitization of guinea-pig taenia coli induced by carbachol or histamine. In this study contractile responses in Ca^{2+}-free medium were measured after a previous potassium-depolarization in Ca^{2+}-containing buffer. The observed contractions are thought to be due to Ca^{2+} release from the intracellular Ca^{2+} pool and have previously been shown to be restricted to rather high agonist concentrations (Casteels & Raeymaekers, 1979; Himpens & Casteels, 1987). However, contractions of visceral smooth muscle in response to muscarinic- and H_1-receptor stimulation are known to be highly dependent on influx of extracellular Ca^{2+} (Best *et al.*, 1985; Morel *et al.*, 1987). Histamine H_1-receptor-mediated contractions of guinea-pig ileum proved to be highly sensitive to blockade by the dihydropyridines and could be potentiated by Bay K 8644 (Best *et al.*, 1985; Morel *et al.*, 1987). These observations indicate that histamine opens dihydropyridine-sensitive Ca^{2+} channels in the plasma membrane, which might be characterized as voltage-gated Ca^{2+} channels. Moreover, H_1-receptor stimulation with low concentrations of histamine has been shown to cause an increase in the frequency of the action potential discharges and a depolarization of the intestinal smooth muscle (Bolton *et al.*, 1981; Yamanaka & Kitamura, 1987). Entry of Ca^{2+} through voltage-gated Ca^{2+} channels is therefore probably one of the main regulatory mechanisms for intestinal smooth muscle contraction after muscarinic- and H_1-receptor activation. Yet, influx of Ca^{2+} through other (receptor-operated?) Ca^{2+} channels is also suggested to be implicated in the contraction (Morel *et al.*, 1987). Based on these findings it can be anticipated that under normal, physiological conditions the contribution of the Ca^{2+}-release from intracellular stores is of relatively minor importance for the observed visceral smooth muscle contraction. The observed desensitization phenomena of the intracellular Ca^{2+}-release (Hishinuma & Uchida, 1988) might therefore not be relevant under physiological conditions. Moreover, the procedure of Ca^{2+} loading via potassium depolarization does not allow the study of the actual time course of desensitization and recovery

of the responses, since, after the desensitization, preparations have to be challenged with a high potassium medium for a rather long time before responses can be measured again. During this period recovery from the desensitization might already have occurred.

Therefore, we have extended the study of Hishinuma & Uchida (1988) and examined the effects of short-term desensitization under conditions that are thought to be more physiologically relevant. We have studied the time courses of desensitization and recovery, and investigated the possible cross-desensitization between the muscarinic- and H_1-receptor system using a superfusion method. With this method we were able to measure responses directly after the removal of the desensitizing agent and return of smooth muscle tension to baseline levels. In this paper we describe observations that reveal three different kinds of desensitization, each developing after a specific stimulus.

Methods

Contraction measurements

Male guinea-pigs (350–450 g) were killed by a blow on the head. Intestine was rapidly removed and suspended in Krebs buffer, composition (mM): NaCl 117.5, KCl 5.6, $MgSO_4$ 1.18, $CaCl_2$ 2.5, NaH_2PO_4 1.28, $NaHCO_3$ 25 and glucose 5.5. Jejunal longitudinal smooth muscle was carefully separated from the circular muscle. Smooth muscle strips (3 mm long, 1 mm wide) were placed in 0.4 ml superfusion chambers and superfused with Krebs buffer, which was continuously gassed with 95% O_2-5% CO_2 and maintained at 37°C, at a flow-rate of 2 ml min^{-1}. Contractions were recorded isotonically under 0.2 g tension.

After 45 min equilibration the longitudinal smooth muscle strips were repetitively stimulated with submaximal concentrations of histamine or methacholine. Contractile agents could be added to the superfusion fluid via a peristaltic pump (Gilson Minipuls II, Villiers-le-bel, France) or a time-controlled infusion-unit (B. Braun-Melsungen AG, Melsungen, W. Germany): the compounds were infused for 30 s, followed by a 90 s washout. Subsequently, the same agent was applied again for 30 s. Experiments were started after stabilization of the initial contractile responses.

Dose-response curves were established by measuring the contractions of single concentrations of agonist. Desensitization was performed with 100 μM methacholine or 100 μM histamine for various durations (30 s–5 min). In these experiments the desensitizing agent was added to the superfusion fluid via the peristaltic pump, while the infusion unit continued to inject low concentrations of the contractile agent for 30 s at 2 min intervals. In this way we were able to measure contractions directly after the removal of the desensitizing agents.

In some experiments caffeine was used to monitor intracellular Ca^{2+} pools. Since caffeine also inhibits adenosine 3':5'-cyclic monophosphate (cyclic AMP)-phosphodiesterase, thereby causing accumulation of cyclic AMP during prolonged infusion, repetitive contractions were not measured with this agent. Only a single contraction after a 10 mM caffeine addition was measured before a 5 min period of desensitization with 100 μM methacholine. After 5 min desensitization with 100 μM methacholine and subsequent return of muscle tension to baseline level, a second response to caffeine was measured. Thereafter responses to 0.3 μM methacholine were recorded again. These muscarinic responses completely recovered, indicating that this procedure (single caffeine addition) did not affect the cyclic AMP levels dramatically. After recovery of the methacholine responses a third and final caffeine-induced contraction was measured. To study the pathway of refilling of the intracellular Ca^{2+} stores, experiments in Ca^{2+}-free Krebs buffer were performed. In this series of experiments, Ca^{2+} was omitted from the buffer, but could be re-added to the superfusing fluid, using the peristaltic pump. In a similar way it was possible to load the intracellular Ca^{2+} store by a 10 min exposure to 40 mM KCl in Ca^{2+}-containing buffer (Casteels & Raeymaekers, 1979). After a 3 min superfusion with Ca^{2+}-free medium, muscle strips were exposed to the contractile agents for 30 s in Ca^{2+}-free buffer. In the experiments with the phorbolesters and H-7 these agents were also added to the superfusion fluid with the peristaltic pump.

Statistics

All data shown are expressed as mean ± s.e.mean of six to eight experiments. EC_{50}-values were obtained by inspection and transformed to logarithmic values for calculation of a mean value. Tissue preparations of at least three different animals were used for each type of experiment. Statistical analysis was carried out by Student's t test: P-values < 0.05 were considered to indicate a significant difference.

Chemicals

Histamine dihydrochloride, methacholine chloride, 4α-phorbol, phorbol-12,13-dibutyrate, H-7(1-(5-isoquinolylsulphonyl)-2-methylpiperazine) and caffeine were obtained from Sigma Chemical Company Ltd. (St. Louis, M.O., U.S.A.). All other reagents were of analytical grade. Phorbol-12,13-dibutyrate and 4α-phorbol were dissolved in dimethylsulphoxide (DMSO), whereas H-7 was dissolved in 10% ethanol. All other reagents were dissolved in distilled water and diluted in Krebs buffer.

Results

Longitudinal smooth muscle strips of guinea-pig jejunum were successively subjected to histamine or methacholine for 30 s as described under Methods. Single applications of various concentrations were used and dose-response-curves were recorded for both agents. This procedure yielded pD_2 values of 6.7 ± 0.1 (mean ± s.e.mean, $n = 6$, $EC_{50} = 0.2\,\mu$M) and 6.9 ± 0.2 (mean ± s.e.mean, $n = 6$, $EC_{50} = 0.13\,\mu$M) for histamine and methacholine respectively. These values did not deviate from pD_2 values, obtained with cumulative dosing schedule in standard non-superfused organ chambers (data not shown).

Based on these results a concentration of 100 μM was chosen for the subsequent desensitization experiments with jejunal longitudinal smooth muscle. Figure 1a shows the effect of a 5 min application of 100 μM histamine on the response to

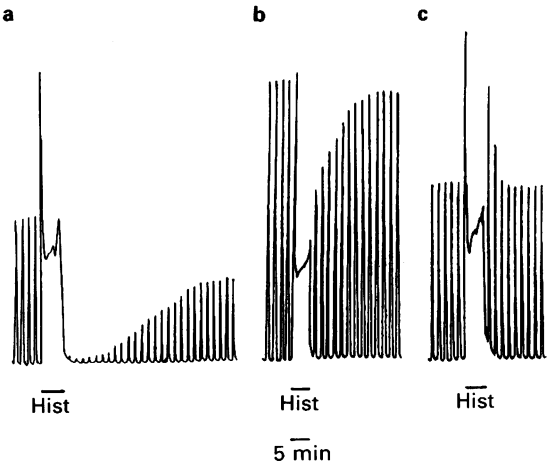

Figure 1 Recorder tracings of experiments, in which responses to (a) 0.3 μM histamine, (b) 3 μM histamine and (c) 0.3 μM methacholine were measured before and after 5 min desensitization with 100 μM histamine (Hist). Longitudinal smooth muscle strips were stimulated every 2 min for 30 s with the applied agent. Typical experiments out of eight are shown.

0.3 μM histamine (30 s, every 2 min). Since 0.3 μM histamine contracted the longitudinal smooth muscle strips approximately half-maximally, this concentration is excellently suited for monitoring changes that might occur during H_1-receptor desensitization (e.g. pD_2-shift).

From Figure 1a it is evident that 5 min stimulation with 100 μM histamine markedly affected the subsequent responses to 0.3 μM histamine. After return of the tension to baseline values only very small contractions were recorded in response to 0.3 μM histamine. Thereafter, contractions slowly increased to reach a maximum after 44 $\pm$ 2 min (mean $\pm$ s.e.mean, $n = 8$). However, the contractions do not reach control levels again within 3 h; after 5 min stimulation with 100 μM histamine responses were depressed by 39 $\pm$ 4% (mean $\pm$ s.e.mean, $n = 8$).

As can be seen in Figure 2, this H_1-receptor desensitization appeared to be time-dependent. Desensitization of the longitudinal smooth muscle strips developed very rapidly: after 2 min application of 100 μM histamine, responses to 0.3 μM histamine were reduced, and a maximal achievable desensitization was obtained after 4 min stimulation with 100 μM histamine.

The period of desensitization did not only affect the maximal level of contraction in response to 0.3 μM histamine. The time course of recovery too, was modulated by the period of desensitization with 100 μM histamine (Figure 3). Longer periods of desensitization with 100 μM histamine resulted in a slower recovery of the subsequent responses. After desensitization with 100 μM histamine, responses to 3 μM histamine (usually a maximal effective concentration) recovered quickly (Figure 1b, Figure 3) and are depressed to a lesser extent, when compared to the 0.3 μM histamine responses (Figure 2). When higher concentrations histamine (100 μM) were tested after desensitization with 100 μM histamine, no reduction in response could be observed (Figure 2). After a 5 min desensitization with 100 μM histamine and recovery of the responses, the pD_2 value for histamine decreased from 6.7 $\pm$ 0.1 ($EC_{50} = 0.2\,\mu$M) to 6.1 $\pm$ 0.1 ($EC_{50} = 0.8\,\mu$M) (mean $\pm$ s.e.mean, $n = 6$, $P < 0.05$ compared to control), whereas the maximal response was not altered (Figure 2).

In order to establish whether these observations were confined to histamine H_1-receptor-mediated contraction, muscle strips were exposed to the muscarinic agonist, methacholine. As can be seen in Figure 1c, muscarinic responses were affected in a totally different way by a 5 min desensitization period with 100 μM histamine. Contractions elicited by 0.3 μM methacholine, a concentration producing approximately half-maximal responses of the muscle strips, were not depressed after desensitization with 100 μM histamine. Moreover, responses to 0.3 μM methacholine were usually slightly augmented directly after muscle tension had returned to baseline levels (Figure 1c).

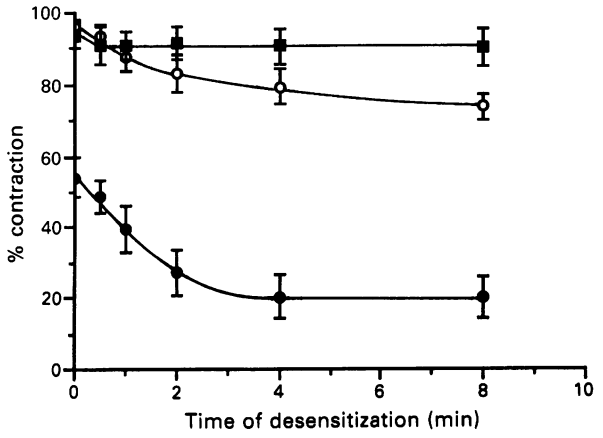

Figure 2 Time-dependency of the desensitization with histamine. Longitudinal smooth muscle strips were stimulated for various periods of time with 100 μM histamine. Responses to 0.3 μM (●), 3 μM (○) and 100 μM histamine (■) were then determined. Data shown are the mean of eight different experiments; s.e.mean shown by vertical bars. Values are expressed as a % of the maximum contractile response of the tissue to histamine under control conditions.

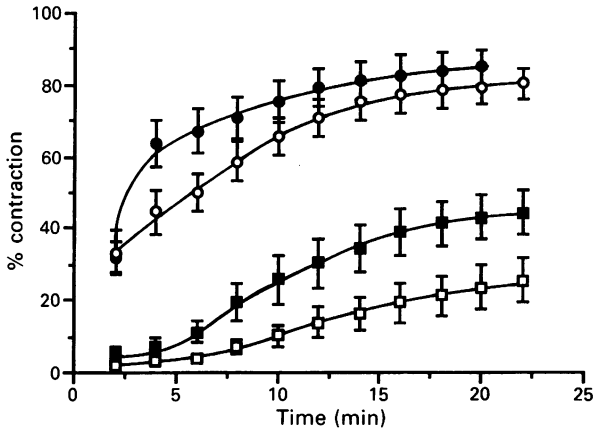

Figure 3 Recovery of contractions of longitudinal smooth muscle strips, stimulated with 0.3 μM (squares) and 3 μM (circles) histamine. Smooth muscle strips were desensitized with 100 μM histamine for 1 min (closed symbols) and 4 min (open symbols). Data shown are the mean of eight different experiments; s.e.mean shown by vertical bars.

Histamine did not contract jejunal smooth muscle in the absence of extracellular Ca^{2+}. Yet, when muscle strips were previously exposed to 40 mM KCl for 10 min in Ca^{2+}-containing medium and washed for 3 min in Ca^{2+}-free buffer, rather high concentrations of histamine were able to elicit transient contractions in Ca^{2+}-free buffer. Under these conditions histamine contracted the smooth muscle strips to 52 $\pm$ 7% of the response in Ca^{2+}-containing medium with a pD_2 value of 5.4 $\pm$ 0.1 ($EC_{50} = 4\,\mu$M) (mean $\pm$ s.e.mean, $n = 4$). Smooth muscle strips were desensitized for 5 min with 100 μM histamine and the intracellular Ca^{2+}-store was loaded with Ca^{2+} via the KCl exposure. The subsequent response to 100 μM histamine under Ca^{2+}-free conditions was inhibited to 67 $\pm$ 8% of the control response (mean $\pm$ s.e.mean, $n = 4$).

When longitudinal smooth muscle strips were desensitized with 100 μM methacholine a completely different picture emerged. As can be seen in Figure 4a, responses to 0.3 μM methacholine were affected by a 5 min desensitization period via the development of a refractory period, in which the muscle did not respond at all. After a few minutes contractions recovered and reached control levels very quickly (Figure 4a). The length of the refractory period proved to be

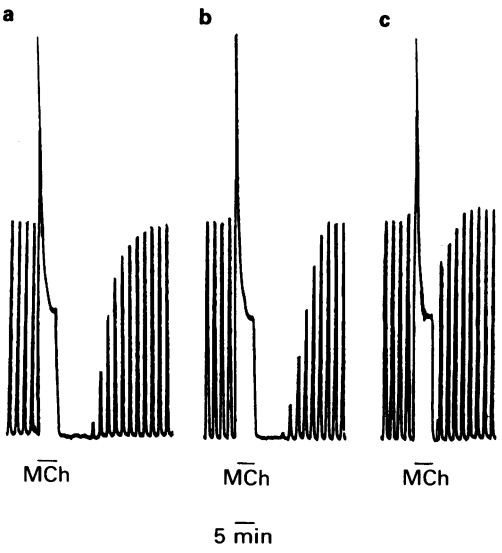

Figure 4 Recorder tracings of experiments, in which responses to (a) 0.3 μM methacholine, (b) 0.3 μM histamine and (c) 40 mM KCl were measured before and after 5 min desensitization with 100 μM methacholine (MCh). Longitudinal smooth muscle strips were stimulated every 2 min for 30 s with the applied agent. Typical experiments out of eight are shown.

dependent on the period of desensitization with 100 μM methacholine. A period of 1 min desensitization with 100 μM methacholine caused a refractory period of 6 ± 1 min (mean $\pm$ s.e.mean, $n = 7$), whereas this refractory period was increased to 11 ± 1 min (mean $\pm$ s.e.mean, $n = 7$) after a 5 min desensitization with 100 μM methacholine.

Besides muscarinic receptor responses, histamine H_1-receptor-mediated contractions were influenced in a similar way. Responses to 0.3 μM histamine proved to be refractory for 10 ± 1 min (mean $\pm$ s.e.mean, $n = 7$) after 5 min stimulation with 100 μM methacholine and were not depressed after reaching steady-state levels (Figure 4b). The duration of the refractory period was not significantly different from the values obtained with 0.3 μM methacholine after a similar methacholine treatment.

To study whether this desensitization was the result of a dysfunction of the contractile apparatus of the smooth muscle, we investigated the effect of methacholine desensitization on responses to 40 mM KCl. This concentration of KCl produced contractions that were almost similar to the responses after 0.3 μM methacholine (i.e. 50% of the maximal methacholine response). The initial contractions after a 5 min desensitization with 100 μM methacholine were only slightly reduced (Figure 4c). It is however clear that contractions after membrane depolarization already occurred at a point of time at which methacholine (Figure 4a) and histamine (Figure 4b) were still unable to elicit any response of the longitudinal smooth muscle. Moreover, contractions after 40 mM KCl application reached control values when the receptor-mediated responses were still very small.

Methacholine did not contract the visceral smooth muscle in Ca^{2+}-free medium. However, after Ca^{2+}-loading with 40 mM KCl, methacholine was able to induce transient contractions in a Ca^{2+}-free medium. These responses reached 74 ± 5% of the response in Ca^{2+}-containing buffer. The pD_2 value for methacholine was 5.6 ± 0.1 ($EC_{50} = 3$ μM) (mean $\pm$ s.e.mean, $n = 4$). Densensitization with 100 μM methacholine for 5 min did not affect the contractions in Ca^{2+}-free medium. After preloading for 10 min with 40 mM KCl and 3 min washing in Ca^{2+}-free buffer, responses to 100 μM methacholine in Ca^{2+}-free medium reached 94 ± 3% of the control response (mean $\pm$ s.e.mean, $n = 4$).

To investigate the involvement of intracellular Ca^{2+} pools more directly, experiments with 10 mM caffeine were performed. Caffeine (10 mM) produced small and transient contractions of jejunal longitudinal smooth muscle strips (Figure 5). After a 1 min desensitization with 100 μM methacholine, caffeine was not able to elicit a contraction when it was added just after the desensitization period (Figure 5). However, when responses to 0.3 μM methacholine had recovered completely, caffeine responses could be observed again (Figure 5).

Recovery of the contractile responses after desensitization with 100 μM methacholine was not dependent on extracellular Ca^{2+}. As can be seen in Figure 6c, responses of smooth muscle strips to 0.3 μM methacholine did also recover when Ca^{2+} was omitted for 10 min from the extracellular medium, just after the 5 min period of desensitization with 100 μM methacholine. Initial responses could be measured again 13 ± 1 min (mean $\pm$ s.e.mean, $n = 6$) after the desensitization period. This refractory period was slightly different from the period observed, when desensitization was allowed to recover in the presence of extracellular Ca^{2+} (Figure 6b). In these experiments a refractory period of 11 ± 1 min (mean $\pm$ s.e.mean, $n = 6$) was observed. This was significantly different from the refractory period in the absence of Ca^{2+} ($P < 0.05$). Nevertheless, since omission of extracellular Ca^{2+} slightly affected initial responses to 0.3 μM methacholine (Figure 6a) when Ca^{2+} was reintroduced, we do not consider this small difference relevant.

Finally, the possible role of protein kinase C in the observed desensitization phenomena was investigated. The protein kinase C activator phorbol-12,13-dibutyrate (PDB) concentration-dependently inhibited contractions to histamine

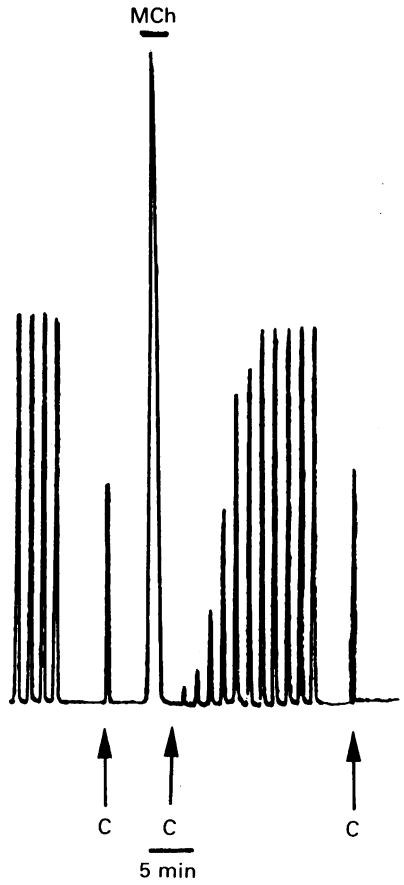

Figure 5 Recorder tracing of experiments, in which responses to 0.3 μM methacholine and 10 mM caffeine (C) were measured before and after 1 min desensitization with 100 μM methacholine (MCh). Longitudinal smooth muscle strips were stimulated every 2 min for 30 s with 0.3 μM methacholine until stable responses were obtained. Thereafter 10 mM caffeine was applied and when tension had returned to baseline levels, 100 μM methacholine was administered for 1 min. After this period 10 mM caffeine was applied in the refractory period and after recovery of the 0.3 μM methacholine responses. A typical experiment out of eight is shown.

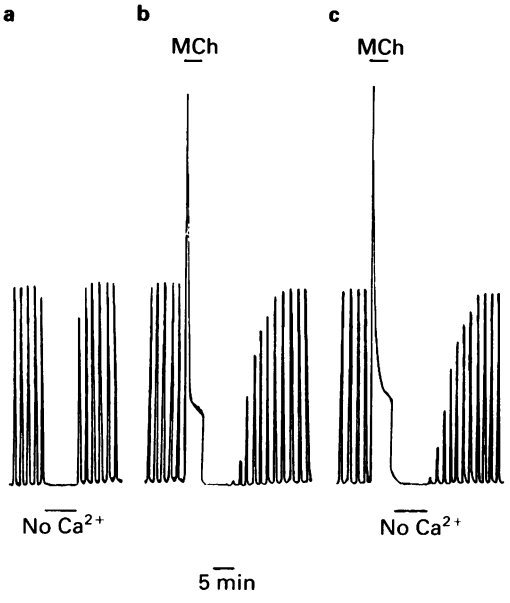

Figure 6 Recorder tracing of experiments, in which responses to 0.3 μM methacholine were measured after omission of extracellular Ca^{2+} (a) and after 5 min desensitization with 100 μM methacholine (MCh) and recovery in the presence (b) or absence (c) of extracellular Ca^{2+}. A typical experiment out of eight is shown.

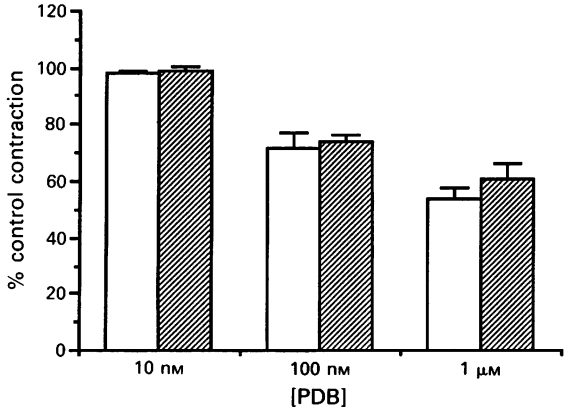

Figure 7 Effects of phorbol-12,13-dibutyrate (PDB) on contractions induced by 0.3 μM histamine (open columns) or 0.3 μM methacholine (hatched columns). Data shown are the mean of 6–8 experiments, with vertical bars showing s.e.mean.

and metacholine (Figure 7). There was no difference in sensitivity towards PDB for the two agents. Responses to both agents were not affected by 10 nM PDB and were reduced for approximately 45% by 1 μM PDB. Contractions were not affected by application of 1 μM 4α-phorbol (data not shown), whereas 1 μM PDB augmented 40 mM KCl responses to 147 $\pm$ 18% (mean $\pm$ s.e.mean, $n = 7$, $P < 0.05$) of the control response. A possible role of protein kinase C in the observed heterologous desensitization was further examined with the protein kinase C inhibitor H-7. Contractions of 0.3 μM methacholine appeared to be rapidly inhibited by 20 μM H-7 for approximately 50%. This effect was not confined to hormonal responses, because 40 mM KCl responses were affected in a similar way (data not shown). When muscle strips were subsequently desensitized with methacholine in the presence of 20 μM H-7, no attenuation of the desensitization dynamics was observed. A lagtime of 10 $\pm$ 1 min (mean $\pm$ s.e.mean, $n = 8$) was still observed in the presence of H-7.

Discussion

Desensitization is a frequently encountered phenomenon, when receptor systems are stimulated for a prolonged period or activated with high concentrations of stimulant. Such a negative feedback process can be the result of various mechanisms and is not necessarily confined to the effects of the stimulant (Hollenberg, 1985a,b).

Much work on desensitization has been performed with the β-adrenoceptor, which is capable of activating adenylate cyclase through its interaction with the stimulatory G-protein G_s (Lefkowitz *et al.*, 1983). The process of desensitization of the β-adrenoceptor has been shown to involve several mechanisms, such as activation of cyclic AMP phosphodiesterase (Taylor, 1987) or phosphorylation of the β-receptor itself (Benovic *et al.*, 1986; Clark *et al.*, 1989).

Yet insights into the mechanisms of desensitization of another major class of receptors, the Ca^{2+}-mobilising receptors, are still lacking. This is mainly due to the fact that these receptors act in a more complex way compared to the cyclic AMP producing receptors (Berridge & Irvine, 1989). At this time all the involved components are not yet known. In visceral smooth muscle the histamine H_1-receptor is considered to be a Ca^{2+}-mobilising receptor, that couples with the phospholipase C, via an as yet unidentified G-protein (G_p) (Best *et al.*, 1985; Bielkiewicz-Vollrath *et al.*, 1987). Previously H_1-receptor desensitization was examined in guinea-pig taenia coli (Hishinuma & Uchida, 1988). However, in this study the effects of short-term desensitization on contractions due to Ca^{2+}-release from intracellular stores was investigated, by use of the procedure of Ca^{2+}-loading via previous KCl depolarization (Casteels & Raeymaekers, 1979). As already discussed in the introduction, the contractions of intestinal smooth muscle are accompanied by a marked membrane depolarization and are highly dependent upon influx of extracellular Ca^{2+} (Bolton *et al.*, 1981; Best *et al.*, 1985; Morel *et al.*, 1987; Yamanaka & Kitamura, 1987). Moreover in Ca^{2+}-free medium only high concentrations of agonist can elicit contractions after preloading the intracellular store (Casteels & Raeymaekers, 1979; this study). However, this observation does not imply that at low concentrations of agonist there is no Ca^{2+}-release. Previously Himpens *et al.* (1989) showed that in the depolarized ileum, intracellular Ca^{2+}-levels and smooth muscle contractions do not always correlate. Based on these findings, measurement of intracellular Ca^{2+}-dependent contractions might not be relevant to the phenomena under physiological conditions. Moreover, since the procedure of Ca^{2+}-loading does not allow the measurement of contractile responses immediately after the period of desensitization, we examined the effects of desensitization with histamine and methacholine in detail under more physiological conditions using a superfusion system which also enabled recovery of the contractions to be monitored very easily.

Responses to 0.3 μM histamine (approximately the EC_{50} concentration) were markedly affected by a short-term desensitization with 100 μM histamine (1–5 min). Initial responses were almost completely abolished just after the desensitization period. Within a few minutes responses recovered and attained a stable level of contraction after approximately 40 min. Nevertheless it should be noted that responses to 0.3 μM histamine did not reach control levels within 3 h. When the effect of desensitization was studied on the responses to different concentrations of histamine, it became clear that maximal responses to histamine were not modified. After desensitization with 100 μM histamine a rightward shift of the histamine dose-response curve was induced. Under Ca^{2+}-free conditions the intracellular Ca^{2+}-dependent contractions induced by a maximally effective concentration of histamine were attenuated after desensitization with histamine. Responses to this concentration were not modified in Ca^{2+}-containing medium, indicating that massive Ca^{2+}-release from the intracellular pool may not be very important for the final contraction.

Since it is known that a receptor reserve for histamine exists in intestinal smooth muscle (Cook *et al.*, 1988), two mechanisms can explain the observed pD_2-shift. First, the resulting effect of the desensitization with 100 μM histamine may be caused by an alteration at the level of the H_1-receptor. A second mechanism could involve the modulation of some steps in the signal transduction, distal to the activated receptor. As desensitization with 100 μM histamine appeared to be homologous with respect to methacholine responses, this possibility is not very likely. Previously it has also been proposed that desensitization of the histamine responses in guinea-pig ileal smooth muscle (Kenakin & Cook, 1979; Bielkiewicz & Cook, 1984), BC3H-1 smooth muscle cells (Brown *et al.*, 1986) and guinea-pig taenia coli (Hishinuma & Uchida, 1988) could be explained by an alteration at the H_1-receptor. In contrast to our findings, Hishinuma & Uchida observed in guinea-pig taenia coli a histamine-induced desensitization that was reflected by a depression of the maximal response. This desensitization totally recovered in time (Hishinuma & Uchida, 1987). These discrepancies probably reflect differences in receptor density and receptor modification in the two tissues.

To our knowledge this is the first observation that the desensitization process of H_1-receptor responses by histamine can be divided in two phases. Just after desensitization responses to 0.3 μM histamine were almost completely inhibited. However, the contractions recovered rather rapidly to a certain extent. Since responses did not completely return to control levels, there appear to be two distinct modifications of the histamine H_1-receptor function, each resulting in a shift of the dose-response relationship and each recovering with a different time course. Although we have no data to support such a hypothesis, we suggest that the H_1-receptor is modified at

two different sites, that are both essential for agonist activity. Modification of one site can easily be reversed, resulting in a rapid recovery of the response. This site is very important for agonist activity, since responses are initially inhibited for approximately 90%. Modification of the second site is not easily reversed and results in a long-lasting change of agonist activity. Recently Hollenberg (1985b) identified a variety of receptor modifications, which potentially play a role in regulating receptor function. Receptor phosphorylation and disulphide-sulphydryl exchange reactions appear to be important protein modifications in this respect (Hollenberg, 1985b). At this time there are no data on phosphorylation of the histamine H_1-receptor, whereas disulphide-sulphydryl exchange has indeed been shown to affect H_1-receptor activity (Donaldson & Hill, 1987; Leurs et al., 1990). Nevertheless, phosphorylation of hormone receptors appears to be a quite common mechanism of receptor regulation (Leeb-Lundberg et al., 1987; Benovic et al., 1989; Clark et al., 1989). Such a hypothetical phosphorylation is probably not caused by a negative feedback via protein kinase C. Although the protein kinase C activator PDB can rapidly reduce histamine-induced responses, methacholine-induced responses were equipotently inhibited. Protein kinase C is therefore probably not implicated in the development of homologous H_1-receptor desensitization.

Although muscarinic receptor-mediated contractions of intestinal smooth muscle are also supposed to involve the mobilisation of Ca^{2+} after IP_3 and DAG formation (Best et al., 1985; Bielkiewicz-Vollrath et al., 1987), a completely different phenomenon occurred after desensitization with methacholine. After a 5 min period of desensitization with 100 μM methacholine longitudinal smooth muscle strips showed heterologous desensitization with respect to histamine H_1-receptor responses. Desensitization with methacholine induced a refractory period, in which H_1-receptor or muscarinic receptor stimulation did not lead to any observable contraction of the smooth muscle strips. The length of this period is dependent on the period of desensitization. During this refractory period the smooth muscle is still able to respond to a membrane depolarization with 40 mM KCl, indicating that the heterologous desensitization cannot be explained by a decrease in the sensitivity of the intestinal smooth muscle to Ca^{2+} (Himpens et al., 1989). These data suggest that after desensitization with methacholine, a step in the signal transduction pathway of the Ca^{2+}-mobilising receptors is modulated. This modulation can be explained by a negative feedback via protein kinase C. The protein kinase C activator, PDB, rapidly reduced both histamine and methacholine responses, but did not inhibit KCl-induced contractions, excluding a non-specific effect of PDB on contractile responses. A role for protein kinase C in the methacholine-induced desensitization was further studied with the protein kinase C inhibitor H-7. This compound already inhibits contractile responses to methacholine. Since KCl-induced responses were also reduced, this action is unrelated to protein kinase C inhibition. H-7 did not attenuate the effect of methacholine desensitization on the remaining responses. This indicates that protein kinase C may not be involved in the heterologous desensitization. However, due to the non-specific effects of H-7 on the contractions the interpretation of the data is difficult. The development of highly specific protein kinase C inhibitors is needed to address the question if protein kinase C is really not implicated in the development of heterologous desensitization.

Responses to methacholine quickly recover after the methacholine desensitization. This recovery is not dependent on the presence of extracellular Ca^{2+}, although the intracellular Ca^{2+}-store is highly dependent on extracellular Ca^{2+}. Hormonal responses are rapidly eliminated after omission of extracellular Ca^{2+}, whereas caffeine (10 mM) was unable to elicit a contraction in Ca^{2+}-free buffer (data not shown). When Ca^{2+} was omitted from the superfusion fluid just after desensitization the refractory period after methacholine desensitization was not altered, indicating that Ca^{2+}-influx is not the rate-determining step in the process of recovery and is therefore probably not altered as a consequence of the desensitization.

Moreover, enhanced Ca^{2+}-efflux from the cytosol to the extracellular space is unlikely to be involved in the desensitization. Contractions after 40 mM KCl application were not affected by methacholine desensitization. This is in contrast with previous findings on guinea-pig taenia coli, where the tonic component of the KCl-induced contractions was inhibited after excessive stimulation with carbachol (Hishinuma & Uchida, 1988). Since the tonic component of the KCl contraction of taenia coli is considered to be dependent on influx of Ca^{2+} (Karaki & Weiss, 1988), these data indicate that a reduction of Ca^{2+}-influx or an enhancement of the Ca^{2+}-efflux might be involved in this preparation.

To study the involvement of the intracellular Ca^{2+} store, the possible target of IP_3, we performed experiments in Ca^{2+}-free medium after KCl depolarization. Under our experimental conditions the intracellular Ca^{2+}-dependent contractions induced by 100 μM methacholine were hardly affected by 5 min desensitization with 100 μM methacholine. This can be explained by a complete recovery of the desensitization during the loading procedure. This is in contrast with the findings in guinea-pig taenia coli (Hishinuma & Uchida, 1988). However, differences in Ca^{2+}-transport kinetics between the two tissues can explain these findings (Casteels & Raeymaekers, 1979). In Ca^{2+}-containing medium the carbachol responses of guinea-pig taenia coli were also not attenuated after previous desensitization (Hishinuma & Uchida, 1988). This shows that in the taenia coli the responses after muscarine receptor desensitization can indeed recover completely as observed in this study and again shows that massive Ca^{2+}-release from the intracellular pool may not be very important for the final contraction.

Due to the rapid recovery of the methacholine-responses, the intracellular Ca^{2+}-dependent contraction appeared not to be very suitable for the study of the role of the intracellular Ca^{2+} store in the methacholine-induced desensitization of guinea-pig jejunum. Therefore experiments with caffeine were performed. Caffeine has been shown to be an activator of the Ca^{2+}-induced Ca^{2+}-release from an intracellular Ca^{2+}-store (Palade et al., 1989). This store is considered to be involved in the IP_3-induced mobilisation of Ca^{2+} (Berridge & Irvine, 1989). Moreover, caffeine has been shown to contract visceral smooth muscle preparations (Hishinuma & Uchida, 1988; Watson et al., 1988), despite its known inhibitory action on cyclic AMP phosphodiesterase. In the present study we showed that 10 mM caffeine produced transient contractions of jejunal longitudinal smooth muscle. However, if caffeine is added just after desensitization with 100 μM methacholine (i.e. during the refractory period) no response of the smooth muscle is observed. After a few minutes methacholine responses quickly recover to the control level; at this time caffeine is also able to elicit contractions again. Previously caffeine-induced contractions were reported to be reduced after heterologous desensitization produced by excessive muscarinic receptor stimulation in guinea-pig taenia caecum (Hishinuma & Uchida, 1988). These data strongly suggest that the heterologous desensitization can be explained by assuming that the intracellular Ca^{2+}-store is depleted or cannot be activated by IP_3. Since caffeine-induced responses are also highly affected, IP_3-receptor desensitization is probably not involved. Ineffective Ca^{2+}-pump or Ca^{2+} release mechanisms of the endoplasmatic reticulum might explain the observed findings. However, these mechanisms recover rather rapidly (within minutes) after desensitization. The findings with caffeine seem to conflict with the previous suggestion that the contribution of the intracellular Ca^{2+}-release is not very important for the contractions under normal conditions. However, we would like to speculate upon a limited, but obligatory role of Ca^{2+}-release from the intracellular store. As is shown in the experiments with caffeine, intracellular Ca^{2+}-

release is impaired after methacholine desensitization. At that time no responses to methacholine can be observed. Yet, there is no direct correlation between the final contraction and the intracellular Ca^{2+}-dependent response. Whereas the intracellular Ca^{2+}-dependent responses to maximally effective concentrations of histamine were attenuated after desensitization, the final response to these concentrations of histamine in Ca^{2+}-containing medium was not reduced. This indicates that only a limited release of Ca^{2+} from the intracellular store is sufficient to elicit maximal responses. This Ca^{2+}-release might be involved in triggering the influx of extracellular Ca^{2+}. Previously this has indeed been suggested as a regulatory mechanism of Ca^{2+}-entry in the guinea-pig ileum (Watson *et al.*, 1988).

In conclusion, in this study we show different profiles of desensitization dynamics of contractile responses of longitudinal smooth muscle of guinea-pig jejunum. After excessive H_1-receptor stimulation a homologous desensitization is observed. This desensitization can be divided into two phases: responses are first blocked to a large extent, but recover rather quickly. Yet, after this recovery, responses to low concentrations of histamine are still inhibited, due to a pD_2-shift of the histamine dose-response curve. We suggest that this desensitization is caused by an alteration at the level of the H_1-receptor, which is not caused by protein kinase C. Currently we are investigating this possibility, using [^{3}H]-mepyramine binding studies and a partial agonist. Yet, if methacholine is used as desensitizing agent a heterologous desensitization occurs, reflected by the development of a refractory period. This desensitization is probably caused by an effect on the IP_3-sensitive intracellular Ca^{2+}-pool.

At this moment it is not clear why such a difference exists in response to excessive stimulation with agents which are both considered to act via the phosphoinositide cascade. A reasonable explanation may be the existence of a system that selectively acts on the H_1-receptor, as has previously been found for the β-adrenoceptor (Benovic *et al.*, 1986). However, there are still no data that can substantiate this suggestion, although the β-receptor kinase is probably a member of a large family of closely related enzymes (Benovic *et al.*, 1989).

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Prostanoid stimulation of anion secretion in guinea-pig gastric and ileal muscosa is mediated by different receptors

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1 The receptors that mediate stimulation of anion secretion by prostanoids in isolated preparations of guinea-pig gastric and ileal mucosa have been compared by use of selective prostanoid agonists and antagonists.

2 In gastric mucosa, the relative potency of agonists suggested that the control of anion secretion in this tissue was complex and may be mediated by EP_2, EP_3, and TP receptors. A role for TP receptors was confirmed with the TP-selective antagonist AH23848 which inhibited short circuit current responses to the TP receptor agonist U-46619 with a pA_2 value of 8.44 but was without effect on responses to prostaglandin E_2 (PGE_2) or the EP selective agonist, sulprostone.

3 In ileal mucosa, the relative potency of agonists differed from that observed in gastric mucosa and was consistent with the view that anion secretion in this region of intestine was controlled by DP and EP_2 receptors.

4 These studies suggest that anion secretion in gastric and ileal mucosa is controlled by different prostanoid receptor subtypes and so provide important informtion for the design of prostanoids which may protect gastric mucosa and that are free from side effects such as diarrhoea.

Introduction

Prostanoids exert many diverse actions in the body, and these effects have been rationalised through the classification of prostanoid receptors. Such classification has been carried out by examining the rank orders of agonist potencies of the natural prostaglandins, and this has resulted in the identification of receptors for thromboxane A_2 (TP receptors), E prostaglandins (EP receptors), F prostaglandins (FP receptors), D prostaglandins (DP receptors) and prostacyclin (IP receptors) (Kennedy *et al.*, 1982). This classification has been consolidated by use of synthetic agonists exhibiting receptor specificity including U-46619 (TP selective), BW245C (DP selective) and fluprostenol (FP selective; Coleman *et al.*, 1985) and selective antagonists such as AH23848 for TP receptors (Brittain *et al.*, 1985). Furthermore studies using the synthetic agonists sulprostone and AY23626 and the antagonists AH6809 and SC19220 have revealed that EP receptors are a heterogeneous population comprising EP_1, EP_2 and EP_3 subtypes (Coleman *et al.*, 1987).

Prostanoids stimulate the gastric secretion of a non-parietal fluid composed mainly of sodium and chloride ions (Miller *et al.*, 1983; Bunce & Clayton, 1987). This non-parietal secretion may be an important component of the mucosal protective properties of prostanoids in the stomach acting to reduce the absorption of noxious substances and diluting them in bulk solution (Moody & Zalewsky, 1981; Thomson, 1984; Pihan & Szabo, 1989). Prostanoids also stimulate electrolyte secretion in the intestine (Racusen & Binder, 1980; Musch *et al.*, 1987) that is responsible for the watery diarrhoea which has tended to limit the clinical use of prostanoids for treatment of peptic ulceration (Brand *et al.*, 1985; Lauritsen *et al.*, 1986).

Using an isolated preparation of guinea-pig gastric mucosa, we have previously demonstrated that gastric non-parietal secretion stimulated by prostaglandin E_2 (PGE_2) is the result of stimulation of electrogenic chloride secretion, a process that is independent of gastric acid secretion (Bunce & Spraggs, 1988). In the present study we have attempted to characterize the receptor types that mediate the secretory effect of prostanoids in gastric mucosa and compare them with the receptors that mediate stimulation of electrogenic anion secretion in the ileal mucosa of this species.

Portions of this work have been communicated to the British Pharmacological Society (Bunce & Spraggs, 1987) and the 13th International Congress of Gastroenterology (Spraggs & Bunce, 1988). These studies suggest that stimulation of electrogenic anion secretion by prostanoids in gastric and ileal mucosa is mediated by different prostanoid receptor types.

Methods

Male guinea-pigs of the Dunkin-Hartley strain weighing 250–350 g were used. For gastric mucosa, guinea-pigs were starved for 18 h to 24 h with free access to water and were anaesthetized by inhalation of a halothane/nitrous oxide/O_2 gas mixture. The muscle layers overlying the fundic (acid-secreting) region of the stomach were removed by a blistering technique as described by Main & Pearce (1978). Two pieces of gastric mucosa were obtained from each stomach and these were mounted in Ussing type chambers (window area 0.8 cm^2).

For ileal mucosa, guinea-pigs with free access to both food and water were killed by cervical dislocation. A 10 cm segment of ileum starting 20 cm from the ileo-caecal junction was removed. Up to four pieces of this segment, each approximately 1.5 cm long were opened along the mesenteric border and pinned, mucosal face down on a wax plate. The overlying muscle layers were removed by dissection with fine forceps and each segment was mounted in an Ussing chamber.

Both preparations were bathed bilaterally with 20 ml of Krebs-Henseleit solution, maintained at 37°C and gassed with 95% O_2:5% CO_2. The composition of this solution (in mM) was: NaCl 117, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 24.8, KH_2PO_4 1.2 and glucose 11.1. Indomethacin (1 μM) was added to the serosal bathing solution to inhibit endogenous prostanoid synthesis.

Tissues were continuously voltage-clamped at zero potential, with compensation for fluid resistance and differences in

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potential between calomel cells using a voltage clamp amplifier (DVC-1000, World Precision Instruments, Connecticut, U.S.A.) and the applied short circuit current (SCC) was continuously recorded as an index of electrogenic anion secretion (Bunce & Spraggs, 1988).

Compounds

Indomethacin (Sigma) was dissolved in 1% $NaHCO_3$ in saline at a concentration of 1 mM. PGE_2 and $PGF_{2\alpha}$ (Upjohn) were diluted for use from stock solutions of 10 mg ml^{-1} and 5 mg ml^{-1} respectively. Fluprostenol (ICI) was diluted from a stock solution of 5 mg ml^{-1}. BW245C (3-(3-cyclohexyl-3-hydroxypropyl)-2,5-heptenoic acid; Wellcome) was dissolved in 1% $NaHCO_3$ at a concentration of 10 mM. AY23626 (11-deoxy PGE_0; Ayerst), 16,16-dimethyl PGE_2 (Cayman), misoprostol (Searle) and enprostil (Syntex) were dissolved in 60% ethanol in distilled water at a concentration of 20 mg ml^{-1}. PGD_2, PGI_2, U-46619 (11,9 epoxymethano PGH_2) and sulprostone were synthesized at Glaxo and dissolved in 60% ethanol in distilled water at a concentration of 20 mg ml^{-1}, except for PGI_2 which was dissolved in Tris buffer (pH 9). AH23848 ([1α(z),2β,5α]-(±)-7-[5-[[(1,1-biphenyl)-4-yl] methoxy]-2-(4-morpholinyl)-3-oxocyclopentyl]-4-heptenoic acid) and AH6809 (6-isopropoxy-9-oxoxanthene-2-carboxylic acid) were also synthesized at Glaxo and dissolved in 1% $NaHCO_3$ in saline at a concentration of 10 mM. All drugs (except for PGI_2, which was diluted in Tris buffer, pH 8) were diluted in saline and added to both bathing solutions in volumes of less than 0.6 ml. The vehicles used to dissolve these drugs did not modify SCC when added to bathing solutions at appropriate concentrations.

Expression of results

SCC was recorded as μA cm^{-2} and expressed as arithmetic mean ± s.e.mean. EC_{50} values were expressed as geometric mean with 95% confidence limits.

Preliminary studies showed that gastric mucosa exhibited tachyphylaxis to repeated administration of PGE_2 and therefore concentration-response curves were constructed by addition of single concentrations of agonist to individual mucosa using a randomised block design. Increases in SCC to agonists were measured as changes in SCC from basal values after a contact time of 60 min. For full agonists, with maximal responses not significantly different from that of PGE_2, EC_{50} values were defined as the concentration to produce an increase in SCC of 38 μA cm^{-2}, this being 50% of the maximal response to PGE_2 (77 ± 6 μA cm^{-2}, $n = 6$). For partial agonists EC_{50} values were calculated at 50% of their own maximum. EC_{50} values were determined from pooled data for individual agonists by least squares regression. For full agonists only equieffective molar ratios (EMR) were determined relative to PGE_2 (EMR = 1) by dividing the mean agonist EC_{50} by the EC_{50} value for PGE_2, and these data were used for receptor classification. In experiments where antagonists were tested, these compounds were added to both bathing solutions 30 min prior to the addition of agonist. Where employed, Schild analysis was performed by determination of concentration-ratios by dividing the EC_{50} for the agonist in the presence of antagonist by the EC_{50} value for the agonist alone. Concentration-ratios were then used to calculate pA_2 and Schild slope values from a plot of the Schild equation.

In ileal mucosa, it was possible to construct repeatable cumulative concentration-response curves for PGE_2. Therefore the effects of agonists on SCC in ileal mucosa were compared with the effects of PGE_2 in individual tissues. Increases in SCC (above basal levels) for agonists were expressed as percentages of the control PGE_2 maximal response in the tissue. EC_{50} and EMR values for agonists were determined graphically in individual tissues and subsequently pooled to provide geometric mean and 95% confidence limits. EC_{50} values for full agonists were determined from the PGE_2 maximum as described above for gastric mucosa. Again, EMRs were only calculated for full agonists (PGE_2 = 1).

Results

Effects of prostanoids on gastric mucosa

The naturally occurring prostanoids and the stable thromboxane A_2-mimetic, U-46619 stimulated increases in SCC in gastric mucosa. Two types of SCC response were observed and are illustrated in Figure 1.

PGE_2 (and PGI_2) stimulated a monophasic rise in SCC with an initial rapid increase followed by a slower rise to a plateau which was achieved approximately 30 min after drug addition. In contrast U-46619 (and PGD_2 and $PGF_{2\alpha}$) produced biphasic responses composed of an initial small reduction in SCC and followed by a slow increase to a plateau 60 min after drug addition. Figure 2 shows concentration-response curves for these prostanoids in gastric mucosa and their effects are summarised in Table 1. PGE_2 was the most potent agonist producing a maximum increase in SCC of 77 ± 6 μA cm^{-2} ($n = 6$). U-46619, PGD_2 and $PGF_{2\alpha}$ were full agonists, with maxima not significantly different from PGE_2 ($P > 0.05$, unpaired t test), but were less potent. PGI_2 was a less potent partial agonist with a maximum response significantly less than that for PGE_2 ($P < 0.05$, unpaired t test).

Since PGE_2 was the most potent of these agonists this suggests that EP receptors are involved in the SCC response in gastric mucosa (Kennedy *et al.*, 1982). The involvement of other receptor types was investigated using the TP receptor antagonist AH23848 and the DP and FP selective agonists BW245C and fluprostenol. AH23848 (30 to 300 nM) inhibited SCC responses to U-46619 with a pA_2 value of 8.44 and a Schild slope value of 0.98 (Figure 3). A higher concentration of AH23848 (1 μM) did not modify SCC responses to PGE_2 (data not shown). BW245C was a less potent stimulant of SCC with an EC_{50} value of 348 (97–853) nM ($n = 6$) and fluprostenol produced no changes in SCC at concentrations up to 1 μM ($n = 4$) (Table 1). The low potency of these selective agonists suggests that DP and FP receptors are not involved in the control of SCC in gastric mucosa.

The EP receptor types mediating the increase in SCC in gastric mucosa were investigated further by use of a range of

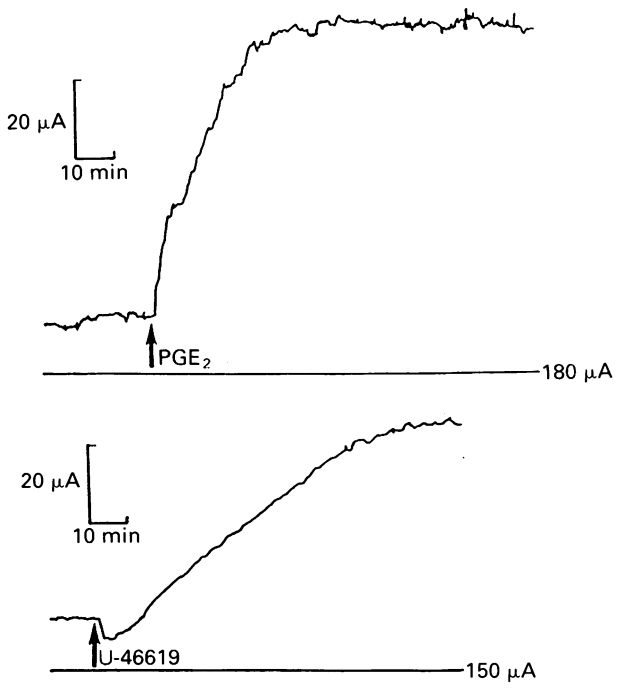

Figure 1 Examples of SCC recordings for prostaglandin E_2 (PGE_2) and U-46619 in guinea-pig isolated gastric mucosa. Records were obtained from 0.8 cm^2 areas of mucosa. Prostanoids were added to both bathing solutions (at arrow) at a concentration of 1 μM. Horizontal lines show SCC values of 180 and 150 μA respectively.

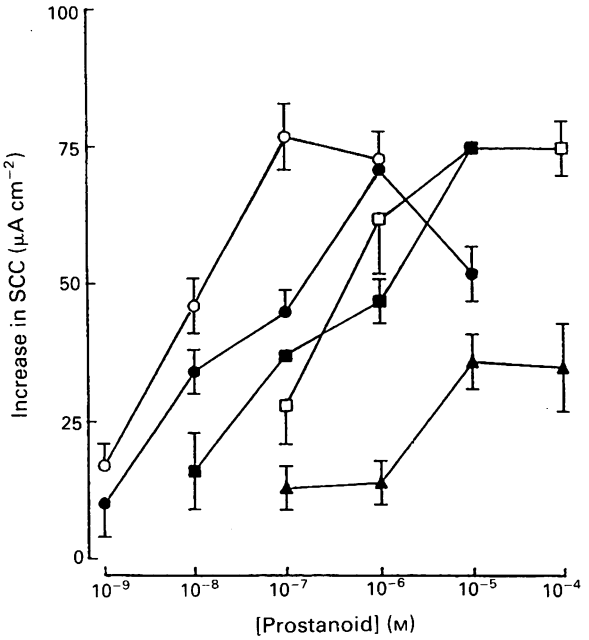

Figure 2 Concentration-response curves for stimulation of SCC by naturally occurring prostanoids and U-46619 in guinea-pig isolated gastric mucosa. Curves were constructed by addition of single concentrations of agonists to individual tissues. Each point is mean, vertical bars represent s.e.mean from 5 to 6 tissues: (○) prostaglandin E_2 (PGE_2); (●) U-46619; (■) PGD_2; (□) $PGF_{2\alpha}$ and (▲) PGI_2.

synthetic PGE analogues; as described above for the naturally occurring prostanoids two types of SCC response were observed. AY23626 and misoprostol produced monophasic rises in SCC, similar to PGE_2, whilst enprostil, 16,16-dimethyl PGE_2 and sulprostone increased SCC in a biphasic manner similar to U-46619. Figure 4 shows concentration-response curves for these agonists in gastric mucosa and their effects are summarised in Table 1. All of the compounds were potent full agonists for stimulation of SCC with equieffective molar ratios (EMR) ranging from 0.4 to 15 (PGE_2 = 1). The possibility that the biphasic responders were acting solely at TP receptors to increase SCC was unlikely since AH23848 (1 μM) did not modify SCC responses to sulprostone (data not shown). In addition sulprostone induced increases in SCC were not inhibited by the EP_1 receptor antagonist AH6809 (10 μM) suggesting that EP_1 receptors did not mediate changes in SCC (data not shown).

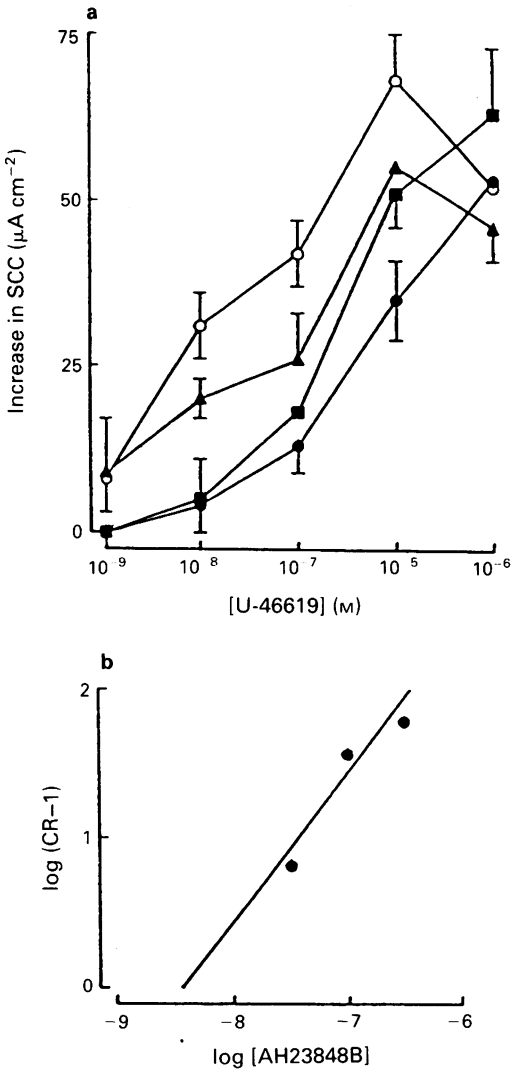

Figure 3 Effect of AH23848 on SCC responses to U-46619 in guinea-pig isolated gastric mucosa. In (a) are shown concentration-response curves for U-46619 alone (○) and in the presence of AH23848, 30 (▲), 100 (■) and 300 nM (●). Concentration-response curves were constructed in the manner described for Figure 2. Each point is the mean, vertical bars represent s.e.mean from 5 to 6 tissues. (b) Schild plot derived from these data. Linear regression analysis produced a pA_2 value of 8.44 and a slope of 0.98.

Table 1 Effects of prostanoid agonists on SCC in guinea-pig isolated gastric mucosa

Agonist	EC_{50}	*EMR* (PGE_2 = 1)	E_{max} (μA cm^{-2})
PGE_2	5 (2–11)	1	77 ± 6
PGD_2	162 (42–523)	31	75 ± 10
$PGF_{2\alpha}$	186 (37–510)	36	75 ± 10
PGI_2	2300 (900–8230)†	ND	36 ± 5*
U-46619	38 (16–96)	7	71 ± 8
BW245C	348 (97–853)	70	47 ± 5 (at 3 μM)‡
Fluprostenol	>1000†	ND	0 ± 0*
AY23626	58 (8–599)	11	65 ± 19
16,16 Dimethyl PGE_2	2 (0.3–19)	0.4	62 ± 4
Enprostil	4 (1–9)	0.8	94 ± 4
Misoprostol	12 (3–65)	2	67 ± 8
Sulprostone	76 (20–293)	15	65 ± 7

EC_{50} (in nM) values are geometric mean (95% confidence limits) and E_{max} values are arithmetic mean ± s.e.mean for 4 to 6 observations at each concentration. Equieffective molar ratios (EMR; PGE_2 = 1) were calculated for full agonists as the ratio of mean EC_{50} values of agonist and PGE_2.

* denotes E_{max} values significantly less than PGE_2 ($P < 0.05$, unpaired t test); for partial agonists EC_{50} values (†) were calculated from their own maximum.

ND denotes that EMR estimates were not determined for these partial agonists.

‡ Maximal response for BW245C was not achieved at the highest concentration tested.

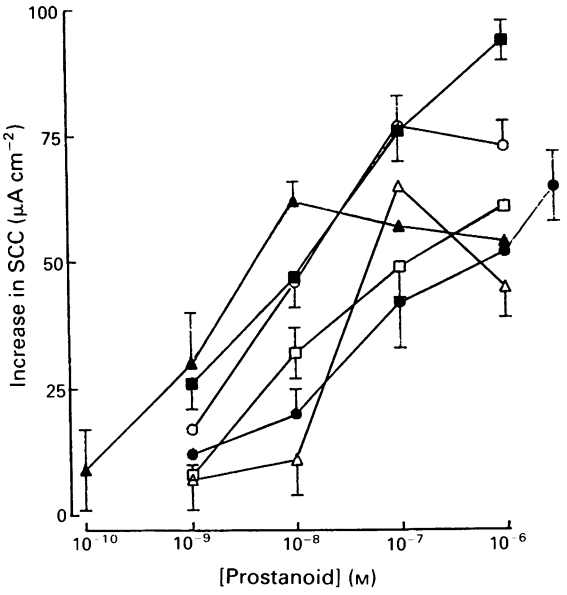

Figure 4 Concentration-response curves for stimulation of SCC by prostaglandin E (PGE) analogues in guinea-pig isolated gastric mucosa. Curves were constructed in the manner described in Figure 2. Each point is mean, vertical bars represent s.e.mean for 6 tissues: (○) PGE_2; (▲) 16,16-dimethyl PGE_2; (■) enprostil; (□) misoprostol; (△) AY23626 and (●) sulprostone.

Effects of prostanoids on ileal mucosa

PGE_2 was a potent stimulant of SCC in ileal mucosa with an EC_{50} value of 18 (5–54) nM and a maximum increase in SCC of $125 \pm 14\,\mu A\,cm^{-2}$ ($n = 6$). This curve could be repeated in tissues after washing with the second PGE_2 concentration-response curve producing an EC_{50} value of 20 (8–49) nM and a maximum increase in SCC of $120 \pm 9\,\mu A\,cm^{-2}$ ($n = 6$) at 100 nM. Therefore test prostanoids were compared with PGE_2 in individual tissues. Figure 5 compares the effects of the naturally occurring prostanoids and U-46619 on SCC and Table 2 summarizes the relative potencies of full agonists in ileal mucosa. In contrast to its activity in gastric mucosa, U-46619 in concentrations up to 1 μM produced a maximum increase in SCC of 14% of the PGE_2 maximum and did not modify subsequent responses to PGE_2 (data not shown). In addition, PGD_2 was 20 times more potent than PGE_2. PGI_2 and $PGF_{2\alpha}$ were only slightly less potent than PGE_2 but displayed non-sigmoid concentration-response relationships. The marked potency of PGD_2 suggested that DP receptors controlled SCC in ileal mucosa and this was confirmed with a selective DP agonist BW245C, which although producing a smaller maximum response than PGE_2 was a potent stimulant of SCC (Table 2).

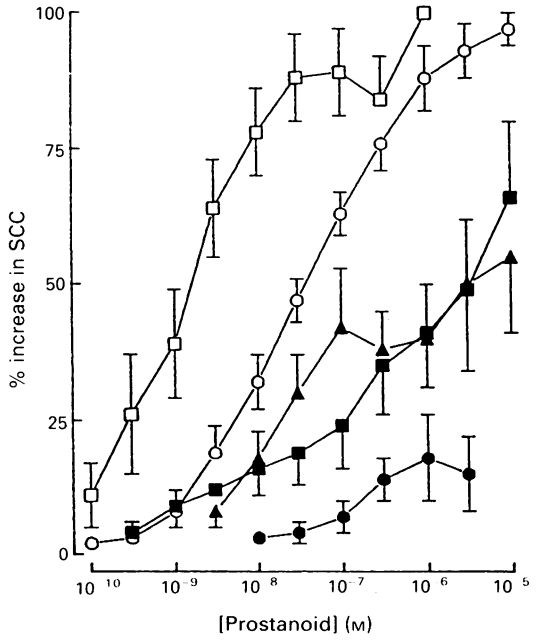

Figure 5 Concentration-response curves for stimulation of SCC by natural prostanoids and U-46619 in guinea-pig isolated ileal mucosa. Data are expressed as percentage of the maximal response to prostaglandin E_2 (PGE_2) in individual tissues. Curves are mean, vertical bars represent s.e.mean from 5 to 6 tissues: (□) PGD_2; (○) PGE_2; (▲) PGI_2; (■) $PGF_{2\alpha}$ and (●) U-46619. Mean EC_{50} values for PGE_2 in this series of experiments ranged from 35 to 57 nM.

The effects of the synthetic PGE analogues on SCC in ileal mucosa are shown in Figure 6. All of these analogues were less potent than PGE_2 and whilst AY23626 and misoprostol were full agonists, enprostil, 16,16-dimethyl PGE_2 and sulprostone were virtually devoid of activity (Table 2, Figure 6). Sulprostone produced no change in SCC and did not display antagonist activity to subsequent additions of PGE_2 at concentrations up to 10 μM (data not shown), showing that it had no measurable affinity for the prostanoid receptor in ileal mucosa.

Discussion

Prostanoids stimulate electrogenic chloride secretion in gastric mucosa and this is reflected by an increase in SCC (Bunce & Spraggs, 1988). In addition it is well established that prostanoid-stimulated anion secretion results in an increase in SCC across ileal mucosa (Musch *et al.*, 1987). In the present study we have attempted to identify the prostanoid receptor types that mediate the stimulation of anion secretion (increases in SCC) in gastric and ileal mucosa. Few selective antagonists for prostanoid receptors exist and receptor classification relies greatly on the relative potencies of selective agonists (Coleman *et al.*, 1985). Receptor classification based on rank order of agonist potency must meet established criteria and may be influenced by factors not related to receptor

Table 2 Effects of prostanoid agonists on SCC in guinea-pig isolated ileal mucosa

Agonist	*EMR* ($PGE_2 = 1$)	E_{max} (%)
PGE_2	1	100
PGD_2	0.05 (0.04–0.08)	98 ± 1
$PGF_{2\alpha}$	ND	66 ± 3 (at 10 μM)‡
PGI_2	ND	55 ± 14 (at 10 μM)‡
U-46619	ND	14 ± 9*
BW245C	ND [EC_{50} = 10 (3–36)]†	65 ± 9*
AY23626	48 (28–86)	94 ± 6
16,16-dimethyl PGE_2	ND	10 ± 8*
Enprostil	ND	15 ± 5 (at 3 μM)‡
Misoprostol	10 (4–23)	80 ± 2
Sulprostone	ND	0 ± 0 (at 10 μM)‡

EMR values are geometric mean (95% confidence limits) calculated from EC_{50} ratios in individual tissues as described in Methods. E_{max} values are % increases in SCC, as arithmetic mean ± s.e.mean in individual experiments, where the maximal response to PGE_2 equalled 100%. Values are means from 4 to 8 tissues. Unlike PGE_2, $PGF_{2\alpha}$ and PGI_2 produced non-sigmoid concentration-response relationships.

* denotes E_{max} values significantly less than PGE_2 ($P < 0.05$, paired t test).

ND denotes that EMR estimates were not determined for these partial agonists.

† EC_{50} value given for BW245C is relative to its maximal response.

‡ Maximal responses for these compounds were not achieved at the highest concentration tested.

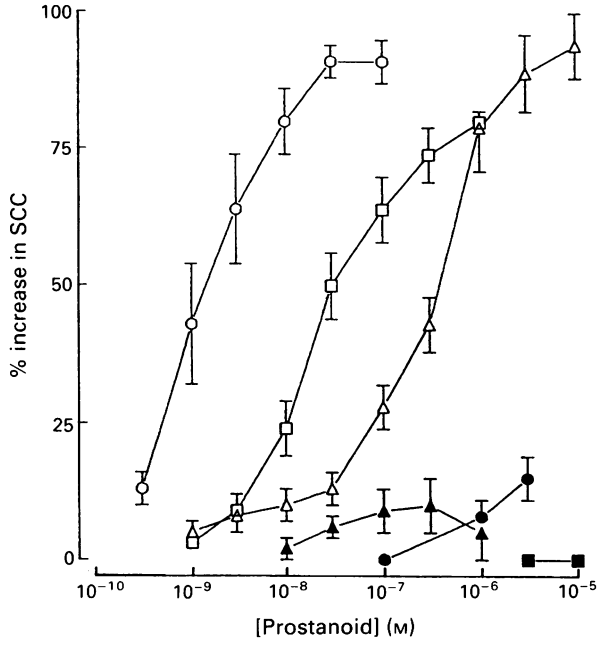

Figure 6 Concentration-response curves for stimulation of SCC by prostaglandin E (PGE) analogues in guinea-pig isolated ileal mucosa. Data are expressed as percentage of the maximal response to PGE_2 in individual tissues. Curves are mean, vertical bars represent s.e.mean from 4 to 8 tissues: (○) PGE_2; (□) misoprostol; (△) AY23626; (▲) 16,16 dimethyl PGE_2; (●) enprostil and (■) sulprostone. Mean EC_{50} values for PGE_2 in this series of experiments ranged from 1 to 30 nM.

differences (Furchgott, 1972). In the present studies some prostanoids were partial agonists or produced concentration-response curves whose slopes differed from PGE_2 and where this occurred, the compounds could not be used to compare responses in the two tissues. Nevertheless, in these studies the different activities of prostanoid agonists in these two tissues were striking and may be suggestive of a number of prostanoid receptor types involved in the control of gastrointestinal secretion.

Tables 1 and 2 show that the relative potencies of the naturally occurring prostanoids and U-46619 for stimulation of SCC in gastric and ileal mucosa are different. Comparison of these with rank orders described for a variety of smooth muscle preparations and platelets (Kennedy *et al.*, 1982) suggests that the effects of prostanoids on SCC may be mediated by multiple prostanoid receptor types in both mucosal tissues.

In gastric mucosa, PGE_2 was the most potent naturally occurring agonist, suggesting an involvement of EP receptors. However U-46619 was more potent in gastric muscosa compared with tissues reported to contain a single population of EP receptors suggesting that TP receptors were also involved in stimulation of SCC (Bunce & Spraggs, 1987). The TP receptor antagonist AH23848 confirmed an involvement of TP receptors. AH23848 inhibited SCC responses to U-46619 in gastric mucosa with a pA_2 value consistent with that for competitive antagonism of TP receptors (Brittain *et al.*, 1985). In contrast, a concentration of AH23848 approximately 300 times its dissociation constant for TP receptors (1 μM) did not modify SCC responses to PGE_2 further suggesting that EP receptors also mediated this response. The stimulation of SCC by PGD_2 and $PGF_{2\alpha}$ could not be explained by the presence of DP or FP receptors since the low potencies of the selective agonists BW245C and fluprostenol excluded an involvement of DP or FP receptors (Coleman *et al.*, 1985). It is possible that PGD_2 and $PGF_{2\alpha}$ stimulate SCC by acting at both EP and TP receptors. In this context, other studies have suggested that PGD_2 (Jones *et al.*, 1982) and $PGF_{2\alpha}$ (Kennedy *et al.*, 1982) display activity at TP receptors in various tissues. These results are consistent with the view that prostanoids stimulate electrogenic chloride secretion in gastric mucosa by actions at EP and/or TP receptors and this may explain the different SCC response profiles encountered with agonists (Figure 1).

In ileal mucosa PGD_2 was more potent than PGE_2, a finding similar to previous observations in this tissue (Baird *et al.*, 1984). These results differ from reports *in vivo* where PGD_2 (and also PGI_2) were weak stimulants of enteropooling in the rat (Robert *et al.*, 1979b). These prostanoids may have different secretory effects across species although PGI_2 has been reported to be a potent stimulant of SCC in rat isolated colonic mucosa (Georg *et al.*, 1984). Since enteropooling *in vivo* reflects net electrolyte and fluid balance, the present studies may not account for additional actions of PGD_2 and PGI_2, such as effects on intestinal blood flow or electroneutral sodium chloride absorption (Bunce *et al.*, 1986). BW245C was also a potent stimulant of SCC, suggesting that stimulation of SCC in ileal mucosa was mediated by DP receptors (Coleman *et al.*, 1985). In contrast to gastric mucosa, U-46619 was virtually inactive as both an agonist and an antagonist in ileal mucosa suggesting that TP receptors were not involved in this response *in vitro*. DP receptors are poorly characterized since tissues that contain them, such as human platelets, also contain other prostanoid receptor types (e.g. IP receptors) which mediate the same response (Miller & Gorman, 1979). However, in ileal mucosa PGE_2 was more potent than expected if the responses were mediated solely by DP receptors. Therefore it is likely that the stimulation of SCC in ileal mucosa by prostanoid receptor agonists is mediated by DP and/or EP receptors.

Since EP receptors appeared to mediate the stimulation of SCC by prostanoids in both gastric and ileal mucosa, more information regarding the EP receptor subtypes that control the effects was obtained with a range of synthetic PGE analogues. The activities of these compounds in the two mucosal preparations may be compared in Tables 1 and 2. In gastric mucosa all of these prostanoids were potent stimulants of SCC. Some of the agonists (sulprostone, enprostil and 16,16-dimethyl PGE_2) produced biphasic SCC response profiles similar to U-46619 (Figure 1). However, although sulprostone produced a similar response profile to U-46619, a high concentration of AH23848 (1 μM) did not modify the stimulation of SCC produced by sulprostone, thus excluding an involvement of TP receptors in this response. In contrast AY23626 and misoprostol produced monophasic increases in SCC similar to that observed for PGE_2 (Figure 1). The differential activities of sulprostone and AY23626 in isolated smooth muscle preparations have suggested a division of EP receptors into 3 subtypes (Coleman *et al.*, 1987). In gastric mucosa, the similar potencies of both sulprostone and AY23626 tended to exclude an involvement of EP_1 receptors. This lack of EP_1 receptor activity was confirmed by the lack of effect of the EP_1 receptor antagonist AH6809, which at 100 times its dissociation constant for EP_1 receptors (Coleman *et al.*, 1985) did not modify SCC responses to sulprostone. In addition, the rank order of agonist potency of the PGE analogues in gastric mucosa was not consistent with those observed for preparations reported to contain a single population of either EP_2 (Coleman *et al.*, 1988) or EP_3 receptors including inhibition of acid secretion in rat isolated gastric mucosa (Reeves *et al.*, 1988). Recent studies have shown that 16,16-dimethyl PGE_2 and enprostil are potent agonists at EP_3 receptors but weak at EP_2 receptors whilst misoprostol is equipotent at both EP_2 and EP_3 receptors (Coleman *et al.*, 1988). The possibility therefore exists that prostanoid agonists stimulate SCC in gastric mucosa by interaction with both EP_2 and EP_3 receptors.

In addition to stimulation of mucosal blood flow (Pihan *et al.*, 1986) and mucus secretion (Allen & Garner, 1980) a prostanoid-stimulated secretion of electrolyte and fluid may have a role in the protective actions of prostanoids in gastric mucosa, as suggested previously (Moody & Zalewsky, 1981; Thomson, 1984). Such a secretion would wash noxious agents away from the mucosal surface. Indeed the promotion of an

unstirred water layer at the surface of an epithelium by fluid secretion results in a reduction in solute absorption (Thomson, 1984; Pihan & Szabo, 1989) and may therefore prevent the absorption of substances damaging to gastric mucosa. In this context prostanoids that stimulate gastric non-parietal secretion such as 16,16 dimethyl PGE_2 (Robert *et al.*, 1979a), enprostil (Rozkowski *et al.*, 1986), misoprostol (Gana *et al.*, 1989) and U-46619 (Bunce & Clayton, 1987) have been shown to protect the gastric mucosa from damage.

The relative potencies of PGE analogues in ileal mucosa differed markedly from their relative potencies in gastric mucosa (Tables 1 and 2), consistent with the view that different EP receptor subtypes control secretion in these two regions of the gastrointestinal tract. The rank order of agonist potencies for these prostanoids in ileal mucosa was very similar to that encountered in cat trachea, a tissue reported to contain EP_2 receptors (Coleman *et al.*, 1988) and therefore it is likely that prostanoid-stimulated SCC in ileal mucosa is mediated by EP_2 receptors. It is perhaps not coincidental that prostanoids displaying potent full agonist activity in ileal mucosa (PGE_2, AY23626 and misoprostol) produced the same shape of response (monophasic) in gastric mucosa, whilst those compounds with little activity on ileal mucosa (sulprostone, enprostil and 16,16 dimethyl PGE_2) were biphasic responders in gastric mucosa. These observations support the contention that SCC in gastric mucosa is controlled by both EP_3 and EP_2 receptors. In view of the high diarrheogenic potency of 16,16 dimethyl PGE_2 and enprostil in rats *in vivo* (Robert *et al.*, 1979b; Rozkowski *et al.*, 1986), the lack of effects of these prostanoids upon SCC in ileal mucosa was surprising. This discrepancy may be related to the high metabolic stability of these analogues *in vivo*. In addition these prostanoids potently inhibit electroneutral NaCl absorption in the rat small intestine (Clayton *et al.*, 1988) and this effect of 16,16 dimethyl PGE_2 on fluid secretion in the colon may be a more important determinant of diarrhoea (Sernka *et al.*, 1982).

This classification of prostanoid receptors that mediate electrogenic anion secretion in the stomach and intestine has been based on the activities of a range of agonists with differing selectivities for prostanoid receptors. The classification has been complicated by the presence of multiple receptors controlling secretion in these different regions of the gastrointestinal tract. The antagonist activity of AH23848 provided unequivocal evidence of an involvement of TP receptors in the stimulation of gastric mucosal electrolyte secretion whilst the differing relative potencies of prostanoids in these tissues provided evidence of an involvement of other receptors. The definitive role of EP and DP receptors in these secretory responses requires the identification of antagonists with selectivity for these receptor types.

In summary, these studies of prostanoid-stimulated electrogenic chloride secretion in gastric mucosa suggest that the control of this secretion is complex. Furthermore comparisons of the effects of prostanoids in gastric and ileal mucosa suggest that secretion by these two regions of gastrointestinal tract may be controlled by different combinations of prostanoid receptors. This information may be important in the development of mucosal protective prostanoids that are free from side effects, such as diarrhoea.

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Role of eicosanoids in PAF-induced increases of the vascular permeability in rat airways

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1 Platelet activating factor (PAF; 1.0 and 5.0 $\mu g\,kg^{-1}$) injected in the tail vein of unanaesthetized rats dose-dependently increased the vascular permeability of the trachea, upper and lower bronchi (up to 400%) as measured by the extravasation of Evans blue dye. The permeability of the parenchyma was not affected by PAF treatment.

2 Pretreatment of the animals with an intravenous injection of the PAF antagonist BN-52021 ($10\,mg\,kg^{-1}$) abolished almost totally the vascular permeability changes elicited by PAF injection ($5.0\,\mu g\,kg^{-1}$).

3 Pretreatment of the animals with intravenous injections of inhibitors of thromboxane formation, indomethacin ($10\,mg\,kg^{-1}$) and compound OKY-046 ($10\,mg\,kg^{-1}$), and thromboxane antagonist, compound L-655,240 ($5\,mg\,kg^{-1}$), partially reduced PAF effects in the airways (from 28 to 69%). The thromboxane mimic U-44069 ($5.0\,\mu g\,kg^{-1}$) did not modify the vascular permeability of rat airways. The effect of a low dose of PAF ($0.1\,\mu g\,kg^{-1}$) on the vascular permeability of the trachea and bronchi (but not of the parenchyma) was potentiated by compound U-44069 ($5.0\,\mu g\,kg^{-1}$) or noradrenaline ($400\,ng\,kg^{-1}$) whereas the effect of a high dose of PAF ($5.0\,\mu g\,kg^{-1}$) was not affected.

4 Neither the peptidoleukotriene antagonist MK-571 ($10\,mg\,kg^{-1}$) nor the 5-lipoxygenase inhibitor, L-663,536 ($10\,mg\,kg^{-1}$) given before the injection of PAF ($5.0\,\mu g\,kg^{-1}$) affected the protein extravasation in rat lung tissues.

5 These data suggest that the effect of PAF on rat vascular permeability is partly modulated by thromboxane formation although thromboxanes have no direct effect on the permeability. Thromboxane may act via a vasoconstriction that increases hydrostatic pressure and potentiates the extravasation elicited by PAF effect on endothelial cells.

6 Leukotrienes do not appear to be involved in the changes of rat airway permeability induced by PAF.

Introduction

Platelet activating factor (PAF), a potent inflammatory mediator, induces airway microvascular leakage by an uncharacterized mechanism. In many species, the effect of PAF seems to be dependent on the activation of platelets and polymorphonuclear leukocytes and is most probably mediated by the release of secondary messengers (for a review, see Braquet *et al.*, 1987). Inarrea *et al.* (1984), demonstrated that rat platelets have no specific binding sites for PAF-acether. This finding correlated well with the reports which showed that the effect of PAF in rats is independent of the presence of platelets (Wedmore & Williams, 1981; Pirotzky *et al.*, 1984) and neutrophils (Pirotzky *et al.*, 1984). Dewar *et al.* (1983) and Bjork & Smedegard (1983) confirmed by microscopy that PAF acts on the vascular endothelium, produces disjunction of endothelial cells of post-capillary venules and increases vascular permeability. Bolin *et al.* (1987), showed that fluid movement across the endothelial membrane into the interstitial space is dependent on hydrostatic and osmotic pressure gradients as well as endothelial permeability. However, the studies quoted above did not distinguish whether the extravasation elicited by PAF was secondary to increased hydrostatic forces or leakage resulting from increased permeability.

The aims of the following experiments were: (1) to study the effects of PAF on the vascular permeability of rat airways; (2) to determine the mediatory role of eicosanoids in PAF effects and, (3) to characterize the mechanisms responsible for PAF activity.

Methods

Unanaesthetized male Wistar rats (225–275 g) were used in these experiments. Protein extravasation, a marker of vascular permeability was evaluated by measuring the extravasation of Evans blue dye (EB). In brief, EB ($25\,mg\,ml^{-1}$ in 0.9% NaCl; $20\,mg\,kg^{-1}$) was injected in the caudal vein with increasing doses of PAF. The animals were decapitated and exsanguinated at different times (5, 10, 30 and 60 min). In some experiments, the animals received pretreatment with an antagonist or an inhibitor 5 and 60 min respectively before the PAF injections and were killed 5 min later. In one group, the thromboxane mimic (U-44069) was injected simultaneously with EB and in another group, the animals were given an injection of PAF together with either U-44069 or noradrenaline, and EB; the animals were killed 5 min later. The thorax was cut open and the lungs were perfused with 15 ml of Krebs solution ($10\,ml\,min^{-1}$) via a cannula inserted in the pulmonary artery to remove the excess of intravascular pulmonary dye. The trachea, upper and lower bronchi and the pulmonary parenchyma were dissected, weighed and a portion of each organ was put in formamide ($4\,ml\,g^{-1}$ wet weight tissue at 20°C for 24 h) while another part was dried at 60°C for 24 h. The upper bronchi corresponded to the parts of the airways that begin at the trachea up to its entry in the lung parenchyma. The lower airways corresponded to major airways surrounded by lung parenchyma which can be easily dissected without magnification.

Evans blue assay

The concentration of EB extracted in formamide was determined by spectrophotometry at 620 nm wavelength using a Titertek Multiskan (Flow Lab.) and 96 well plates. The results were plotted on a standard curve of EB ($0.5–25\,\mu g\,ml^{-1}$). Evans blue content of each sample was expressed as EB $\mu g\,g^{-1}$ dry weight of tissue. The expression of our results as a function of dry weight of tissue avoided underevaluation of changes due to oedema formation.

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Drugs used

The following drugs were used: Evans blue and noradrenaline hydrochloride (Sigma Chem., St Louis, U.S.A.), PAF(1-O-hexadecyl - 2 - O - acetyl - sn - glycero - 3 - phosphorycholine (Bachem, Switzerland), U-44069 15(S)-hydroxy-9α,11α-(epoxymethano)-prosta-5-cis,13-trans-dienoic acid (gift from Dr J. Pike, Upjohn, Kalamazoo, U.S.A.), BN-52021 (3-*t*-butyl-hexahydro - 4,7b,11 - trihydroxy - 8 - methyl - 9H - 1,7a -epoxy - methano - 1H,6aH - cyclopenta[c]furo[2,3 - b]furo- [3',2':3,4] cyclopenta[1,2-d] furan-5,9,12(4H) trione; L-655,240 (3 - [1 - (4 - chlorobenzyl) - 5 - fluoro - 3 - methyl - indol - 2 - yl]2,2 - dimethyl propanoic acid), MK-571 (3-(3-(2-(7-chloro-2-quinolinyl)ethenyl) phenyl ((3-dimethyl amino-3-oxo propyl) thio) methyl) thio) propanoic acid), indomethacin, L-663,536 3-[1-(4-chlorobenzyl)-3-t-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid (gifts from Merck Frosst Lab., Montreal, Canada), OKY-046 ((E)-3-[4-(1-imidazolylmethyl) phenyl]-2-propenoic acid hydrochloride monohydrate; gift from ONO Pharmaceuticals, Japan).

Statistical analysis

Results are expressed as means ± s.e.mean and significance has been determined by analysis of variance. P values <0.05 were considered as significant.

Results

Effects of PAF on protein extravasation in rat airways

In the first series of experiments, EB dye was injected with increasing doses of PAF (1.0 and 5.0 $\mu g\,kg^{-1}$) in the caudal vein of rats that have been killed 5, 10, 30 and 60 min after the injection. A group of rats received EB only and were used as controls. The following organs were collected: trachea, upper and lower bronchi and the pulmonary parenchyma. As shown in Figure 1, basal levels of EB (control) were fairly stable as a function of time and ranged from 50 to 140 $\mu g\,g^{-1}$ of dry weight in all tissues. A slight increase as a function of time was noted in the pulmonary parenchyma. The injection of PAF (1.0 $\mu g\,kg^{-1}$) increased the content of EB in the trachea, upper and lower bronchi by 99%, 170% and 156% respectively, and the maximal effect was noted at 60 min. The highest concentration of PAF (5.0 $\mu g\,kg^{-1}$) increased the EB extravasation by 204%, 755% and 397% in the same three tissues. The maximal effect was reached at 5 min (Figure 1). PAF injections up to 5.0 $\mu g\,kg^{-1}$ did not affect the permeability of lung parenchyma (Figure 1b).

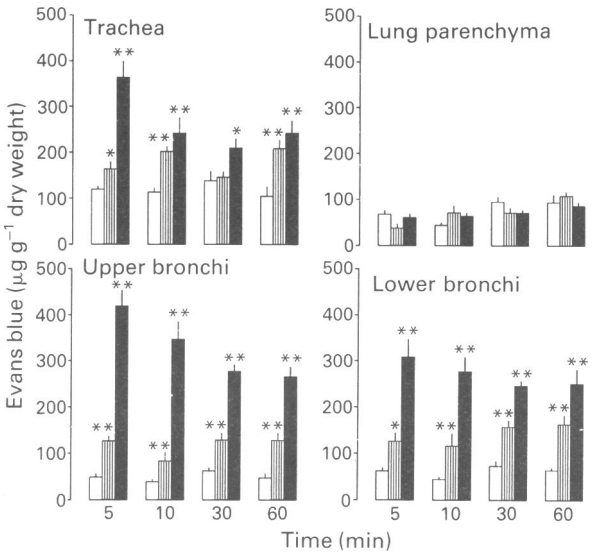

Figure 1 Evans blue dye contents ($\mu g\,g^{-1}$ dry tissue) as a function of time after the intravenous injection of increasing concentrations of PAF. (□) Control, (▥) 1.0 $\mu g\,kg^{-1}$, (■) 5.0 $\mu g\,kg^{-1}$ together with Evans blue dye (20 $mg\,kg^{-1}$). * $P < 0.05$ and ** $P < 0.01$ as compared with controls in the absence of PAF. Columns are means of 4–6 experiments; vertical lines show s.e.mean.

Effects of the PAF antagonist BN-52021 on PAF-induced vascular permeability

In the second series of experiments, the effects of the PAF antagonist BN-52021 (10 $mg\,kg^{-1}$) were studied on the vascular permeability changes elicited by PAF injection to conscious rats. The antagonist was injected in the tail vein 5 min before the injection of PAF (5.0 $\mu g\,kg^{-1}$) and EB. The animals were decapitated 5 min later. As shown in Figure 2, compound BN-52021 reduced by 80, 92 and 100% the protein extravasation in the trachea, upper and lower bronchi, respectively. Compound BN-52021 did not affect the level of permeability of the parenchyma. Given in the absence of PAF, this antagonist did not modify either the basal protein extravasation of the trachea, upper and lower bronchi.

Effects of selected antagonists and inhibitors of thromboxane formation

The present series of experiments was undertaken to evaluate the role of thromboxane A_2 in the lung response to PAF and to determine the role of constriction in the increase of vascular permeability. As shown in Figure 3, the treatment of rats with indomethacin (10 $mg\,kg^{-1}$), an inhibitor of prostaglandin and thromboxane formation, injected 60 min before PAF (5.0 $\mu g\,kg^{-1}$), reduced by 49, 28 and 46% protein extravasation in the trachea, upper and lower bronchi respectively. No significant effect was noted in the parenchyma.

The compound OKY-046 (10 $mg\,kg^{-1}$), a thromboxane synthetase inhibitor injected 60 min before the PAF injection (5.0 $\mu g\,kg^{-1}$), reduced by 69 and 36% the protein extravasation of the trachea and upper bronchus respectively but did not affect significantly PAF-induced vascular permeability of the lower bronchus and parenchyma. Similarly, compound L-655,240 (5 $mg\,kg^{-1}$), a novel thromboxane antagonist, injected 5 min before the PAF injection (5.0 $\mu g\,kg^{-1}$), reduced

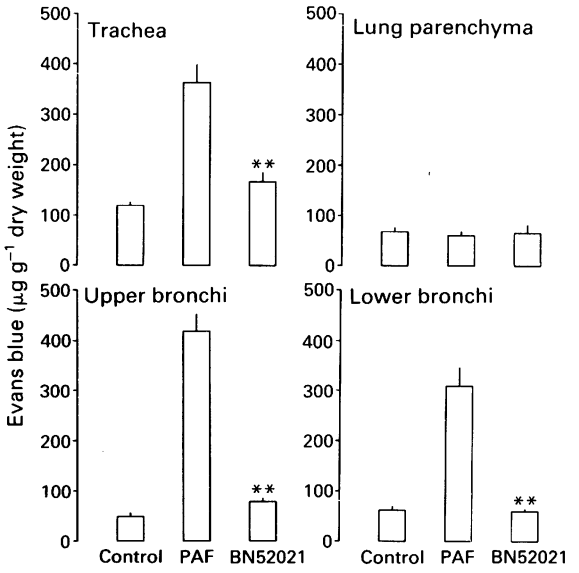

Figure 2 Effects of pretreating the rats with compound BN-52021 (10 $mg\,kg^{-1}$) 5 min before a PAF injection (5.0 $\mu g\,kg^{-1}$) together with Evans blue dye (20 $mg\,kg^{-1}$). The rats were killed 5 min after the PAF treatment. The contents of Evans blue dye ($\mu g\,g^{-1}$ dry tissue) are means of 4–6 experiments; vertical lines show s.e.mean. * $P < 0.05$ and ** $P < 0.01$ respectively as compared with PAF.

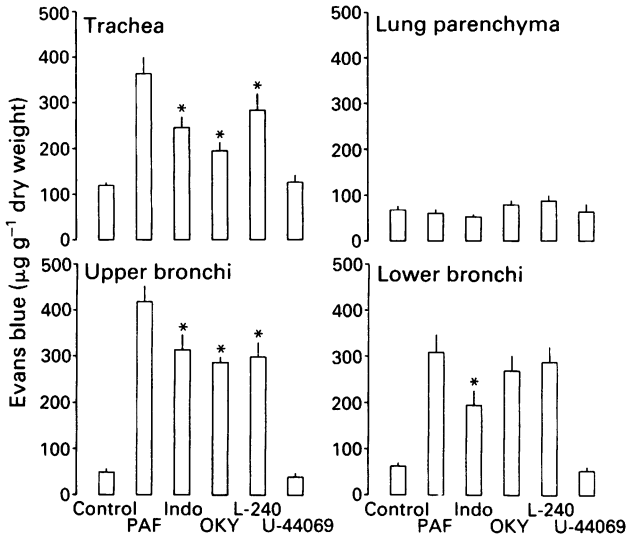

Figure 3 Effects of pretreating the rats with indomethacin (Indo; 10 mg kg^{-1}), compound OKY-046 (OKY; 10 mg kg^{-1}) and compound L-655,240 (L-240; 5 mg kg^{-1}) on the effect of PAF (5.0 μg kg^{-1}). The animals were pretreated with the inhibitors indomethacin and OKY-046 60 min before the injection of PAF and Evans blue dye (20 mg kg^{-1}) whereas the antagonist of thromboxane, compound L-655,240 was given 5 min before the injection of PAF. The rats were killed 5 min after the PAF treatment. The thromboxane mimic (U-44069; 5.0 μg kg^{-1}) was injected together with Evans blue dye (20 mg kg^{-1}) and the rats were killed 5 min later. Values are means of 4–6 experiments; vertical lines show s.e.mean. * and ** as in Figure 2.

protein extravasation in the trachea and upper bronchi by 33 and 32%, respectively, whereas it did not interfere with the permeability of the lower bronchus and the parenchyma.

Based on these results that suggested a role for thromboxane in PAF-induced protein extravasation, compound U-44069 (5.0 μg kg^{-1}), a thromboxane mimic, was used. As shown on the right hand side of each panel of Figure 3, a large dose of this compound (5 μg kg^{-1}) did not induce protein extravasation from the selected lung tissues 5 min after its injection.

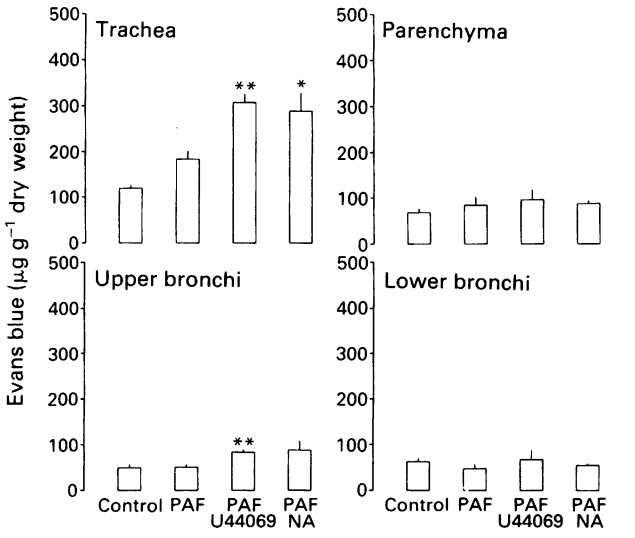

Figure 4 Effects of compound U-44069 (5.0 μg kg^{-1}) or noradrenaline (NA, 400 ng kg^{-1}) combined with a low dose of PAF (0.1 μg kg^{-1}) on protein extravasation by rat airways. Evans blue dye (20 mg kg^{-1}) was injected alone as a control. The rats were killed 5 min after the treatment. Values are means of 4–6 experiments; vertical lines show s.e.mean. * and ** as in Figure 2.

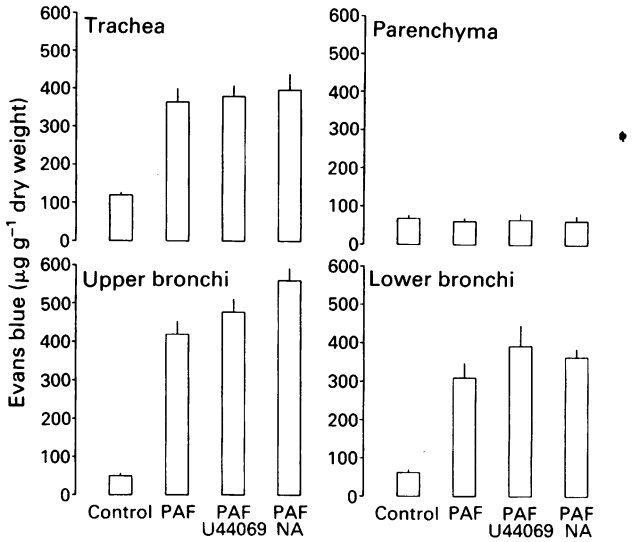

Figure 5 Effects of compound U-44069 (5.0 μg kg^{-1}) or noradrenaline (NA, 400 ng kg^{-1}) combined with a high dose of PAF (5.0 μg kg^{-1}) on protein extravasation by rat airways. Evans blue dye (20 mg kg^{-1}) was injected alone as a control. The rats were killed 5 min after the treatment. Values are means of 4–6 experiments; s.e.mean shown by vertical lines.

Effect of noradrenaline and compound U-44069 on PAF-induced microvascular leakage

In order to understand better the basic mechanisms underlying microvascular leakage and especially the role of vasoconstriction in this process, the effects of noradrenaline and U-44069 on PAF-induced increases in EB extravasation were studied. PAF was used at doses of 0.1 μg kg^{-1} and 5 μg kg^{-1}. As shown in Figure 4, the low dose of PAF produced only slight changes on EB extravasation in airway and lung tissues.

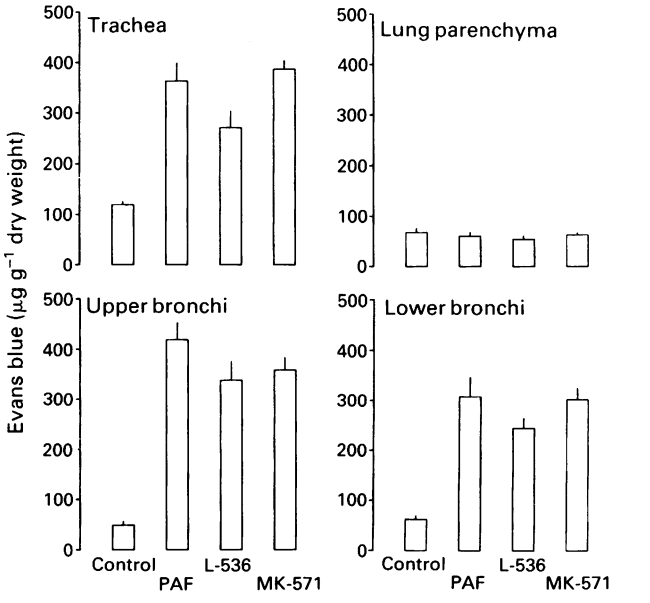

Figure 6 Effects of pretreating rats with compounds L-663,536 (L-536; 10 mg kg^{-1}) and MK-571 (10 mg kg^{-1}) 60 and 5 min respectively before the PAF injection (5.0 μg kg^{-1}) together with Evans blue dye (20 mg kg^{-1}). The rats were killed 5 min after the PAF treatment. The content of Evans blue dye (μg g^{-1} dry tissue) are means of 4–6 experiments; s.e.mean shown by vertical lines.

The simultaneous injection of PAF ($0.1\,\mu g\,kg^{-1}$) and U-44069 ($5\,\mu g\,kg^{-1}$) increased by 67 and 65% the protein extravasation in the trachea and upper bronchi respectively, as compared with the effect of PAF alone, but did not affect significantly the extravasation in the lower bronchi and parenchyma. Similarly, the combined injection of the low dose of PAF ($0.1\,\mu g\,kg^{-1}$) and noradrenaline ($400\,ng\,kg^{-1}$) increased by 57 and 74% the extravasation in the trachea and lower bronchi and did not affect significantly the permeability of the lower bronchi and parenchyma.

Figure 5 shows the effects of noradrenaline and compound U-44069 on the effects of a higher dose of PAF ($5.0\,\mu g\,kg^{-1}$) on vascular permeability. The results on the effect of PAF are identical to those presented in Figure 1. They show that PAF strongly increased the protein extravasation in the trachea, upper and lower bronchi but not in the parenchyma. Compound U-44069 ($5.0\,\mu g\,kg^{-1}$) as well as noradrenaline ($400\,ng\,kg^{-1}$) given in combination with PAF ($5.0\,\mu g\,kg^{-1}$) did not act in synergism and the total EB extravasation in all 4 tissues was similar to the extravasation produced by the injection of PAF alone.

Effects of an antagonist and an inhibitor of leukotriene formation

In the last series of experiments, the role of leukotrienes as possible modulators of PAF activities was evaluated with the use of two novel molecules: compound L-663,536, a lipoxygenase inhibitor, and compound MK-571, a peptido-leukotriene antagonist. Given at a dose of $10\,mg\,kg^{-1}$, 60 and 5 min, respectively, before the injection of PAF, compound L-663,536 and compound MK-571 did not block the increases of protein extravasation in lung tissues (Figure 6).

Discussion

These results show that intravenous injections of PAF dose-dependently increased protein extravasation in rat trachea, upper and lower bronchi, but did not modify that of pulmonary parenchyma. The effect of PAF on protein extravasation was almost totally inhibited with compound BN-52021 pretreatment. These results are in agreement with those of Wedmore & Williams (1981) and Page *et al.* (1983) who demonstrated that PAF increases vascular permeability in rabbit and guinea-pig skin and paw. Evans *et al.* (1986; 1987) and O'Donnell & Barnett (1987) also described PAF-mediated protein extravasation in guinea-pig airways at a dose 1000 times less than in rats. Bjork & Smedegard (1983) suggested that the difference in PAF doses needed to induce an effect in various species is related to platelet receptor sensitivity to this mediator. The absence of effect of PAF noted in the lung parenchyma as compared to the trachea and bronchi could be related to the microcirculation under study. Lung parenchyma is perfused by the pulmonary circulation whereas the tracheal and bronchial tissues are perfused by the systemic circulation.

Our results also show that the thromboxane/prostaglandin endoperoxide antagonist L-655,240, as well as the inhibitors indomethacin and compound OKY-046, decreased the protein extravasation induced by PAF injection. The partial inhibition of the protein extravasation produced either by a pretreatment with a thromboxane antagonist or by inhibitors of thromboxane formation, suggests that intravenous PAF treatment stimulates thromboxane secretion which potentiates or modulates protein extravasation. However, Bjork & Smedegard (1983) and Evans *et al.* (1987) did not reach the same conclusions since indomethacin did not modify PAF-induced increases in vascular permeability in the hamster cheek pouch and in guinea-pig airways.

The thromboxane mimic, U-44069, was not shown to affect vascular permeability by itself but it potentiated the effect of low dose of PAF. Similarly, noradrenaline potentiated the effects of a low dose of PAF on EB extravasation. These results appear paradoxical. However, vascular permeability is controlled by complex mechanisms which include the opening of permeability channels by disjunction of endothelial cells and increase of capillary hydrostatic pressure. A number of studies have shown that the effects of PAF on vascular permeability are secondary to disjunction of endothelial cells (Dewar *et al.*, 1983; Bjork & Smedegard, 1983; Handley *et al.*, 1984). Heffner *et al.* (1983) showed that PAF induces the release of thromboxanes which raises microvascular pressure. The increment in microvascular pressure does not itself cause changes in vascular permeability since neither compound U-44069 nor noradrenaline, two potent vasoconstrictors, increased vascular permeability in our study. However, it is possible that in the presence of PAF-induced cell disjunction, vasoconstrictors may raise hydrostatic pressure by post-capillary vasoconstriction and consequently, potentiate plasma leakage. Our data, as well as those presented by Heffner *et al.* (1989), strongly support this hypothesis. It appears that vascular permeability is only modulated by changes in blood pressure. For instance, peptido-leukotrienes are vasoconstrictors and increase vascular permeability (Dahlen *et al.*, 1981) while histamine is a potent dilator of the microcirculation but also increases vascular permeability (Majno & Palade, 1961). Drugs acting on cholinoceptors and adrenoceptors have potent effects on blood pressure but do not affect plasma leakage (reviewed by Grega & Adamski, 1988). Vasoconstrictors such as thromboxane A_2 could potentiate the plasma leakage by an increase of hydrostatic pressure only in the presence of compounds that open the permeability channels.

Neither compound MK-571, a new specific leukotriene D_4 (LTD_4) antagonist (Jones *et al.*, 1989) nor compound L-663, 536, a new 5-lipoxygenase inhibitor (Gillard *et al.*, 1989) reduce PAF-induced protein extravasation. These results strongly suggested that the effects of PAF are independent of leukotriene synthesis in rat airways. This is in agreement with the results of Handley *et al.* (1986), who demonstrated that FPL-55712, an antagonist of LTC_4 and LTD_4, was ineffective in reducing the haemoconcentration mediated by a PAF injection.

In conclusion, intravenous injection of PAF causes massive protein extravasation which is partly mediated by thromboxane secretion. Exogenous thromboxane may potentiate the effects of PAF by producing a vasoconstriction at the post-capillary sites which increases the hydrostatic pressure and potentiates the extravasation. PAF effects seem to be independent of leukotriene generation. The reason for the absence of effect of PAF on the pulmonary parenchyma is under investigation.

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Effects of several potassium channel openers and glibenclamide on the uterus of the rat

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1 The ability of several potassium (K^+) channel openers to inhibit spasm of the uterus of the non-pregnant rat and their susceptibility to antagonism by glibenclamide was assessed *in vitro* and *in vivo*.

2 In the isolated uterus exposed to oxytocin (0.2 nM), cromakalim, RP 49356 and pinacidil were of similar potency (mean $pD_2 = 6.4$, 6.0 and 6.2 respectively) while minoxidil sulphate was of lower potency ($pD_2 = 4.7$). Glibenclamide antagonized cromakalim and RP 49356 with the interactions consistent with competitive antagonism (mean pA_2 of 6.57 and 7.00 respectively). Glibenclamide also antagonized pinacidil ($pA_2 = 6.22$) but the slope of the Schild plot was significantly greater than -1. Neither salbutamol nor minoxidil sulphate was antagonized by glibenclamide (10 μM).

3 Cromakalim (1 and 10 μM), RP 49356 (1 and 10 μM), pinacidil (1 μM) and minoxidil sulphate (100 μM) suppressed spasm evoked by low (<40 mM) but not high (≥40 mM) KCl concentrations. Glibenclamide (10 μM) prevented cromakalim (10 μM)-, RP 49356 (10 μM)- and pinacidil (10 μM)-induced suppression of KCl (20 mM)-evoked spasm. Pinacidil (10 and 100 μM), cromakalim (100 μM) and salbutamol (0.01–1 μM) inhibited spasm evoked by all concentrations of KCl (10–80 mM). Suppression of spasm evoked by KCl (10–80 mM) by cromakalim (100 μM) and pinacidil (100 μM) was insensitive to glibenclamide (10 μM).

4 Cromakalim (0.1 mg kg^{-1}) and RP 49356 (0.1 mg kg^{-1}), given by i.v. bolus injection, inhibited uterine contractions, produced a fall in blood pressure and a slight tachycardia in the conscious ovariectomized rat. Glibenclamide (20 mg kg^{-1}), given by i.v. infusion, antagonized the vascular and uterine smooth muscle relaxant properties of cromakalim and RP 49356.

5 Several K^+ channel openers are uterine relaxants. The antagonism of cromakalim, RP 49356 and pinacidil, at low concentrations, by glibenclamide suggests their actions may involve an ATP-sensitive K^+ channel. High concentrations of pinacidil (10 and 100 μM) and cromakalim (100 μM) may exert an additional action in the uterus. The low potency of minoxidil sulphate and its insensitivity to glibenclamide in the isolated uterus suggests that its mechanism of action may differ from that of the other K^+ channel openers.

Introduction

Cromakalim, RP 49356, pinacidil and minoxidil sulphate are relaxants of vascular smooth muscle, actions suggested to be mediated by the opening of potassium (K^+) channels in the plasma membrane (Cook, 1988; Mondot *et al.*, 1988; Hamilton & Weston, 1989; Winquist *et al.*, 1989; Newgreen *et al.*, 1990). Cromakalim is a relaxant of the isolated uterus of the term-pregnant rat (Hollingsworth *et al.*, 1987; 1989) where it exhibits similar properties to those seen in other smooth muscles in terms of potency, rate of onset of action, reversibility and sensitivity to non-selective blockers of K^+ channels such as tetraethylammonium. However, in the uterus of the term-pregnant rat, in contrast to other smooth muscles, cromakalim does not increase the efflux of $^{86}Rb^+$ or $^{42}K^+$, produce a marked hyperpolarization or augment the outward K^+ current in isolated myometrial cells (Hollingsworth *et al.*, 1987; 1989). Recently, it has been shown that glibenclamide is a selective antagonist of these K^+ channel openers *in vitro* (Buckingham *et al.*, 1989; Cavero *et al.*, 1989; Eltze, 1989; Wilson, 1989; Winquist *et al.*, 1989; Newgreen *et al.*, 1990). Glibenclamide has been demonstrated to inhibit the opening of ATP-dependent K^+ channels in the pancreas (Sturgess *et al.*, 1988), the heart (Escande *et al.*, 1989) and the mesenteric artery (Standen *et al.*, 1989). We have, therefore, studied the relaxant properties of cromakalim and other K^+ channel openers in the isolated uterus of the non-pregnant rat and their sensitivity to glibenclamide.

Cromakalim is also a relaxant of the uterus of the non-pregnant and pregnant rat *in vivo*, although at doses which lower blood pressure (Downing *et al.*, 1989). Tolerance has been observed to cromakalim both as a uterine relaxant and as a vasodilator (Downing *et al.*, 1989; Downing & Hollingsworth, 1989). The ability of glibenclamide to antagonize the vasodilator effects of cromakalim and RP 49356 (Mondot *et al.*, 1988; Buckingham *et al.*, 1989; Cavero *et al.*, 1989; Quast & Cook, 1989a,b) supports the idea that these drugs act by K^+ channel opening in the cardiovascular system *in vivo*. We have, therefore, investigated whether RP 49356 is also a uterine relaxant and examined the selectivity of cromakalim and RP 49356 between the uterus and the cardiovascular system and the sensitivity of these two relaxants to antagonism by glibenclamide *in vivo*. Preliminary data have been presented to the British Pharmacological Society (Piper & Hollingsworth, 1989; Sadraei *et al.*, 1989).

Methods

Non-pregnant Sprague-Dawley rats (200–250 g) were purchased from Charles River Ltd.

In vitro *studies*

Uterine horns were obtained from rats pretreated 24 h earlier with 17-β oestradiol benzoate (100 μg kg^{-1} s.c.) and cut into longitudinal strips of approximately 1 cm length. Strips were mounted for isometric recording under 1 g tension in physiological salt solution (PSS) maintained at 37°C, gassed with 95% O_2/5% CO_2 and equilibrated for 1 h. The mechanical responses were measured as integrated tension by the method of Granger *et al.* (1985).

Effects of relaxants on spasm evoked by oxytocin Uteri were exposed to PSS containing oxytocin (0.2 nM) to induce phasic

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spasm. After 15 min equilibration, relaxants were added in a cumulative manner to the bath at 10 min intervals. Their relaxant effect was assessed by expressing the integrated tension during 5–10 min after addition of each drug concentration as a percentage of the tension in the 5 min period before drug addition. In antagonism studies, the effects of increasing concentrations of glibenclamide (0.3, 1.0 and 10.0 μM) or the equivalent vehicle were obtained by use of consecutive concentration-effect curves. Initial concentration-effect curves to the relaxant were constructed and once maximal inhibition had been obtained strips were washed until predrug spasm returned. Glibenclamide (0.3 μM) or vehicle was then incubated in the bath for 30 min before the concentration-effect curve to relaxant was repeated. This protocol was repeated in the presence of glibenclamide (1 and 10 μM) or equivalent vehicle. To avoid desensitization of the uterus to oxytocin, the equilibration period to glibenclamide or vehicle consisted of 15 min with PSS plus glibenclamide or vehicle followed by 15 min with PSS plus oxytocin (0.2 nM) and glibenclamide or vehicle. In studies with minoxidil sulphate only a single concentration of glibenclamide (10 μM) or vehicle was studied. Minoxidil sulphate is chemically very unstable in storage, therefore its potency was also assessed against spontaneous spasm of the isolated portal vein of the rat (Hamilton *et al.*, 1986).

Effects of relaxants on spasm evoked by KCl Two concentration-effect curves were constructed to KCl (10–80 mM), with KCl added in a cumulative manner every 10 min. Uterine strips were then incubated with relaxant or vehicle for 10 min before further concentration-effect curves to KCl were obtained. In further experiments, after the effect of a relaxant was tested, glibenclamide (10 μM) or vehicle was incubated for 30 min. During the last 10 min of incubation relaxant was added to the bath before the KCl concentration-effect curve was repeated. Controls to assess the reproducibility of KCl-evoked spasm with time, of relaxant-induced suppression of KCl-evoked response(s) and of the effect of glibenclamide (10 μM) on KCl concentration-effect curves were carried out. Responses were measured as integrated tension for the 10 min period after each KCl addition and expressed as a percentage of the maximum integral in the second control concentration-effect curve. Stated concentrations of KCl refer to the KCl added to the bath and do not include the concentration of K^+ present in the PSS.

In vivo *studies*

The surgical technique was based on that of Downing & Hollingsworth (1988) and Downing *et al.* (1989). Rats were anaesthetized with tribromoethanol (240 mg kg^{-1}, i.p.), bilaterally ovariectomized, the right jugular vein cannulated and an intrauterine balloon inserted. In a second group of rats, the left carotid artery was cannulated instead of uterine balloon placement. Morphine sulphate (5 mg salt kg^{-1}, s.c.) was given postoperatively and animals were allowed 24 h for recovery.

Intrauterine pressure and its integral or blood pressure and heart rate were measured in conscious unrestrained rats and recorded on a Grass polygraph as described previously (Downing & Hollingsworth, 1988; Downing *et al.*, 1989). Uterine contractions were recorded as changes in intrauterine pressure for 30 min before and 1.5–3.0 h after an i.v. bolus dose of cromakalim (0.1 mg kg^{-1}) or RP 49356 (0.1 mg kg^{-1}). Glibenclamide (20 mg kg^{-1}) or vehicle (5 ml kg^{-1}) was then infused over 5 min. The same dose of cromakalim or RP 49356 was repeated 30 min after the start of glibenclamide infusion. Uterine contractions were measured as the integral of area under the pressure curve in 15 min periods. The integrals in each 15 min period after injection of cromakalim or RP 49356 were expressed as a % of the mean integral of the two 15 min periods before the drug injection.

The same experimental design was adopted to measure the effects of cromakalim (0.1 mg kg^{-1}) and RP 49356 (0.1 mg kg^{-1}) on blood pressure (BP) and heart rate (HR) before and after glibenclamide or vehicle infusion. Data are expressed as % change from pre-injection control values.

Drugs and solutions

The following drugs were used: cromakalim (SmithKline/Beecham), RP 49356 ((±)-N-methyl-2-(3-pyridyl)-tetrahydrothiopyran-2-carbothiamide-1-oxide, Rhône-Poulenc), pinacidil monohydrate (Leo), minoxidil sulphate (Upjohn), glibenclamide (Hoechst), salbutamol sulphate (Glaxo), oxytocin acetate (grade X, Sigma), 17-β oestradiol benzoate (Sigma), tribromoethanol (Fluka Chemicals), morphine sulphate (Evans). The stock solutions (10 mM *in vitro* and 5 mg ml^{-1} *in vivo*) of cromakalim, RP 49356 and pinacidil were prepared in 70% (w/v) ethanol: isotonic saline and that of minoxidil sulphate in 50% (w/v) ethanol: isotonic saline. Glibenclamide (10 mM) was prepared in 95% ethanol (*in vitro*) and as a 5 mg ml^{-1} solution in 0.02 N sodium hydroxide in 4% w/v glucose (*in vivo*). Salbutamol (10 mM) was prepared in 0.1 N hydrochloric acid, oxytocin in twice distilled water and 17-β oestradiol benzoate in arachis oil. Minoxidil sulphate and glibenclamide stock solutions were made fresh daily. The PSS had the following composition (mM): Na^+ 143.0, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.55, $H_2PO_4^-$ 1.2, SO_4^{2-} 1.2, Cl^- 128.0, HCO_3^- 25.0 and glucose 11.0.

Analysis of results

The pD_2 values of relaxants in isolated uterus were calculated by linear regression of probit of response v $\log_{10}$ M concentration. The calculation of pA_2 values in studies with glibenclamide was by the method of Arunlakshana and Schild (1959) where $\log_{10}$ (DR − 1) was plotted against $\log_{10}$ M glibenclamide concentration. DR is the dose ratio at any given molar concentration of glibenclamide corrected for any significant shift observed in time-matched vehicle controls (as determined by one-way analysis of variance). Values are quoted as means ± s.e.mean with n in parentheses. The significance of differences between means was calculated by two-tailed paired Student's t test or by one-way analysis of variance and the least significant difference test.

Results

In vitro

Effects of relaxants on spasm evoked by oxytocin Cromakalim (0.1–1.6 μM), RP 49356 (0.2–6.4 μM), pinacidil (0.2–3.2 μM), minoxidil sulphate (3.2–51.2 μM) and salbutamol (0.5–16 nM) were all able to inhibit phasic spasm induced by oxytocin (0.2 nM) in a concentration-dependent manner (Figures 1 and 2). The pD_2 values were 6.39 ± 0.07, 6.03 ± 0.06, 6.19 ± 0.10, 4.70 ± 0.17 and 8.89 ± 0.05 respectively (n = 12–23). The rank order of potency was, therefore, salbutamol ≫ cromakalim = pinacidil = RP 49356 > minoxidil sulphate. In each case relaxants showed a rapid onset of action (time to equilibrium < 5 min) and reversibility. Minoxidil sulphate was a potent relaxant of spontaneous spasm of the portal vein of the rat (pD_2 = 6.02 ± 0.16, n = 6).

Control experiments demonstrated the reproducibility of the concentration-effect curves for cromakalim, RP 49356, pinacidil and salbutamol over four curves (data not shown). In the case of minoxidil sulphate, the concentration-effect curve was reproducible over two curves only. Glibenclamide (0.3, 1.0 and 10.0 μM) caused a progressive parallel rightward shift of the concentration-effect curves for cromakalim, RP 49356 and pinacidil indicative of competitive-like antagonism (Figure 1). Schild analysis yielded pA_2 values for the interaction of glibenclamide with cromakalim, RP 49356 and pinacidil of 6.57 ± 0.18 (9), 7.00 ± 0.11 (6) and 6.22 ± 0.12 (8), respectively. The slopes of the Schild plots for the interaction

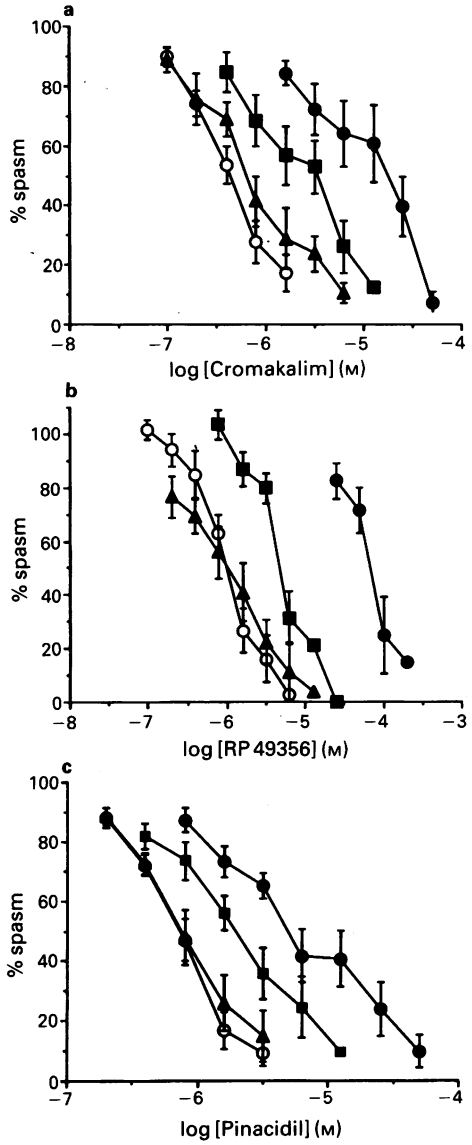

Figure 1 Effect of (a) cromakalim, (b) RP 49356 and (c) pinacidil on tension development in the isolated uterus of the non-pregnant rat treated with oxytocin (0.2 nM). Effects are shown in the absence (○) and in the presence of glibenclamide (0.3 μM, ▲; 1 μM, ■; 10 μM, ●). Ordinate scales: spasm remaining as a % of the spasm prior to relaxant addition. Abscissa scales; $\log_{10}$ molar concentration of relaxant. The points are the means and the vertical lines show the s.e.means (n = 6–9).

of glibenclamide with cromakalim and RP 49356 were not significantly different from negative unity (-1.19 ± 0.11, -1.08 ± 0.12 respectively, $P > 0.05$). However, the slope of the Schild plot for the interaction of glibenclamide with pinacidil was significantly different from negative unity (-1.33 ± 0.08, $P < 0.05$). In the presence of glibenclamide (10 μM) there was no significant displacement of the concentration-effect curve for minoxidil sulphate (pD_2 in absence of glibenclamide = 4.7 ± 0.17, pD_2 in the presence of 10 μM glibenclamide = 4.8 ± 0.18 [$n = 6$], $P > 0.05$; Figure 2). Glibenclamide (0.3, 1.0 and 10.0 μM) had no effect on the position of the concentration-effect curve for salbutamol ($P > 0.05$; Figure 2) when account was taken of the significant leftward-shift observed in time-matched vehicle controls. Glibenclamide (0.3, 1.0 and 10.0 μM) did not alter the resting tone of the uterus or modify the spasm to 0.2 nM oxytocin ($P > 0.05$). For example, phasic spasm to oxytocin 25–30 min after incubation in glibenclamide (10 μM) was $117.0 \pm 13.2\%$ ($n = 12$) of the integral prior to any glibenclamide incubation, which was not different ($P > 0.05$) from the integral ($120.4 \pm 13.9\%$, $n = 12$) after incubation in vehicle time-matched controls.

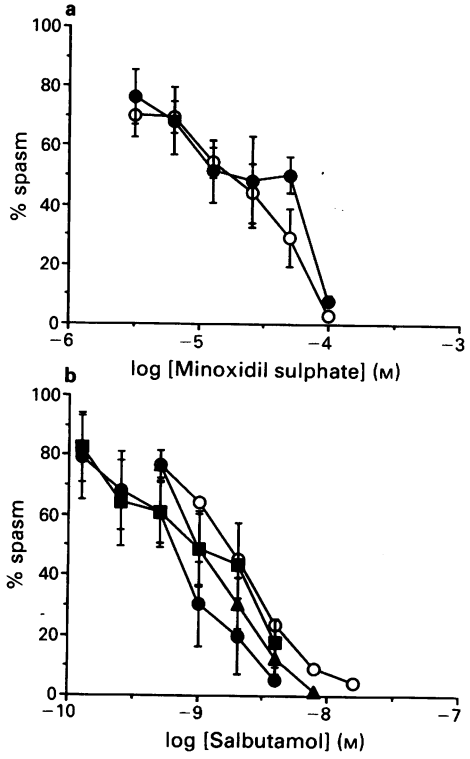

Figure 2 Effect of (a) minoxidil sulphate and (b) salbutamol sulphate on tension development in the isolated uterus of the non-pregnant rat treated with oxytocin (0.2 nM). Effects are shown in the absence (○) and in the presence of glibenclamide (0.3 μM, ▲; 1 μM, ■; 10 μM, ●). Ordinate scales: spasm remaining as a % of the spasm prior to relaxant addition. Abscissa scales: $\log_{10}$ molar concentration of relaxant. The points are the means and the vertical lines show the s.e.means (n = 6–10).

Effects of relaxants on spasm evoked by KCl Cromakalim (1 and 10 μM), RP 49356 (1 and 10 μM), minoxidil sulphate (100 μM) and pinacidil (1 μM) were able to reduce significantly ($P < 0.05$) spasm evoked by 20 μM KCl but had no effect on spasms evoked by 40 and 80 mM KCl ($P > 0.05$) (Figure 3). Higher concentrations of pinacidil (10 and 100 μM) and cromakalim (100 μM) were able to reduce significantly ($P < 0.05$) spasm evoked by 40 and 80 mM KCl (Figures 3 and 4). The significant suppression of KCl (20 mM)-induced spasm by cromakalim (10 μM), RP 49356 (10 μM) and pinacidil (10 μM) was prevented by prior administration of glibenclamide (10 μM) ($P > 0.05$; Figure 4a–c). Pinacidil (10 μM)-induced suppression of KCl (40 mM)-evoked spasm was also prevented by glibenclamide (10 μM) (Figure 4c), but cromakalim (100 μM)- and pinacidil (100 μM)-induced suppressions of spasms to all KCl concentrations (20–80 mM) were insensitive to glibenclamide (10 μM) (Figure 4d,e).

Salbutamol was able to inhibit spasm evoked by 20 mM KCl and significantly reduced spasms evoked by 40 and 80 mM KCl ($P < 0.01$) at all concentrations tested (Figure 3). In control experiments the position and shape of the KCl concentration-effect curve was unaffected with time, vehicle for the relaxants or glibenclamide (10 μM). Relaxant-induced suppression of KCl-evoked spasm(s) were repeatable and were not modified by vehicle for glibenclamide (data not shown).

In vivo

The vehicle for cromakalim and RP 49356 (1 ml kg^{-1} of 1.4% ethanol in saline) produced only a transient (<5 min) suppression of uterine contractions and fall in blood pressure.

The first bolus i.v. dose of cromakalim (0.1 mg kg^{-1}, Figure 5) or RP 49356 (0.1 mg kg^{-1}, Figure 6) produced an immediate and nearly complete inhibition of uterine contractions ($78.5 \pm 5.5\%$ and $73.8 \pm 5.7\%$, $n = 16$, inhibition over 0–

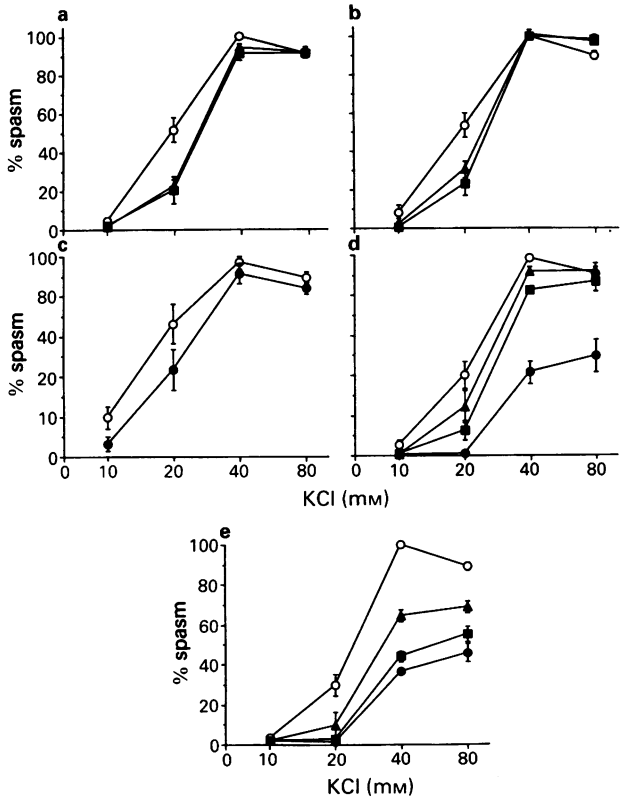

Figure 3 Effect of (a) cromakalim, (b) RP 49356, (c) minoxidil sulphate, (d) pinacidil and (e) salbutamol sulphate on tension development to KCl in the isolated uterus of the non-pregnant rat. Effects are shown in the absence (○) and in the presence of a potassium channel opener (1 μM, ▲; 10 μM, ■; 100 μM, ●) or salbutamol (0.01 μM, ▲; 0.1 μM, ■; 1 μM, ●). Ordinate scales: responses expressed as a % of the maximum response to KCl in the initial concentration-effect curve. Abscissa scales: concentrations of KCl. The points are the means and the vertical lines show the s.e.means (n = 6–8).

15 min respectively). Uterine contractions started to reappear 20–30 min after injection of RP 49356 and its effect had largely ceased by 1 h. For cromakalim there was some resumption of uterine contractions after approximately 30 min but there was still some inhibition (42.8 ± 10.3%) at 2 h. Cromakalim (0.1 mg kg^{-1}) or RP 49356 (0.1 mg kg^{-1}) repeated after glibenclamide vehicle produced peak inhibitions of uterine contractions (85.8 ± 6.4% and 64.3 ± 9.1%, n = 8, over 0–15 min) which were not significantly different ($P > 0.05$) from the effects seen with the first injection of these drugs (Figures 5 and 6). Infusion of glibenclamide (20 mg kg^{-1}) significantly ($P < 0.01$) reduced the uterine inhibitory effects of cromakalim (0.1 mg kg^{-1}) and of RP 49356 (0.1 mg kg^{-1}) (Figures 5 and 6). Inhibitions over 0–15 min after injection of cromakalim and RP 49356 were 27.1 ± 9.3% (n = 8) and 4.3 ± 13.8% (n = 8), respectively. The integrals of uterine contractions in the 30 min after the commencement of glibenclamide or vehicle infusion were 135.3 ± 14.9% (n = 17) and 109.7 ± 12.4% (n = 15), respectively, of the integrals in the 30 min before their infusion. Therefore, glibenclamide had no significant effect on spontaneous uterine contractions ($P > 0.05$).

In other groups of rats, cromakalim (0.1 mg kg^{-1}) and RP 49356 (0.1 mg kg^{-1}) produced falls in mean blood pressure (BP) and a small tachycardia (Table 1). The peak fall in blood pressure produced by cromakalim and RP 49356 (Table 1) was less than the peak inhibitions of uterine contractions (Figures 5 and 6). However, the time courses of offset of vasodepressor and uterine effects were similar. The effects of cromakalim were significantly less ($P < 0.05$) when repeated after vehicle, presumably because BP and HR had not fully returned to pre-drug values before the second dose of cromakalim was given. The vasodilator effect and tachycardia produced by cromakalim and RP 49356 were reduced ($P < 0.05$) after glibenclamide infusion (Table 1).

Discussion

We have demonstrated that three K^+ channel openers (cromakalim, RP 49356 and pinacidil) are potent relaxants of the isolated uterus of the non-pregnant rat and are antagonized by glibenclamide. Also, we have shown that cromakalim and RP 49356 are relaxants of the uterus of the rat *in vivo* and are similarly antagonized by glibenclamide. These effects were observed under two hormonal states, namely oestrogen-treated (isolated uterus) and after ovariectomy (*in vivo*).

Effects of low concentrations of cromakalim, RP 49356 and pinacidil in the isolated uterus

The potencies of cromakalim, RP 49356 and pinacidil in the isolated uterus against oxytocin-induced spasms were similar to those observed in vascular smooth muscle (Southerton *et al.*, 1988; Cavero *et al.*, 1989; Eltze, 1989). Low concentrations of cromakalim (1 and 10 μM), RP 49356 (1 and 10 μM), pinacidil (1 μM) and minoxidil sulphate (100 μM) all showed characteristic inhibition of spasm evoked by low but not high concentrations of KCl in uterus. This observation is consistent with observations in other smooth muscles and with the idea that these drugs open K^+ channels in this tissue.

Glibenclamide is a blocker of ATP-dependent K^+ channels in pancreatic β-cells (Sturgess *et al.*, 1988) and in smooth muscle (Standen *et al.*, 1989). Glibenclamide has been used in smooth muscle as a pharmacological tool to demonstrate that cromakalim and related compounds act via ATP-dependent K^+ channels (Quast & Cook, 1989b). In the isolated uterus glibenclamide exhibited selectivity as it antagonized cromakalim, RP 49356 and pinacidil but not salbutamol as relaxants of oxytocin-induced spasm. Glibenclamide produced parallel rightward shifts of the concentration-effect curves for cromakalim and RP 49356 against oxytocin-evoked spasm, with no suppression of the maximum inhibition and with the Schild slopes not different from −1 indicating competitive-like antagonism. Schild analysis of the interaction of glibenclamide with cromakalim and RP 49356 yielded pA_2 values similar to those found in vascular smooth muscle (Cavero *et al.*, 1989; Eltze, 1989; Quast & Cook, 1989a; Wilson, 1989). The pA_2 value for the interaction of glibenclamide with pinacidil was lower than that for cromakalim or RP 49356 and there was a Schild slope of greater than −1. It is possible that the ability to measure the potency of glibenclamide against pinacidil for its K^+ channel opening action is affected by an additional mechanism of pinacidil (see below).

The inhibitory effects of lower concentrations of cromakalim (10 μM), RP 49356 (10 μM) and pinacidil (10 μM) against spasm evoked by KCl (20 mM) were prevented by glibenclamide (10 μM). Collectively these observations suggest that cromakalim, RP 49356 and pinacidil have an action involving glibenclamide-sensitive mechanisms in uterus, possibly ATP-dependent K^+ channels. However, it should be noted that there is no direct evidence to support the involvement of K^+ channels in the actions of these drugs in the uterus of the oestrogen-treated rats. A similar situation with regard to the uterus of late-pregnant rat has been discussed previously (Hollingsworth *et al.*, 1987; 1989).

Effects of minoxidil sulphate in the isolated uterus

Minoxidil sulphate showed a low potency in uterus against oxytocin-induced spasm (pD_2 = 4.7) when compared with its potency in the isolated portal vein of the rat against spontaneous spasm (pD_2 = 6.02). The possibility that minoxidil sulphate had become inactive, due to its chemical instability, was

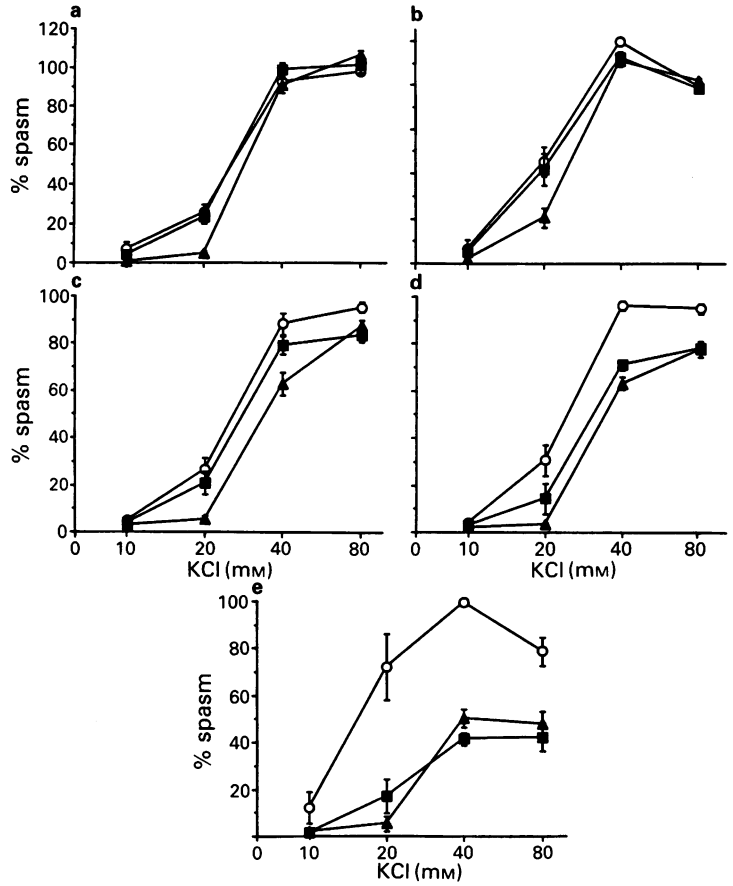

Figure 4 Effect of (a) cromakalim (10 μM), (b) RP 49356 (10 μM), (c) pinacidil (10 μM), (d) cromakalim (100 μM) and (e) pinacidil (100 μM) on tension development to KCl in the isolated uterus of the non-pregnant rat. Effects are shown in the absence of relaxant or glibenclamide (○), in the presence of relaxant (▲) and in the presence of relaxant plus glibenclamide (10 μM, ■). Ordinate scales: responses expressed as a % of the maximum response to KCl in the initial concentration-effect curve. Abscissa scales: concentration of KCl on a log scale. The points are the means and the vertical lines show the s.e.mean (n = 4–9).

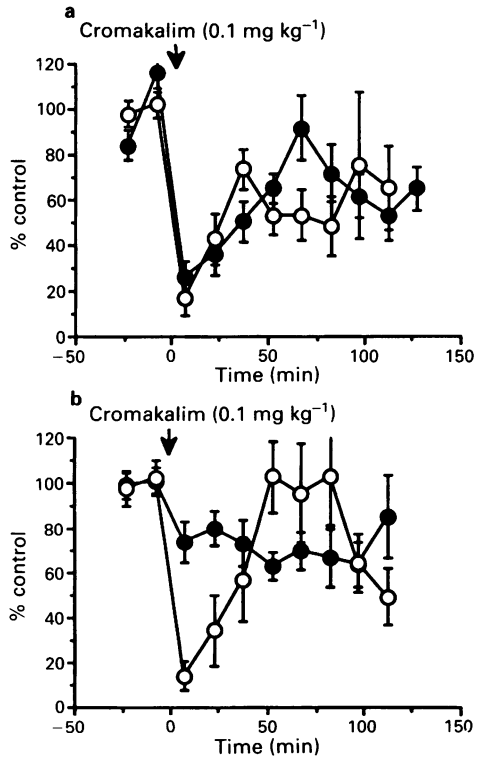

Figure 5 Effect of cromakalim (0.1 mg kg^{-1}) on uterine contractions before (a) and after (b) either a 5 min infusion of glibenclamide (20 mg kg^{-1}) (●) or of vehicle (○). The points are the means and the vertical lines show the s.e.means (n = 8). Integrals of uterine contractions were expressed as a % of the integrals in the 30 min before each cromakalim injection.

discounted since its potency as an inhibitor of the spontaneous spasm of isolated portal vein of the rat in the present study (pD_2 = 6.02) was comparable to that in a previous study (pD_2 = 6.2, Newgreen *et al.*, 1990). Thus, although the two situations, a hormone-treated uterus with tone induced by oxytocin and the spontaneously active portal vein are clearly different, the results obtained here may indicate selectivity of minoxidil sulphate for vascular compared with uterine smooth muscle.

In vascular smooth muscle the interaction between glibenclamide and minoxidil sulphate exhibited features of non-competitive antagonism (Winquist *et al.*, 1989; Newgreen *et al.*, 1990). In contrast, in the isolated uterus glibenclamide did not antagonize minoxidil sulphate. This latter observation plus the low potency of minoxidil sulphate in uterus, lends strong support to the idea that the site of action of minoxidil sulphate differs from that of cromakalim, RP 49356 and pinacidil (Quast & Cook, 1989b). The data in the present study also suggest that the site of interaction of minoxidil sulphate present in vascular smooth muscle is not present in the uterus, or that the channel activated in vascular smooth muscle is not active in the uterus.

Effects of high concentrations of cromakalim and pinacidil in the isolated uterus

The ability of pinacidil (10 and 100 μM) and cromakalim (100 μM) to reduce uterine spasm evoked by high KCl concentrations could be indicative of a mechanism of action other than K^+ channel opening. Similar effects have been previously described for pinacidil in both guinea-pig trachea and rabbit aorta (Nielsen-Kudsk *et al.*, 1988; Cook *et al.*, 1989) and for cromakalim in rat aorta (Quast *et al.*, 1990).

The mechanisms by which these effects of high concentra-

Table 1 The effects of cromakalim (0.1 mg kg^{-1}) and RP 49356 (0.1 mg kg^{-1}) on mean blood pressure (BP) and heart rate (HR) measured 30 min after bolus i.v. injection

	% *fall in mean BP*		% *increase in HR*	
	Pretreatment	*Posttreatment*	*Pretreatment*	*Posttreatment*
Cromakalim				
Control	36.3 ± 3.0	16.0 ± 2.5** (7)	14.5 ± 2.7	0.5 ± 2.4* (6)
Test	35.1 ± 4.8	1.2 ± 3.0** (7)	18.1 ± 2.8	2.6 ± 4.7** (7)
RP 49356				
Control	18.8 ± 4.9	12.7 ± 2.8 (5)	15.9 ± 3.2	8.2 ± 3.4 (4)
Test	24.0 ± 4.0	−0.4 ± 3.8* (4)	10.7 ± 2.0	1.5 ± 3.5* (3)

Treatment was glibenclamide (20 mg kg^{-1}) in test animals, vehicle in controls. Values are means ± s.e.means, *n* in parentheses. Significantly different from pretreatment values, * $P < 0.05$, ** $P < 0.01$.

tions of pinacidil and cromakalim are achieved is as yet unclear. Glibenclamide (10 μM) was unable to prevent the suppression of KCl-induced spasm seen with pinacidil (100 μM) and cromakalim (100 μM). One explanation is that these actions do not involve glibenclamide-sensitive mechanisms. Alternatively, the blockade caused by glibenclamide (10 μM) could have been overcome by the high concentrations of the relaxants. The limited solubility of glibenclamide precluded the use of more than 10 μM glibenclamide without the effect of the solvent. This effect of high concentrations of cromakalim and pinacidil could well be a common feature of K^+ channel openers.

Effects of cromakalim and RP 49356 in vivo

RP 49356, like cromakalim (Downing *et al.*, 1989 and present study) was a uterine relaxant at a dose which also lowered BP and produced slight tachycardia, indicating that the compound is not very uteroselective. However, it should be noted that 100% inhibition of uterine contractions could be achieved for short periods at a dose which produced <50% fall in BP. These observations were after bolus i.v. injection and experience has shown that selectivity of drugs for the uterus can vary with the nature of the dosage regimen and hormonal status (Hollingsworth & Downing, 1988). Downing (unpublished) has demonstrated that the ID_{50} of cromakalim for inhibition of uterine contractions of the non-pregnant rat is about one tenth of the ID_{50} for lowering BP. Both cromakalim and RP 49356 were antagonized by glibenclamide as uterine relaxants and as vasodilators in the present study (see also Mondot *et al.*, 1988; Buckingham *et al.*, 1989; Cavero *et al.*, 1989; Quast & Cook, 1989a) suggesting that there are similarities both between the mechanisms of action of the drugs and their sites of action in uterine and vascular tissue.

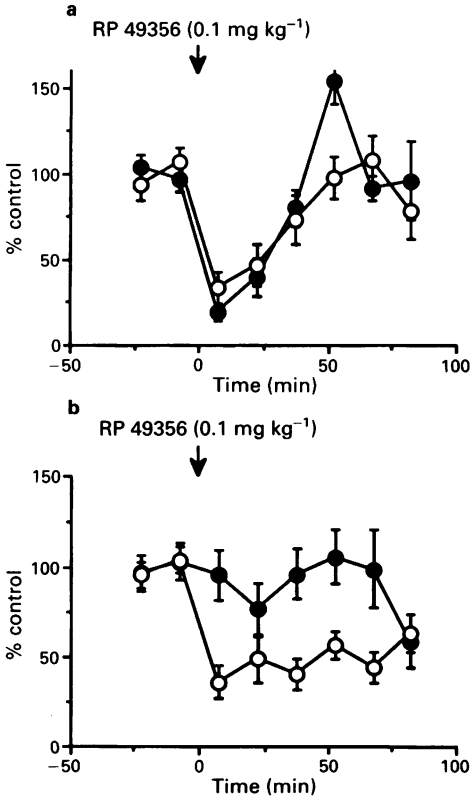

Figure 6 Effect of RP 49356 (0.1 mg kg^{-1}) on uterine contractions before (a) and after (b) either a 5 min infusion of glibenclamide (20 mg kg^{-1}) (●) or of vehicle (○). The points are the means and the vertical lines show the s.e.means ($n = 8$). Integrals of uterine contractions were expressed as a % of the integrals in the 30 min before each RP 49356 injection.

Glibenclamide and cromakalim receptors

Glibenclamide reduced the opening of ATP-dependent K^+ channels and induced insulin secretion in isolated pancreas at low concentrations (50–500 nM) (Geisen, 1988; Sturgess *et al.*, 1988; Garrino *et al.*, 1989). These effects will explain the hyperinsulinaemia and hypoglycaemia seen after glibenclamide *in vivo* (doses 0.1–0.5 mg kg^{-1}, Bosboon *et al.*, 1973; Geisen, 1988). However, the concentrations of glibenclamide required *in vitro* (1–10 μM) and doses needed *in vivo* (20 mg kg^{-1}) to antagonize the uterine actions of the K^+ channel openers, in the present study, are 1–2 orders of magnitude higher than the doses of glibenclamide required to block ATP-sensitive K^+ channels in the pancreas. This comparison suggests that there are differences between the pancreas and the uterus with regard to glibenclamide, presumably either in the mechanism of action of glibenclamide or that the receptors in the two tissues are not identical.

The doses (*in vivo*) and concentrations (*in vitro*) required for the antagonist actions of glibenclamide in uterus are similar to those described in other smooth muscles (Mondot *et al.*, 1988; Buckingham *et al.*, 1989; Cavero *et al.*, 1989; Quast & Cook, 1989a) and in the heart (Escande *et al.*, 1989). By contrast, cromakalim at doses which had uterine relaxant and vasodilator actions had minimal effects on plasma glucose and insulin concentrations *in vivo* (Wilson *et al.*, 1988; Quast & Cook, 1989a). Garrino *et al.* (1989) found that cromakalim and pinacidil only inhibited insulin secretion from mouse pancreatic islets at very high concentrations. These observations suggest that if glibenclamide and the above K^+ channel openers interact with a common site then there may be two sub-classes of receptor, one sub-class existing in pancreas and another in smooth muscle.

Glibenclamide alone did not modify spontaneous uterine contractions (*in vivo*) or resting tone, oxytocin-driven or KCl-stimulated spasm in the isolated uterus at concentrations which antagonized cromakalim, RP 49356 and pinacidil. Such observations suggest that the channel(s) opened by these relaxants is not open under these conditions and does not contribute to the maintenance of resting tone. Similar conclusions have been drawn in vascular and tracheal smooth muscle (Hamilton *et al.*, 1986; Murray *et al.*, 1989; Quast & Cook, 1989a).

In conclusion, several K^+ channel openers are relaxants of the uterus both *in vitro* and *in vivo*. Whether other K^+ channel openers will exhibit significant selectivity for the uterus remains to be seen.

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Effects of putative neurotransmitters and related drugs on withdrawal contractures of guinea-pig isolated ileum following brief contact with [Met^5]enkephalin

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1 Brief exposure for 2 min of guinea-pig isolated ileum to [Met^5]enkephalin (MEnk) and noradrenaline has been shown previously to produce withdrawal contractures on washout of the agonist or addition of naloxone (MEnk) or phentolamine (noradrenaline).

2 The present study was undertaken to investigate firstly, whether other putative neurotransmitters and/or related drugs which inhibit transmitter release also produced withdrawal responses following 2 min contact with the ileum and secondly, whether they affected the opioid withdrawal response.

3 Adenosine (1–5 μM), but not U-50,488H (1–5 μM), somatostatin (0.01–5 μM), ocreotide (1–5 μM), baclofen (1–25 μM) or dopamine (5, 50 μM), produced a contracture on washout following 2 min contact with the ileum. The adenosine (5 μM) washout contracture, in common with MEnk and noradrenaline washout contractures, was inhibited by the substance P antagonist, spantide (10 μM).

4 Added 30 s before washout at a concentration of 5 μM, noradrenaline, U-50,488H, adenosine, somatostatin and ocreotide significantly inhibited the washout withdrawal response following 2 min contact of the ileum with MEnk, 1 μM. A higher concentration of baclofen, 250 μM, also inhibited this response.

5 The naloxone (1 μM)-precipitated withdrawal response following contact of the ileum with MEnk, 1 μM, for 2 min, was inhibited only by noradrenaline (5 μM) and U-50,488H (5 μM).

6 It is concluded that during naloxone-precipitated opioid withdrawal an additional population of enteric motor neurones is recruited which is not involved in the washout withdrawal response and these neurones have less diversity of presynaptic receptors mediating inhibition of transmitter release than cholinergic motor neurones.

Introduction

Brief exposure for 2 min to [Met^5]enkephalin (MEnk), several enkephalin analogues and noradrenaline produced dependence in guinea-pig ileum as measured by withdrawal contractures on washout (Chahl, 1983; 1985; 1986). Withdrawal responses were also precipitated by naloxone following 2 min contact with several opioids including morphine, β-endorphin and dynorphin A- (1–13), and by phentolamine following contact with clonidine (Chahl, 1985). Furthermore it has been shown that the α_2-adrenoceptor agonist clonidine (Chahl, 1985), and both μ and κ-opioid receptor agonists inhibited the opioid withdrawal response of the guinea-pig ileum (Chahl, 1986).

Opioids and α-adrenoceptor agonists have in common an action to inhibit release of acetylcholine (ACh) and substance P (SP) from enteric neurones (see Barthó & Holzer, 1985). Several other putative enteric neurotransmitters, including adenosine (see Fredholm & Dunwiddie, 1988), somatostatin (Guillemin, 1976; Yau *et al.*, 1986) and γ-aminobutyric acid (GABA) (Cherubini & North, 1984), have also been reported to inhibit transmitter release from enteric neurones. In order to obtain further information on possible mechanisms involved in withdrawal responses, the present study was undertaken to investigate firstly, whether these putative neurotransmitters and/or related drugs produced dependence as manifest by a withdrawal response following brief contact with the ileum, and secondly whether they affected the opioid withdrawal response.

Methods

Adult guinea-pigs of either sex were killed by a blow to the head and a 2 cm segment of ileum was removed and suspended under 1 g tension in a 2 ml organ bath containing oxygenated Tyrode solution at 37°C. Responses were recorded with a force transducer and Grass polygraph. At the start of each experiment a maximum response to ACh (5 μM) was obtained so that all responses could be expressed as percentages of the ACh maximum. Standard responses were then obtained to ACh (0.1 μM), SP (2.5 nM), and to washout following 2 min contact with MEnk, 1 μM.

Effects of agonists and antagonists

Neurotransmitters and related drugs, were each tested for their effects on the ileum on contact, and on washout following 2 min contact. The effect of the SP antagonist, spantide, 10 μM, added 30 s before washout, was tested on the washout response to adenosine.

The effect of the somatostatin antagonist, cyclo(7-aminoheptanoyl-Phe-D-Trp-Lys-Thr[Bzl]) (Fries *et al.*, 1982) added 2 min after addition of somatostatin to the organ bath was tested. The effect of naloxone, 1 μM, was tested following 2 min contact of the ileum with the κ-opioid agonist, U-50,488H. Likewise the effect of the $GABA_B$ antagonist, phaclofen (Kerr *et al.*, 1987), was tested following 2 min contact of the ileum with baclofen.

Effects of agonists and related drugs on the opioid withdrawal response

The effects of the neurotransmitters and related drugs were tested on the washout withdrawal response to MEnk. In these experiments MEnk, 1 μM was added to the bath for 2 min before washout and the substance to be tested was added 0.5 min before washout (1.5 min after addition of MEnk). In some experiments ocreotide was added 5 min before addition of MEnk. Control responses to MEnk washout were obtained on the same preparations.

In some experiments the effects of the substances were tested as above on the naloxone-precipitated withdrawal response to MEnk. In these experiments MEnk, 1 μM, was added to the bath and naloxone, 1 μM, was added 2 min later. Since only one response to naloxone was obtained on each preparation, control responses were obtained on other preparations from the same animals.

Statistics

Responses were compared by Student's t tests for unpaired observations, or paired t tests for paired observations. The n values represent preparations from different animals.

Drugs

Drugs used were: acetylcholine chloride (Sigma); adenosine (Merck); baclofen (Ciba-Geigy); dopamine hydrochloride (Sigma); γ-aminobutyric acid (Sigma); [Met^5]enkephalin (Peptide Institute, Osaka, Japan); naloxone hydrochloride (Sigma); neurotensin (Peptide Institute); (−)-noradrenaline bitartrate (Sigma); ocreotide (Sandostatin, Sandoz); phaclofen (Professor David Kerr and Dr Jennifer Ong); 8-phenyltheophylline (Research Biochemicals Inc.); somatostatin (Sigma); cyclo(7-aminoheptanoyl-Phe-D-Trp-Lys-Thr[Bzl]) (somatostatin antagonist) (Sigma); substance P (Peptide Institute); [D-Arg^1,D-Trp^{7,9}, Leu^{11}] substance P Spantide, Bachem); U-50,488H (trans-(±)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide methane sulphonate Upjohn) (Stephen Johnson); vasoactive intestinal polypeptide (Peptide Institute).

Results

Effects of agonists on guinea-pig ileum

The effects of putative neurotransmitters and related drugs on the tension of the guinea-pig ileum are shown in Table 1. On preparations where tone was slightly elevated, some fall in tone was observed on addition of U-50,488H, adenosine, somatostatin and its analogue ocreotide, baclofen, and dopamine, in the concentrations shown in Figures 1, 2 and 4 respectively. None of these drugs produced contraction of the ileum. However, GABA, 5 μM, neurotensin, 0.05 μM, and VIP, 0.1 μM, produced marked contractions on contact with the ileum. At a concentration of 0.1 μM, GABA produced no effect on the ileum whereas at 1 μM it produced relaxation only. The contractions to VIP, and to a lesser extent neurotensin, exhibited tachyphylaxis on subsequent additions.

Table 1 Effects of 2 min contact of guinea-pig ileum with putative neurotransmitters and related drugs and the effect of washout

Drug	*Effect on ileum*	*Response on washout*
[Met^5]enkephalin (Chahl, 1985)	relaxation	contraction (1 μM) 20 ± 2† (20)
Noradrenaline (Chahl, 1985)	relaxation	contraction (5 μM) 22 ± 4 (8)
U-50,488H 1–5 μM	relaxation	nil (9) (5 μM)
Adenosine 1–5 μM	relaxation	contraction (5 μM) 32 ± 4 (15)
Somatostatin 0.01–5 μM	relaxation	nil (6) (5 μM)
Ocreotide 1–5 μM	relaxation	nil (5) (5 μM)
GABA		
0.1 μM	nil	nil (3)
1 μM	relaxation	nil (3)
5 μM	'spike' contraction 60;50	nil (2)
Baclofen 1–25 μM	relaxation	nil (7) (5 μM)
Dopamine		
5 μM	slight relaxation	nil (5)
50 μM	slight relaxation	nil (11)
VIP 0.1 μM	maximal contraction (tachyphylaxis) (5)	return to baseline
Neurotensin 0.05 μM	maximal contraction (5)	return to baseline

† Percentage of ACh maximum ± s.e.
Numbers in parentheses are the numbers of preparations from different animals.

Effects of washout

On washout following 2 min contact, adenosine, 1–5 μM, was the only drug tested which produced a contracture (Table 1, Figure 1). The washout contracture to adenosine, 5 μM, was abolished in seven preparations (from seven animals) by addition of the SP antagonist, spantide, 10 μM, 30 s before washout of the bath (Figure 1). Thus adenosine produced similar actions on the ileum to those previously described for MEnk and noradrenaline (Chahl, 1983; 1985). The lack of response to washout of somatostatin, 5 μM, is shown in Figure 2.

Effects of addition of antagonists

Addition of the somatostatin antagonist, cyclo(7-aminoheptanoyl-Phe-D-Trp-Lys-Thr[Bzl]), at a concentration of 5 μM, following 2 min contact of the ileum with concentrations of somatostatin of 1 μM ($n = 5$) and 0.1 μM ($n = 3$) did not produce contraction of the ileum. In one experiment the somatostatin antagonist at a concentration of 50 μM did not produce contraction following 2 min contact of the ileum with somatostatin, 5 μM. Naloxone, 1 μM, added 2 min after addition of U-50,488H, 1 or 5 μM, did not result in contracture of the ileum ($n = 9$). Higher concentrations of naloxone were not tested as previous experiments indicated that naloxone was not specific at these concentrations (Chahl, 1983). The $GABA_B$ antagonist, phaclofen, 500 μM, produced a small contraction (23 ± 6% of the ACh maximum, $n = 7$) following 2 min contact of ileum with the $GABA_B$ agonist, baclofen, 250 μM. However, this response was not significantly greater than the contraction produced by phaclofen alone on the ileum (16 ± 3%, $n = 8$). The effect of the adenosine A_1 antagonist, 8-phenyltheophylline (5 μM), was tested on two preparations following 2 min contact with adenosine 5 μM and found to produce contractions (14% and 25%).

Effects of agonists on the opioid withdrawal response

The effects of the drugs tested on the withdrawal response following 2 min contact with MEnk, 1 μM, precipitated by washout or addition of naloxone, 1 μM, are shown in Table 2.

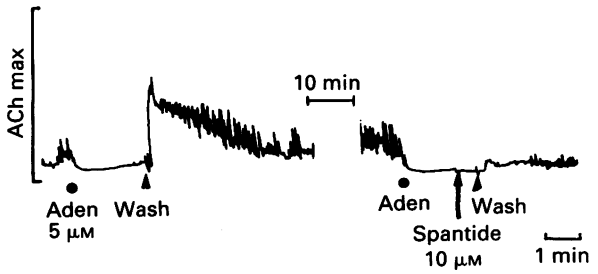

Figure 1 Inhibition by the substance P antagonist, spantide, 10 μM, of the washout withdrawal response of guinea-pig ileum to adenosine (Aden), 5 μM. Adenosine was washed out after 2 min contact with the tissue. Spantide was added 30 s before washout and replaced immediately after washout.

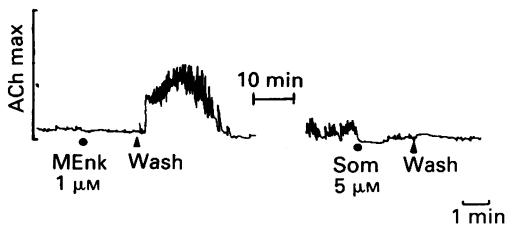

Figure 2 Comparison of the effects of washout of [Met^5]enkephalin (MEnk) 1 μM, and of somatostatin (Som) 5 μM, on guinea-pig ileum. Each drug was present in the bath for 2 min before washout.

Table 2 Effect of putative neurotransmitters and related drugs on the withdrawal responses precipitated either by washout or naloxone (1 μM) following 2 min contact with [Met^5]enkephalin (1 μM)

	MEnk washout withdrawal	*MEnk naloxone-precipitated withdrawal*
Control	44 ± 10 (7)	62 ± 9 (12)
Noradrenaline (5 μM)	*19 ± 6 (7)	***3 ± 2 (9)
Control	27 ± 4 (5)	56 ± 7 (8)
U-50,488H (5 μM)	**0 ± 0 (5)	***0 ± 0 (8)
Control	50 ± 9 (7)	62 ± 9 (12)
Adenosine (5 μM)	***6 ± 4 (7)	43 ± 17 (6)
Control	47 ± 7 (8)	52 ± 9 (8)
Somatostatin (5 μM)	***11 ± 6 (8)	47 ± 9 (7)
Control	31 ± 4 (5)	
Ocreotide (5 μM)	**10 ± 6 (5)	
Control	50 ± 7 (9)	52 ± 9 (8)
Ocreotide (5 μM, added 5 min before MEnk)	***7 ± 4 (9)	51 ± 12 (6)
Control	40 ± 11 (5)	56 ± 7 (8)
Baclofen (5 μM)	36 ± 9 (5)	60 ± 19 (5)
Control	48 ± 7 (7)	62 ± 9 (12)
Baclofen (250 μM)	***11 ± 2 (7)	76 ± 13 (8)

Except where indicated, drugs were added 30 s before washout or naloxone.
Responses are expressed as mean percentages of the ACh maximum ± s.e.mean. The numbers of preparations from different animals are shown in parentheses. Asterisks indicate statistically significant differences from control values, obtained in paired t tests (washout withdrawal) or Student's t tests (naloxone-precipitated withdrawal). *$0.05 > P > 0.01$; **$0.01 > P > 0.001$; ***$P < 0.001$.

At a concentration of 5 μM, noradrenaline, U-50,488H, adenosine, somatostatin (Figure 3) and ocreotide, added 30 s before washout, significantly inhibited the MEnk washout withdrawal response. None of these agonists inhibited standard responses to SP or ACh. Ocreotide was more effective added 5 min before addition of MEnk, indicating that it took time to reach equilibrium with the receptors (Figure 4). A higher concentration of baclofen, 250 μM, inhibited the MEnk washout withdrawal response. However, the naloxone-precipitated MEnk withdrawal response was inhibited only by noradrenaline and U-50,488H (Table 2). Lack of effect of ocreotide on naloxone-precipitated MEnk withdrawal is shown in Figure 4. In three preparations somatostatin at a concentration of 50 μM, did not inhibit the naloxone-precipitated MEnk withdrawal response, but in another three preparations, baclofen, 500 μM, abolished the naloxone-precipitated MEnk response. This concentration of baclofen produced little effect on the SP standard response (10% reduction).

Discussion

The present study has demonstrated that following brief (2 min) contact with guinea-pig ileum, adenosine, in common with MEnk and noradrenaline, produced a washout withdrawal contracture which was inhibited by an SP antagonist. The basis of the common actions of these three agonists is most probably due to their ability to inhibit transmitter release from enteric cholinergic neurones following activation of their own specific receptors (see Introduction). Although U-50,488H, somatostatin, ocreotide, baclofen and dopamine all produced relaxation of the ileum presumably due to inhibition of ACh release, none of these agonists produced a washout withdrawal contracture. A likely explanation for the lack of withdrawal responses to these agonists is that they were less readily removed from their receptors on washout than MEnk, noradrenaline and adenosine. This possibility is supported by the observation that those agonists which did not exhibit a washout withdrawal response tended to produce a prolonged inhibition of tone and spontaneous movements after washout. However, it is also possible that the two groups of agonists did not have similar actions on neurotransmitter release (see below).

In order to produce withdrawal responses to substances that are not rapidly removed from receptors by washout it would be necessary to use a specific receptor antagonist to precipitate withdrawal. For example, naloxone has been shown to precipitate withdrawal following 2 min contact of ileum with morphine (Chahl, 1986), and phentolamine precipitated withdrawal following 2 min contact with clonidine (Chahl, 1985). In the present experiments concentrations of naloxone (1 μM) which precipitated withdrawal from μ-receptor agonists (Chahl, 1986), did not produce a withdrawal contracture from the κ-agonist, U-50,488H. Higher concentrations of naloxone were not tested as previous experiments indicated that naloxone was not specific at higher concentrations (Chahl, 1983). The finding that the adenosine A_1 antagonist, 8-phenyltheophylline, produced contraction of the ileum following 2 min contact with adenosine confirms previous findings in which withdrawal of ileum following 16–21 h incubation with adenosine was demonstrated by 8-phenyltheophylline challenge (Collier & Tucker, 1983). The lack of effect of the somatostatin antagonist and the $GABA_B$ receptor antagonist, phaclofen, in producing withdrawal responses from somatostatin and baclofen respectively, probably reflected the low potency of these antagonists.

It has been proposed previously (Chahl, 1983) that a feedback circuit of neurones might be responsible for mediating the opioid withdrawal response and that the contracture response might involve release of transmitters (SP and ACh) from more than one type of neurone. If such a circuit is indeed

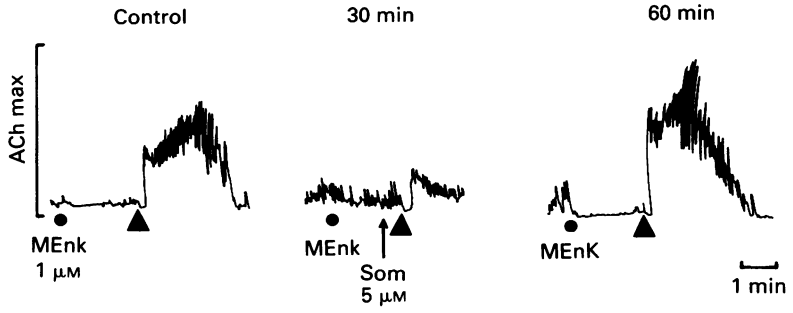

Figure 3 Reversible inhibition of the washout withdrawal response to [Met^5]enkephalin (MEnk) 1 μM, by somatostatin (Som) 5 μM, added 30 s before washout. Washout shown as triangles.

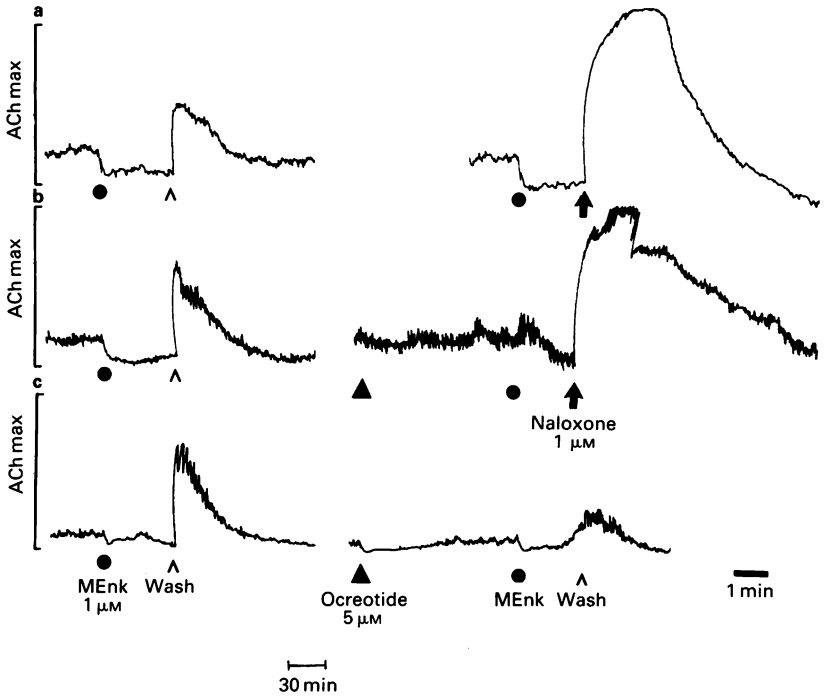

Figure 4 Effect of ocreotide on [Met^5]enkephalin (MEnk) withdrawal responses on three ileum preparations from the same animal. On each preparation a control withdrawal response was obtained by washout following 2 min contact with MEnk, 1 μM. On preparation (a) a control naloxone (1 μM)-precipitated withdrawal response to MEnk (1 μM) was obtained. Naloxone was added 2 min after MEnk. On preparation (b) a MEnk naloxone-precipitated withdrawal response was obtained 5 min after addition of the somatostatin analogue, ocreotide, 5 μM. On preparation (c), a MEnk washout withdrawal response was obtained 5 min after addition of ocreotide, 5 μM. No marked difference between the heights of responses to MEnk washout and naloxone-precipitated withdrawal (as in these preparations) was consistently observed over a number of preparations (see Table 2 for mean values), although the naloxone-precipitated response was often slightly larger and usually more prolonged.

involved, it is not known whether the other agonists tested here acted in the same circuit and on the same neurones in the circuit as the opioids. The agonists used in this study reduce transmitter release from neurones by different mechanisms. Thus μ-opioid and α_2-adrenoceptor agonists, and possibly adenosine and somatostatin, increase potassium conductance (see North, 1986; Fredholm & Dunwiddie, 1988), whereas κ-opioid and $GABA_B$ receptor agonists decrease an inward calcium conductance (see North, 1986; Cherubini & North, 1984). The relative proportions of these receptors on different neuronal types in the guinea-pig ileum and their relative effectiveness in inhibiting transmitter release are unknown. Nevertheless, if the receptors for all agonists tested were present in similar numbers on the same population of enteric neurones and acted through the same intracellular mechanism to inhibit transmitter release to a similar extent, it would be expected that each agonist could substitute for any one of the others and thus prevent its withdrawal response. This was tested in the present experiments by examining whether each agonist would inhibit the MEnk washout and naloxone-precipitated withdrawal responses. The results obtained for the MEnk washout withdrawal response were as expected, that is, each agonist tested inhibited this response. Thus it would seem reasonable to conclude that the population(s) of neurones involved in the MEnk washout withdrawal response had not only μ-opioid receptors, but also κ-opioid receptors, α-adrenoceptors, somatostatin, adenosine and $GABA_B$ receptors, stimulation of which resulted in the same functional response.

Results for the experiments on MEnk naloxone-precipitated withdrawal, however, differed from those for the MEnk washout withdrawal. Only noradrenaline and U-50,488H inhibited the MEnk naloxone-precipitated withdrawal at concentrations that inhibited the washout withdrawal response. A two fold higher concentration of baclofen inhibited the MEnk naloxone precipitated withdrawal response, a finding which agreed with previous findings on the inhibition by baclofen (1 μM) of the naloxone contracture in morphine-tolerant guinea-pig ileum (Luzzi *et al.*, 1985). However, even a ten fold increase in the concentration of somatostatin was ineffective in reducing the response. Two possible explanations for these findings should be considered. Firstly, since the MEnk naloxone-precipitated withdrawal response was much more resistant to inhibition by some agonists than others, at concentrations which markedly inhibited the MEnk washout withdrawal response, it might be concluded that an additional population(s) of neurones is involved in the MEnk naloxone-precipitated response, and that this population(s) is equipped with μ- and κ-opioid receptors, α-adrenoceptors and perhaps $GABA_B$ receptors, but with few or no adenosine or somatostatin receptors. Alternatively, it could be postulated that the number/effectiveness of receptors for adenosine and somatostatin is less than that of α_2- and κ-receptors and thus adenosine and somatostatin were unable to inhibit the naloxone-precipitated response, which was often slightly larger and more prolonged than the washout response. This latter proposal seems less likely in view of the fact that no reduction or delay in response was noted with a ten times higher concentration of somatostatin than that which inhibited the washout response.

A difference in the pharmacology of the MEnk washout and naloxone-precipitated withdrawal responses was reported previously (Chahl, 1983). Although both responses were inhibited by antagonism of SP, the MEnk washout response was more atropine-sensitive than the naloxone-precipitated response. It is also known that SP (and ACh) are present in more than one type of neurone in the myenteric plexus (Costa *et al.*, 1981). It is therefore possible that a population of non-cholinergic, SP-containing neurones might play a greater role in MEnk naloxone-precipitated withdrawal than in washout withdrawal, and that these neurones do not have as extensive a range of receptor types as cholinergic neurones. In support of

the concept of differing receptor types on myenteric neurones, Yau *et al.* (1986) found that MEnk but not somatostatin inhibited the efflux of ACh evoked by SP in the myenteric plexus. Nevertheless, it is difficult to explain why withdrawal induced by naloxone should recruit additional neurones to those recruited by washout, unless naloxone more effectively reaches certain compartments of the preparation than washout.

In conclusion, the present study has elucidated a pharmacological difference between washout and naloxone-precipitated withdrawal, in their different sensitivities to agonists which inhibit ACh release from enteric cholinergic neurones. Although these agonists have in common the ability to inhibit release from cholinergic neurones, it is by no means clear that their action is limited to these neurones, or that these neurones are the only type involved in withdrawal responses. It is now proposed that in naloxone-precipitated withdrawal, not only cholinergic motor neurones are involved but an additional, distinct population of motor neurones containing SP (see Barthó & Holzer, 1985) is recruited which has less diversity of presynaptic receptors.

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Endotoxin-induced impairment of vasopressor and vasodepressor responses in the pithed rat

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1 Effects of *E. coli* endotoxin on vascular responsiveness to a variety of agents were compared with those of the calcium channel blocking drug nicardipine in pithed rats.

2 Infusion of endotoxin ($250\,\mu g\,kg^{-1}\,h^{-1}$) produced a fall in mean arterial blood pressure (8 mmHg). A similar fall (11 mmHg) was seen in rats receiving nicardipine ($1.0\,mg\,kg^{-1}$).

3 Endotoxin impaired responsiveness to vasopressin, phenylephrine and cirazoline, producing a shift to the right in the dose-response curves without any change in the maximum response. Responsiveness to 5-hydroxytryptamine (5-HT) and to the α_2-adrenoceptor agonists clonidine and BHT 933, was also impaired with a marked reduction in their maximum responses. The dose-response curve to the pressor effects of endothelin was not significantly modified.

4 Nicardipine produced a similar pattern of impairment of responsiveness to these agents to that produced by endotoxin. However, nicardipine also shifted the pressor dose-response curve to endothelin to the right with no significant alteration in its maximum response.

5 The pressor responses to endothelin and to 5-HT were, respectively, preceded and followed by dose-dependent depressor responses, which were markedly reduced by endotoxin and nicardipine.

6 The concomitant infusion of arginine vasopressin ($0.64\,iu\,kg^{-1}\,h^{-1}$) prevented endotoxin-induced hypotension and also prevented the impairment in responsiveness to cirazoline and to BHT 933.

7 The similarity of the pattern of impaired pressor responsiveness (except in relation to endothelin) and depressor responsiveness produced by endotoxin and nicardipine may be consistent with a common mechanism of action.

Introduction

Impaired responsiveness to vasoconstrictor catecholamines may contribute importantly to the unrelenting hypotension of septic shock (Parratt, 1989). Attenuation of pressor responses to catecholamines has been demonstrated in animal models of sepsis (Fink *et al.*, 1985) and endotoxaemia (Parratt, 1973; Auclair & Schmitt, 1987). Responsiveness to α_2-adrenoceptor agonists and to vasopressin, angiotensin II, calcium, the calcium channel activating drug Bay K 8644 and sympathetic nerve stimulation is also impaired (McCaig & Parratt, 1980; Schaller *et al.*, 1985; Gray *et al.*, 1987; 1990b). Since these agents produce vasoconstriction through a variety of mechanisms and have different requirements for intracellular versus extracellular calcium (van Zwieten *et al.*, 1985), endotoxin may impair the contractile machinery itself. If this is the case then all vasoconstrictors should be affected similarly. The present work was undertaken to investigate this possibility using a wide variety of agonists in a rat model of endotoxaemia. Additionally, the role of endotoxin-induced hypotension in impairing reactivity was considered in experiments in which this hypotension was prevented by vasopressin infusion. Agonists used were vasopressin, Bay K 8644, 5-hydroxytryptamine (5-HT), endothelin, the selective α_1-adrenoceptor agonists phenylephrine and cirazoline and the selective α_2-adrenoceptor agonists clonidine and BHT 933. The pithed rat was used to circumvent problems due to reflex release of catecholamines (Jones & Romano, 1984; Schaller *et al.*, 1985; McKechnie *et al.*, 1985). Preliminary accounts of some of these data have been presented to the British Pharmacological Society (Guc *et al.*, 1989; 1990).

Methods

Animals

Male Sprague-Dawley rats (250–300 g) fed on standard laboratory pellet diet (Oxoid) were used throughout. The animals were allowed free access to food and water until the experiment.

Rats were anaesthetized with ether and pithed by the method of Gillespie *et al.* (1970). The animals were ventilated artificially via a tracheal cannula (Palmer pump, 48 strokes min^{-1}; 100% O_2; 1 ml $100\,g^{-1}$, to maintain arterial P_{CO_2} at about 30 mmHg). Catheters were placed in the aortic arch via the left carotid artery and in both jugular veins. Blood pressure was recorded continuously via a Gould pressure transducer coupled to a Mingograph chart recorder. The electrocardiogram was recorded continuously from subcutaneous leads on the fore-limbs and one hind-limb. Body temperature was maintained at 37 ± 0.5°C by an incandescent lamp placed over the abdomen and controlled by a thermistor probe inserted into the rectum. After a 30 min equilibration period, infusion of endotoxin ($250\,\mu g\,kg^{-1}\,h^{-1}$) or saline ($0.1\,ml\,h^{-1}$) was started. In some experiments, endotoxin infusion was replaced by injection of nicardipine (0.3 or $1\,mg\,kg^{-1}$ or its vehicle) given slowly over about 5 min. Pressor dose-response curves to agonists were started at 60 min into the infusion, or at 60 min after nicardipine injection, usually by the cumulative administration of agonists, each subsequent dose of agonist being injected at the peak of the response to the previous dose. Only 5-HT was administered as single doses. The use of cumulative injections allowed the entire dose-response curve to be constructed in 4–30 min, depending on the agonist. This was especially important in the case of endothelin, where the pressor response was very long lasting. Previous studies with noradrenaline and angiotensin II (Gray, 1989) and preliminary experiments with clonidine and phenylephrine (Figure 1) showed dose-response curves constructed in this way to be superimposable on curves constructed by conventional, single-dose injection of drugs.

In some experiments the fall in blood pressure in endotoxin-treated rats was prevented by the concomitant infusion of vasopressin ($0.64\,iu\,kg^{-1}\,h^{-1}$).

The increase in mean arterial blood pressure (mmHg) was determined for each dose of agonist and the dose of agonist producing 50% of the maximum response (E_{max50}) was calculated. Pressor dose-response curves were analysed in terms of

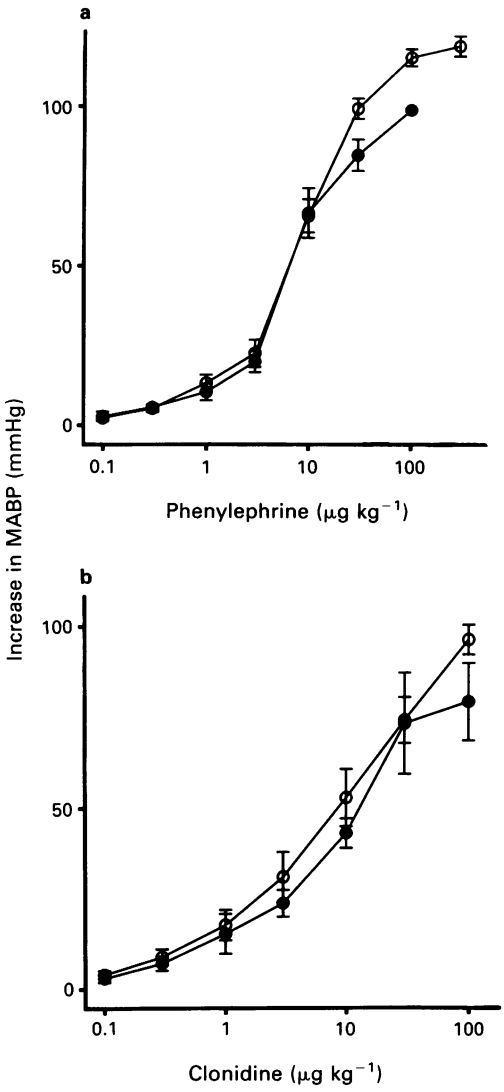

Figure 1 Pressor dose-response curves to (a) phenylephrine and (b) clonidine in pithed rats obtained by cumulative injection (○) and single dose administration (●). Each point is the mean of (a) 2–11 or (b) 3–12 observations; vertical lines indicate s.e.mean. MABP = mean arterial blood pressure.

the E_{max50} and the maximum responses. Depressor dose-response curves to 5-HT and to endothelin could not be analysed in this way, because true maximum depressor responses to endothelin and 5-HT could not be obtained.

All values are expressed as arithmetic mean ± s.e.mean. Results from pressor dose-response curves (E_{max50} values and maximum responses) were analysed by Student's t test for unpaired observations, or two way analysis of variance, as appropriate. Student's t test was also used to compare depressor responses in treated rats with those in controls. In these cases, comparisons were made only at the doses giving apparent maximum responses in control rats. Statistical significance was accepted where $P < 0.05$.

Drugs used

Endotoxin (Difco Laboratories U.K., 055 B5, Boivin preparation) was suspended in 0.9% w/v sodium chloride solution. (−)-Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoro-methylphenyl) pyridine-5-carboxylate, Dr M. Schramm, Bayer, Wuppertal) was prepared (1 mg ml^{-1}) in a solvent which consisted of glycerol (60 g, Sigma), distilled water (100 ml) and polyethylene glycol 400 (1129 g, B.D.H.); further dilutions were made in saline in darkened containers to avoid light-induced degradation. Nicardipine (Syntex) was dissolved with the solvent used for Bay K 8644. Phenylephrine (Sigma), clonidine (Sigma), cirazoline (Synthelabo), BHT 933 (azepexol HCl, Boehringer Ingelheim), 5-hydroxytryptamine (Sigma), endothelin I (human, Novabiochem), arginine vasopressin (Grade V, Sigma) were all dissolved in 0.9% (w/v) sodium chloride solution.

Results

Effect of endotoxin

Pithed rats infused with saline throughout the experiment were stable with mean arterial blood pressure changing only little from the initial value (62.5 mmHg). Infusion of endotoxin (250 μg kg^{-1} h^{-1}) produced a progressive decline in mean arterial blood pressure, which reached a nadir at 1 h (endotoxin 49.5 ± 0.4 mmHg ($n = 77$); control 57.9 ± 0.5 mmHg ($n = 94$); $P < 0.01$).

Effects of endotoxin on pressor responses to various agonists

In saline-infused control rats phenylephrine, cirazoline, clonidine, BHT 933, Bay K 8644, vasopressin, 5-HT and endothelin each produced dose-dependent pressor responses. The pressor responses to endothelin (Figure 2) and to 5-HT (Figure 3) were preceded and followed, respectively, by dose-dependent depressor responses.

Infusion of endotoxin modified these pressor responses, although a different pattern was observed with the different agonists. Pressor dose-response curves to phenylephrine, cirazoline and vasopressin were shifted to the right as evidenced by a significant increase in their E_{max50} values (Figure 4 and Table 1). However, the maximum responses to these agonists were not significantly modified. On the other hand, maximum pressor responses to clonidine, BHT 933 and especially to 5-HT appeared to be significantly depressed (Figure 4). In some cases it was assumed, from the shape of the dose-response curves, that the largest dose of agonist used produced a maximum response in endotoxin-treated rats. This was necessitated by the lethal effects of higher doses. The dose-response curve to Bay K 8644 was shifted to the right but responses to higher doses were complicated by the pronounced pressor effect of the vehicle. It was therefore not possible to determine the effect of endotoxin on the maximum response to this agent. Neither the maximum pressor response to endothelin nor its E_{max50} value was significantly modified by endotoxin (Figure 4 and Table 1).

Effects of nicardipine on pressor responses to agonists

Administration of nicardipine (0.3 and 1.0 mg kg^{-1}) by slow intravenous injection produced an immediate and sustained decrease in mean arterial blood pressure (nicardipine 0.3 mg kg^{-1}, 46.8 ± 0.4 mmHg ($n = 40$, $P < 0.01$ vs control), nicardipine 1.0 mg kg^{-1}, 46.6 ± 0.4 mmHg ($n = 43$, $P < 0.01$ vs control); control 57.9 ± 0.5 mmHg ($n = 94$)).

Like endotoxin, nicardipine shifted to the right the pressor dose-response curves to phenylephrine, cirazoline and vasopressin, without any change in the maximum responses (Figure 5). The pressor response to endothelin was similarly affected by nicardipine, in contrast to the ineffectiveness of endotoxin. Again, the maximum responses to Bay K 8644 were complicated by the effects of the solvent. On the other hand, the maximum responses to clonidine, BHT 933 and 5-HT were significantly reduced (Figure 5). The effect of nicardipine appeared to be dose-dependent in that a more pronounced effect was usually seen with the higher dose.

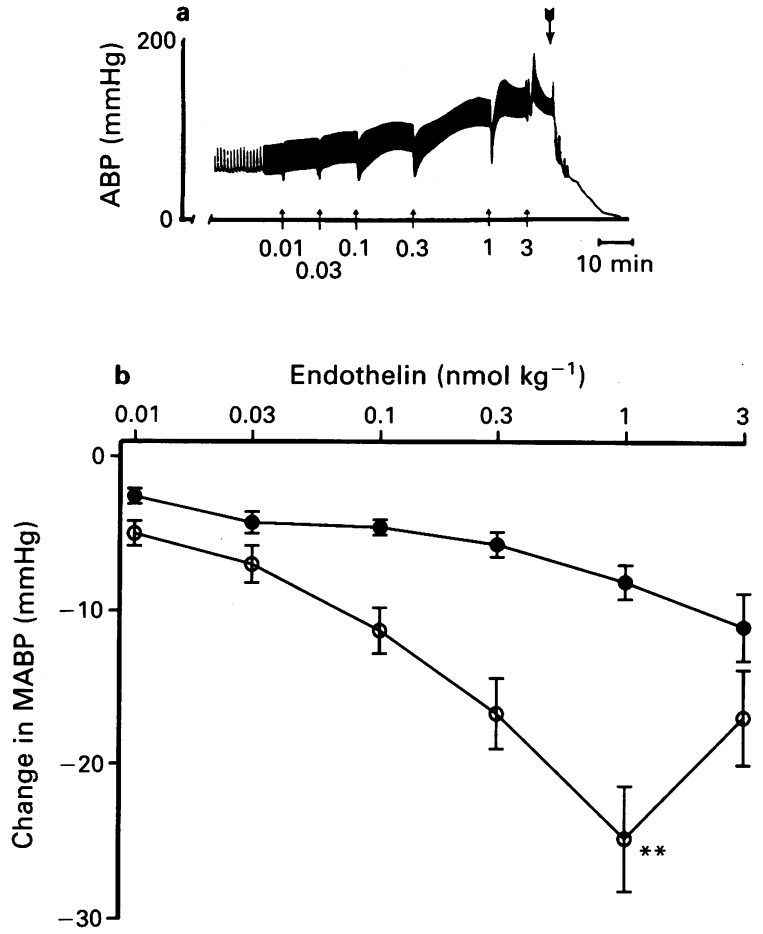

Figure 2 (a) Typical recording of the effects of cumulative administration of endothelin (0.01–10 nmol kg^{-1}) on arterial blood pressure (ABP) in a pithed rat. Endothelin was injected at the arrows. (b) Dose-depressor response curves to endothelin in pithed rats receiving an infusion of either endotoxin (250 $\mu g\,kg^{-1}\,h^{-1}$, ●) or saline (○). Each point represents the mean of 14–16 observations and vertical lines show s.e.mean. ** $P < 0.01$.

Effects of vasopressin infusion on endotoxin-induced changes in vascular reactivity

The concomitant infusion of vasopressin (0.64 iu $kg^{-1}\,h^{-1}$) prevented endotoxin-induced hypotension measured 1 h after commencement of the infusion (endotoxin alone 48 ± 3 mmHg; endotoxin + vasopressin 59 ± 2 mmHg; control, saline infusion 61 ± 3 mmHg). This treatment also prevented the endotoxin-induced impairment of vascular responsiveness to cirazoline (Figure 6). E_{max50} values ($\mu g\,kg^{-1}$) for cirazoline in control, endotoxin-infused, endotoxin + vasopressin-infused rats and saline + vasopressin-infused rats were, respectively, 3.1 ± 0.7, 14.2 ± 3, 6.4 ± 1 and 3.2 ± 0.8 (effect of endotoxin, $P < 0.01$, effect of vasopressin, $P < 0.05$, interaction, $P < 0.01$). In saline-infused animals, vasopressin infusion elevated the mean arterial blood pressure to

Table 1 Effects of endotoxin (250 $\mu g\,kg^{-1}\,h^{-1}$) or nicardipine (0.3, 1.0 mg kg^{-1}) on the E_{max50} values of various agonists, expressed in $\mu g\,kg^{-1}$ except in the case of vasopressin (iu kg^{-1})

				Nicardipine	
Agonist	*Control*	*Endotoxin*	*Control*	0.3	1.0
Phenylephrine	8.5 ± 1.6 (*n* = 6)	28.5 ± 7.1* (*n* = 6)	10.8 ± 1.9 (*n* = 6)	16.4 ± 1.5 (*n* = 6)	30.0 ± 0.5* (*n* = 5)
Cirazoline	2.14 ± 0.4 (*n* = 6)	14.5 ± 3.8* (*n* = 6)	2.1 ± 0.4 (*n* = 6)	7.1 ± 2.3 (*n* = 5)	4.8 ± 0.3** (*n* = 6)
Clonidine	8.7 ± 0.9 (*n* = 12)	16.5 ± 2.4** (*n* = 6)	8.7 ± 0.9 (*n* = 12)	46.2 ± 5.4** (*n* = 6)	51.2 ± 1.7** (*n* = 6)
BHT 933	225.0 ± 38.4 (*n* = 10)	192.0 ± 45.6 (*n* = 8)	225.0 ± 38.4 (*n* = 10)	310 ± 48.7 (*n* = 4)	300.0 ± 39.7 (*n* = 4)
Endothelin	2.4 ± 0.3 (*n* = 11)	2.99 ± 0.37 (*n* = 14)	2.4 ± 0.3 (*n* = 11)	ND	12.2 ± 1.5** (*n* = 6)
5-HT	169.0 ± 27.0 (*n* = 6)	208.0 ± 24.0 (*n* = 6)	169.0 ± 27.0 (*n* = 6)	118.0 ± 38.0 (*n* = 6)	134.0 ± 30.0 (*n* = 6)
Vasopressin	0.08 ± 0.01 (*n* = 6)	0.52 ± 0.08** (*n* = 5)	0.08 ± 0.014 (*n* = 6)	0.27 ± 0.05** (*n* = 4)	0.88 ± 0.12** (*n* = 5)

Each value is the mean ± s.e.mean of the numbers in parentheses. ND – not determined.
* Indicates $P < 0.05$ vs appropriate control; ** $P < 0.01$.

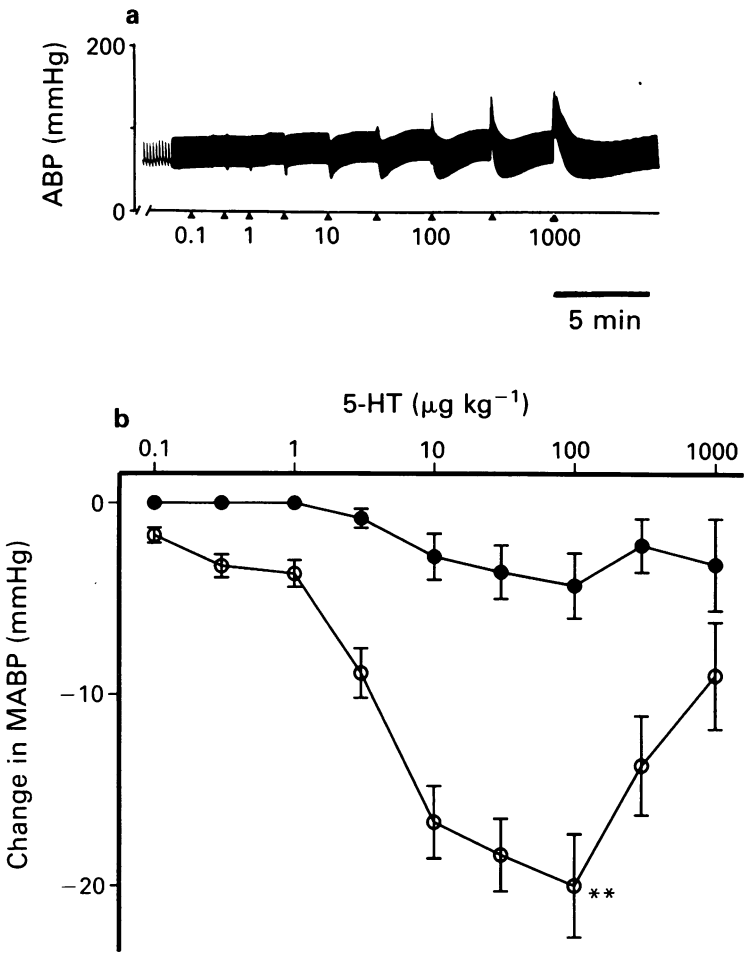

Figure 3 (a) Typical recording of the effect of single dose administration of 5-hydroxytryptamine (5-HT, 0.1–1000 $\mu g\,kg^{-1}$) on arterial blood pressure (ABP) in the pithed rat. (b) Vasodepressor effects of 5-HT (0.1–1000 $\mu g\,kg^{-1}$) in pithed rats receiving an infusion of endotoxin (250 $\mu g\,kg^{-1}\,h^{-1}$, ●) or saline (○). Each point represents the mean of 6–8 observations and vertical lines show s.e.mean. ** $P < 0.01$.

70 ± 4 mmHg (Figure 6). The apparent reduction in the maximum response produced by vasopressin in control rats may be explained by the elevated resting blood pressure. The endotoxin-induced reduction in the maximum response to BHT 933 was prevented by vasopressin infusion (Figure 6). Vasopressin-infusion in saline-treated rats was without effect on the pressor responsiveness to BHT 933 (data not shown).

Effects of endotoxin on vasodepressor responses to endothelin and to 5-HT

The initial depressor response to endothelin was significantly reduced (Figure 2) and the depressor component of the 5-HT response was also markedly and significantly attenuated (Figure 3) by endotoxin infusion. These responses were also markedly diminished by nicardipine. Thus the control maximum responses (mmHg) to endothelin (1.0 $nmol\,kg^{-1}$, 24.8 ± 3.4, $n = 16$) and to 5-HT (100 $\mu g\,kg^{-1}$, 20.0 ± 2.7, $n = 7$) were reduced, respectively, to 4.0 ± 0.7 ($n = 5$, $P < 0.01$) and 3.8 ± 1.3 ($n = 6$, $P < 0.01$) by nicardipine (1.0 $mg\,kg^{-1}$).

Discussion

This is the first account of the effects of endotoxin on the vascular responsiveness to a wide variety of agonists under the same experimental conditions. Moreover, a more detailed examination of the effects of endotoxin on the different agonists was made, wherever possible, by using complete pressor dose-response curves. The pithed rat was used to avoid the problems of reflex compensation and endotoxin-induced release of catecholamines (McKechnie *et al.*, 1985; Schaller *et al.*, 1985). This release is not seen in the pithed rat (Gray, 1989).

The present findings are broadly in agreement with previous studies showing that endotoxin impairs vascular responsiveness to vasopressor agents. Thus we have confirmed that responsiveness to selective α_2-adrenoceptor agonists clonidine and BHT 933 and to vasopressin is impaired in endotoxin-treated rats (Auclair & Schmitt, 1987; Schaller *et al.*, 1985). Additionally, we have shown that endotoxin administration impairs responsiveness to the selective α_1-adrenoceptor agonists (phenylephrine, cirazoline) and to 5-HT. In contrast to the findings of Ives *et al.* (1986), who demonstrated increased responsiveness to Bay K 8644, we found impaired responsiveness to this calcium channel activating drug, as obtained by Gray *et al.* (1990b).

The mechanisms underlying the loss of reactivity remain uncertain. Previous studies have suggested a down-regulation of α-adrenoceptors (Carcillo *et al.*, 1988), but this cannot be a fundamental mechanism in view of the impairment of responsiveness to a variety of agonists acting through a number of different mechanisms. Although responsiveness to a variety of agents is impaired this does not indicate a non-specific loss of reactivity through endotoxin-induced hypotension, since pressor responses to endothelin were not significantly modified. Moreover, endotoxin-induced impairment of responsiveness to vasopressor substances follows two patterns. In the case of α_1-adrenoceptor agonists and vasopressin, the E_{max50} values were increased without any change in the maximum response. On the other hand, a more marked effect was seen

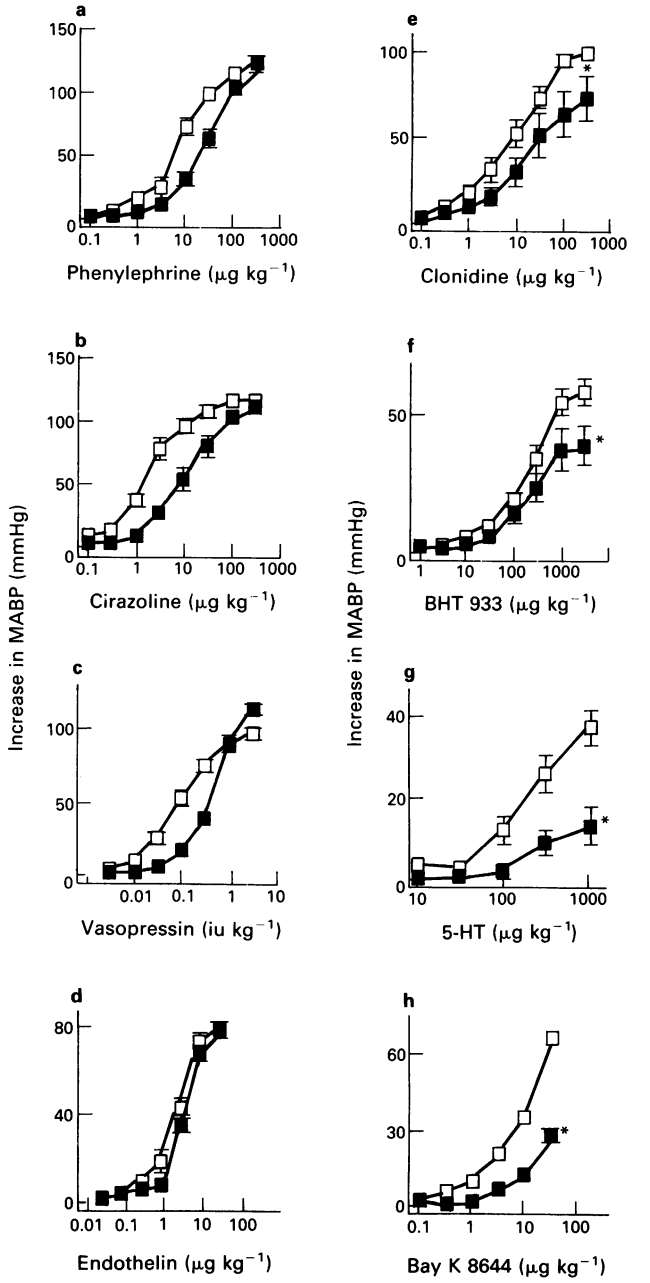

Figure 4 Pressor dose-response curves to (a) phenylephrine, (b) cirazoline, (c) vasopressin, (d) endothelin, (e) clonidine, (f) BHT 933, (g) 5-hydroxytryptamine (5-HT) and (h) Bay K 8644 determined in pithed rats 1 h after commencement of an infusion of either endotoxin ($250\,\mu g\,kg^{-1}\,h^{-1}$, ■) or saline (□). Each point represents the mean of n number of observations and vertical lines indicate s.e.mean (see Table 1 for E_{max50} values and statistics). In (a), (b), (c), (f) and (g), $n = 6$; in (d) $n = 14$–17; in (e) $n = 6$–12 and in (h) $n = 9$–11. * Indicates a significant difference between the maximum response obtained in control and endotoxin-treated rats ($P < 0.05$).

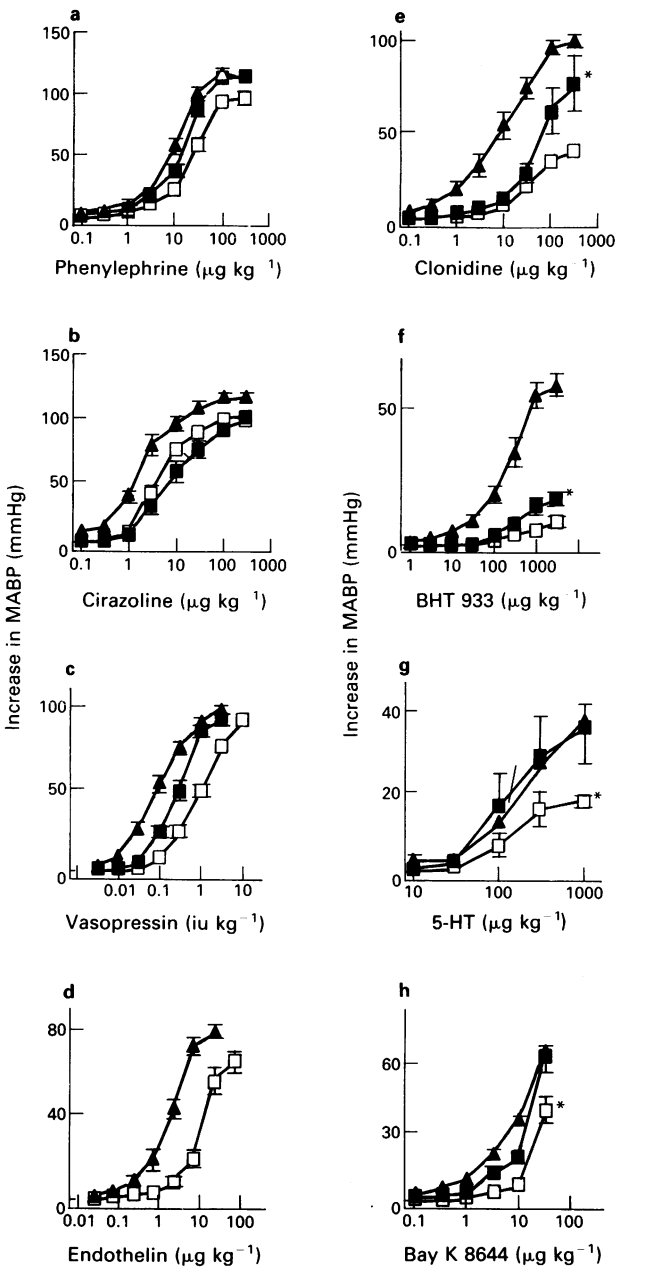

Figure 5 Pressor dose-response curves to (a) phenylephrine, (b) cirazoline, (c) vasopressin, (d) endothelin, (e) clonidine, (f) BHT 933, (g) 5-hydroxytryptamine (5-HT) and (h) Bay K 8644 determined in pithed control rats (▲) or in pithed rats 20 min after the injection of nicardipine ($0.3\,mg\,kg^{-1}$, ■ or $1\,mg\,kg^{-1}$, □). Each point represents the mean of n number of observations; vertical lines indicate s.e.mean (see Table 1 for E_{max50} values and statistics). In (a), (b), (c) and (g) $n = 6$; in (d), (e) and (h) $n = 6$–12; in (f) $n = 6$–8. * Indicates a significant difference between the maximum response obtained in control and endotoxin-treated rats ($P < 0.05$).

on responses to α_2-adrenoceptor agonists and especially to 5-HT, where the maximum responses were depressed in addition to an increase in their E_{max50} values. Vascular responses to 5-HT and α_2-adrenoceptor agonists show a marked dependency upon entry of calcium through voltage-sensitive channels (Nakaki *et al.*, 1985). Responses to α_1-adrenoceptor agonists and vasopressin, on the other hand, appear to have a greater dependency upon mobilization of calcium from intracellular stores via activation of phosphatidylinositol metabolism and production of inositol triphosphate (IP_3, Chuang, 1989). Thus the more marked effect of endotoxin on responses to α_2-adrenoceptor agonists and to 5-HT may be due to some effect on calcium entry. In this context it is interesting that the calcium channel blocking drug nicardipine impaired responsiveness to the various agonists in a pattern which was strikingly similar to that produced by endotoxin. The pattern was similar to that found by Nicols *et al.* (1989) for the modification of the pressor effects of α_1- and α_2-adrenoceptor agonists by calcium channel blocking drugs. It could therefore be speculated that endotoxin acts in an analogous manner. However, the response to endothelin was not modified by endotoxin despite being modified by nicardipine in a manner similar to that seen with the α_1-adrenoceptor agonists and vasopressin. The depressor response to endothelin was, on the other hand, markedly impaired by endotoxin. It is thus possible that the reduction in an opposing vasodilator response masked any impairment of the pressor response. This is

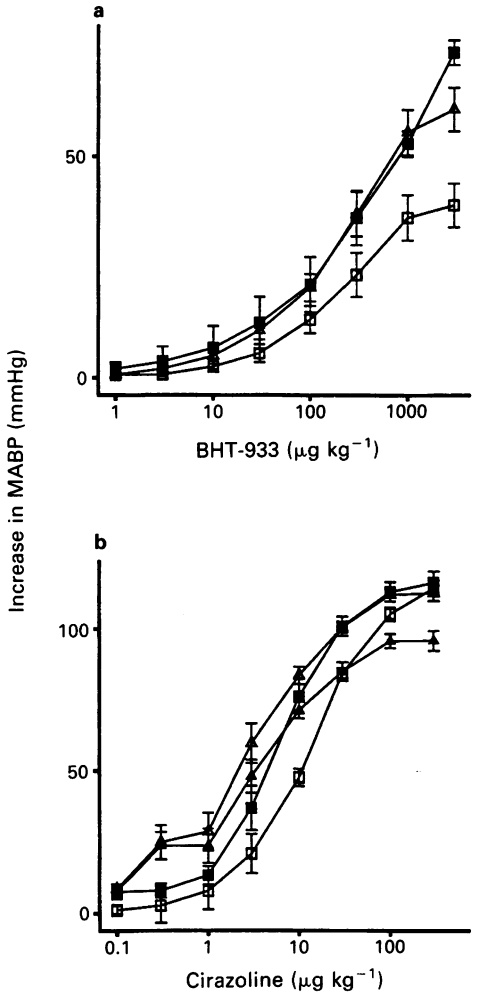

Figure 6 (a) Vasopressor dose-response curves to BHT 933 in pithed rats infused with saline (△), endotoxin ($250\,\mu g\,kg^{-1}\,h^{-1}$) (□), endotoxin + vasopressin ($0.64\,iu\,kg^{-1}\,h^{-1}$) (■). Each point is the mean of 6–7 observations. * $P < 0.05$ compared to control. (b) Vasopressor dose-response curves to cirazoline in pithed rats infused with saline (△), endotoxin ($250\,\mu g\,kg^{-1}\,h^{-1}$) (□), saline + vasopressin ($0.64\,iu\,kg^{-1}\,h^{-1}$) (▲) or endotoxin + vasopressin (■). Each point is the mean of 6–7 observations (ANOVA, effect of endotoxin, $P < 0.01$, effect of vasopressin, $P < 0.05$, interaction, $P < 0.01$). In (a) and (b) vertical lines show s.e.mean.

unlikely to be the complete explanation, since the depressor response to 5-HT was almost completely suppressed concomitantly with a marked impairment of the pressor response. Moreover, nicardipine markedly depressed the vasodepressor responses to both endothelin and 5-HT, while also reducing the pressor responses. It is unclear why nicardipine had such a marked effect on the vasodepressor responses to endothelin and to 5-HT. These responses are endothelium-dependent and, although early work suggested the involvement of L-type channels in the release of endothelium derived relaxant factor (EDRF) (Singer & Peach 1982), this is not supported by more recent work (Angus & Cocks, 1989). The small fall in resting blood pressure produced by either endotoxin or nicardipine is unlikely to be the sole explanation, since large doses of 5-HT did produce the typical hypotensive response.

Previous studies suggested a role for cyclo-oxygenase products of arachidonic acid metabolism in endotoxin-induced impairment of vascular reactivity, since responsiveness to noradrenaline and angiotensin II was restored by flurbiprofen and BW755C. Moreover, the effect of endotoxin was mimicked by infusion of prostaglandin E_2 (PGE_2, Gray *et al.*, 1990b). Additional factors may, however, be involved, as L-NMMA was also found to restore responsiveness to noradrenaline *in vivo* and *ex vivo*, suggesting a role for nitric oxide (EDRF) (Fleming *et al.*, 1990; Gray *et al.*, 1990a). Endotoxin was also shown to increase production of nitric oxide from bovine aortic endothelial cells (Salvemini *et al.*, 1989). An enhanced production of EDRF in response to endotoxin must be somehow reconciled with the marked impairment of the vasodepressor responses to endothelin and to 5-HT, which are known to be endothelium-dependent (de Nucci *et al.*, 1988; Mylecharane & Phillips, 1989). Endotoxin is known to activate macrophages (Morrison & Ryan, 1987), which are themselves an important source of nitric oxide (Moncada *et al.*, 1989). Thus macrophage-derived nitric oxide may contribute to the impaired pressor responsiveness. On the other hand, as endotoxin may produce endothelial damage (McGrath & Stewart, 1969; McKenna *et al.*, 1986) this may explain impaired vasodepressor responsiveness to endothelium-dependent vasodilators. However, the explanation may be more complex in the light of recent evidence (Gardiner *et al.*, 1989) that the vasodilator effects of endothelin are mediated by neither nitric oxide nor prostacyclin, the two known endothelium-derived vasodilators.

Finally, it is interesting that endotoxin-induced impairment of pressor responsiveness to cirazoline or BHT 933 was prevented by infusion of vasopressin at a rate that just prevented endotoxin-induced hypotension. This infusion did not augment pressor responses to cirazoline in non-endotoxin-treated (saline-infused) animals. The vasoconstrictor effect of vasopressin involves activation of phosphatidylinositol turnover with increased formation of IP_3 and diacylglycerol (Chuang, 1989). This will lead to elevated cytosolic Ca^{2+} and to sensitization of the contractile apparatus to Ca^{2+}. Such effects would be expected to restore reactivity if endotoxin acts through a reduction in the availability of intracellular Ca^{2+}. We do not yet know if the effect of vasopressin is specific or if the same action will be seen with other pressor agents. Moreover, complete interpretation of the interaction between vasopressin and endotoxin, and indeed of the effects of endotoxin itself, requires an examination of the effects of endotoxin on agonist-induced changes in regional blood flow.

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Benzodiazepine analogues inhibit arachidonate-induced aggregation and thromboxane synthesis in human platelets

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1 Benzodiazepine analogues inhibit human platelet aggregation induced by arachidonate with an EC_{50} value of 0.68 μM for PK 11195, the most potent analogue used.

2 There was a highly significant correlation between the inhibition of arachidonate-induced aggregation and the affinity for the peripheral-type of benzodiazepine binding sites.

3 There was no significant correlation between the inhibition of the platelet activating factor (PAF)-induced aggregation and the binding to the peripheral-type of benzodiazepine binding sites.

4 The inhibition of platelet aggregation seems to result from the inhibition of arachidonic acid cyclo-oxygenation, since the synthesis of thromboxane and 12-hydroxy-heptadecatrienoic acid, both cyclo-oxygenase products, was reduced.

5 Our results suggest that peripheral-type of benzodiazepine binding sites on human platelets could be linked to cyclo-oxygenase.

Introduction

The central-type benzodiazepine receptor displays a high affinity binding for classical benzodiazepines. This receptor is linked to the γ-aminobutyric acid (GABA)-receptor-chloride-channel complex and it is responsible for many of the centrally-mediated effects of benzodiazepines. Besides this central-type receptor, peripheral-type binding sites for benzodiazepines have been identified both in the periphery and the central nervous system (Marangos *et al.*, 1982). Peripheral-type binding sites are distinct from the central-type receptors in their subcellular location and structural requirements (Anholt *et al.*, 1986). Porphyrins have been proposed as potential endogenous ligands of the peripheral-type sites located on mitochondria (Verma *et al.*, 1982).

The precise physiological function of the peripheral-type benzodiazepine binding sites is still not clear despite the fact that benzodiazepines exert many pharmacological activities at the periphery. Among these activities, some benzodiazepines inhibit platelet aggregation. Triazolam and alprazolam inhibit platelet activating factor (PAF)-induced aggregation (Kornecki *et al.*, 1984), and the inhibition of PAF binding to platelets has been proposed to explain the anti-aggregatory effect (Chesney *et al.*, 1987). This is however controversial for diazepam has been shown either to inhibit arachidonate-induced aggregation (Romstedt & Huzoor-Akbar, 1985) or to have no effect (Kornecki *et al.*, 1984).

Thus, despite the fact that benzodiazepines may alter platelet aggregation, several questions concerning their mechanism of action need to be answered (i) are the benzodiazepines acting in competition with PAF? (ii) are trizolobenzodiazepines the only active products? (iii) are the anti-aggregatory effects linked to the peripheral-type sites of benzodiazepines? In the present study, we compared the efficiency of a series of benzodiazepines for inhibiting PAF- or arachidonate-induced platelet aggregation and we attempted to correlate this inhibition with the affinity of those benzodiazepines for platelet peripheral binding sites.

Methods

Platelet preparation

Human platelets were obtained from healthy donors who had not taken any drug for at least ten days. Platelets were isolated from plasma as previously described (Lagarde *et al.*, 1985) and resuspended in a Tyrode HEPES buffer (pH 7.4) without albumin.

Aggregation studies

Platelet aggregation was monitored by the turbidimetric method of Born (1962) with a Chrono-log dual aggregometer. The aggregation was induced by either arachidonic acid (2.5 μM) in ethanol (less than 0.5%), PAF (0.4 μM) or U 46619 (0.1 μM).

Binding studies

[^{3}H]-Ro 4864, [^{3}H]-PK 11195 and [^{3}H]-diazepam have been used by different authors to measure the binding of these molecules. The use of [^{3}H]-Ro 4864 or [^{3}H]-PK 11195 provided grossly similar results (K_d in the range of 10 nM, non saturable binding at 10% of total binding) (Benavides *et al.*, 1984; Moingeon *et al.*, 1984; Weizman *et al.*, 1987), whereas the use of [^{3}H]-diazepam led to contradictory results concerning the affinity (Wang *et al.*, 1980; Moingeon *et al.*, 1984; O'Beirne & Williams, 1984). This discrepancy probably comes from highly non saturable binding that we have found to occur with the latter ligand (results not shown). Therefore, we used [^{3}H]-PK 11195 as the radioactive ligand in our study.

Platelet suspension (200 μl) was incubated in 1 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 100 mM NaCl and [^{3}H]-PK 11195 (0.01 μM). After 30 min incubation at 4°C, the incubate was filtered on GF/C glass filters (Whatman), the filter was washed four times with 5 ml of cold buffer and the radioactivity on the filter was determined by liquid scintillation counting. Non saturable binding was measured in the presence of 100 μM cold PK 11195. We have checked that this value was the same as the value obtained in the absence of cold ligand with boiled platelet suspension.

Arachidonic acid metabolism

Platelets were incubated in the aggregometer for 3 min and [1-^{14}C]-arachidonic acid (2.5 μM) was added. The aggregation was monitored and the reaction was terminated after 4 min by addition of 1.2 ml ethanol. Lipids were extracted with 2.4 ml chloroform in the presence of 5×10^{-5} M butylated hydroxy-toluene as an anti-oxidant. The lipid extraction procedure was repeated once and the organic phase was concentrated by evaporation and spotted on silica gel plates (Merck 60G

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5721). The first elution with the mixture hexane/diethylether/ acetic acid (60:40:1) allowed the separation of 12-hydroxy-heptadecatrienoic acid (HHT) (R_F 0.13), 12-hydroxy-eicosatetraenoic acid (12-HETE)(R_F 0.23) and arachidonic acid (R_F 0.43) from the origin. A second elution with diethylether/ methanol/acetic acid (90:1:2) allowed the separation of thromboxane B_2 (R_F 0.27) from the origin (Lagarde *et al.*, 1985). The radioactivity of each spot was determined with a Berthold thin layer chromatography (t.l.c.) analyser.

Data analysis

IC_{50} was defined as the concentration of benzodiazepine analogue which induced a 50% inhibition of the [^{3}H]-PK 11195 binding, with the concentration of radioactive ligand being 0.01 μM. This value was determined by use of at least 4 concentrations of drug and the analysis was carried out with the Enzfitter programme from Elsevier Biosoft. The equation was taken as bound $c.p.m._i = c.p.m._0 * IC_{50}/(IC_{50} + i)$ where $c.p.m._0$ is bound radioactivity without inhibitor, cpm_i is radioactivity in presence of inhibitor and i the inhibitor concentration. In an attempt to characterize further the binding, we substituted $(i)^h$ for i in the previous equation and determined this coefficient. The estimation of K_i from IC_{50} was determined by the method of Bennett (1978). EC_{50} was defined as the concentration of benzodiazepine analogue which induced 50% inhibition of the increased light transmission elicited by the aggregating agent (arachidonic acid or PAF). As the relationship relating the aggregation inhibition to the drug concentration is not known, the EC_{50} was obtained by successive assays of drug concentration until 50% inhibition was reached.

Drugs and materials

Ro 16-6028 (tert-butyl(S)-8-bromo-11,12,13,13a-tetrahydro-9-oxo-9H-imidazo[1,5-a]-pyrrolo[2,1-c][1,4]benzodiazepine-1 - carboxylate), Ro 15-4513 (ethyl 8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate), Ro 15-1788, flumazenil (ethyl 8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate) and midazolam (8-chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo[1,5-a][1,4]benzodiazepine) were gifts from Hoffmann-la Roche, Basle, Switzerland.

CGS 8216 (2-phenylpyrazolo-[4,3-c]-quinolin-3 (5H)-one) was a gift from Ciba-Geigy, Basle, Switzerland.

RP 31264 and PK 11195 (1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinoline-carboxamide) were gifts from Rhone Poulenc, Vitry-s-Seine, France.

Alprazolam (8-chloro-1-methyl-6-phenyl-4H-[1,2,4] triazolo[4,3-a][1,4] benzodiazepine) and triazolam (8-chloro-6-(2-chlorphenyl)-1-methyl-4H-[1,2,4]triazolo[4,3-a][1,4] benzodiazepine) were gifts from Upjohn, Paris, France.

Ro 5-4864 (7-chloro-5-(4-chloro-phenyl)-1,3-dihydro-1-methyl-2H-1,4-benzodiazepine-2-one) and diazepam were from Fluka and Sigma laboratories respectively.

ACA 6 (3,3',4',5,7-penta-O-ethylquercetin) and MP 37 (3-carboxymethyl-3',4,5,7-tetra-O-ethylquercetin) were synthesized in our laboratory by Dr M. Picq.

[^{3}H]-PK 11195 was from CEA, France (reference TMM-261, specific activity 2.44 MBq mmol^{-1}).

Results

The binding of [^{3}H]-PK 11195 was studied in human platelets (Figure 1). Scatchard and Hill plots revealed straight lines indicating the occurrence of a single class of sites which do not interact. The values of K_d determined by the two different curves were 39 and 33 nM, respectively. The B_{max} value was 6.14 pmol/assay which corresponds to 41 fmol/10^6 platelets.

The 13 drugs that we tested exhibited various potencies in inhibiting [^{3}H]-PK 11195 binding (Table 1). These drugs were chosen to represent the different classes of benzodiazepine ligands, peripheral-type and central-type (including agonist, antagonist, partial inverse agonist). We also used two flavones (ACA6 and MP37) which exhibit inhibitory effects on platelet aggregation (Beretz & Cazenave, 1988). In addition, we have shown that these two flavones have some affinity for peripheral-type benzodiazepine sites (M. Picq and P. Fonlupt, unpublished results). The IC_{50} varied from 0.05 μM to values greater than 50 μM. We confirmed that PK 11195, Ro 4864 and diazepam have the greatest affinities for the peripheral-type binding sites (IC_{50}: 0.05, 0.38, 0.70 μM, respectively).

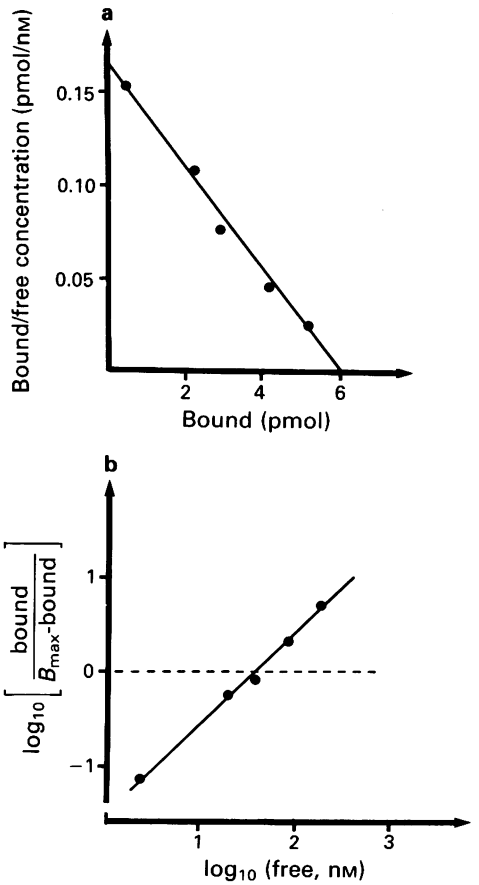

Figure 1 [^{3}H]-PK 11195 binding on human platelets. PK 11195 concentrations ranged from 2.4 to 200 nM. Each point was determined by use of 3 batches of blood platelets. (a) Scatchard analysis. The parameters were determined using the Enzfitter programme: B_{max} = 6136 ± 185 fmol/assay (41 fmol/10^6 platelets), K_d = 39 ± 3 nM. (b) Hill plot analysis. The parameters were determined using the Enzfitter programme: Hill coefficient = 0.93 ± 0.9, K_d = 33 ± 7 nM.

In an attempt to compare these values with the previously published ones (Wang *et al.*, 1980; Le Fur *et al.*, 1983; Benavides *et al.*, 1984) we calculated the corresponding K_i from IC_{50} values (Table 1). We found a slightly lower affinity for both PK 11195, Ro 4864 and diazepam (K_i: 39, 299, 551 nM, respectively) than those previously described (K_i: 4, 22, 194 nM, respectively). However, the relative K_i ratio (K_i of Ro 4864 or diazepam compared to K_i of PK 11195) was preserved. For the 13 products studied, the decreasing order of potency was: PK 11195 > Ro 4864 > diazepam > triazolam > alprazolam > midazolam > ACA 6 > Ro 16-6028 > Ro 19-4513 > MP 37 > suriclone > CGS 8216 > Ro 15-1788. When ligands of the peripheral-type benzodiazepine binding sites were added to platelet suspensions, a decrease in arachidonate-induced aggregation was observed (Figure 2). The potencies, in decreasing order, were PK 11195 > Ro 4864 > midazolam > diazepam (EC_{50}: 0.68, 2.4, 4.0, 8.5 μM, respectively) as shown in Table 1.

Table 1 Inhibition of [^{3}H]-PK 11195 binding, and of arachidonic acid- and PAF-induced aggregation by various agents

Name	Type	n	Binding inhibition IC_{50} (μM)	h	Aggregation inhibition Arachidonate K_i (nM)	Arachidonate EC_{50} (μM)	PAF EC_{50} (μM)
PK 11195	peripheral	5	0.05 ± 0.02	(1.13)	39 ± 16	0.68 ± 0.19	0.14 ± 0.03
Ro 4864	peripheral	5	0.38 ± 0.09	(1.05)	299 ± 71	2.4 ± 0.6	3.3 ± 0.8
Diazepam	periph/central	5	0.70 ± 0.15	(0.97)	551 ± 118	8.5 ± 2.1	1.7 ± 0.4
Midazolam	agonist	3	2.28 ± 0.51	(1.08)	1795 ± 401	4.0 ± 1.2	nd
CGS 8216	antagonist	2	15.8 ± 3.43	(0.95)	12440 ± 2700	36 ± 15	nd
Ro 15-1788	antagonist	4			no inhibition at 38 μM		
Ro 19-4513	inverse agonist	3	5.3 ± 1.64	(1.01)	4173 ± 1291	36 ± 12	nd
Alprazolam	agonist	5	1.63 ± 0.35	(1.08)	1283 ± 273	14 ± 3	0.35 ± 0.07
Triazolam	agonist	3	1.14 ± 0.27	(0.93)	897 ± 212	15 ± 4	0.07 ± 0.02
Suriclone	agonist	2	11.0 ± 4.30	(0.95)	8661 ± 3385	21 ± 8	30 ± 15
Ro 16-6028	partial agonist	2	3.50 ± 0.68	(1.08)	2756 ± 535	35 ± 11	11 ± 4
MP 37	flavone	3	7.00 ± 1.86	(1.10)	5512 ± 1464	16 ± 5	nd
ACA 12	flavone	3	3.02 ± 0.60	(1.03)	2378 ± 472	12 ± 3	nd

The pharmacological profiles of these drugs have been reviewed by Gardner (1988).
Experiments and calculations were conducted as described in the Methods section where n is the number of different blood samples. Each was used for the binding, in triplicate, of [^{3}H]-PK 11195 in the presence of 4 different concentrations of each analogue. IC_{50} is the concentration of drug which induced 50% inhibition of binding. The value reported in the table is the mean ± s.d. of values obtained in each individual experiment. The h value (determined as described in Methods) is given in parentheses. K_i values were calculated from IC_{50} values according to Bennett (1978) ($K_i = IC_{50}/(1 + [^3H]\text{-PK 11195 concentration}/K_d)$). EC_{50}s were obtained by successive assays of drug concentration until 50% inhibition was reached. Values given in the table are the mean ± s.d. of values obtained in each individual experiment.

When log EC_{50}s were plotted versus log IC_{50}s a clear correlation appeared between these two groups of values ($\alpha < 0.001$) (Figure 3).

Benzodiazepines also inhibit PAF-induced aggregation, and we confirmed that triazolobenzodiazepines (triazolam and alprazolam) were potent inhibitors of PAF-induced aggregation (EC_{50} 0.07, 0.35 μM, respectively) whereas the other analogues tested, except PK 11195 (EC_{50} 0.14 μM), exhibited EC_{50}s greater than 1 μM (Table 1). However, when log EC_{50}s were plotted versus log IC_{50}s no significant correlation appeared (Figure 4).

Arachidonate is metabolized in platelets by lipoxygenase to 12-hydroxy-eicosatetraenoic acid (12-HETE) and by cyclooxygenase to mainly 12-hydroxy-heptadecatrienoic acid (HHT) and thromboxane (Tx) A_2, the latter interacting with platelet receptors to induce aggregation. As benzodiazepines counteracted the effect of arachidonate, a possible mechanism is the inhibition of thromboxane synthesis. We have measured the synthesis of TxB_2 (the stable metabolite of TxA_2), HHT and 12-HETE, and the arachidonate incorporation into phospholipids, in the presence or absence of benzodiazepine analogues, at concentrations which inhibit aggregation. Concentrations were chosen for obtaining 5 to 100% inhibition. Figure 5 shows that the benzodiazepine analogues inhibited TxB_2 and HHT synthesis and that the inhibition of both products was correlated with the degree of inhibition. Moreover, we did not find any decrease of 12-HETE synthesis and even a slight increase was observed in the presence of some benzodiazepine analogues. No relevant modification of arachidonate incorporation into phospholipids occurred (Table 2).

Another mechanism for explaining the inhibition of arachidonate-induced aggregation of platelets is the inhibition of prostaglandin H_2/thromboxane A_2 binding on their receptor sites. In the presence of PK 11195, the U-46619-induced aggregation (U-46619 is a stable TxA_2 analogue) was inhibited but the effect appeared for a concentration of PK 11195 more than 10 fold higher than that needed for inhibiting arachidonate-induced aggregation. Moreover, the inhibition of U-46619-induced aggregation was quite different from that induced by arachidonate. When induced by U-46619, the time course of aggregation was altered but not its extent (Figure 2). This result agrees with the fact that PK 11195, diazepam and Ro 4864 did not inhibit the binding of [^{125}I]-PTA-OH, a labelled analogue of thromboxane (unpublished results).

Discussion

The results of the present study show that when peripheral-type benzodiazepine sites were occupied by appropriate ligands, inhibition of arachidonate-induced aggregation occurred, which was presumably mediated by the inhibition of thromboxane synthesis.

Benzodiazepines bind to platelets of various species, rat,

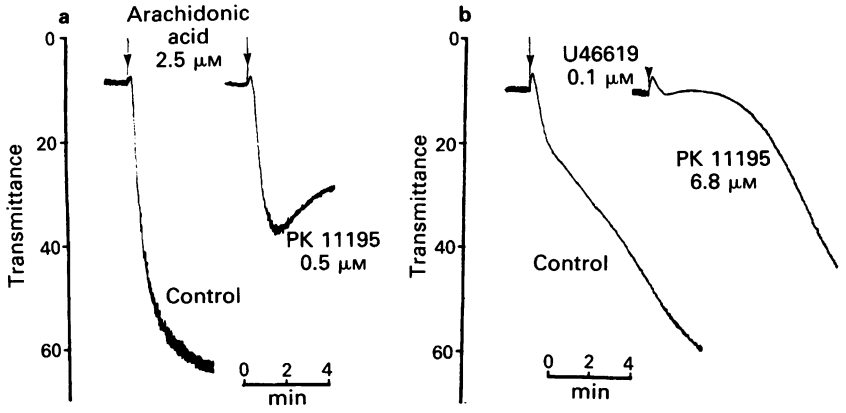

Figure 2 Effect of PK 11195 on human platelet aggregation induced by arachidonic acid (a) or U-46619 (b). Concentrations used were 2.5 and 0.1 μM, respectively, of the aggregating agents.

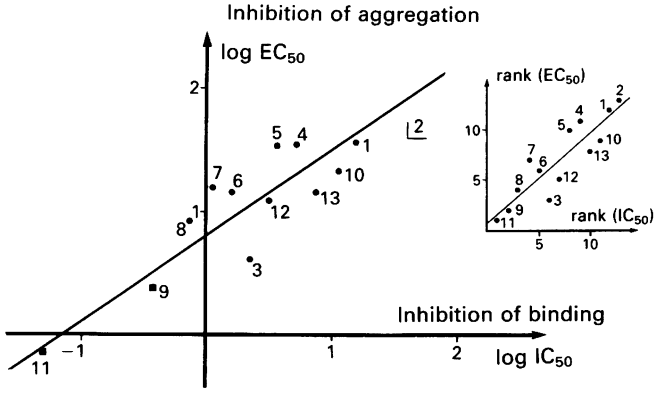

Figure 3 Relationship between the inhibition of arachidonate-induced aggregation by benzodiazepine analogues and their affinity for the binding sites. The EC_{50} (μM) is the concentration of analogue which inhibited 50% of aggregation; IC_{50} (μM) is the concentration of analogue which inhibited 50% of [^{3}H]-PK 11195 binding (0.01 μM). The line was determined by linear regression analysis; $n = 12$ (values corresponding to compound 2 are not available since it did not inhibit even at 10^{-4} M), $r = 0.868$ (r limit for an $\alpha_{0.01}$ risk: 0.71), $\exp_{10}$(slope) = 4.5. The inset shows a linear regression analysis of drug ranks according to their potencies (Spearman's test). Abscissa scale: ranks for IC_{50}; ordinates: ranks for EC_{50}. The higher the potency, the lower the rank. $n = 13$, $r = 0.89$, (r = limit for an $\alpha_{0.01}$ risk: 0.68). 1 CGS 8216; 2 Ro 15-1788; 3 midazolam; 4 Ro 19-4513; 5 Ro 16-6028; 6 alprazolam; 7 triazolam; 8 diazepam; 9 Ro 4864; 10 suriclone; 11 PK 11195; 12 ACA 6; 13 MP 37.

mouse and man (Moingeon *et al.*, 1984; O'Beirne & Williams, 1984). Concerning the binding of benzodiazepine analogues to human platelets, our results are in accordance with previous studies reporting PK 11195, Ro 4864 and diazepam as being the most potent agents. This confirms that the affinity for the peripheral-type binding sites is totally different from the affinity for the central-type binding sites (Sieghart & Shuster, 1984; Le Fur *et al.*, 1983). As expected, because of the absence of GABA sites in platelets, we did not find any difference in the behaviour of agonists, antagonists or partial inverse agonists of central-type sites.

Some previous reports have shown that benzodiazepines are able to inhibit platelet aggregation. Kornecki *et al.* (1984)

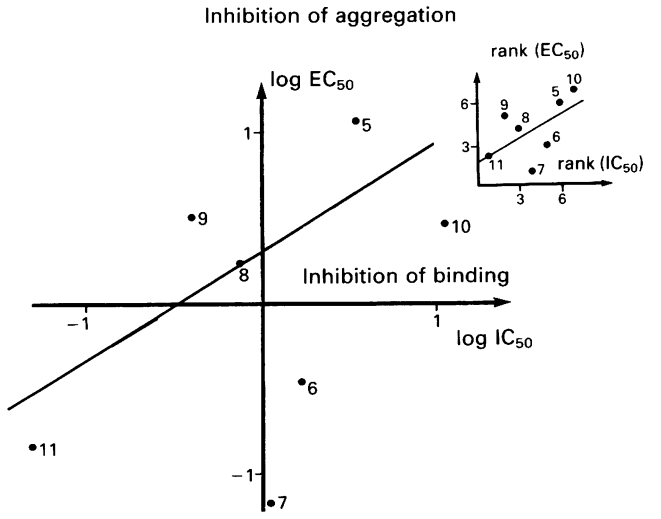

Figure 4 Relationship between the inhibition of PAF-induced aggregation by benzodiazepine analogues and their affinity for the binding sites. EC_{50} (μM) is the concentration of analogue which inhibited 50% of aggregation; IC_{50} (μM) is the concentration of analogue which inhibited 50% of [^{3}H]-PK 11195 binding (0.01 μM). The line was determined by linear regression analysis; $n = 7$, $r = 0.64$ (r limit for an $\alpha_{0.10}$ risk: 0.71). The inset shows the linear regression analysis of ranks as stated in Figure 3: $n = 7$, $r = 0.57$, (r limit for an $\alpha_{0.10}$ risk: 0.68). 5 Ro 16-6028; 6 alprazolam; 7 triazolam; 8 diazepam; 9 Ro 4864; 10 suriclone; 11 PK 11195.

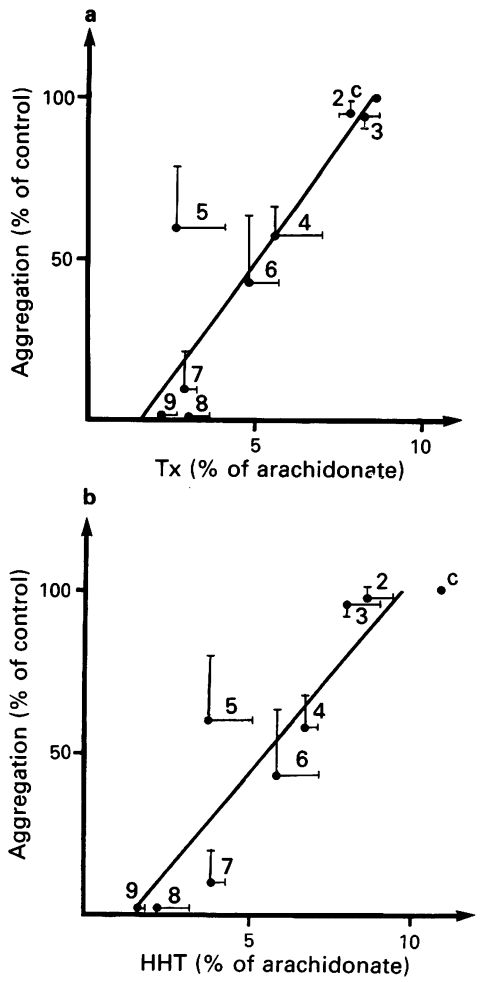

Figure 5 Relationship between platelet aggregation and thromboxane (Tx) (a) or 12-hydroxyheptadecatrienoic acid (HHT) (b) synthesis in the presence of benzodiazepines. Aggregation was expressed as % of control; thromboxane and HHT as % of initial [^{14}C]-arachidonate. 1 Control; 2 alprazolam 6.9 μM; 3 diazepam 4 μM; 4 alprazolam 13.9 μM; Ro 4864 1.37 μM; 6 diazepam 8 μM; 7 pK 11195 0.6 μM; 8 Ro 4864 3.4 μM; 9 PK 11195 1.28 μM. Each point is the mean of 3 to 6 determinations; s.d. shown by bars. The line was determined by linear regression analysis; $n = 9$, $r = 0.90$ for (a), $r = 0.91$ for (b) (r limit for an $\alpha_{0.01}$ risk: 0.80).

showed that alprazolam and triazolam inhibited PAF-induced aggregation whereas diazepam did not. The latter result is questionable since a high arachidonate concentration (170 μM) was used (Kornecki *et al.*, 1984), which is far from the minimal concentration needed (2 μM). Other authors reported the inhibition of arachidonate-induced aggregation by diazepam or flurazepam (Romstedt & Huzoor-Akber, 1985). More recently, evidence was produced for a direct action of triazolobenzodiazepines on PAF binding sites whereas diazepam has no affinity for these sites (Kornecki *et al.*, 1984). In our study, we showed that the occupancy of peripheral-type benzodiazepine binding sites and the inhibition of arachidonate-induced aggregation are significantly correlated. Furthermore, there was no significant correlation between the binding on benzodiazepine sites and PAF-induced aggregation. We suggest that benzodiazepine sites are responsible for the inhibition of arachidonate-induced aggregation without any link to PAF binding sites. The inhibition of PAF-induced aggregation might then result from some structural feature, with the triazolo structure being the most efficient.

The mechanism by which peripheral-type benzodiazepine sites are coupled to platelet aggregation remains unclear. We suggest blockade of calcium influx may be involved because benzodiazepines have been described as inhibitors of calcium channels (Rampe & Triggle, 1986). However, Anwer *et al.*

Table 2 Metabolism of [^{14}C]-arachidonic acid in platelet suspensions: effect of ligands of peripheral-type benzodiazepine sites

	TxB_2	*HHT*	*12-HETE*	*PL*
Control	8.6 ± 1.7	10.9 ± 1.3	15.6 ± 0.8	13.9 ± 1.6
PK 11 195 0.64 μM	2.9 ± 0.2*	3.9 ± 2.0*	20.0 ± 10.9	13.8 ± 2.0
PK 11 195 1.28 μM	2.1 ± 0.2*	1.7 ± 0.1*	14.4 ± 1.6	11.0 ± 0.9
RO 4864 1.7 μM	2.7 ± 1.5*	3.8 ± 1.5*	20.6 ± 1.2	12.9 ± 0.7
RO 4864 3.4 μM	3.0 ± 1.1*	2.2 ± 1.3*	22.9 ± 0.2*	10.6 ± 0.2
Diazepam 4 μM	7.7 ± 0.2	7.9 ± 1.2	22.5 ± 1.8	11.5 ± 0.7
Diazepam 8 μM	4.8 ± 0.9*	5.8 ± 1.3*	27.8 ± 0.9*	13.5 ± 1.0
Alprazolam 6.9 μM	8.2 ± 1.2	8.5 ± 0.8	19.1 ± 3.1	18.7 ± 0.9
Alprazolam 13.9 μM	5.5 ± 1.4*	6.7 ± 0.8*	22.7 ± 2.2	14.5 ± 0.8

Radioactivity associated to thromboxane B_2 (TxB_2), 12-hydroxyheptadecatrienoic acid (HHT), 12-hydroxyeicosatetraenoic acid (12-HETE) and phospholipids (PL) was determined as described in the experimental section.
The results are expressed as % of initial radioactive [^{14}C]-arachidonic acid. Each value is the mean ± s.d. from 3 to 6 determinations.
As the distribution of the values was not known, statistical analysis of the results was carried out by using the Kruskall-Wallis test. When H value showed significant differences within the data, comparison of the mean values from control and each analogue concentration assays was performed by protected t test. * Statistically different from control with an α risk lower than 0.05.

(1988) reported the inhibition of ionomycin-induced aggregation by flurazepam in blocking the calcium entry suggesting that benzodiazepines act at a step proximal to the influx of calcium into platelets. In the present work, we studied the metabolism of arachidonate, in particular its oxygenation by platelet cyclo-oxygenase and lipoxygenase, and found that benzodiazepines inhibit the formation of cyclo-oxygenase products, TxB_2 and HHT. Indirect evidence has been previously reported with flurazepam and diazepam, which inhibited the formation of malondialdehyde (Romstedt & Huzoor-Akber, 1985). The link between benzodiazepine sites and cyclo-oxygenase remains unknown but a hypothesis is suggested by the work of Verma *et al.* (1987) who have shown that porphyrins are potent ligands for peripheral-type benzodiazepine sites. Since cyclo-oxygenase uses a porphyrin reversibly linked to the enzyme as a cofactor (Kulmacz & Lands, 1984), we hypothesize that benzodiazepine might bind to the porphyrin site, thus inducing a loss of activity.

We conclude that our study indicates a possible link between peripheral-type benzodiazepine sites and cyclo-oxygenase in human platelets. This observation raises two questions: (i) are brain peripheral-type sites also linked to brain cyclo-oxygenase which is involved in cholinergic neurotransmission? and (ii) are benzodiazepines able to modify other porphyrin-associated enzymes? Answering these questions will probably be helpful in understanding the physiological role of peripheral-type benzodiazepine sites and in explaining the pharmacological effects of benzodiazepines at the periphery.

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Functional characterization of neuronal pre and postsynaptic α_2-adrenoceptor subtypes in guinea-pig submucosal plexus

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1 The α_2-adrenoceptors on cell bodies of submucosal neurones, on presynaptic cholinergic nerve terminals innervating submucosal neurones, and on presynaptic sympathetic fibres innervating submucosal arterioles were characterized in functional studies by use of subtype selective ligands.

2 Both membrane hyperpolarization and presynaptic inhibition of nicotinic excitatory synaptic potentials (e.p.s.ps) produced by UK 14304 were similarly antagonized by idazoxan, yohimbine, SKF 104078, WB 4101 and ARC-239. Antagonism was competitive and dissociation equilibrium constants were the same for both effects.

3 Vasoconstriction of submucosal arterioles in response to stimulation of the sympathetic nerves (20 Hz for 2 s) was inhibited by UK 14304 and clonidine; concentrations producing half-maximum responses were 6 nM and 10 nM respectively. Idazoxan, yohimbine, WB 4101 and SKF 104078 antagonized this action, with dissociation constants similar to those for antagonism of the postsynaptic membrane hyperpolarization and presynaptic inhibition of nicotinic e.p.s.ps.

4 Oxymetazoline was a partial agonist when membrane hyperpolarization or presynaptic inhibition of nicotinic e.p.s.ps were measured but a full agonist when presynaptic inhibition of sympathetically-mediated arteriolar vasoconstriction was measured. As an agonist, oxymetazoline produced half maximum responses at 80–120 nM; the dissociation constant for oxymetazoline as an antagonist was 130 nM.

5 Neither prazosin nor chlorpromazine (up to 30 μM) altered any of the three responses to α_2-adrenoceptor agonists.

6 It is concluded that α_2-adrenoceptors present on submucosal neuronal cell bodies, on presynaptic cholinergic nerve terminals and on presynaptic sympathetic nerve terminals are the α_{2A} subtype. However, functional characterization of this subtype differs from that provided by ligand binding studies.

Introduction

Ligand binding studies generally have indicated that at least two α_2-adrenoceptor subtypes can be distinguished; these are termed α_{2A} and α_{2B} adrenoceptors (Cheung *et al.*, 1982; Bylund, 1988). Cases have also been made for the presence of additional, pharmacologically distinct, α_2-adrenoceptor binding sites (Bylund, 1988; Michel *et al.*, 1989b). Three genes that encode α_2-adrenoceptors have been cloned and COS cells transfected with these genes show different binding site profiles consistent with the α_{2A} and α_{2B} classification (Kobilka *et al.*, 1987; Regan *et al.*, 1988; Zeng *et al.*, 1990). The main criteria for α_{2A} adrenoceptors are high affinity binding of oxymetazoline and WB 4101 and low affinity binding of prazosin, chlorpromazine and ARC-239; the converse criteria apply for α_{2B}-adrenoceptors (Bylund, 1988; Bylund *et al.*, 1988; Michel *et al.*, 1989b).

There is ample evidence for the existence of multiple α_2-adrenoceptors from functional studies, in particular those in which presynaptic adrenoceptors mediating the inhibition of neurotransmitter release are compared with postsynaptic α_2-adrenoceptors mediating vascular contractility or platelet aggregation (Bylund & U'Prichard, 1983; Limbird & Sweatt, 1985; Ruffolo *et al.*, 1987; Bylund, 1988). It has been suggested that the α_2-adrenoceptor on cholinergic nerve terminals in the guinea-pig ileum differs from the α_2 receptor on sympathetic nerve terminals in the same tissue (Kapocsi *et al.*, 1987).

Three separate responses to noradrenaline can be distinguished in the submucosal plexus at the guinea-pig ileum. Noradrenaline hyperpolarizes submucosal neurones, inhibits the release of acetylcholine from (cholinergic) submucosal neurones as well as the release of sympathetically released neurotransmitter onto submucosal arterioles (North & Surprenant, 1985; Suprenant, 1989; Hirst, 1989). The postsynaptic adrenoceptor mediating membrane hyperpolarization in submucosal neurones has been shown to be an α_2-adrenoceptor (North & Surprenant, 1985; Surprenant & North, 1988); presynaptic adrenoceptors mediating both inhibition of acetylcholine release onto submucosal neurones and inhibition of sympathetically released transmitter onto submucosal arterioles are also likely to be α_2-adrenoceptors (Surprenant, 1989). We were interested in determining whether postsynaptic neuronal α_2-adrenoceptors were identical to presynaptic α_2-adrenoceptors and whether these adrenoceptors could be classified with ligands which have been shown to be selective in binding studies. Therefore we examined the effects of several ligands on each of these three adrenoceptor-mediated actions.

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Methods

All experiments were carried out on preparations of the submucosal plexus obtained from the small intestine of young guinea-pigs (200–300 g). Methods of dissection, intracellular recording from submucosal neurones, stimulation of interganglionic nerve fibres and optical measurements of arteriolar diameter have been described in detail previously (Surprenant, 1984; Neild *et al.*, 1990). The following physiological saline solution was used in all experiments (mM): NaCl 126, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.5, KCl 5, $NaHCO_3$ 25, glucose 11; gassed with 95% O_2 and 5% CO_2. Temperature was 35–37°C throughout.

Concentration-response curves for membrane hyperpolarizations

Hyperpolarizations in response to cumulative additions of the α_2-receptor agonist, UK 14304, were recorded in the absence and then presence of at least three concentrations of antago-

nist; previous studies have shown that cumulative and non-cumulative applications of UK 14304 produce similar hyperpolarizations in submucosal neurones (North & Surprenant, 1985; Surprenant & North, 1988). Schild plots were obtained from data on individual neurones and the dissociation equilibrium constants (K_Ds) and slopes so obtained were then averaged.

Concentration response curves for inhibition of nicotinic e.p.s.p.

Membrane potential was held at −90 to −95 mV (i.e. approximately the potassium equilibrium potential) while single nerve stimuli were applied once every 30 s. The stimulus threshold for activation of the nicotinic e.p.s.p. is much lower than for activation of the adrenergic inhibitory synaptic potential or the peptidergic slow excitatory synaptic potential (Surprenant, 1984); in all these experiments stimulus parameters were used which elicited only the nicotinic e.p.s.p. The time course of decay of the e.p.s.p. recorded in these experiments ($\tau = 52 \pm 3$ ms, $n = 32$) was about three times slower than the membrane time constant ($\tau = 18 \pm 4$ ms, $n = 29$); this difference is most likely the result of asynchronous release from multiple fibre inputs (see Bornstein *et al.*, 1987). In some cells, both the amplitude and duration of the e.p.s.p. were reduced by UK 14304 (e.g. Figure 3): the decreased duration could result from either (or both) a reduction in the membrane time constant (due to the decreased membrane resistance) or failure of some fibres to release transmitter. Therefore, in those cells in which the duration was altered by UK 14304, e.p.s.ps were measured in two ways, as peak amplitude only, or as peak amplitude multiplied by the half-duration; results obtained with either measure were the same (values were within 5% of each other). UK 14304 was applied in a cumulative fashion and five consecutive e.p.s.ps were averaged in control solution and 2–5 min after addition of each concentration of agonist; agonist effects reached a steady-state in this time. All antagonists were present for 5–10 min prior to construction of dose-response curves. Average values for antagonist K_Ds and slopes of Schild plots were obtained in the same way as for the experiments on membrane hyperpolarization.

Concentration-response curves for inhibition of nerve-evoked vasoconstriction

A focal stimulating electrode was placed onto the surface of a submucosal ganglion from which bundles of nerve fibres could be seen to cross the vessel directly. Outside diameter of the submucosal arteriole was monitored with Diamtrak software (Neild, 1989; Neild *et al.*, 1990). Vasoconstriction in response to electrical stimulation at 20 Hz for 2 s was measured as the peak amplitude multiplied by the half-duration of the evoked response; stimulation was delivered once every 5 min. Responses to this stimulation protocol were constant for periods of up to 4 h. Tetrodotoxin (0.5 μM) inhibited the stimulation-evoked vasoconstriction by 95–100% in all experiments. The vasoconstriction in response to nerve stimulation is due solely to neurotransmitter released from sympathetic nerves because it is abolished by guanethidine (10–20 μM) and by prior surgical denervation of the sympathetic nerve supply to the intestine (Neild *et al.*, 1990; Galligan *et al.*, 1990). Preliminary experiments were carried out to determine whether the actions of UK 14304 on the nerve-evoked vasoconstriction differed when agonist was applied in a cumulative or non-cumulative fashion. IC_{50} values and maximum effective concentrations were the same in either case and so cumulative applications of agonist were used in all experiments with antagonists. The competitive receptor antagonists used significantly enhanced the response to nerve stimulation (see Figure 6d); therefore, vasoconstrictions were plotted as a percentage of maximum vasoconstriction elicited.

Drugs

Drugs used were UK 14304 (5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline, gift from Pfizer), SKF 104078 (gift from SK&F), yohimbine and tetrodotoxin (Sigma), clonidine, oxymetazoline, WB 4101 (2-(2,6-dimethoxyphenoxyethyl) aminomethyl-1,4-benzodioxane), prazosin and chlorpromazine (RBI), guanethidine (Ciba), ARC-239 (2-[2-[4(O-methoxyphenyl) piperazine-1-yl]ethyl] 4,4 dimethyl-1,3,(2H-4H) isoquinolinedione, gift from Karl Thomae) and idazoxan (gift from Reckitt). Tests of significance were carried out with Student's *t* test; means are shown with standard errors of means (s.e.mean).

Results

Hyperpolarization of submucosal neurones

SKF 104078, ARC-239 and WB 4101 reduced the hyperpolarization caused by UK 14304 in a competitive fashion; each antagonist produced a parallel, rightward shift in the concentration-hyperpolarization curve (Figure 1). Slopes obtained from Schild plots of these data were not significantly different from unity (Figure 1, Table 1). There were no direct postsynaptic effects of these antagonists (at concentrations up to 30 μM) on the membrane potential, input resistance or directly evoked action potential.

Neither prazosin (0.1–10 μM, $n = 9$) nor chlorpromazine (0.1–10 μM, $n = 5$) directly altered resting membrane properties of submucosal neurones; concentrations ≤ 10 μM did not alter the UK 14304 concentration-hyperpolarization curves ($n = 4$ for each substance). Higher concentrations (30–100 μM) did inhibit UK 14304 hyperpolarizations but they also depressed hyperpolarizations produced by somatostatin and therefore were considered not to represent a selective action on postsynaptic α_2-adrenoceptors.

Oxymetazoline acted as a partial agonist to activate the postsynaptic α_2-adrenoceptor (Figure 2). Oxymetazoline produced a membrane hyperpolarization in eight of ten neurones

Table 1 Antagonist K_D values and slopes obtained from Schild plots of data for α_2-adrenoceptor-mediated postsynaptic hyperpolarization, presynaptic inhibition of nicotinic e.p.s.p. and presynaptic inhibition of sympathetic vasoconstriction

	Hyperpolarization		*Presynaptic inhibition of e.p.s.p.*		*Presynaptic inhibition of vasoconstriction*	
	K_D (nM)	slope	K_D (nM)	slope	K_D (nM)	slope
Idazoxan	6 ± 3	1.0 ± 0.1	10 ± 4	1.09 ± 0.12	5 ± 1	1.05 ± 0.1
Yohimbine	25 ± 9	0.98 ± 0.18	40 ± 7	1.16 ± 0.18	10 ± 3	1.09 ± 0.2
SKF 104078	1000 ± 350	1.2 ± 0.2*	800 ± 98	0.84 ± 0.17*	450 ± 112	0.89 ± 0.2
Oxymetazoline	125 ± 52	0.9 ± 0.12	130 ± 30	0.82 ± 0.2*	80 ± 24**	
WB 4101	50 ± 14	1.08 ± 0.05	45 ± 12	0.97 ± 0.12	40 ± 9	1.02 ± 0.19
ARC-239	320 ± 86	1.03 ± 0.1	500 ± 95	1.09 ± 0.09	—	
Prazosin	> 10,000		> 10,000		> 10,000	
Chlorpromazine	> 10,000		> 10,000		—	

$n = 3$–7 for each value. * Slope significantly different from one. ** Agonist EC_{50} value.

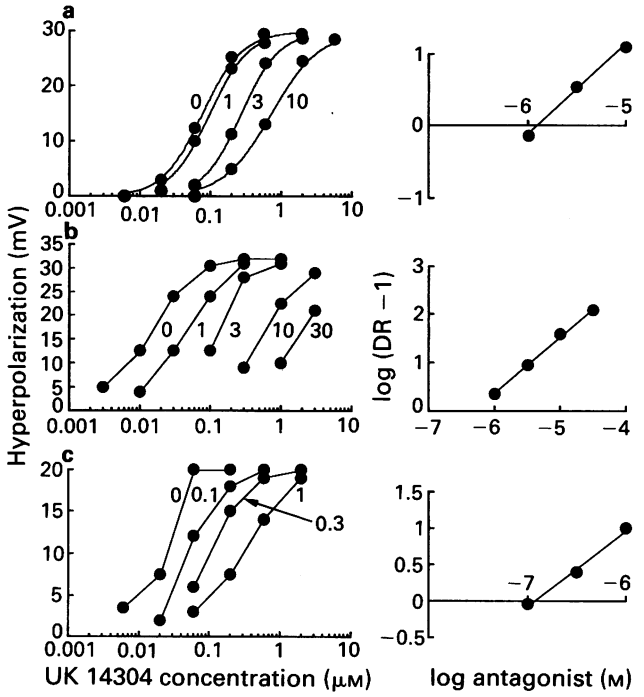

Figure 1 Postsynaptic α_2-adrenoceptor response in submucosal neurones. Hyperpolarization in response to superfusion with UK 14304 recorded from three different cells in the absence and then presence of SKF 104078 (a), ARC-239 (b) and WB 4101 (c); all concentrations shown as μM. Schild plots of these data are shown to the right of the individual dose-response curves; slopes were 1.06, 1.07 and 1.1 respectively.

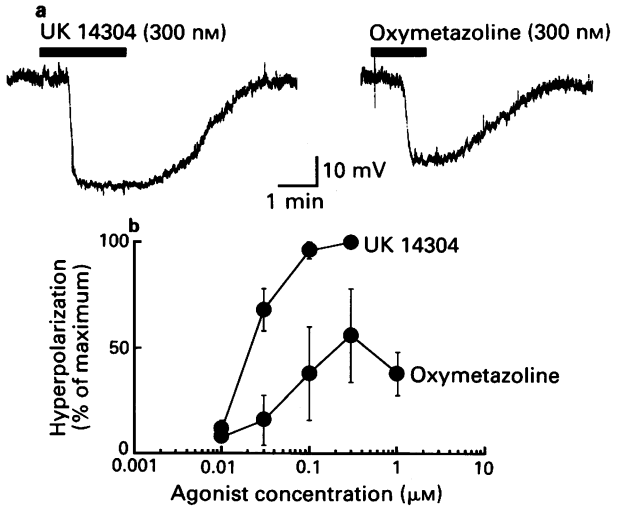

Figure 2 Agonist action of oxymetazoline at postsynaptic α_2-adrenoceptors in submucosal neurones. (a) Hyperpolarization recorded from one cell in response to superfusion with UK 14304 (left recording) and oxymetazoline (right recording); these were maximum responses to both agonists. Filled bar above traces indicate duration of agonist superfusion. (b) Concentration-hyperpolarization curve for UK 14304 and oxymetazoline obtained from the same cells ($n = 4$ for each point).

examined and the maximum hyperpolarization was variable from cell to cell, ranging from 25–85% of the maximum hyperpolarization to UK 14304. EC_{50} for oxymetazoline as an agonist was 120 nM (Figure 2). In the two neurones in which oxymetazoline had no direct postsynaptic effect, it acted as a competitive antagonist of the hyperpolarization produced by UK 14304; the K_D values (and slopes) from Schild plots were 120 nM (0.96) and 111 nM (1.06, data not shown).

We have previously determined the K_Ds of idazoxan and yohimbine for the inhibition of noradrenaline-, clonidine- and UK 14304-induced hyperpolarizations in these neurones (North & Surprenant, 1985; Crist & Surprenant, 1987). These experiments were repeated in the present study as internal controls; K_Ds for idazoxan and yohimbine (6.3 nM and 25 nM respectively, see Table 1) are very similar to those determined in our earlier studies.

Inhibition of acetylcholine release

The nicotinic e.p.s.p. is a sensitive assay for acetylcholine release onto neurones (Kretz *et al.*, 1984). UK 14304 inhibited the nicotinic e.p.s.p. in a dose-dependent manner and the concentration which produced half-maximum inhibition of the e.p.s.p. ranged from 20–80 nM ($n = 15$); UK 14304 inhibited the e.p.s.p. by 85–100% in all neurones (Figures 3, 4).

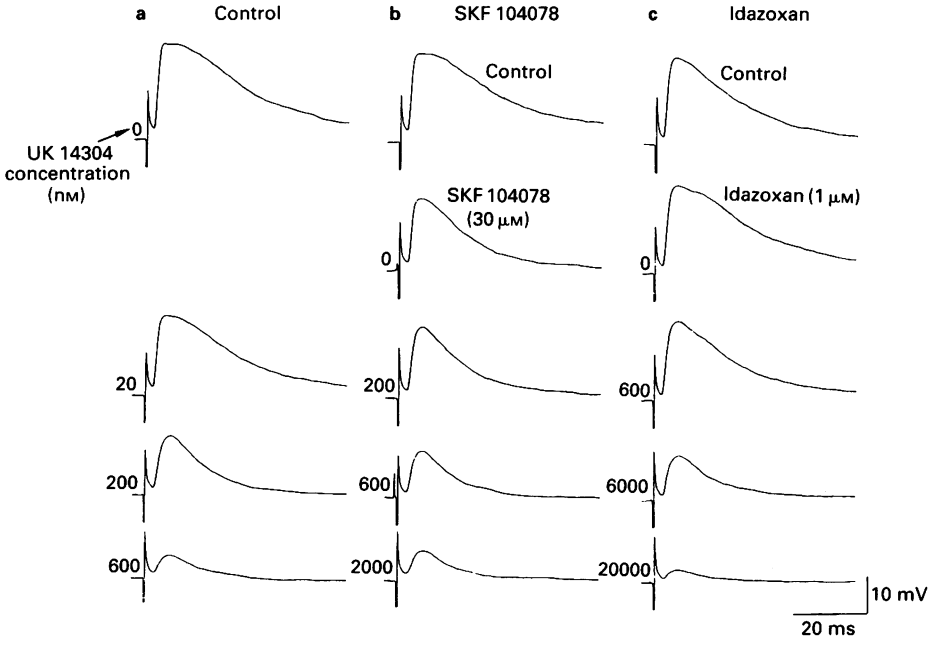

Figure 3 Presynaptic inhibition of nicotinic e.p.s.p. by UK 14304 (a) and its antagonism by SKF 104078 (b) and idazoxan (c). Each e.p.s.p. is the average of five consecutive responses; all recordings were obtained from the same cell. Note SKF 104078 alone decreased the e.p.s.p. amplitude; idazoxan (1 μM) alone enhanced the e.p.s.p. amplitude and duration.

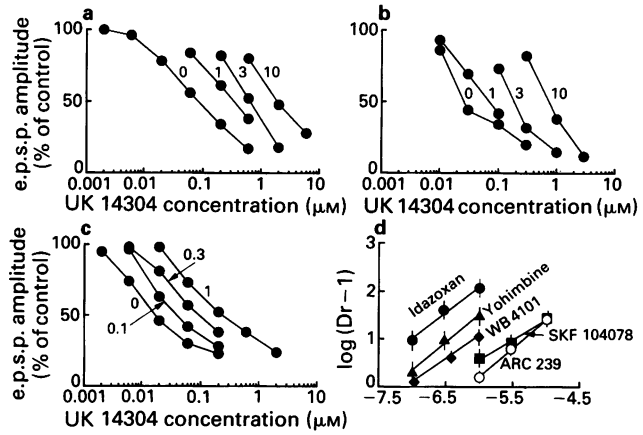

Figure 4 Presynaptic α_2-adrenoceptor response to inhibit e.p.s.ps in submucosal neurones. E.p.s.p. amplitude recorded in individual cells in control solution and in the presence of increasing concentrations of SKF 104078 (a), ARC-239 (b) and WB 4101 (c); all concentrations shown as μM. (d) Summary of data obtained from all experiments performed as in (a)–(c); results are pooled from cells in which three doses of antagonists were examined (n = 3–7 for each antagonist). Slopes are 1.09 for idazoxan, 1.16 for yohimbine, 0.97 for WB 4101, 0.84 for SKF 104078 and 1.09 for ARC-239; only the slope for SKF 104078 differed significantly from unity.

Idazoxan, yohimbine, WB 4104, SKF 104078 and ARC-239 all antagonized UK 14304 when inhibition of the e.p.s.p. was measured (Figure 4). Slopes of Schild plots obtained for these antagonists were not significantly different from unity except in the case of SKF 104078 (Figure 4, Table 1). This may be because SKF 104078 exhibited weak partial agonist actions (Figure 3); it inhibited the e.p.s.p. at concentrations of 10 or 30 μM by 8 ± 1.5% and 14 ± 3.2% respectively (n = 14).

Oxymetazoline inhibited the nicotinic e.p.s.p. in all submucosal neurones although its actions were more variable than those of UK 14304, with the maximum inhibition of the e.p.s.p. by oxymetazoline ranging from 25–100% (Figure 5a). IC_{50} for oxymetazoline inhibition of the e.p.s.p. was 110 nM (Figure 5b). When oxymetazoline had little agonist action on its own it acted as an antagonist of UK 14304 (Figure 5c); the K_D was 130 ± 30 nM (n = 4) but slopes of these Schild plots were significantly different from one (Figure 5d; Table 1).

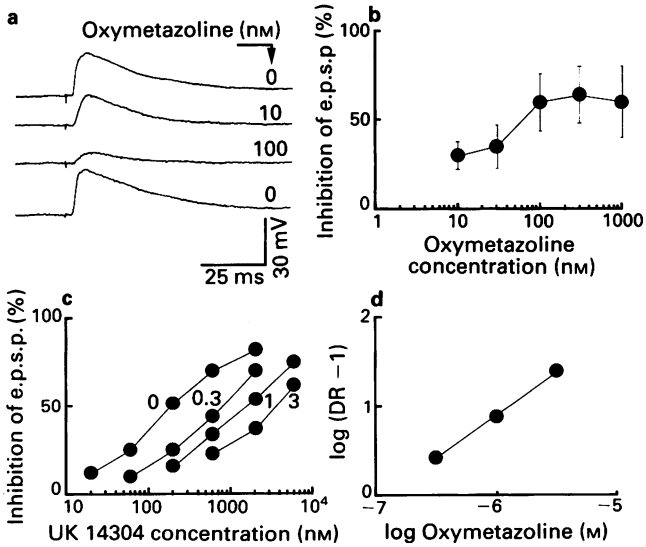

Figure 5 Agonist (a,b) and antagonist (c,d) actions of oxymetazoline at presynaptic cholinergic adrenoceptors on submucosal neurones. (a) E.p.s.p. recorded in control solution, in 10 nM and 100 nM oxymetazoline and after washout. (b) Concentration-response curve for oxymetazoline as an agonist to inhibit the nicotinic e.p.s.p.; EC_{50} is 115 nM. (c) Antagonism by oxymetazoline of UK 14304 inhibition of the nicotinic e.p.s.p.; all values were obtained from a single neurone; all concentrations of oxymetazoline μM. (d) Schild plot of data shown in (c); slope of the line is 0.82.

Prazosin and chlorpromazine (10–30 μM) did not significantly reduce the inhibition of the nicotinic e.p.s.p. by UK 14304 (n = 4 for each substance).

Inhibition of sympathetic neurotransmitter

Neither UK 14304 (1 nM–10 μM) nor clonidine (1 nM–1 μM) altered the outside diameter of submucosal arterioles, indicating the absence of α_2-adrenoceptors on the vasculature itself. UK 14304 did inhibit nerve-evoked vasoconstrictions with an EC_{50} of 6 ± 0.5 nM (n = 8); thus, UK 14304 is some 3–10 fold more potent in inhibiting the sympathetically mediated vasoconstriction than in producing neuronal membrane hyperpolarization (EC_{50} approximately 30 nM) or inhibiting acetylcholine release onto submucosal neurones (EC_{50} 20–60 nM). Clonidine was also effective in inhibiting the nerve-evoked vasoconstriction with an EC_{50} of 3 ± 0.4 nM (n = 3); however, the maximum inhibition by clonidine was only 50%, in contrast to UK 14304 which inhibited nerve-evoked vasoconstrictions by greater than 90% (Figure 7).

Idazoxan, SKF 104078, yohimbine and WB 4101 significantly increased the amplitude and duration of the nerve-evoked vasoconstriction (Figure 6); this action was dose-dependent with maximum effects being observed at 100 nM idazoxan, 3 μM SKF 104078 and 1 μM WB 4101 (Figure 6d). These susbtances also acted as competitive antagonists of UK 14304 to inhibit the nerve-evoked vasoconstrictions (Figure 7); K_Ds were 5 nM for idazoxan, 10 nM for yohimbine, 450 nM for SKF 104078 and 40 nM for WB 4101 (Table 1).

Oxymetazoline mimicked the actions of UK 14304; the EC_{50} for inhibition by oxymetazoline of the nerve-evoked vasoconstriction was 80 ± 9 nM (n = 4); the maximum inhibition was the same as that produced by UK 14304.

ARC-239 acted as neither agonist nor antagonist of the neurally evoked vasoconstriction in submucosal arterioles (n = 6). However, low concentrations (1 and 3 nM) shifted the UK 14304 or clonidine concentration curve to the left such that the EC_{50} for inhibition of the response by UK 14304 was

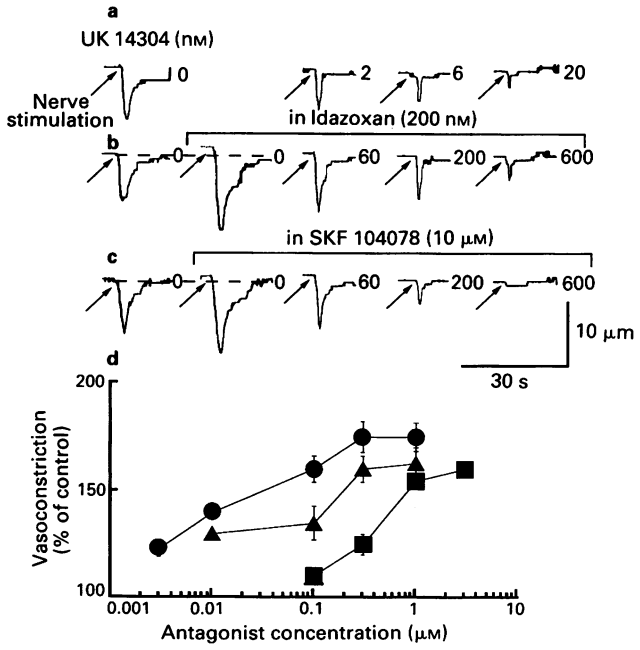

Figure 6 Presynaptic α_2-adrenoceptor response mediating inhibition of sympathetic vasoconstriction in submucosal arterioles. Vasoconstrictions in response to nerve stimulation (20 Hz for 2 s at arrow) in control solution (leftmost traces in a–c) and in the presence of UK 14304; responses in (a) and (b) are from the same arterioles, those in (c) from another vessel. Idazoxan (b) or SKF 104078 (c) alone increased the vasoconstriction produced by nerve stimulation and antagonized the UK 14304 inhibition. (d) Summary of actions of idazoxan (●), WB 4101 (▲) and SKF 104078 (■) to increase nerve-evoked vasoconstrictions (n = 3–5 for each point).

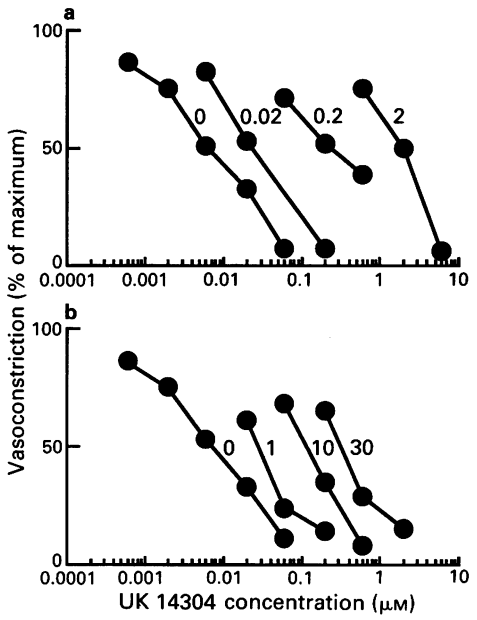

Figure 7 Concentration-response curves for inhibition of stimulation evoked vasoconstriction by UK 14304 and antagonism by idazoxan (a) and SKF 104078 (b); all concentrations shown as μM. Vasoconstriction is expressed as percentage of maximum response evoked in the absence of agonist; in both cases this was the response evoked in the presence of antagonist alone.

4 nM in the absence of ARC-239 and 0.8 nM in the same arterioles when 1 nM ARC-239 was present ($n = 4$). We have not examined this action of ARC-239 further.

Prazosin (1–30 μM) did not alter the nerve evoked vasoconstriction ($n = 9$); prazosin has previously been shown to be ineffective in blocking excitatory junction potentials recorded from submucosal arterioles in response to sympathetic nerve stimulation (Hirst, 1989). Prazosin (10 μM) also did not alter the inhibition of the nerve-evoked vasoconstriction by UK 14304 ($n = 4$).

Discussion

The main purpose of this study was to characterize the α_2-adrenoceptor subtype(s) present presynaptically and postsynaptically in peripheral autonomic neurones. It was of particular interest to examine the actions of ligands which have been shown to be α_2 subtype selective in radioligand binding studies because these ligands, ARC-239, oxymetazoline, prazosin and chlorpromazine (Bylund, 1988; Michel *et al.*, 1989b), are most commonly used in functional assays as ligands selective for α_1-rather than α_2-receptors (Starke, 1981; Minneman, 1988).

Presynaptic and postsynaptic α_2-adrenoceptors on cholinergic neurones

Previous determinations of K_D values for idazoxan, yohimbine and phentolamine for the receptors involved in postsynaptic hyperpolarization indicated that a homogeneous population of α_2-adrenoceptors was present on submucosal neurones (North & Surprenant, 1985; Surprenant & North, 1988). Slopes of Schild plots for idazoxan, yohimbine, WB 4101 and ARC-239 to antagonize the UK 14304 inhibition of the nicotinic e.p.s.p. (Table 1) were not significantly different from unity, consistent with a single population of α_2-adrenoceptors present on cholinergic nerve terminals. The finding that the slopes for oxymetazoline and SKF 104078 differed from one (Table 1) at the presynaptic cholinergic site seems more likely to be due to their partial agonist actions than to heterogeneity of α_2-adrenoceptors. Oxymetazoline acted as a partial agonist at both pre and postsynaptic sites (Figures 2, 5) while SKF 104078 exhibited agonist actions at the presynaptic cholinergic site but only antagonist actions at the postsynaptic site. SKF 104078 shows weak partial agonist activity in several functional assays, such as the α_2-mediated contraction of human saphenous vein and presynaptic inhibition of (nicotinic) stimulation-evoked contractions in guinea-pig ileum (Connaughton & Docherty, 1990).

The nicotinic e.p.s.p. recorded from submucosal neurones in the guinea-pig ileum is due to the release of acetylcholine from two sources; approximately half the nicotinic input is from cholinergic interneurones in the submucosal plexus and the other half derives from cholinergic neurones in the myenteric plexus (Bornstein *et al.*, 1987). Because the majority of submucosal neurones are hyperpolarized by α_2-agonists (Surprenant & North, 1988), many of the cell bodies from which hyperpolarizations were recorded in the present study will have been those that provide the cholinergic terminals. Thus, it might be expected that the postsynaptic adrenoceptor on the cholinergic cell body would be the same as the presynaptic adrenoceptor on the cholinergic nerve terminal. The results of the present study give credence to this expectation; the identity of these α_2-receptors is apparent in Figure 8a, which shows the correlation between pA_2 values determined for α_2-receptors on the postsynaptic membrane and on the presynaptic cholinergic nerve terminal.

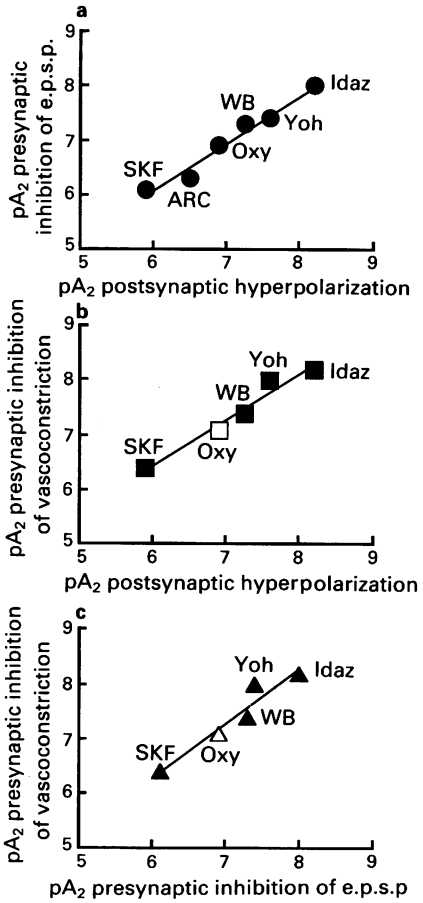

Figure 8 (a) Correlation between pA_2 values obtained at the neuronal postsynaptic α_2-adrenoceptor and the cholinergic presynaptic α_2-adrenoceptor; slope is 0.87 ($r = 0.97$). (b) Correlation between pA_2 values at the neuronal postsynaptic α_2-receptor and the sympathetic presynaptic α_2-adrenoceptor; slope is 0.85 ($r = 0.97$). (c) Correlation between pA_2 values at the cholinergic presynaptic site and the sympathetic presynaptic α_2-adrenoceptor; slope is 0.97 ($r = 0.99$). The values for oxymetazoline in (b) and (c) are shown as open symbols because the EC_{50} value for this ligand as an agonist of presynaptic inhibition of nerve-evoked vasoconstriction was used. SKF = SKF 104078; ARC = ARC-239; Oxy = oxymetazoline; WB = WB 4101; Yoh = yohimbine; Idaz = idazoxan.

Can the receptor identified in this study (Table 1) be placed into the α_{2A} or α_{2B} subtype classification? Our K_Ds for the non-selective α_2 antagonists, idazoxan, yohimbine and SKF 104078 are the same as those obtained in other tissues by both radioligand binding and functional assays (Starke, 1981; Ruffolo et al., 1987; Bylund, 1988; Bylund et al., 1988; Michel et al., 1989b; Connaughton & Docherty, 1990). ARC-239 was a competitive antagonist at both postsynaptic and cholinergic presynaptic nerve terminal sites, with a K_D of approximately 300 nM (Figures 1, 2, Table 1). This ligand shows a 10–100 fold selectivity for α_{2B} (K_Ds of 1–50 nM) over α_{2A} (K_Ds 85–200 nM) adrenoceptors in ligand binding studies (Bylund et al., 1988; Michel et al., 1989b); the K_D for ARC-239 obtained in the present study is in accord with the α_{2A}-adrenoceptor values.

Bylund (1988) has suggested that oxymetazoline can be used as a selective ligand for α_{2A}- over α_{2B}-adrenoceptors with K_Ds at this site ranging from 1 to 10 nM compared to K_Ds of 50–250 nM at the α_{2B}-site (Bylund et al., 1988; Michel et al., 1989b); based on this information, the K_D for oxymetazoline at adrenoceptors on submucosal neurones ($\sim$125 nM) might be placed in the α_{2B}-adrenoceptor category. The K_D of oxymetazoline as an agonist is 120–150 nM in functional studies on rat vas deferens (Diaz-Toledo & Marti, 1988) and hippocampus (Curet & deMontigny, 1988); based on these values it has been suggested that these two tissues express the α_{2B}-adrenoceptor (Bylund et al., 1988). However, there are several reasons to question the interpretation that oxymetazoline is acting at α_{2B}-receptors on submucosal cells. Anomalous binding affinities have been observed with oxymetazoline, even in tissues with otherwise well-defined subtypes of α_2- as well as α_1-adrenoceptors (Michel et al., 1989a,b). Oxymetazoline exhibits partial agonist actions, not only in this study, but also in a majority of other functional assays in several tissues (Minneman, 1988; Curet & deMontigny, 1988; Diaz-Toledo & Marti, 1988); thus large differences in apparent K_D values may be expected to occur in comparisons among tissues and/or species (see Kenakin, 1984). Indeed, there do not appear to be any reports with functional assays in which oxymetazoline, as either agonist or antagonist, exhibits a K_D or EC_{50} value in the range of those reported for the α_{2A}-subtype in binding studies (e.g. 1–5 nM; Bylund, 1988; Bylund et al., 1988; Michel et al., 1989b).

Probably the most convincing data which suggest that the α_2-receptor on submucosal neurones is most likely to be the α_{2A}-subtype are the lack of antagonistic actions of prazosin and chlorpromazine. Prazosin and chlorpromazine K_Ds in binding experiments are 5–20 nM at the α_{2B}-site and 0.5–5 μM at the α_{2A}-site. Neither of these ligands was effective at concentrations up to 30 μM in the present study. There are several other examples where α_2-adrenoceptor responses, such as presynaptic inhibition of transmitter release, are completely insensitive to antagonism by prazosin (Starke, 1981; Kapocsi et al., 1987). It may be illuminating to consider results obtained in electrophysiological experiments on the NG108-15 neuroblastoma cell line; these cells are considered to possess prototypic α_{2B}-subtype binding sites (Bylund et al., 1988). Noradrenaline inhibits the calcium current in NG108-15 cells and this action is antagonized in a competitive manner by yohimbine (K_D 100 nM) and prazosin (K_D 3 μM) (Docherty & McFadzean, 1989). Thus, in the case of the NG108-15 neuroblastoma cells, prazosin K_D values from binding experiments and from electrophysiological experiments differ by 100 fold. If similar differences occur at the α_{2A}-site, one would expect prazosin to have no functional antagonistic action.

α_2-Adrenoceptor on sympathetic nerve terminals innervating submucosal arterioles

Excitatory junction potentials (e.j.ps) recorded from submucosal arterioles in response to sympathetic nerve stimulation are insensitive to blockade by high concentrations of prazosin (Hirst, 1989). These e.j.ps have been attributed to the release of ATP from sympathetic nerves (Burnstock & Griffith, 1988) or to activation of a distinct noradrenaline receptor (Hirst, 1989). In the present study, the nerve-evoked vasoconstriction of submucosal arterioles was similarly insensitive to prazosin; 10–30 μM prazosin did not alter the amplitude or time course of the nerve evoked vasoconstriction. On the other hand, submucosal arterioles readily constrict when noradrenaline or phenylephrine is applied (Hirst, 1989; Neild & Kotecha, 1989) and this vasoconstriction is antagonized by prazosin with a K_D of 1 nM (Vanner et al., 1990). Thus, it appears that noradrenaline released from sympathetic nerves to submucosal arterioles by even high frequency stimulation does not activate the α_1-adrenoceptors present on the vascular smooth muscle. Nevertheless, the vasoconstriction we observed can be attributed solely to the release of transmitter from sympathetic fibres because the response is abolished by surgical sympathectomy and by application of guanethidine (Neild et al., 1990; Galligan et al., 1990; see Methods).

The results we obtained with idazoxan, yohimbine, SKF 104078 and WB 4101 are similar to those of previous studies in which overflow of tritiated noradrenaline from blood vessels has been measured (Starke, 1981); that is, these antagonists all increased the nerve-evoked vasoconstriction. Such enhancement of the effects of nerve stimuli by α_2-receptor antagonists is the hallmark feature of negative feedback inhibition by presynaptic α_2-adrenoceptors, or autoreceptors (Starke, 1981). Thus, whether or not it is noradrenaline, ATP, or another transmitter, that is responsible for the vasoconstriction in submucosal arterioles, it is clear that autoreceptors are present and functional on the sympathetic terminals to this vascular bed.

There are several examples of the presence of α_2-adrenoceptors present on vascular smooth muscle, activation of which causes contraction (McGrath, 1982; Medgett & Ruffolo, 1987; 1988; Nielson et al., 1989; Connaughton & Docherty, 1990); it has been suggested that postjunctional vascular α_2-receptors are more common in small arteries and arterioles than in larger vessels (Nielson et al., 1989). We found no evidence for vascular α_2-adrenoceptors in these resistance vessels. The antagonism by idazoxan, yohimbine, WB 4101 and SKF 104078 of the action of UK 14304 to reduce nerve-evoked vasoconstriction conformed to a simple competitive model with Schild plots of unit slope: K_D values for these antagonists at this site confirm it is an α_2-adrenoceptor. Oxymetazoline, whose clinical use is second only to phenylephrine as the most widely used vasoconstrictor in nasal decongestants (Barnhart, 1988), had no (α_1) vasoconstrictor action on submucosal arterioles. It acted as a full agonist at the presynaptic α_2-site with an agonist EC_{50} that was similar to its K_D as an antagonist at the presynaptic adrenoceptor on cholinergic submucosal neurones (Table 1).

Figures 8b and 8c show the correlation between pA_2 values determined for pre and postsynaptic α_2 receptors on submucosal neurones and for presynaptic α_2-adrenoceptors on sympathetic nerve terminals innervating submucosal arterioles. These strong correlations indicate the α_2-adrenoceptors on sympathetic nerve terminals are the same as those on the submucosal cholinergic nerve terminals and submucosal cell bodies. Accordingly, we suggest this adrenoceptor is most probably the α_{2A}-subtype.

Pharmacological characterizations of ligand binding sites have provided a consensus for the existence of two predominant α_2-adrenoceptor subtypes (Neylon & Summers, 1985; Alabaster et al., 1986; Bylund, 1988; Bylund et al., 1988; Michel et al., 1989b); upwards of four additional, distinct, subtypes have also been identified in radioligand binding profiles (Murphy & Bylund, 1988; Regan et al., 1988; Michel et al., 1989b). The binding profiles obtained on cells transfected with each of the three cloned cDNAs encoding for α_2-adrenoceptors are consistent with one α_{2A}-adrenoceptor and two structurally distinct α_{2B}-adrenoceptors (Kobilka et al., 1987; Regan et al., 1988; Zeng et al., 1990). Definitive correlation

between α_2-adrenoceptor binding sites and structurally identified α_2-adrenoceptors remains incomplete; however, it is clear from this study and others that there is an acute need for further functional studies directed at determining more precise functional correlates for the α_2-adrenoceptor binding sites. It is particularly apparent from this study that identification and correlation of functionally distinct α_2-adrenoceptor subtypes with current ligand binding profiles will require subtype selective ligands other than prazosin, chlorpromazine, ARC-239 and oxymetazoline.

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A salivary vasodilator in the blood-sucking bug, *Rhodnius prolixus*

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1 Salivary gland homogenates of the blood-sucking bug, *Rhodnius prolixus* induced transient, dose-dependent relaxation of rabbit aortic preparations pre-constricted with 200 ng ml^{-1} noradrenaline, 1 μg ml^{-1} histamine or 20 ng ml^{-1} angiotensin II. Such relaxations were less marked when the aorta was constricted by 60 mM KCl. These effects were observed with as little as 0.2 μg ml^{-1} of crude salivary gland protein.

2 The vasodilator effect was endothelium-independent, abolished by 50 μM hydroquinone or 50 μM methylene blue, and potentiated by 30 u ml^{-1} superoxide dismutase.

3 Salivary homogenates generated a coloured compound when reacted with sulfanilic acid in the presence of N-(1-naphtyl)-ethylediamine, indicating the presence of reactive nitrogen groups, equivalent to 35 $\pm$ 3 ng of sodium nitrite per pair of glands.

4 Molecular sieving high performance liquid chromatography of salivary gland homogenates generated a single peak of vasorelaxant activity which coincided with the presence of platelet antiaggregating and spasmolytic (guinea-pig ileum contracted with histamine) activities, as well as with reactive nitrogen groups.

5 It is concluded that a protein of molecular weight 16,500 daltons in the salivary glands of *R. prolixus* contains reactive nitrogen groups which assist the bug during a blood meal. It is suggested that saliva of blood sucking arthropods is a natural resource of novel pharmacological activities.

Introduction

The task of blood-feeding by arthropods is facilitated by saliva which is injected into the host skin during probing and feeding. This role of saliva is mediated by a number of different pharmacologically active compounds, including anti-coagulants, anti-platelet activities, anti-inflammatory agents, immunosuppressants and vasodilators that counteract the host's defense mechanisms that could prevent blood finding or blood feeding (Ribeiro, 1987; 1989). Indeed, triatomine bugs or mosquitoes that were surgically deprived of salivary function had a much decreased success in obtaining a blood meal from a living host, but were successful when feeding from an artificial membrane feeder (Ribeiro & Garcia, 1981a; Ribeiro *et al.*, 1984).

The salivary glands of the blood-sucking bug *Rhodnius prolixus* contain a number of different components. An anti factor VIII (Hellmann & Hawkins, 1964; 1965) delays clotting. Anti-platelet activity prevents aggregation of citrated platelet-rich plasma induced by either adenosine diphosphate (ADP), collagen or arachidonic acid (AA) (Ribeiro & Garcia, 1981b; Ribeiro & Sarkis, 1982). A potent salivary apyrase (ATP-diphosphohydrolase) may help to counteract ADP-induced platelet aggregation (Ribeiro & Garcia, 1980; Sarkis *et al.*, 1986), and an anti-thromboxane activity antagonizes aggregation induced by AA and collagen suspensions, as well as the vasoconstrictor effect of thromboxane A_2 (Ribeiro & Sarkis, 1982). Saliva also antagonizes the effects of histamine and 5-hydroxytryptamine on smooth muscle preparations (Ribeiro, 1982). This bug appears to be well equipped to counteract their host's haemostasis.

A very potent salivary vasodilator peptide was described in the sand fly *Lutzomyia longipalpis* (Ribeiro *et al.*, 1989), and the vasodilator prostaglandin E_2 was found in the saliva or salivary glands of *Boophilus microplus*, *Hyaloma anatolicum* and *Ixodes dammini* ticks (Higgs *et al.*, 1976; Shemesh *et al.*, 1979; Ribeiro *et al.*, 1985). To our knowledge, no vasodilators have been studied from any other of the thousands of haematophagous arthropod species. Yet salivary vasodilators should be common among blood-sucking animals due to the selective advantage these compounds offer. Vasodilators would increase the supply of food at the arthropod's mouthparts and decrease the time necessary for completing the meal (Gillet, 1967; Edman & Kale, 1971; Edman *et al.*, 1974). A fifth instar nymph of *R. prolixus*, weighing about 30 mg, typically takes 300 μl of blood in 15 min from a single cannulated skin venule or arteriole (Lavoipierre *et al.*, 1959). A salivary vasodilator would presumably help the bug to accomplish this feat. In this paper, we investigated and characterized a salivary vasodilator in *R. prolixus*, and discuss the possibility that the saliva of blood sucking arthropods is a rich natural source of pharmacologically active substances.

Methods

Rhodnius prolixus bugs, fed on anaesthetized guinea-pigs, were reared at the Harvard School of Public Health. Salivary glands were removed from adult male and female *Rhodnius prolixus* at one week of age or older (Ribeiro & Garcia, 1980). Groups of 25 pairs of glands were dissected, pooled together in 1.5 ml conical plastic tubes and frozen at −70°C until use. Frozen salivary glands were suspended in 250 μl phosphate buffered saline (150 mM NaCl and 10 mM sodium phosphate, pH 7.4), homogenized in a glass homogenizer and centrifuged at 10,000 *g* for 5 min. The supernatant, containing 1.8 $\pm$ 0.3 mg salivary gland protein (mean $\pm$ s.e.mean, $n = 3$), was collected and used for the experiments. The supernatants could be kept at −70°C and submitted to several freeze and thaw cycles without apparent loss of activity.

Vasodilator activity was measured on 3 mm wide rabbit aortic strips or rings, in a 5 ml glass bath kept at 37°C. The preparation was immersed in Tyrode solution (composition in mM: NaCl 137, KCl 2.68, $CaCl_2$ 1.8, $MgCl_2$ 0.49, NaH_2PO_4

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0.362, $NaHCO_3$ 11.9, disodium EDTA 0.03). The pH of the solution, gassed with 95% O_2 and 5% CO_2, was adjusted to 7.5 (Webster & Prado, 1970). An initial tension of 2 g was applied and the preparation was allowed to rest for 1.5 h with changes in the bathing solution every 30 min. The tension generated was recorded isometrically. After this resting period, 200 ng ml^{-1} noradrenaline (NA) was added and when a plateau was obtained, the various experiments were performed. In certain experiments, the aorta was contracted with 60 mM K^+ instead of NA. When 60 mM K^+ was utilized, the final concentration of Na^+ was reduced 60 mM to maintain osmolarity (Karaki, 1987).

For smooth muscle spasmolytic activity a guinea-pig terminal ileum preparation was used and treated as for aortic preparations, except that isotonic contractions against a load of 1.5 g were recorded. Histamine (200 ng ml^{-1}) was added to the bath at 3 min intervals and allowed to act for 20 s before being washed.

Anti-platelet activity was measured in a Born aggregometer (Born & Cross, 1963) by use of 50 μl human citrated platelet-rich plasma and 50 μl of column eluates in 150 mM NaCl + 10 mM phosphate buffer pH 6.8. Aggregation was triggered by adenosine diphosphate (10 μM).

Reactive nitrogen groups (such as NO or NO_2^-) were measured colorimetrically by a slight modification of published techniques (Bell *et al.*, 1963; Ignarro *et al.*, 1987). Briefly, 40 μl of 40% trichloroacetic acid was added to 200 μl of sample containing one or two salivary glands in phosphate buffered saline. The tube was vortexed and spun at 10,000 *g* for one minute, which produced a visible precipitate containing the salivary pigment. To 200 μl of the clear supernatant, 25 μl of sulfanilic acid (2 mg ml^{-1}) were added, vortexed, and 25 μl of N-(1-naphtyl)-ethylenediamine (NEDA, 1 mg ml^{-1}) were then added. After 30 min, the colour was measured at 560 nm on an ELISA reader. Controls lacking either the sulfanilic acid or NEDA gave the same optical density reading as those lacking the salivary homogenate.

Molecular sieving high performance liquid chromatography (h.p.l.c.) was performed on salivary gland homogenates with a TSK 125 column (300 × 7.5 mm) and pre-column (75 × 7.5 mm, obtained through Bio-Rad, U.S.A.), with 150 mM NaCl and 10 mM sodium phosphate, pH 6.8. The absorbance at 280 nm was recorded. Molecular weight markers used were human IgG, bovine serum albumin, ovalbumin, myoglobin and cyanocobalamin which were supplied by Bio-Rad (U.S.A.). A 4000 series Milton Roy pump, detector and integrator were utilized.

Organic reagents, histamine, angiotensin II, noradrenaline and superoxide dismutase were purchased from the Sigma Chemical Co. (U.S.A.). Other salts were of A.C.S. or analytical grade. Deionized water was used throughout the work. Protein was determined by the Bio-Rad Commassie-blue kit following the manufacturer's instructions.

Results

To investigate whether salivary glands of *Rhodnius prolixus* contained vasodilator activity, we added different amounts of salivary gland homogenates to the rabbit aortic strip preparation pre-constricted with NA. Addition of 1% of one pair of glands (about 1.8 μg of protein) to a 5 ml bath gave a noticeable transient relaxation of the preparation. The phenomenon was dose-dependent (Figure 1a). Repeated additions of the same dose gave reproducible transient relaxations on the same preparation (Figure 1b). Relaxation was also observed when the aorta was contracted with 1 μg ml^{-1} histamine or 20 ng ml^{-1} angiotensin II (data not shown).

We tested the effects of the homogenate on aortic strips from which the endothelium was removed by rubbing the intima with filter paper. Addition of salivary homogenates continued to elicit relaxation on endothelium-free preparations (Figure 2).

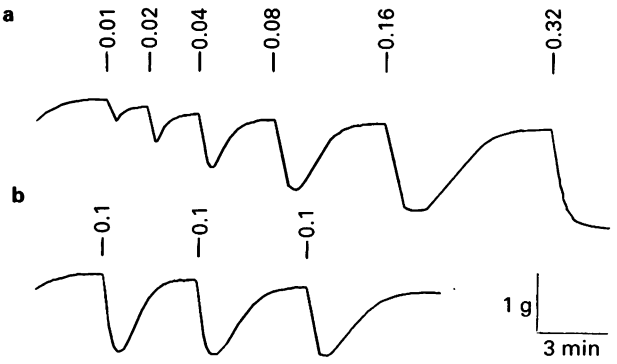

Figure 1 Transient relaxation of the rabbit aortic strip induced by *Rhodnius prolixus* salivary gland homogenates. The preparation was constricted with 200 ng ml^{-1} noradrenaline and the additions made are of fractions of one pair of glands to a 5 ml organ bath.

The action of the salivary vasodilator was inhibited by 50 μM methylene blue added at least 10 min before saliva addition (Figure 3a), and by 50 μM hydroquinone, added 3–5 min before saliva addition (Figure 3b). The vasodilator activity was, however, potentiated by superoxide dismutase (30 u ml^{-1}), added 3–5 min before saliva (Figure 3c). Similar results were obtained on 3 different aortic preparations with 3 different batches of salivary glands.

The vasodilator was less effective at relaxing potassium-induced (60 mM) contractions than those induced by 200 ng ml^{-1} NA (Figure 4).

To test whether *R. prolixus* salivary gland homogenates contained reactive nitrogen compounds, we treated the acidified homogenate with sulfanilic acid and NEDA. A coloured product developed indicating the presence of 35 ± 3 ng of sodium nitrite equivalents per pair of salivary glands (mean ± s.e.mean, $n = 4$ in pools of 25 pairs of glands).

To determine the molecular weight of the substance(s) responsible for the effects of saliva, we fractionated *R. prolixus* salivary homogenates on a molecular sieving column and measured its relaxant or inhibitory effects on NA-induced contractions of the rabbit aorta, histamine-induced contractions of guinea-pig ileum and adenosine diphosphate-induced aggregation of human citrated platelet-rich plasma. Additionally, reactive nitrogen groups were measured colorimetrically. Vasodilator, spasmolytic, anti-platelet and reactive nitrogen activities co-eluted with the major 280 nm absorption peak, with an apparent molecular weight of 16,500 daltons (Figure 5).

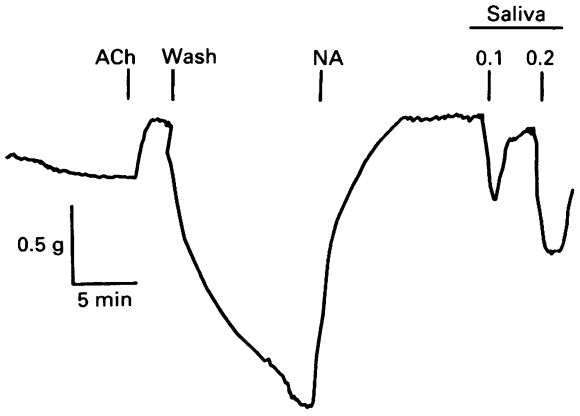

Figure 2 Endothelium-independence of the effect of *Rhodnius prolixus* salivary homogenate on the rabbit aortic ring constricted with noradrenaline (200 ng ml^{-1}). The efficiency of removal of the endothelium, accomplished by rubbing the intima with a filter paper, was determined by the failure of acetylcholine (ACh, 1 μg ml^{-1}) to elicit vasodilatation. The numbers indicate the fraction of one salivary gland added to a 5 ml organ bath. Two other preparations yielded similar results.

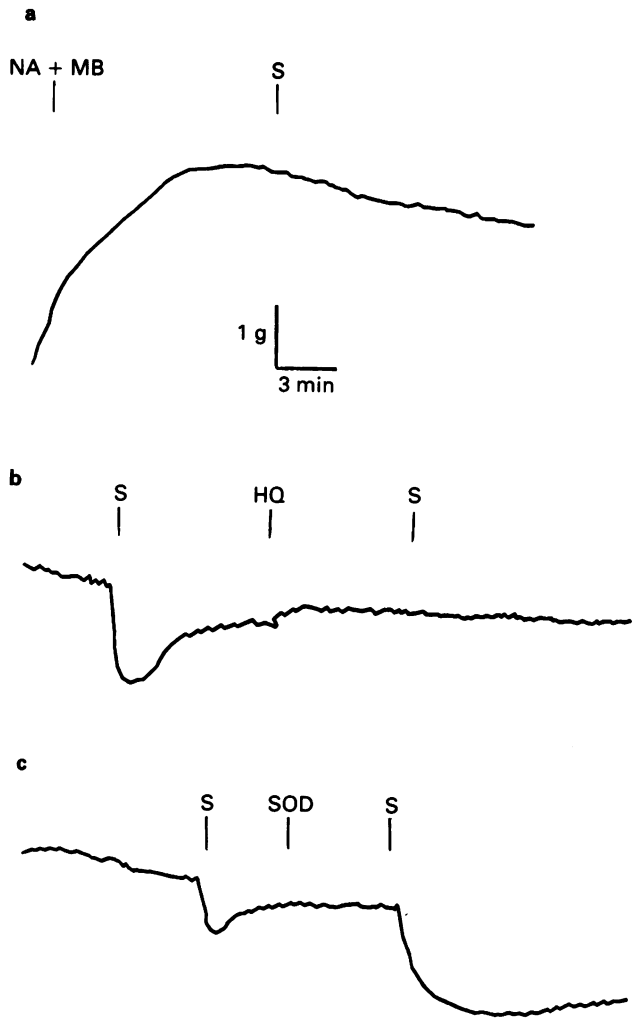

Figure 3 Sensitivity of *Rhodnius prolixus* salivary vasodilator to oxidants and protection by superoxide dismutase. Rabbit aortic strips were constricted with 200 ng ml^{-1} noradrenaline (NA) and 10% (a and b) or 3% (c) of one pair of glands was then added (indicated by S). (a) Inhibition of the vasodilator effect by 50 μM methylene blue (MB) added with noradrenaline. (b) Inhibition of vasodilator effect by 50 μM hydroquinone (HQ). (c) Enhancement of the vasodilator effect by superoxide dismutases (SOD, 30 u ml^{-1}). Two other experiments, with different rabbit aortic strips and different batches of salivary gland homogenate, yielded similar results.

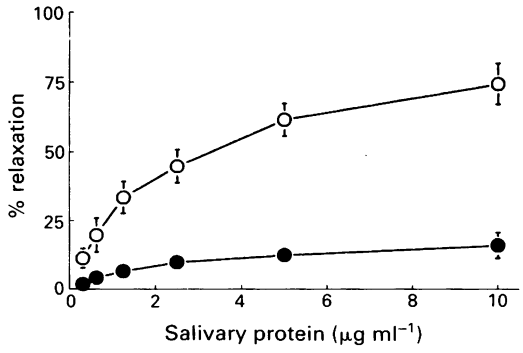

Figure 4 Dose-response curves showing the vasodilator activity of *Rhodnius prolixus* salivary gland homogenates on rabbit aortic strips constricted with 200 ng ml^{-1} noradrenaline (○) or with 60 mM KCl (●). Symbols and vertical bars show the mean and s.e.mean of 4 determinations representing 4 different aortic preparations and 4 different pools of salivary glands.

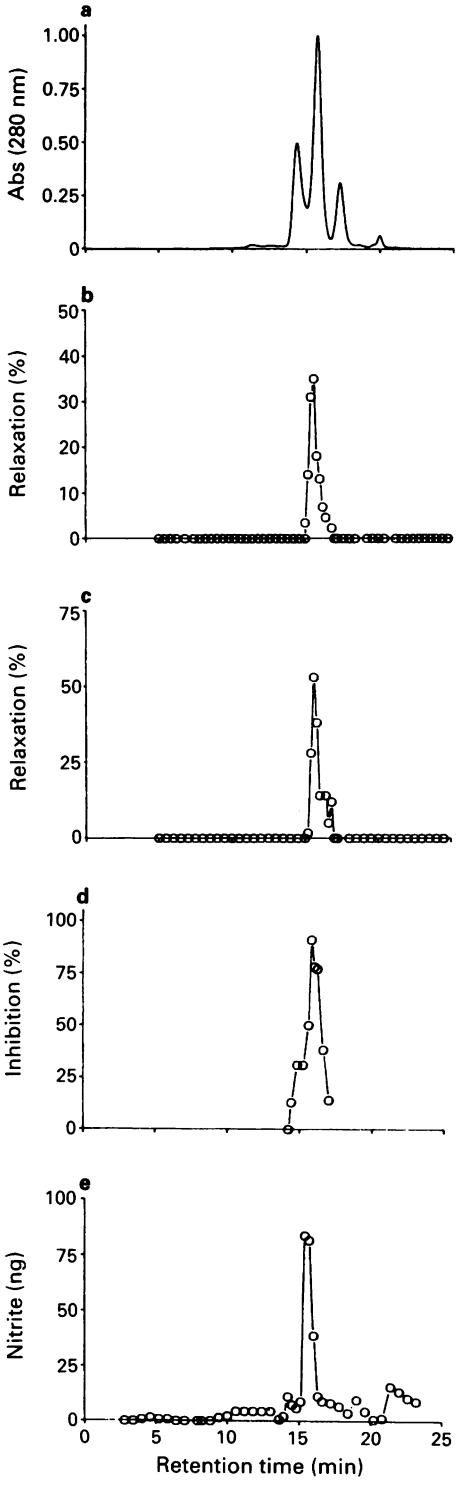

Figure 5 Molecular sieving liquid chromatography of 10 homogenized pairs of salivary glands of *Rhodnius prolixus* in a TSK 125 column, run at 0.8 ml min^{-1} with 150 mM NaCl and 10 mM phosphate buffer, pH 6.8. Results shown are combined from two runs under identical conditions. Fractions were collected at every 0.2 min except for reactive nitrogen group determinations, where it was at 0.3 min intervals. (a) The absorbance (Abs) at 280 nm (optical density). (b) Vasodilatation was measured by adding 10 μl of each fraction to a rabbit aortic strip constricted with 200 ng ml^{-1} noradrenaline. (c) Spasmolytic activity was measured by adding 50 μl of each fraction to a guinea-pig ileum constricted with 200 ng ml^{-1} histamine. Contractions were elicited before application of each experimental fraction. (d) Anti-platelet activity was determined by mixing 50 μl of indicated fractions with 50 μl of human citrated platelet-rich plasma, and measuring the resulting aggregation 5 min after addition of 10 μM ADP. (e) Reactive nitrogen groups were measured in 0.2 ml samples from each fraction to determine nitrite equivalent.

Discussion

The transient nature of the *Rhodnius prolixus* salivary gland-induced aortic relaxation (Figure 1) is similar to relaxation seen with some nitrovasodilators and nitric oxide (Gruetter *et al.*, 1980). The actions of nitrovasodilators are endothelium-independent and are inhibited by oxidant compounds such as methylene blue (Gruetter *et al.*, 1979; 1980) and hydroquinone (Rapoport & Murad, 1983). In addition, the actions of nitric oxide are enhanced by superoxide dismutase (SOD) (Gryglewski *et al.*, 1986). These same characteristics were observed with the *R. prolixus* salivary vasodilator (Figure 2 and 3).

Further similarities between *R. prolixus* salivary vasodilator activity and nitrovasodilators were obtained when comparisons were made between the relaxation induced in aortic strips preconstricted with either NA or KCl. As is the case with nitric oxide (Ignarro *et al.*, 1988), saliva-induced relaxation was more marked in NA pre-constricted aortic strips than in KCl pre-constricted strips (Figure 4).

Nitrovasodilators also relax guinea-pig ileum and inhibit platelet aggregation (Azuma *et al.*, 1986; Moncada *et al.*, 1988; Buga *et al.*, 1989). *R. prolixus* salivary gland homogenates also possess these activities. Additionally, when submitted to molecular sieving fractionation, the spasmolytic and anti-platelet aggregating activities of homogenates co-eluted with the vasodilator activity. Furthermore, reactive nitrogen groups were found in the same fractions. Taken together, these results strongly support the hypothesis that the vasodilator in *R. prolixus* salivary glands is a nitrovasodilator.

Because nitrovasodilators have multiple actions (Moncada *et al.*, 1988), the presence of a salivary nitrovasodilator could explain the inhibitory effects on the actions of histamine, 5-hydroxytryptamine and thromboxane previously obtained in the saliva of *R. prolixus* (Ribeiro, 1982; Ribeiro & Sarkis, 1982), and thus a single substance could account for multiple actions, all of potential benefit for blood feeding.

If it is assumed that there is a nitrovasodilator in *R. prolixus* salivary glands, the nature of the carrier molecule of the reactive nitrogen groups, as well as how these groups are released when in contact with the tissues remain to be investigated. It is possible that a protein with an apparent molecular weight of 16,500 daltons present in saliva is the parent molecule from which the nitric oxide-like material is released. *R. prolixus* salivary glands have a deep cherry colour, first studied by Wigglesworth (1942–3), who determined that the spectroscopic properties of the pigment were similar to those of haemalbumin (haeme groups bound to human serum albumin). However, Wigglesworth did not measure the molecular weight of the pigments. Indeed all three major 280 nm absorbing peaks shown in Figure 5 absorb at 405 nm (the haeme Soret band) more intensely than at 280 nm, and their spectra is consistent with a haeme group (data not shown). Haeme proteins are known to bind nitric oxide, some with very high affinity (Ignarro & Kadowitz, 1985). It is therefore possible that the 16,500 molecular weight peptide in saliva binds haeme with high affinity and thus is able to bind nitric oxide when the peptide is at a high concentration, such as when stored in the salivary glands. The nitric oxide could then dissociate from the haeme when the concentration of the protein-haeme-nitric oxide complex is lowered upon contact with tissue. Alternatively, a reducing or enzymatic activity of the tissues could lead to the release of the nitric oxide from the haeme. It is also possible that the putative nitrogen groups are bound to a non-haeme group.

Blood feeding evolved independently in several orders of insects and ticks, and also in annelids and mammals. Thus, different genetic backgrounds became the stage for the evolution of pharmacologically active salivary compounds that could help these diverse life-forms to obtain food efficiently. It is perhaps not surprising that prostaglandins, peptides and possibly nitrovasodilators were found as the solution to a common problem (Ribeiro, 1987). There are, however, many as yet unexplored blood-sucking species which may have developed alternative pharmacological mechanisms for feeding success. Thus, it is likely to expect that research in the saliva of blood sucking animals should be productive in yielding novel pharmacologically active substances.

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The effect of *E. coli* STa enterotoxin on the absorption of weakly dissociable drugs from rat proximal jejunum *in vivo*

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1 The effect of *E. coli* heat stable (STa) enterotoxin on the absorption of radio-labelled weak electrolytes and their appearance in peripheral blood was assessed *in vivo* by use of an intestinal recirculation procedure.

2 STa reduced the luminal disappearance ($P < 0.02$) and peripheral blood appearance ($P < 0.02$) of label from salicylic acid as well as the luminal disappearance ($P < 0.02$) of diphenylhydantoin.

3 In contrast, STa increased the appearance in peripheral blood and disappearance from the lumen of label from morphine ($P < 0.05$), amphetamine ($P < 0.01$) and lignocaine ($P < 0.01$).

4 Increased weak base (lignocaine) absorption can also be achieved by a combination of forskolin and theophylline which resembles STa in its ability to neutralise the usually acid surface pH of the proximal jejunum.

5 Increased weak base absorption and hindered weak acid absorption occurs despite a uniform reduction in net fluid absorption after STa exposure, making it unlikely that variations in fluid absorption account for the variations in drug absorption.

6 The ability of STa to elevate the mucosal surface pH (or acid microclimate) to neutral values, thereby altering the proportion of uncharged weak-electrolyte, may explain its different effects on weak acids and bases; neutralisation of the acid microclimate would increase the amount of undissociated weak base available for uptake.

Introduction

Early work on the absorption of weak electrolytes (Schanker *et al.*, 1957; Hogben *et al.*, 1957) indicated that dissociable drugs cross gastric epithelium by diffusion of the undissociated, lipid-soluble form. From these and similar studies on kidney transport, a pH-partition hypothesis emerged (Shore *et al.*, 1957; Milne *et al.*, 1958) whereby absorption was determined by the dissociation constant of a weak electrolyte and the pH of the medium. While in the small intestine, where most drug absorption occurs, this principle seemed valid, benzoic and salicylic acid were absorbed at rates faster than predicted from their dissociation constants (Schanker *et al.*, 1958). At the prevailing intraluminal pH, the dissociated form would predominate, which was difficult to reconcile with an unmodified pH-partition process. For this reason, a zone at the jejunal surface of pH lower than the lumen was proposed, termed an 'acid microclimate', which had a 'virtual' pH which did not correspond with the luminal pH (Hogben *et al.*, 1959). This acid-microclimate can be detected by pH electrode *in vivo* in human (Rawlings *et al.*, 1987) and in rat proximal jejunum (McEwan *et al.*, 1988).

A 'microclimate' hypothesis (Figure 1) can therefore be formulated which predicts that the rate of absorption of weakly dissociable compounds will be determined primarily by the pH at the absorptive surface, rather than the bulk phase pH of the luminal solution or *in vitro* bathing medium. One test of this hypothesis would be to alter the microclimate pH and measure the effect on weak electroyte absorption. Changing luminal pH has little effect on microclimate pH (Lei *et al.*, 1977) and this explains why relatively large changes in luminal pH are required before significant alterations in absorption can be detected. However, a recent study (McEwan *et al.*, 1988) has demonstrated the ability of *E. coli* heat-stable STa enterotoxin to neutralise the acid microclimate in rat small intestine *in vivo*, offering the possibility of using this enterotoxin to test the microclimate hypothesis. In the presence of STa, weak acid absorption should be reduced and weak base absorption should be increased by the pH shift which occurs at the mucosal surface though not in the lumen. The present study investigated the effect of STa enterotoxin on the jejunal absorption of two weakly acidic and three weakly basic drugs.

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Methods

Surgical procedures

Adult male Wistar rats (245–255 g) were anaesthetized by intraperitoneal injection (80 mg kg^{-1} body weight) of pentobarbitone (Sagatal; May & Baker, U.K.). Body temperature was maintained at 37°C by a rectally placed thermistor controlling a heating table assembly. After the trachea had been cannulated, a midline abdominal incision was made and the ligament of Treitz located. A 15 cm length of proximal jejunum was isolated along with its mesenteric vasculature by

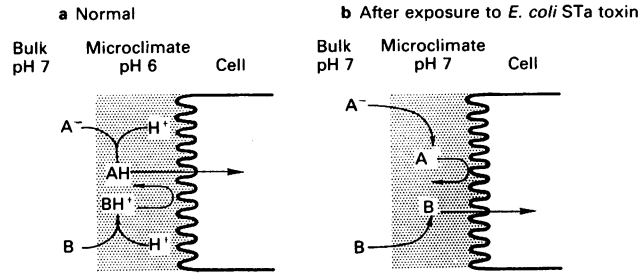


Figure 1 Conceptual model of the influence of the microclimate pH in rat proximal jejunum on weak electrolyte permeation. (a) Weak electrolyte anion (A^-) is converted to neutral, undissociated form (AH) at the microclimate pH and is absorbed well. In contrast, undissociated weak electrolyte base (B) is protonated (BH^+) at the microclimate pH and is less well absorbed. (b) After *E. coli* heat stable enterotoxin (STa) exposure, weak acid anion (A^-) is no longer converted to the neutral form, as the microclimate pH approximates the bulk phase pH and is less well absorbed, whilst undissociated base undergoes less protonation at the mucosal surface. After STa exposure, compared to normal tissue, weak acid absorption is hindered and weak base absorption is enhanced.

ligatures, cannulated at both ends and returned to the peritoneal cavity. The abdominal wall was closed around the cannulae by loose sutures. To obtain peripheral blood samples, the left carotid artery was cannulated and connected via a 3-way tap to a syringe filled with a heparinised 0.9% saline solution.

Perfusion details

The jejunum was perfused by the standard recirculation procedure (Schanker *et al.*, 1958). The cannulae from the intestinal loop were connected by flexible silicon tubing to the perfusing solution reservoir maintained at 37°C. A peristaltic perfusion pump (82334; Crouzet, U.K.) recirculated solution aborally through the intestinal loop at a flow rate of $1\,ml\,min^{-1}$. The perfusing solution was Krebs-bicarbonate buffer (Krebs & Henseleit, 1932) gassed with 95:5% $O_2:CO_2$ (v/v) to maintain Pco_2 levels, additionally containing $5\,mg\,ml^{-1}$ of polyethylene glycol (PEG) 4000 (Sigma, U.K.) with 1 μCi [^{3}H]-PEG 4000 (New England Nuclear, F.R.G.) as a non-absorbable marker for fluid transport. Individual drugs were added to this solution in a concentration of $1\,mmol\,l^{-1}$ with 0.5 μCi ^{14}C-labelled drug as a marker for drug absorption.

Experimental protocol

Before the experiment, 15 ml of the appropriate perfusing solution was added to the reservoir and the pH adjusted to 7.40. Perfusing solution was then pumped through the intestine for 15 min to equilibrate with residual fluid in the loop and to allow for any initial adsorption of PEG 4000 on to the mucosal surface. At the end of this equilibration period the 'zero time' pH was measured and a 50 μl perfusate sample was withdrawn from the reservoir. This and all subsequent samples were dissolved in 10 ml of Ecoscint in polythene vials before the ^{3}H and ^{14}C activity was counted in a Packard Tricarb 2425 liquid scintillation spectrophotometer. Perfusate samples and pH measurements were then taken at 15 min intervals for the duration of the 3 h perfusion. In addition, 100 μl carotid blood samples were withdrawn at 'zero-time' and at subsequent 30 min intervals for the duration of the perfusion. The withdrawn blood was replaced by an equal volume of an isotonic heparinised 0.9% saline solution. Blood samples were prepared for liquid scintillation counting of ^{14}C activity by an oxidation method (Moore, 1981). At the end of the experiment the animals were killed by anaesthetic overdose and the intestinal loop was removed. Apparent hydrogen ion secretion was calculated from back-titration of the perfusate. The tissue was then dried overnight to constant weight in an oven at 100°C. All results were standardised for a tissue dry weight of 100 mg.

Treatment of data and subsequent statistical procedures

Disappearance from the intestinal lumen and appearance in the peripheral blood was calculated by a standard method (Schanker *et al.*, 1958), which assumes that (1) changes in the ^{14}C-activity from the labelled drug in the perfusate reflect the movement of the unlabelled drug and (2) changes in the ^{3}H activity of labelled PEG 4000 represent changes in perfusate volume. Peripheral blood drug concentrations were calculated by assuming that no change in specific activity occurred during absorption. The lines of best fit for perfusate drug amount and volume against time were calculated for each experiment. By definition, these provided the rates of luminal disappearance of drug and of fluid absorption both of which were standardised for a loop dry weight of 100 mg. This treatment of the data provided an empirical analysis in the form of rates of disappearance from the intestinal lumen. In addition, a single exponential process was fitted to the change in luminal concentration over time to derive first order absorption rate constants. Two methods of estimating the rate constant (dimensions of inverse time) and associated standard error were used. The first procedure estimated the rate constant for each experiment from which pool of estimates the mean rate constant was calculated. The second procedure estimated the mean rate constant, by use of all experimental data points, by means of a non-linear statistical software (BMDP) programme (Dixon *et al.*, 1981). Luminal concentration values over time were integrated to provide the area under the curve (AUC lumen) as were blood isotope levels, giving a similar blood AUC.

Where lignocaine was investigated, an analysis of variance was undertaken since STa and forskolin/theophylline treatment were compared to the same control group. The repeated measure, multiple comparison procedure (P1V) of BMDP was used. When the F-test was significant, comparison between mean treatments was by multiple comparison procedures. In all other cases, statistical comparisons between control and test conditions were made by Student's t test.

Drugs

The drugs investigated were: salicylic acid (Sigma, U.K.), phenytoin (gift from May & Baker, U.K.), amphetamine, morphine (gift from Dr W. Wilson, Pharmacology, Glasgow University) and lignocaine (gift from Boots plc, U.K.). [^{14}C]-morphine and [^{14}C]-phentyoin were purchased from Amersham International, U.K.; [^{14}C]-lignocaine and [^{14}C]-salicylic acid from New England Nuclear, F.R.G.; [^{14}C]-amphetamine from Commissariat a l'Energie Atomique, France.

Results

The effect of selected drugs on luminal acidification and fluid transport

The effect of the presence of the selected drug on the STa effect on luminal acidification was investigated. The STa induced reduction in luminal acidification (Table 1) was not affected by the presence of any of the drugs, indicating that none inhibited this aspect of STa action. In contrast, net fluid movement was affected by the presence of drug alone but in all cases, STa abolished net absorption or induced even greater secretion. The effect of STa on drug absorption was therefore always investigated under circumstances of moderate to severe net fluid secretion, a factor which would tend to reduce net drug absorption.

Effect of E. coli *STa toxin on morphine absorption*

When morphine was perfused, there was a very small but significant ($P < 0.01$) absorption of drug from the intestinal lumen. The rate of luminal disappearance (Figure 2a) was 0.4 ± 0.1 (6) $nmol\,min^{-1}\ 100\,mg^{-1}$ tissue dry wt and represented 0.5% absorption of the administered dose, by far the lowest absorption rate measured for any of the drugs. Exposure to STa increased the rate of absorption significantly ($P < 0.01$) to 2.8 ± 0.6 (6) $nmol\,min^{-1}\ 100\,mg^{-1}$ tissue dry wt, which represented more than a seven fold increase in luminal disappearance. The estimated absorption rate constant (Table 2) for toxin experiments of $2.8 \pm 0.6\ (6) \times 10^{-2}\,h^{-1}$, was significantly ($P < 0.01$) higher than the value of $0.3 \pm 0.3\ (6) \times 10^{-2}\,h^{-1}$ estimated from control experiments. The integrated luminal concentration (AUC) was significantly reduced ($P < 0.01$) when compared with the control value, indicating a lower mean concentration after STa exposure. The peripheral blood isotope AUC was also increased (Table 2) but not significantly after STa challenge. However, inspection of the peripheral blood isotope levels for control and toxin experiments (Figure 3a) showed that there was a significant ($P < 0.05$) increase in blood drug concentration in the presence of toxin after 165 min of perfusion.

Table 1 Effect of native *E. coli* STa enterotoxin (56 μg ml^{-1}) and forskolin (0.1 mM)/theophylline (20 mM) on luminal acidification and net fluid absorption in rat proximal jejunum *in vivo* in the presence of the weak electrolytes (1 mM) selected for investigation

Weak electrolyte present (1 mM)	*Acidification* (μequiv min^{-1} 100 mg^{-1} tissue dry wt)		*Net fluid absorption* (μl min^{-1} 100 mg^{-1} tissue dry wt)	
	Control	*Enterotoxin*	*Control*	*Enterotoxin*
Salicylic acid	0.54 ± 0.08 (8)	−0.54 ± 0.12 (7)***	11.0 ± 1.9 (8)	1.4 ± 2.7 (7)**
Phenytoin	0.46 ± 0.09 (7)	−0.15 ± 0.04 (7)***	−9.3 ± 0.9 (7)	−22.0 ± 3.8 (7)***
Amphetamine	0.46 ± 0.05 (6)	−0.04 ± 0.03 (6)***	2.4 ± 0.9 (6)	−25.9 ± 3.2 (6)***
Morphine	0.56 ± 0.05 (6)	−0.01 ± 0.02 (6)***	5.6 ± 6 (6)	−2.8 ± 0.7 (6)***
Lignocaine	0.53 ± 0.07 (9)	−0.12 ± 0.03 (9)***	4.4 ± 0.9 (9)	−8.3 ± 2.1 (9)***
	Control	*Forskolin/ theophylline*	*Control*	*Forskolin/ theophylline*
Lignocaine	0.53 ± 0.07 (9)	−0.08 ± 0.02 (5)***	4.4 ± 0.9 (9)	−23.2 ± 2.9 (5)***

Results are expressed as means ± s.e.mean with number of animals in parentheses. Statistical significance: * $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$.

Effect of E. coli *STa toxin on amphetamine absorption*

The rate of luminal disappearance of amphetamine (Figure 2b) was calculated to be 5.0 ± 0.4 (6) nmol min^{-1} 100 mg^{-1} tissue dry wt. In the presence of STa, luminal disappearance of amphetamine increased significantly ($P < 0.01$) to values of 9.7 ± 1.0 (6) nmol min^{-1} 100 mg^{-1} tissue dry wt. Similarly, the absorption rate constant estimated for the toxin experiments (Table 2) of 8.7 ± 0.7 (7) × 10^{-2} h^{-1}, was significantly ($P < 0.02$) higher than the value of 6.2 ± 0.6 (7) × 10^{-2} h^{-1} estimated for control experiments, when all experiments were combined. Mean luminal drug concentrations after STa treatment were significantly lower than control values after 75 ($P < 0.05$) and 120 ($P < 0.05$) min. Because of the small extent of amphetamine absorption, no discernible changes in the luminal concentration AUC were noted (Table 2).

This increased rate of amphetamine absorption was paralleled by a significant ($P < 0.02$) increase in the blood AUC concentrations after toxin challenge, rising from 35 ± 5 (7) μM . h in control experiments to 51 ± 3 (7) μM . h after exposure to STa. From the blood isotope profile (Figure 3b) it can be seen that the blood levels became significantly ($P < 0.02$) higher in STa experiments after 105 min of perfusion.

Effect of E. coli *STa toxin on salicylic acid absorption*

In control experiments, 8.65 ± 0.92 (8) μmol of drug were absorbed over the three hour perfusion period. This amounted to 58% of the total administered dose and represented (Figure 2c) a mean rate of absorption (luminal disappearance) of 33.0 ± 3.4 (8) nmol min^{-1} 100 mg^{-1} tissue dry wt. With *E. coli* STa enterotoxin in the perfusate, luminal disappearance was significantly ($P < 0.02$) less (Figure 2c), averaging 20.2 ± 3.4 (7) nmol min^{-1} 100 mg^{-1} tissue dry wt. This reduced absorption in the presence of STa was paralleled by a significant ($P < 0.01$) reduction in the blood AUC (Table 2) from control values of 540 ± 53 (5) to 326 ± 44 (6) μM . h after enterotoxin challenge. Levels of drug isotope in the peripheral blood became significantly ($P < 0.02$) lower than control values after 105 min of perfusion (Figure 3c) and remained so for the remainder of the perfusion period.

Effect of E. coli *STa toxin on phenytoin absorption*

The rate of luminal disappearance of drug in control loops was 21.5 ± 2.7 (7) nmol min^{-1} mg^{-1} tissue dry wt. Thus approximately 26% of the initial 15 μmol dose was absorbed over the three hour perfusion period. Challenge of the jejunal mucosa with STa reduced phenytoin absorption (Figure 2d) to values of 16.3 ± 1.0 (7) nmol min^{-1} mg^{-1} tissue dry wt, which was not significantly different from the control rate. However, the absorption rate constant of 18.5 ± 3.1 (7) × 10^{-2} h^{-1} after STa challenge (Table 2) was significantly ($P < 0.02$) lower than the control value of 32.3 ± 3.5 (7) × 10^{-2} h^{-1}. Similarly, the integrated luminal concentration (AUC) also demonstrated that significantly more ($P < 0.01$) phenytoin was left in the luminal solution after enterotoxin challenge (Table 2). A reduction in the peripheral blood drug AUC observed after STa challenge just failed to be significant. Inspection of the mean blood drug concentrations at each sample time (Figure 2d) shows a significant ($P < 0.05$) difference between control and toxin treatment after 45 min of perfusion.

Effect of E. coli *STa toxin on lignocaine absorption*

In control experiments, lignocaine (Figure 4a) was absorbed at a rate of 16.7 ± 1.7 (9) nmol min^{-1} 100 mg^{-1} tissue dry wt, with 20% of the initial dose disappearing from the lumen over

Table 2 First order rate constants (k) for luminal disappearance, integrated luminal and blood concentration (AUC^{l}_{0}) of weak electrolyte absorbed *in situ* from rat proximal jejunum in the presence and absence of *E. coli* (STa) enterotoxin

Weak electrolyte		10^2 k (h^{-1})[a]	10^2 k (h^{-1})[b]	*AUC (lumen)* (mM . h)	*AUC (blood)* (μM . h)
Salicylic acid	Control (8)	28.7 ± 6.7	24.8 ± 2.6	7.52 ± 0.47	540 ± 53 (5)
	STa (6)	20.4 ± 3.6	18.0 ± 2.2	8.18 ± 0.41	326 ± 44 (6)***
Phenytoin	Control (7)	32.3 ± 3.5	31.4 ± 1.5	8.54 ± 0.36	108 ± 14 (7)
	STa (7)	18.5 ± 3.1**	17.9 ± 1.3***	10.20 ± 0.39***	82 ± 6 (7)
Amphetamine	Control (7)	6.3 ± 0.7	6.2 ± 0.6	11.97 ± 0.19	35 ± 5 (7)
	STa (7)	8.8 ± 1.4	8.7 ± 0.7**	11.53 ± 0.22	51 ± 3 (7)**
Morphine	Control (5)	0.4 ± 0.1	0.3 ± 0.3	13.34 ± 0.11	37 ± 2 (5)
	STa (6)	2.7 ± 0.4***	2.8 ± 0.6***	12.46 ± 0.21***	48 ± 5 (6)
Lignocaine	Control (11)	18.0 ± 1.2	17.4 ± 0.9	9.72 ± 0.27	53 ± 6 (8)
	STa (10)	35.1 ± 5.0***	32.1 ± 2.1***	7.90 ± 0.48***	85 ± 11 (10)***
	F/T (5)	22.3 ± 0.3***	22.2 ± 1.4**	10.0 ± 0.25	87 ± 11 (5)**

[a] Rate constants calculated from each experiment and averaged.
[b] Rate constant calculated from pooled experiments. Results are expressed as mean ± s.e.mean. * $P < 0.05$. ** $P < 0.02$. *** $P < 0.01$.
F/T = effect of forskolin/theophylline.

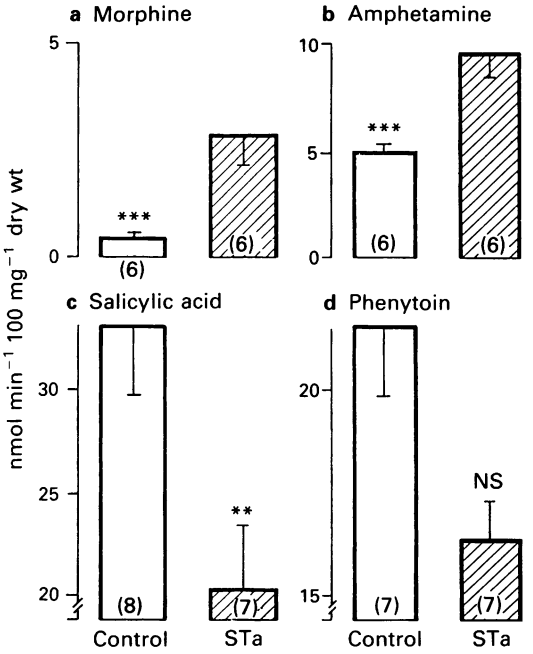


Figure 2 The effect of 56 μg ml^{-1} *E. coli* heat stable (STa) enterotoxin (hatched columns) on the rate of luminal disappearance of 1 mM ^{14}C-labelled (a) morphine (spec. act. 56 mCi mmol^{-1}), (b) amphetamine (spec. act. 49 mCi mmol^{-1}), (c) salicylic acid (spec. act. 52 mCi mmol^{-1}) and (d) phenytoin (spec. act. 58 mCi mmol^{-1}), expressed as nmol min^{-1} 100 mg^{-1} tissue dry weight from 15 cm loops of rat proximal jejunum *in vivo*. Results are expressed as means with bars representing s.e.mean; the number of animals (equals the number of experiments) is given in parentheses. Statistical significance, ** $P < 0.02$; *** $P < 0.01$. NS, not significant.

the three hour perfusion period. Exposing the jejunal mucosa to STa significantly ($P < 0.01$) increased lignocaine absorption to values of 25.4 ± 2.0 (9) nmol min^{-1} 100 mg^{-1} tissue dry wt. This finding was confirmed by non-linear regression, as the absorption rate constant (Table 2) of 35.1 ± 5.0 (10) × 10^{-2} h^{-1} for the toxin experiments was significantly ($P < 0.001$) greater than the control value of 18.0 ± 1.2 (11) × 10^{-2} h^{-1}. This increased absorption was mirrored by a significant ($P < 0.01$) increase in the peripheral blood isotope AUC from values of 53 ± 6 (8) μM . h in con-

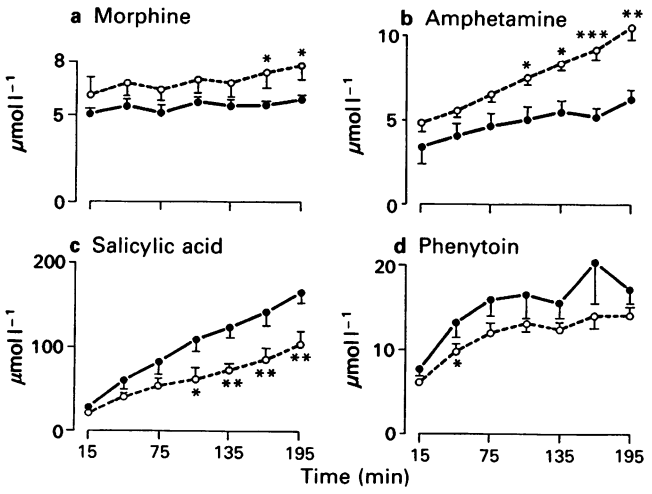
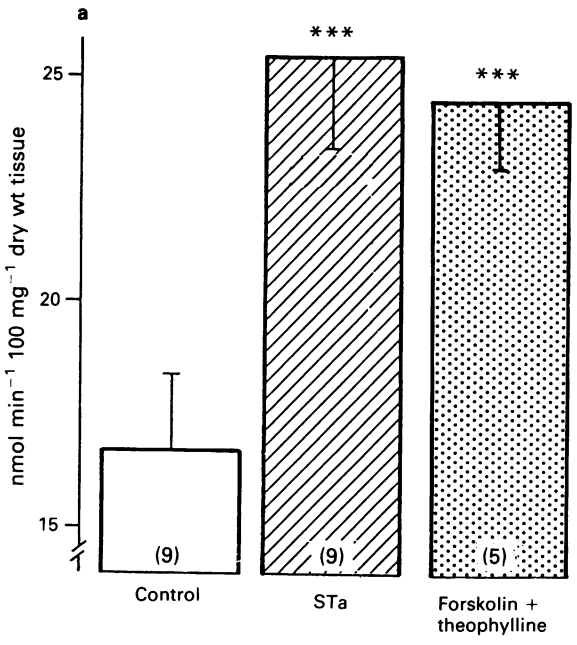


Figure 3 The appearance of (a) morphine, (b) amphetamine, (c) salicylic acid and (d) phenytoin in carotid artery blood samples (^{14}C d.p.m. expressed as micromolar equivalents) with time in control (●) and *E. coli* heat stable enterotoxin (○) experiments. Results are expressed as means and vertical lines show s.e.mean. Statistical significance, * $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$.



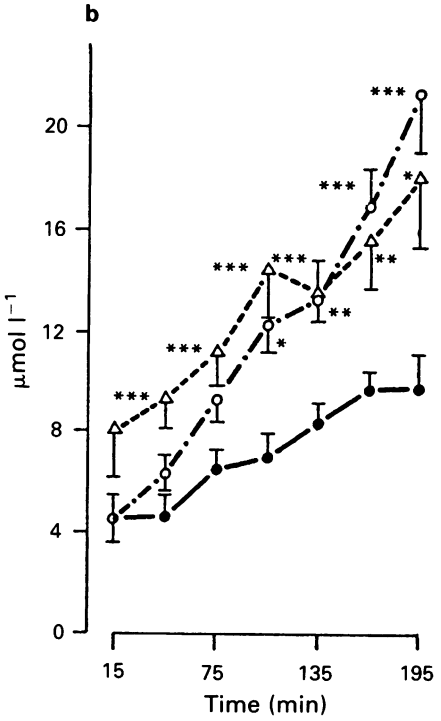


Figure 4 The effect of 56 μg ml^{-1} of *E. coli* heat stable enterotoxin (STa) and 1 mM forskolin/20 mM theophylline on (a) the rate of luminal disappearance of 1 mM ^{14}C-labelled lignocaine (spec. act. 52 mCi mmol^{-1}) expressed as nanomol min^{-1} 100 mg^{-1} tissue dry weight from 15 cm loops of rat proximal jejunum *in vivo* and (b) the appearance of label in carotid artery blood samples (^{14}C d.p.m. expressed as micromolar equivalents) over the duration of the experiment in control (●), STa (○) and forskolin/theophylline (△) experiments. Results are expressed as means and vertical lines show s.e.mean; the number of animals (equals the number of experiments) is indicated in parentheses. Statistical significance, * $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$, by analysis of variance

trols to 85 ± 11 (10) μM . h in the presence of STa. From the peripheral blood profiles (Figure 4b), it can be seen that the blood concentrations in the presence of STa became significantly ($P < 0.01$) elevated over control values after 45 min and remained higher ($P < 0.001$) throughout the remainder of the experiment.

Effect of forskolin in combination with theophylline on lignocaine absorption

A combination of forskolin and theophylline elevates the jejunal mucosal surface pH in a manner very similar to that observed after STa challenge (McEwan *et al.*, 1988). In the presence of forskolin ($1\,mmol\,l^{-1}$) and theophylline ($20\,mmol\,l^{-1}$), lignocaine absorption (Figure 4a) increased from 16.7 ± 1.7 (9) $nmol\,min^{-1}\ 100\,mg^{-1}$ tissue dry wt in control experiments to 24.4 ± 1.3 (5) $nmol\,min^{-1}\ 100\,mg^{-1}$ dry wt, which was significantly higher ($P < 0.01$) than control values but not significantly different from the rate after *E. coli* STa exposure. Confirming the empirical analysis, the absorption rate constant of 22.3 ± 0.3 (5) $\times 10^{-2}\,h^{-1}$ from the forskolin/theophylline experiments, was significantly ($P < 0.01$) higher than the control value of 18.0 ± 1.2 (11) $\times 10^{-2}\,h^{-1}$. This significant but smaller increase than that seen with STa also meant that the area under the luminal concentration curve was not significantly different, unlike that with STa (Table 2). Increased luminal drug absorption was paralleled by an increased ($P < 0.01$) peripheral blood isotope AUC of 87 ± 11 (5) μM . h, similar to that found with STa. These elevated blood isotope concentrations (Figure 4b) in the presence of forskolin and theophylline became significant ($P < 0.05$) after 75 min and remained higher for the duration of the experiments.

Physicochemical characteristics and absorption parameters of the various weak electrolytes

For ease of reference, various physicochemical parameters e.g. partition coefficient are presented for the weak-electrolytes (Table 3), together with other calculated values. The partition coefficients (b) for the undissociated form between water and octanol were calculated from published data (Ritschel & Hammer, 1980) after correction for dissociation. The amount of undissociated weak electrolyte [ni] in a 1 mM solution at pH 6.2, the mean mucosal surface pH (McEwan *et al.*, 1988), as well as at pH 7.0, the mean surface pH after STa exposure was calculated, as was the ratios of STa to normal undissociated amounts. Similarly, the ration of mean luminal absorption after STa treatment to the control values were calculated, for comparison with the ionisation changes.

Discussion

As proposed by Hogben and co-workers (1959), the 'microclimate' hypothesis predicts that the intestinal surface pH controls the rate of weak electrolyte absorption by determining the proportion of unionised species at the site of absorption. Previous attempts at investigating the hypothesis assumed that changing luminal pH would result in similar change at the enterocyte surface (Nogami & Matsuzawa, 1961; 1962; Crouthamel *et al.*, 1971; Bridges *et al.*, 1976; Hollander *et al.*, 1981; Prieto *et al.*, 1987). As luminal pH has little influence on the microclimate pH (Lei *et al.*, 1977; Hoegerle & Winne, 1983; Shiau *et al.*, 1985; Iwatsubo *et al.*, 1986), relatively large changes in luminal pH are necessary before alterations in absorption can be detected. This difficulty was overcome by using *E. coli* STa enterotoxin which neutralises the acid surface pH of rat jejunum *in vivo* (McEwan *et al.*, 1988). According to the microclimate hypothesis, alkalinisation of the mucosal surface should reduce weak acid absorption but more importantly should also enhance weak base uptake (Figure 1). With both STa and with the combination of forskolin and theophylline, the jejunal mucosal surface pH of 6.2 can be elevated to pH 7.0. Consequently, these values have been used to calculate the expected change in undissociated form of weak electrolyte after STa exposure.

Two weakly acidic drugs were selected for study, salicylic acid with dissociable carboxylic group of low pKa (3.0) and phenytoin with a phenol group (after keto-enol tautomerism) of high pKa (8.3). The presence of STa impaired the luminal disappearance of both weak acids. Similarly reduced levels of isotope in the peripheral blood were consistent with a decreased total transfer rather than decreased cellular uptake. The apparent change in undissociated form can be calculated (Table 3) to be greater for salicylic acid than for phenytoin, although both weak electrolytes have comparable lipid solubility. While in accordance with the microclimate concept, reduced weak acid uptake can also be attributed to the marked reduction in fluid absorption after STa challenge. Salicylic acid absorption is known to be altered by osmotic loads which induce fluid movement through the paracellular pathway (Ochsenfahrt & Winne, 1974; Karino *et al.*, 1982a,b). However, in the presence of phenytoin the magnitude of STa-induced fluid secretion was very large. If fluid entrainment made an important contribution to the total absorption of these weak acids, a pronounced reduction in absorption would be expected. Given that the reduction in phenytoin absorption is associated with an extremely small change in the concentration of the undissociated form, (Table 3) it seems more likely that in this case reduced fluid absorption caused the reduced weak electrolyte absorption. For salicyclic acid, both the change in microclimate pH and the reduction in fluid absorption could have contributed to the reduced absorption.

While the results of experiments with weak acids are consistent both with the microclimate hypothesis and with reduced fluid entrainment, the elevation of surface pH by STa should increase weak base absorption, provided that the unfavourable changes in net fluid transport have little effect on net weak electrolyte absorption. For this reason, the jejunal absorption of amphetamine, morphine and lignocaine was investigated. Amphetamine was selected as a small but relatively strong weak base (pKa 9.9) and lignocaine as a similarly small and lipophilic molecule of weaker dissociation constant (pKa 7.9). Morphine was chosen as a larger more hydrophobic weak base with dissociation constant (pKa 8.05) similar to lignocaine. After exposure to STa, the luminal absorption rate of the three weak bases was significantly enhanced. Increased drug uptake was mirrored by similar increases in the peripheral blood radioactivity. Label in the peripheral blood includes metabolites from intestinal and liver metabolism, as

Table 3 Dissociation constant (a), log octanol: water partition coefficient of undissociated form of weak electrolyte (b), concentration of weak electrolyte [ni] at normal mucosal surface pH of 6.2 (c), concentration of weak electrolyte at surface pH of 7.0 after STa treatment (d), concentration-ratio (d/c) of undissociated weak electrolyte (e) for STa and control mucosal surface pH, mean absorption rate of luminal weak electrolyte disappearance after STa treatment relative to rate for untreated jejunum (f)

Weak electrolyte	(a) *pKa*	(b) *log P*	(c) *[ni] at pH 6.2* ($\mu mol\,l^{-1}$)	(d) *[ni] at pH 7.0* ($\mu mol\,l^{-1}$)	(e) *Ratio* [ni]	(f) *Ratio absorption*
Salicylic acid	3.0	2.87	0.63	0.13	0.21	0.61
Phenytoin	8.3	2.52	992	962	0.97	0.57
Amphetamine	9.9	1.79	0.20	1.00	5.0	1.94
Lignocaine	7.9	2.27	19.6	91.0	4.64	1.52
Morphine	8.05	0.89	13.9	66.1	4.75	7.0

well as the intact weak-electrolyte, and this complicates the interpretation. The concentrations of the drugs presented to the mucosa were relatively high such that intestinal metabolism was likely to account for a small percentage of the total peripheral blood label. Liver metabolism is substantial but this is not the site of action of STa enterotoxin (Guerrant *et al.*, 1980). Changes in peripheral blood radioactivity of all weak-electrolytes always paralleled the change in luminal disappearance, consistent with the view that STa affected total drug transfer. It is also unlikely that the changes in the level of blood radioactivity were due to altered renal clearance, since STa has no effect on the kidney (Guerrant *et al.*, 1980; Rao *et al.*, 1980). In all cases, increased absorption occurred despite considerable net fluid secretion, indicating that mucosal surface pH was likely to have been a major determinant of the rate of drug absorption.

The observed effects on drug absorption may have been peculiar to the STa-treated jejunum. To investigate this, lignocaine absorption was measured after exposure to a combination of forskolin and theophylline, which causes an elevation of the jejunal mucosal surface virtually identical in time course and magnitude to the alkalinisation caused by STa (McEwan *et al.*, 1988). Forskolin and theophylline enhanced lignocaine absorption to the levels observed after STa-challenge. The increased blood radioactivity was similarly not significantly different from that obtained with STa-treated animals. These observations support the view that mucosal surface pH changes were the cause of the promoted lignocaine absorption. In contrast, the forskolin/theophylline combination induced a fluid secretion almost three times greater than that induced by STa, indicating that surface pH is a much more important determinant of permeation than the direction or extent of fluid movement.

According to the Henderson-Hasselbalch equation, the measured elevation of mucosal surface pH after STa-challenge could cause a four to five fold change in the ratio of ionised to unionised weak electrolyte at the mucosal surface (Table 3). With the exception of morphine, the changes in drug absorption were considerably less than this. This is not unexpected since the unstirred layer should prevent the microclimate from exerting its full effect. Surface pH changes alter the extent to which the amount of weak-electrolyte can partition into the lipid membrane after diffusion through the unstirred layer. The extent to which surface pH change will modify total permeation will depend on the relative contributions of membrane and unstirred layer to total permeability. If diffusion across the unstirred layer is rate-limiting, then enhanced membrane permeability will barely enhance the total permeation. The degree of change in absorption rate therefore cannot exceed and will probably be less than the change expected from pH calculations.

It has been proposed (Thomson & Dietschy, 1984) that the unstirred water layer can account for all deviations of weak electrolyte absorption from the pH partition hypothesis. Reduced weak acid uptake could be explained by a thicker unstirred layer in the presence of enterotoxin. However, the same enterotoxin would have to thin the unstirred layer to enhance weak base absorption. It seems unlikely that STa would have specific effects on unstirred layer thickness depending on whether the absorbed molecule is a weak acid or weak base. Another possibility is that STa in some way alters the permeability of the brush border membrane to the ionised species, if ionised permeation can occur as has been proposed. While increased cation permeability might explain the observed effects of STa on weak base absorption, there would also need to be reduced anion permeability to explain the reduced weak acid uptake. These assumptions seem unnecessarily complex when the measured mucosal surface pH change can adequately account for the altered permeation of both weak acids and weak bases.

There are few studies on the influence of surface pH on weak electrolyte absorption and none on the ability of bacterial toxins to influence surface pH and hence alter weak electrolyte absorption. However, earlier work did link the ability to acidify the lumen with the extent of drug absorption. Increased amphetamine and decreased salicylic acid absorption was detected in the duodenum and ileum after acetazolamide treatment (Schnell & Miya, 1970), which reduced luminal acidification in the duodenum and enhanced ileal alkalinisation. The present studies indicate that the absorption of both drugs is influenced by the mucosal surface pH. In addition, nicotine and salicylic acid absorption corresponds closely to that expected from pH-partition *in vitro* (Elbert *et al.*, 1986) and *in vivo* (Hoegerle & Winne, 1983), when allowance is made for the extent of dissociation occurring at the pH of the surface. It is premature to conclude that the rate of absorption of all weak-electrolytes is determined by the microclimate pH or that a change in this will discernibly alter their absorption rates – relatively few weak electrolytes have been studied. Water-soluble weak-electrolyte dissociation will be determined by changes in the microclimate pH, but this may be insufficient to alter the absorption rate if the latter is limited by diffusion across the unstirred layer. However, it seems probable that the microclimate should be relevant for moderately lipophilic substances with a dissociation constant reasonably close to the microclimate pH. In view of the phenytoin experiments, the pKa of a weak electrolyte should not have 'crossed over' the microclimate pH such that change in the latter has only a minor effect on the percentage of undissociated form. One might anticipate that STa treatment should not affect the absorption of bases of pKa less than 5 or weak acids of pKa greater than 8.0. However, this point needs to await further investigation. The results of this study are consistent with the microclimate hypothesis and support the view that the predominating pH at the intestinal mucosal surface determines the rate of absorption of weakly dissociable compounds from the small intestine.

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A series of novel, highly potent and selective agonists for the κ-opioid receptor

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1 This paper describes the opioid receptor pharmacology and *in vivo* activity of several novel benzeneacetamidopiperidine and benzeneacetamidopiperazine analogues.

2 These compounds all showed potent, naloxone-reversible, full agonist activiy in the field-stimulated rabbit vas deferens, indicating that they are κ-opioid agonists; but showed very little activity in the rat or hamster vas deferens, indicating good selectivity with regard to μ- and δ-opioid receptors.

3 They were all potent antinociceptive agents, the most potent compound, GR103545, having an ED_{50} value in the mouse abdominal constriction test of 0.25 μg kg^{-1} s.c. The compounds also produced sedation and diuresis, but had little effect on respiration rate or gastrointestinal motility.

4 It is concluded that the seven novel compounds described are all potent and selective agonists for the κ-opioid receptor.

Introduction

There is considerable evidence to support the existence of three opioid receptor types – μ, κ and δ (Martin *et al.*, 1976; Lord *et al.*, 1977). It has been suggested that agonists selective for the κ-receptor will produce good analgesic activity, without many of the side-effects associated with the use of μ-receptor agonists, particularly physical dependence, constipation and respiratory depression (Cowan & Gmerek, 1986). Thus, considerable effort has been expended in attempting to discover novel agents with selectivity for this receptor subtype.

The greatest degree of κ-agonist selectivity has been achieved in compounds belonging to the 2-benzeneacetamidocyclohexylamine class, including U-50,488H (Von Voigtlander *et al.*, 1983), U-62,066E (Lahti *et al.*, 1985) and PD 117,302 (Leighton *et al.*, 1987) and more recently in 2-benzeneacetamidoalkylamines (Costello *et al.*, 1988).

This paper describes the *in vitro* and *in vivo* pharmacology of several novel benzeneacetamidopiperidine and benzeneacetamidopiperazine analogues with κ-opioid agonist activity, some of whose potencies are considerably greater than existing compounds. The structures of these analogues are given in Figure 1.

Methods

Isolated tissue experiments

Vasa deferentia from PVG Hooded rats (150–250 g), California rabbits (2.5–3.5 kg), Golden Syrian hamsters (250–300 g) and CRH mice (50–75 g) were removed and suspended between platinum stimulating electrodes, in 5 ml organ baths containing Krebs-Henseleit solution of the following composition (mM): NaCl 118, $NaHCO_3$ 29, glucose 11.1, KCl 4.7, $CaCl_2$ 1.25 and KH_2PO_4 1.2, gassed with 95% O_2/5% CO_2 and maintained at 37°C. Contractions of mouse vas deferens and hamster vas deferens were evoked by field stimulation with trains of 3 rectangular pulses of 0.5 ms duration, 200 ms apart, at a frequency of 0.1 Hz and 1 V above maximal voltage (Hayes *et al.*, 1985a; Sheehan *et al.*, 1986). Contractions of rat vas deferens and rabbit vas deferens were evoked by a single pulse of width, frequency and voltage as above (Hayes & Kelly, 1985; Sheehan *et al.*, 1988). Resting tensions were 0.2 g (mouse and hamster), 0.5 g (rabbit) and 0.75 g (rat).

Cumulative concentration-response curves were constructed for agonists, from which the IC_{50} concentration for 50% inhibition of twitch height was determined. Tissues were always initially checked for viability with a standard agonist (ethylketocyclazocine in rabbit vas deferens, [D-Ala^2, D-Leu^5] enkephalin (DADLE) in hamster vas deferens, [D-Ala^2, N-MePhe^4, Gly(ol)^5] enkephalin (DAMGO) in rat vas deferens,

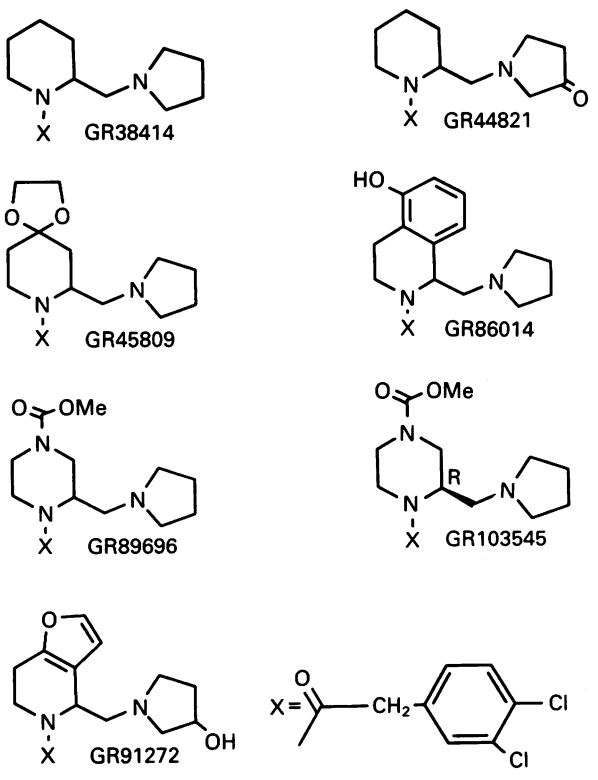

Figure 1 Chemical structures of GR38414, GR44821, GR45809, GR86014, GR89696, GR103545 and GR91272.

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and DAMGO and U69593 in mouse vas deferens). In antagonist experiments, the tissues were incubated for 30 min with antagonists before the agonist concentration-response curve was re-determined. In the experiments with GR89696 and norbinaltorphimine, several antagonist concentrations were used in each tissue and the resulting dose-ratios were used to obtain a pA_2 value and Schild slope (Arunlakshana & Schild, 1959). Values from different tissues were then averaged together. In all the other antagonist experiments only a single antagonist concentration was used and a pK_B value was calculated from the equation: $pK_B = \log(\text{dose-ratio} - 1) - \log[\text{antagonist}]$.

In vivo *experiments*

Mouse experiments Male CRH mice (17–25 g) were used. Agonist drugs were administered subcutaneously (s.c.) in a dose volume of 10 ml kg^{-1} or orally in a dose volume of 20 ml kg^{-1} and 30 and 60 min respectively before testing. Naloxone was administered s.c. at 20 min before testing. Antinociception was measured by the acetylcholine-induced abdominal constriction test in the mouse (details of which are given in Hayes *et al.*, 1987a). Potential side-effects were assessed by studying drug effects in the rotarod test, on respiration rate and on gastrointestinal propulsion, all in the mouse (details of the measurement of these parameters are given in Hayes & Tyers, 1983).

Individual tests were carried out with dose groups of 6 mice. Either data from one individual experiment were used, or data were combined from 2 experiments i.e. $n = 12$. Each dose was randomised between cages and animals and drug solutions were colour coded such that the operators were unaware of which treatment the animals were receiving. The methods of Finney (1978) were used to determine regression slopes, linearity and parallelism for dose-response curves and also antinociceptive activities (ED_{50} values) and potency ratios, with 95% confidence limits.

Urine output of the rat Male PVG rats (125–200 g) were used. They were deprived of food overnight on the eve of an experiment and were water-loaded (25 ml kg^{-1}) 10 min before s.c. administration of the opioid agonist and/or antagonist, which were given in a dose volume of 4 ml kg^{-1}. On injection, rats were placed in individual stainless steel metabolism cages and voided urine was collected in glass separators. Cumulative urine volumes were recorded hourly for up to 6 h; during this test period rats were deprived of food and water.

Drugs

Doses of salts refer to the weight of parent compound. The following drugs were used – GR38414 (1-[(3,4-dichlorophenyl) acetyl]-2-(1-pyrrolidinylmethyl) piperidine fumarate), GR89696 (methyl 4-[(3,4-dichlorophenyl)acetyl]-3-(1-pyrrolidinylmethyl)-1-piperazinecarboxylate fumarate), GR103545 ((R)-methyl 4-[(3,4-dichlorophenyl)acetyl]-3-(1-pyrrolidinylmethyl)-1-piperazinecarboxylate fumarate), GR45809 (8-[(3,4-dichlorophenyl)acetyl]-7-(1-pyrrolidinylmethyl)-1,4-dioxa-8-aza[4.5]spirodecane hydrochloride), GR91272 (5-[(3,4-dichlorophenyl)acetyl]-4,5,6,7-tetrahydro-4-[(3-hydroxy-1-pyrrolidinyl)methyl]furo[3,2-c]pyridine hydrochloride), GR44821 (1-[(3,4-dichlorophenyl)acetyl]-2-[(3-oxo-1-pyrrolidinyl)methyl]piperidine maleate) and GR86014 (2-[(3,4-dichlorophenyl)acetyl]-1,2,3,4-tetrahydro-1-(1-pyrrolidinylmethyl)-5-isoquinolinol maleate); all these compounds were synthesised in the Medicinal Chemistry Department, Glaxo Group Research Ltd., Ware. Ethylketocyclazocine (EKC) methane sulphonate (Sterling-Winthrop), morphine hydrochloride (Macfarlan-Smith), fentanyl citrate (Janssen), [D-Ala^2, D-Leu^5] enkephalin (DADLE, Bachem), [D-Ala^2, N-MePhe^4, Gly(ol)^5] enkephalin (DAMGO, Bachem), naloxone hydrochloride (Sterling-Winthrop), naltrexone hydrochloride (Endo), 16-methyl cyprenorphine (M8008, synthesized in the Medicinal Chemistry Department, Glaxo Group Research Ltd., Ware), U69593 (5α,7α,8β-(−)-N-methyl-N-(7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl)benzeneacetamide) (Upjohn), norbinaltorphimine (norBNI, synthesized in the Medicinal Chemistry Department, Glaxo Group Research Ltd., Ware).

Results

Isolated tissue experiments

The data from the isolated tissue experiments are shown in Table 1. All of the compounds were extremely potent, full

Table 1 Effects of novel κ-agonists in the field-stimulated rabbit vas deferens, rat vas deferens and hamster vas deferens *in vitro*

	Potency in isolated tissue (mean IC_{50} ± s.e.mean) in nM		
Compound	*Rabbit vas deferens*	*Rat vas deferens*	*Hamster vas deferens*
GR38414	10.6 ± 2.3 (6)	NT	NT
GR44821	0.2 ± 0.1 (3)	NSE at concentrations up to 10 μM (2)	
GR45809	0.1 ± 0.06 (3)	No agonist effect at concentrations up to 10 μM	
		pK_B v DAMGO = 5.4 (2)	pK_B v DADLE = 5.2 (2)
GR86014	0.6 ± 0.4 (4)	4,400 / 10,000 (2)	4,200 / 12,000 (2)
GR89696	0.09 ± 0.03 (4)	No agonist effect up to 10 μM	No agonist effect up to 30 μM
		pK_B v DAMGO = 5.6 (2)	pK_B v DADLE = 6.1 (2)
GR103545	0.02 / 0.02 (2)	No agonist effect up to 10 μM	1,900 / 160 (2)
		pK_B v DAMGO = 6.4 (2)	
GR91272	2.7 ± 2.3 (3)	NSE at concentrations up to 10 μM (2)	
U69593	300 / 540 (2)	No agonist effect up to 10 μM	NSE at concentrations up to 10 μM
		pK_B v DAMGO = 5.2 (4)	

NT = not tested, NSE = no agonist effect and no antagonism of [D-Ala^2, N-MePhe^4, Gly(ol)^5]enkephalin (DAMGO) (RVD) or [D-Ala^2, D-Leu^5]enkephalin (DADLE) (HVD). *n* values are given in parentheses.

Table 2 *In vivo* activity of novel κ-agonists in comparison with U69593

Compound	*ED_{50} in abdominal constriction (95% confidence limits)* ($mg\,kg^{-1}$)	*Rotarod ED_{50} (95% confidence limits)* ($mg\,kg^{-1}$)	*Depression of respiration rate* ($mg\,kg^{-1}$)	*Inhibition of gut propulsion* ($mg\,kg^{-1}$)	*Effect on urine output* ($mg\,kg^{-1}$)
GR38414	0.03 s.c. (0.02–0.05) 1.2 p.o. (0.8–1.6)	2.7 s.c. (0.3–17)	Shallow DRC at 0.1–19 s.c. E_{max} = 28%	Shallow DRC at 0.1–10 s.c. E_{max} = 46%	Diuresis at 0.08–6 s.c.
GR44821	0.06 s.c. (0.03–0.09) 1.9 p.o. (1.3–3)	0.3 s.c. (0.002–1)	Shallow DRC at 0.3–3 s.c. E_{max} = 18%	Shallow DRC at 0.3–3 s.c. E_{max} = 46%	Diuresis at 0.01–2 s.c.
GR45809	0.001 s.c. (0.0003–0.002)	NT	NT	NT	NT
GR86014	0.003 s.c. (0.001–0.004)	NT	NT	NT	NT
GR89696	0.0005 s.c. (0.0003–0.0009) 0.04 p.o. (0.02–0.12)	0.052 s.c. (0.04–0.008)	Shallow DRC at 0.0004–0.1 s.c. E_{max} = 16%	Shallow DRC at 0.0004–0.1 s.c. E_{max} = 46%	Diuresis at 0.008–0.12 s.c.
GR103545	0.00025 s.c. (0.00012–0.00045) 0.012 p.o. (0.005–0.035)	0.022 s.c. (0.017–0.028)	NSE at doses up to 1 s.c.	Shallow DRC at 0.004–0.25 s.c. E_{max} = 53%	Diuresis at 0.001–0.03 s.c.
GR91272	0.002 s.c. (0.001–0.004)	0.05 s.c. (0.012–0.099)	NT	NT	Diuresis at 0.08–1.2 s.c.
U69593	0.14 s.c. (0.09–0.22)	1.2 s.c. (0.8–1.8)	NSE at doses up to 6 s.c.	Shallow DRC at 0.2–6 s.c. E_{max} = 28%	Diuresis at 2.5 and 10 s.c.

DRC = dose-response curve; NT = not tested; For all values $n > 6$.

agonists for inhibiting twitch responses in the rabbit vas deferens. The effects of (R,S)-GR89696 and its (R)-enantiomer, GR103545, in this tissue were antagonized by naloxone with mean pK_B values of 7.7 ± 0.04 ($n = 4$) and 7.5 ($n = 2$) respectively, indicating that these effects are indeed mediated via κ-receptors. None of the compounds showed any effects at μ-receptors in the rat vas deferens or at δ-receptors in the hamster vas deferens at concentrations less than 1 μM, indicating that they are all very selective κ-agonists. Furthermore, GR89696 inhibited twitch responses of the mouse vas deferens with an IC_{50} of 0.12 ± 0.03 nM ($n = 8$), an effect which was antagonized by the κ-antagonist, norBNI, with a mean pA_2 value of 9.92 (slope = 0.95, $n = 2$); the mean pK_B for antagonism of U69593 by norBNI was 10.4 ($n = 2$) and for antagonism of DAMGO was 7.8 ($n = 2$).

In vivo *experiments*

The *in vivo* activities of the seven novel κ-opioid agonists are shown in Table 2. Their profiles of activity in the whole animal are very similar to that of the known κ-agonist U69593. Thus, they produced very potent antinociceptive activity in the mouse abdominal constriction test, with GR103545 being the most potent (ED_{50} = 0.25 $\mu g\,kg^{-1}$ s.c.). The antinociceptive effect of GR38414 in the mouse was antagonized by naloxone (Table 3), at doses very similar to those necessary to antagonize the effect of the κ-agonist U69593 and considerably higher than those needed to antagonize the effects of the μ-agonist morphine. All the compounds produced marked sedative effects, as noted by visual observation and quantified with the mouse rotarod test. They produced very little depression of respiration rate or inhibition of gut propulsion in the mouse, any effects seen being of low maximum amplitude. The compounds which were tested for effects on urine output in the rat all produced a marked diuresis. The diuretic effect at higher doses was often preceded by an initial antidiuretic effect, a property that was also shared by U69593 (Figure 2). In an attempt to investigate the mechanism of this antidiuretic effect, its reversal by the opioid antagonists, naltrexone and M8008, was studied. Naltrexone, 5 $mg\,kg^{-1}$ s.c., reduced both the initial decrease and the subsequent increase in urine output produced by U69593, indicating that both are mediated via opioid receptors. M8008 produced a small diuretic effect on its own, but did not antagonize the antidiuretic effects of U69593 or GR89696 at a

Table 3 Naloxone reversal of the antinociceptive effects of morphine, U69593 and GR38414 in the mouse abdominal constriction test

Naloxone dose ($mg\,kg^{-1}$ s.c.)	*Potency ratio for naloxone antagonism (95% confidence limits)* *Morphine*	*U69593*	*GR38414*
6	—	40.2 (24.1–29.3)	56.8 (35.3–92.9)
2	—	17.8 (10.3–29.6)	32.0 (19.0–54.8)
0.6	93.6 (38.6–232)	6.8 (4.0–11.3)	17.3 (10.7–28.1)
0.2	28.8 (11.8–71)	—	—
0.07	10.4 (4.3–25.8)	—	—

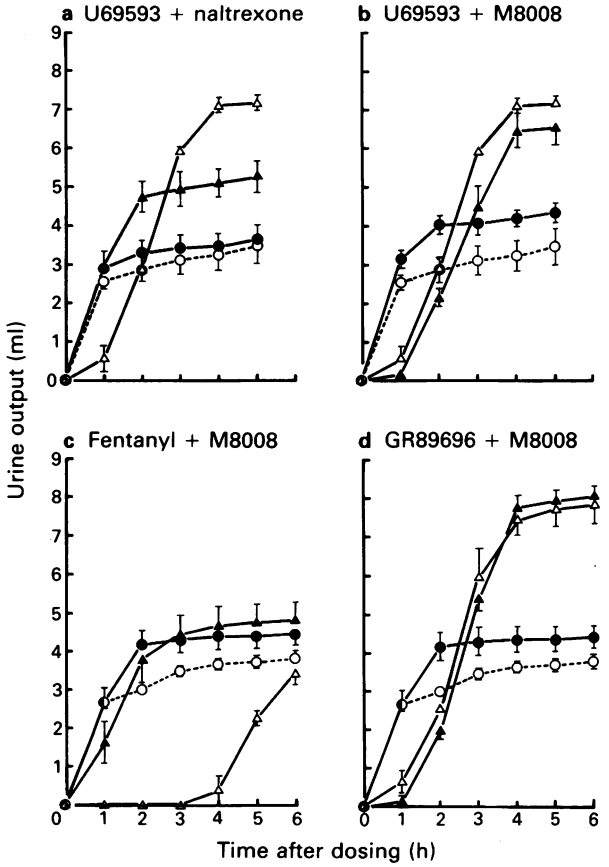

Figure 2 Reversal of the effects of (a and b) U69593, (c) fentanyl and (d) GR89696 on urine output in the rat by the opioid antagonists, naltrexone and M8008. U69593 was given at $10\,mg\,kg^{-1}$ s.c., GR89696 at $0.03\,mg\,kg^{-1}$ s.c., fentanyl at $0.9\,mg\,kg^{-1}$ s.c., naltrexone at $5\,mg\,kg^{-1}$ s.c. and M8008 at $4\,mg\,kg^{-1}$ s.c. Each point represents the mean value calculated from at least 5 different rats; vertical lines show s.e.mean. (○) Represents vehicle-treated animals, (●) represents animals treated with antagonist alone, (△) represents animals treated with agonist alone and (▲) represents animals treated with agonist plus antagonist.

dose, $4\,mg\,kg^{-1}$ s.c., which completely abolished the decrease in urine output produced by the μ-agonist, fentanyl.

Discussion

The novel compounds described in this paper were all extremely potent agonists in the rabbit vas deferens, a tissue which contains opioid receptors mainly of the κ-type (Oka *et al.*, 1981; Hayes & Kelly, 1985). However, the compounds showed very little activity in the rat vas deferens, a tissue which contains predominantly μ-opioid receptors (Smith & Carter, 1986; Sheehan *et al.*, 1988), and very little activity in the hamster vas deferens, a tissue which contains predominantly δ-opioid receptors (McKnight *et al.*, 1985; Miller & Shaw, 1985; Sheehan *et al.*, 1986). These data from isolated tissues thus suggest that the compounds are very potent and selective κ-opioid agonists. This was confirmed for one of the compounds, GR89696, in the mouse vas deferens in that its agonist effect was antagonized by the κ-selective antagonist, norBNI, with a pA_2 value within the range previously obtained for its action at κ-receptors (Birch *et al.*, 1987) and very similar to that obtained against U69593, but about 2 orders of magnitude higher than that obtained against the μ-agonist, DAMGO.

The potency and κ-agonist selectivity of the compounds was confirmed in the experiments *in vivo*. All of the compounds were potent antinociceptive agents, with GR103545 being the most potent κ-agonist described so far. In common with other κ-agonists, the compounds were generally highly depressant in the mouse. Sedation is also known to occur with κ-agonists in man (Peters *et al.*, 1987) and may be accompanied by psychotomimetic effects, although the latter are less well documented and do not appear to be detected in animals. GR89696 and GR103545 appeared to show a greater separation between sedative and antinociceptive doses in the mouse than the other compounds. However, this separation is not as good in other species (e.g. GR89696 has identical ED_{50} values in the rat paw pressure and rat rotarod tests – unpublished observations). The compounds produced very little respiratory depression or inhibition of gastrointestinal motility; the lack of such effects with κ-agonists is well documented in the literature (Tavani *et al.*, 1983; Porreca *et al.*, 1984; Hayes *et al.*, 1985b).

The compounds described here all produced large increases in urine output in the water-loaded rat. Such diuretic effects of κ-agonists have been extensively described previously (Leander, 1983; Slizgi *et al.*, 1984) and are thought to be due primarily to the presence of κ-receptors in the posterior pituitary, whose function is to inhibit the release of vasopressin (Bicknell *et al.*, 1987; Oiso *et al.*, 1988). Our own compounds and U69593 each produced an initial decrease in urine output before the large increase. Such decreases in urine output have generally been associated with activation of μ-receptors (Leander, 1984; Hayes *et al.*, 1987b), but this seems unlikely to account for the effect seen with these κ-agonists, as the antidiuretic effect, unlike that of the μ-agonist fentanyl, was not antagonized by the μ-antagonist M8008. The antidiuretic effect of U69593 was reduced by naltrexone and is therefore opioid in origin; the most likely explanation is that it is a κ-receptor-mediated effect and may, for example, be a manifestation of the marked behavioural depression seen with these compounds.

In summary, we have described here a novel series of highly potent and selective κ-agonists. These compounds should be useful probes for ascertaining further the functional significance of the κ-opioid receptor.

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Electrophysiological characterization of potent agonists and antagonists at pre- and postsynaptic $GABA_B$ receptors on neurones in rat brain slices

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1 Intracellular recordings were made from neurones in striatum (caudate-putamen) and substantia nigra pars compacta in rat brain slices. Three $GABA_B$ agonists, baclofen, 3-aminopropylphosphinic acid (3-APPA) and 3-aminopropyl(methyl)phosphinic acid (SK&F 97541), depressed excitatory postsynaptic potentials (e.p.s.ps) mediated by glutamate in the striatum, and hyperpolarized neurones in the substantia nigra. The ability of 3-aminopropyl(diethoxymethyl)phosphinic acid (CGP 35348), 3-aminopropyl (hexyl)phosphinic acid (3-APHPA) and phaclofen to antagonize these responses was assessed.

2 Striatal e.p.s.ps, studied in the presence of bicuculline (30 μM), were reduced in amplitude by 92% with 6,7-dinitroquinoxaline-2,3-dione (DNQX; 30 μM). These e.p.s.ps were depressed by up to 95% by SK&F 97541 and baclofen with EC_{50}s of 0.092 μM and 1.25 μM respectively. The maximal effect of 3-APPA was 67% with an EC_{50} of 0.83 μM. Agonist concentration-effect data fitted a single-site logistic model. $GABA_B$ agonists were without effect on striatal neurone membrane potential, input resistance or depolarizations induced by applied glutamate.

3 The depression of striatal e.p.s.ps by SK&F 97541 was reversibly antagonized by CGP 35348, 3-APHPA and phaclofen with estimated equilibrium dissociation constants (K_B) of 11.2 ± 1.7 μM ($n = 4$), 13.3 ± 0.4 μM ($n = 3$), and 405 ± 43 μM ($n = 3$) respectively. CGP 35348 and 3-APHPA appeared to act competitively (Schild plot slopes of 0.99 and 1.01 respectively).

4 Nigral neurones were hyperpolarized by up to 25 mV by SK&F 97541 and baclofen with EC_{50}s of 0.15 μM and 3.6 μM respectively. The maximum hyperpolarization by 3-APPA was only 84% that of the other agonists, with an EC_{50} of 9.0 μM. Agonist concentration-effect data fitted a single-site logistic model.

5 The SK&F 97541-induced hyperpolarization was reversibly antagonized by CGP 35348, 3-APHPA and phaclofen with estimated K_Bs of 17.6 ± 4.4 ($n = 3$), 14.0 ± 1.5 ($n = 4$), and >400 μM ($n = 1$) respectively. CGP 35348 appeared competitive (Schild plot slope of 0.99). Antagonists were also tested with baclofen as agonist, yielding similar K_B estimates as for SK&F 97541.

6 It is concluded that at both the presynaptic and postsynaptic sites examined, SK&F 97541 was about 10 fold more potent than baclofen or 3-APPA. The antagonists CGP 35348 and 3-APHPA (K_B 10–20 μM) were about 20 fold more potent than phaclofen. The similarities in relative agonist potency and estimated antagonist affinity between these two functionally distinct $GABA_B$ receptors renders them pharmacologically indistinguishable at present.

Introduction

The initial descriptions of the γ-aminobutyric acid $(GABA)_B$ binding site in mammalian brain (Bowery *et al.*, 1980; Hill & Bowery, 1981) have prompted examination of the functional role of $GABA_B$ receptors at cellular and behavioural level, together with their consideration as targets for centrally acting therapeutic agents (see Bowery 1989, for review). Functional studies of $GABA_B$ receptors have largely relied on (4-amino-3-(4-chlorophenyl)butyric acid (baclofen) as the prototypic selective $GABA_B$ receptor agonist (Bowery *et al.*, 1980) and it is only recently that phaclofen, the phosphono- analogue of baclofen and the first selective $GABA_B$ antagonist, became widely available (Kerr *et al.*, 1987; Figure 1).

Several different neuronal types in the central nervous system (CNS) are hyperpolarized by baclofen through a direct postsynaptic action due to an increase in membrane potassium conductance. These include hippocampal pyramidal cells (Newberry & Nicoll, 1984), neocortical pyramidal neurones (Howe *et al.*, 1987; McCormick, 1989) and neurones in the dorsolateral septal nucleus (Hasuo & Gallagher, 1988), lateral geniculate nucleus (Soltesz *et al.*, 1988) and substantia nigra pars compacta (Lacey *et al.*, 1988). Slow inhibitory postsynaptic potentials (i.p.s.ps), as well as the action of baclofen, are weakly inhibited by phaclofen (>0.5 mM; Dutar & Nicoll, 1988a; Hasuo & Gallagher, 1988; Soltesz *et al.*, 1988; McCormick, 1989), indicating that they are also mediated by $GABA_B$ receptors.

Baclofen also depresses fast synaptic transmission in hippocampus (Dutar & Nicoll, 1988b; Harrison, 1990), neocortex (Howe *et al.*, 1987), dorsal raphe nucleus (Colmers & Williams, 1988), nucleus accumbens (Uchimura & North, 1990), dorsal striatum (Cordingley & Weight, 1986; P. Calabresi, personal communication) and on spinal motorneurones (Wang & Dun, 1990). These effects appear to be presynaptic and dissociable from the $GABA_B$ hyperpolarization, which is clearly a postsynaptic effect. In contrast to the postsynaptic action of baclofen, its presynaptic effects have been reported to be insensitive to phaclofen (Dutar & Nicoll, 1988b; Harrison, 1990; Wang & Dun, 1990). Furthermore, postsynaptic $GABA_B$ hyperpolarizations are prevented by pretreatment with pertussis toxin, the ADP-ribosylator of certain guanosine triphosphate-binding (G-) proteins, whereas presynaptic $GABA_B$ inhibition is unaffected (Dutar & Nicoll, 1988b; Thalmann, 1988; Colmers & Williams, 1988; Harrison, 1990). These findings suggested that there might be pharmacologically distinct $GABA_B$ receptors on central neurones, possibly with different receptor-effector coupling, and with correspondingly different functional roles (Harrison, 1990).

We have used electrophysiology in rat brain slices to characterize further the pharmacology of two $GABA_B$ receptor-mediated actions. These were (1) the postsynaptic

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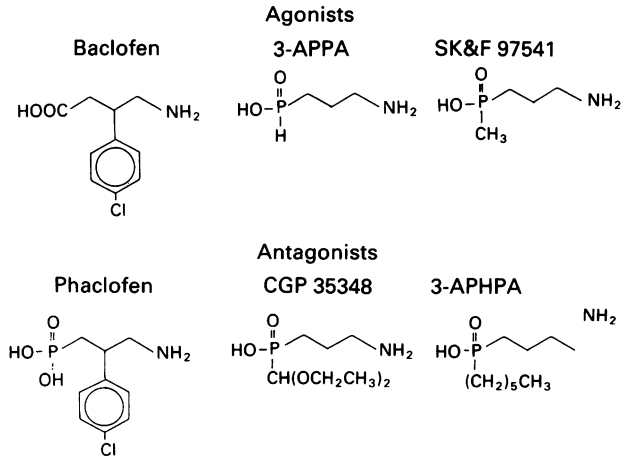


Figure 1 Chemical structures of $GABA_B$ ligands used in the present study, given with their names as used in the text.

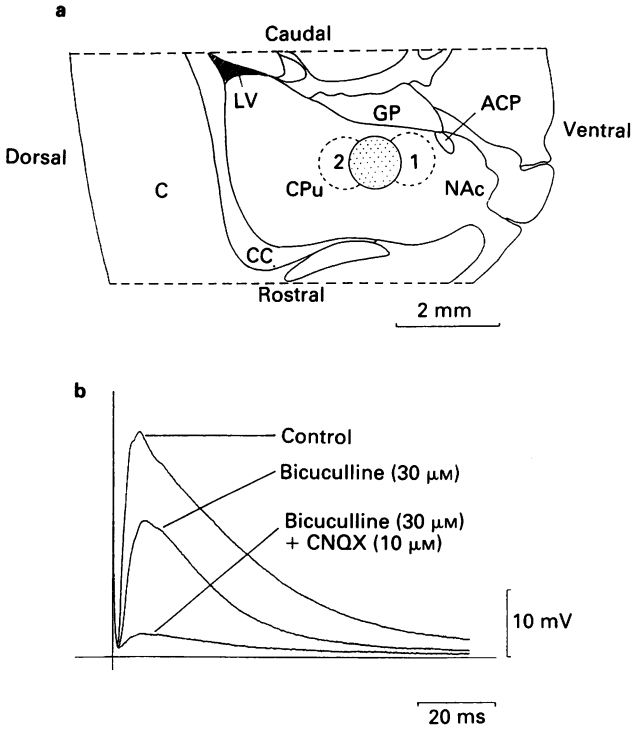


Figure 2 Stimulation within 500 μm of recording site in striatum (caudate putamen) elicits a synaptic potential partly blocked by bicuculline (30 μM) and almost completely blocked by further addition of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM). (a) Diagrammatic representation of sagittal slice preparation containing striatum (CPu), showing areas of recording (shaded) and both ventro-caudal (1) and dorso-rostral (2) areas of stimulation. C, cerebral cortex; CC, corpus callosum; GP, globus pallidus; NAc, nucleus accumbens; ACP, anterior commisure (posterior), LV, lateral ventricle. (b) Striatal synaptic potential, preceded by stimulus artefact, evoked by stimulation in ventro-caudal region (area 1 in (a)) shown before application of drugs (control), in the presence of bicuculline (30 μM) and in bicuculline plus CNQX (10 μM). Bicuculline reduced the synaptic potential to 59% of control amplitude; the residuum was reduced by 85% following application of CNQX. E.p.s.ps evoked by stimulation of dorso-rostral region (area 2 in (a)) were largely insensitive to bicuculline. In this and subsequent figures, each waveform consists of 10 averaged potentials evoked at 0.1 Hz. Resting membrane potential was -82 mV.

hyperpolarization of substantia nigra compacta neurones, an effect robustly activated by baclofen (Lacey *et al.*, 1988), and (2) presynaptic inhibition of glutamate-mediated synaptic potentials in dorsal striatum (caudate-putamen), where baclofen is not only effective, but does not cause a concomitant postsynaptic hyperpolarization (P. Calabresi, personal communication). In addition to baclofen and phaclofen, the actions of the recently described $GABA_B$ agonist 3-aminopropylphosphinic acid (3-APPA; Dingwall *et al.*, 1987) and the antagonists 3-aminopropyl-(diethoxymethyl)-phosphinic acid (CGP 35348) and 3-aminopropyl-hexylphosphinic acid (3-APHPA; Baylis *et al.*, 1989; Bittiger *et al.*, 1990) were quantified, together with that of a novel $GABA_B$ agonist, 3-aminopropyl-methylphosphinic acid (SK&F 97541; Hills & Howson, 1990). The chemical structures of these compounds are shown in Figure 1.

Methods

Experimental preparations and recording techniques

Intracellular recordings were made from neurones in either dorsal striatum (caudate-putamen) or substantia nigra pars compacta within slices (300–350 μm thick) of rat brain. The techniques for preparation of, recording from, and application of substances to substantia nigra compacta neurones have been described previously (Lacey *et al.*, 1988; 1989). In brief, coronal slices of substantia nigra were submerged in a tissue chamber (volume 1 ml) by a continuously superfused (at 1.5 ml min^{-1}) artificial cerebrospinal fluid medium (ACSF) kept at 36°C. The ACSF contained (in mM): NaCl 126, KCl 2.5, NaH_2PO_4 1.2, $MgCl_2$ 1.3, $CaCl_2$ 2.4, $NaHCO_3$ 26 and glucose 10, and was saturated with 95% O_2/5% CO_2. Intracellular recordings were made with 40–80 MΩ resistance glass microelectrodes filled with KCl (2 M). Membrane potential was amplified with an Axoclamp 2A amplifier and displayed on a Gould RS 3200 chart recorder.

Sagittal slices of striatum (caudate-putamen) were made approximately 3 mm lateral to the midline, lateral to the anterior portion of the anterior commissure. Slices were cut obliquely at 20 degrees to midline (rostral portion more lateral than caudal portion) in an attempt to preserve neuronal projections to globus pallidus (see Figure 2a). Synaptic potentials were evoked by focal stimulation of the slice with bipolar electrodes positioned 200–500 μM from the recording electrode (Figure 2a). Stimuli (duration 0.1 ms, frequency 0.1 Hz, 1–10 V) were applied with a Master-8 stimulator (A.M.P.I.). Amplified membrane potential transients were filtered (3–10 kHz; Frequency Device Filters model 902) and recorded on digital tape (Biologic DTR-1200) for subsequent analysis. Synaptic potential waveforms were averaged ($n = 10$) after digital acquisition (> 10 kHz) with a CED 1401 interface linked to a Compaq 386 computer using software developed by J. Dempster (SCAN). Other methods for experiments on the striatum were as described for those on substantia nigra neurones.

Analytical techniques

Drug effects were expressed as either % inhibition of e.p.s.ps with respect to control amplitude (in striatum) or as mV hyperpolarization (substantia nigra pars compacta). Concentration-effect data were fitted by computer by least squares analysis of variance (ANOVAR) to a single-site logistic equation, weighted according to the number of replicates for each point. For data pooled from a number of experiments, curves were fitted without constraint on the maximum value (as in Figures 3b, 7b). For data obtained from individual cells, fitted curves were constrained to either 100% inhibition

of the excitatory postsynaptic potentials (e.p.s.ps) (striatum; as in Figure 5b) or, in substantia nigra compacta, to the fitted maximum of the control data (as in Figure 8b). From the logistic fit, half maximally-effective agonist concentrations (EC_{50}) were derived and subsequently used to calculate dose ratios for the quantification of antagonist effects.

Data are expressed as mean ± standard error of the mean (s.e.mean).

Drugs

Drugs were applied in the superfusion medium in known concentrations, reaching the recording chamber after a delay of 20–30 s. The following drugs were used: (±)-baclofen, (−)-bicuculline methiodide and (±)-2-amino-5-phosphono-valeric acid (2-APV) (all from Sigma); 6,7-dinitroquinoxaline-2,3-dione (DNQX), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 3-amino-2-(4-chlorophenyl)propylphosphonic acid (phaclofen; all Tocris Neuramin); 3-aminopropylphosphinic acid (3-APPA; prepared by the method of Dingwall *et al.*, 1987), 3-aminopropyl(diethoxymethyl)phosphinic acid (CGP 35348) and 3-aminopropyl(hexyl)phosphinic acid (3-APHPA), both prepared as described by Baylis *et al.* (1989); 3-aminopropyl(methyl)phosphinic acid (SK&F 97541), prepared as described by Hills & Howson (1990). The structures of the $GABA_B$ receptor ligands are shown in Figure 1. L-Glutamate (Sigma) was applied to striatal neurones by pressure ejection (100 kPa for 10–50 ms) of a small volume of solution (100 mM in ACSF) from the submerged tip of a pipette positioned close to the site of recording.

Results

Striatum: properties of neurones and synaptic potentials

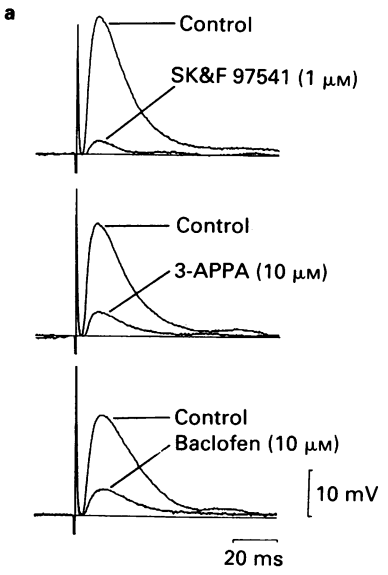
Intracellular recordings were made from 66 cells with mean membrane potentials of −76.9 ± 1.3 mV. Depolarization of the membrane to a threshold of −30 to −40 mV with intracellular current pulse injection (0.5–1 nA, 200 ms) elicited action potential firing. Apart from occasional spontaneous, transient depolarizations probably of synaptic origin, cells were otherwise quiescent. Cell input resistance, measured by passage of hyperpolarizing current pulses (0.1–0.2 nA, 200 ms) from rest, was 55.1 ± 5.7 MΩ (n = 10; see Figures 4 and 6).

Bipolar stimulation within 200–500 μm of the recording site (Figure 2a) elicited a depolarizing synaptic potential (Figure 2b). The amplitude of the synaptic potential was dependent upon stimulus intensity and would elicit action potential firing if the threshold of around −35 mV was exceeded. When stimulation was caudal and ventral to the site of recording (area 1 in Figure 2a), the synaptic potential was reversibly reduced by between 10 and 80% of the maximum by application of the $GABA_A$ receptor antagonist (−)-bicuculline methiodide (30 μM; 23 cells; Figure 2b). In contrast, synaptic potentials evoked by stimulation rostral and dorsal to the site of recording (area 2 in Figure 2a) were only minimally sensitive to bicuculline. In the presence of bicuculline (30 μM), synaptic potentials evoked by stimulation in both regions were reduced by 92.0 ± 2.5% with DNQX (30 μM, 6 cells) and by 85.9 ± 2.5% with CNQX (10 μM; 5 cells; Figures 2b, 6a), two selective glutamate receptor antagonists (Honore *et al.*, 1988). The bicuculline- and CNQX-resistant component of the synaptic potential was not blocked by the selective NMDA receptor antagonist 2-APV (10–30 μM; n = 4).

In all experiments subsequently described, synaptic potentials evoked by stimulation in both areas 1 and 2 (Figure 2a) were examined in the presence of bicuculline methiodide (30 μM). These e.p.s.ps were presumably the result of activation of postsynaptic excitatory amino acid receptors by glutamate released from presynaptic terminals.

Effect of $GABA_B$ agonists on e.p.s.ps

The amplitudes of e.p.s.ps were depressed in a concentration-dependent manner from their initial value of 10–25 mV by application of SK&F 97541 (0.01–3 μM), baclofen and 3-APPA (0.1–30 μM; Figure 3a). The chemical structures of these compounds are shown in Figure 1. Maximal effects of all three compounds occurred within 3 min of commencing their application, reversing within 5–15 min of their washout from the recording chamber. The effect of baclofen was often slower to



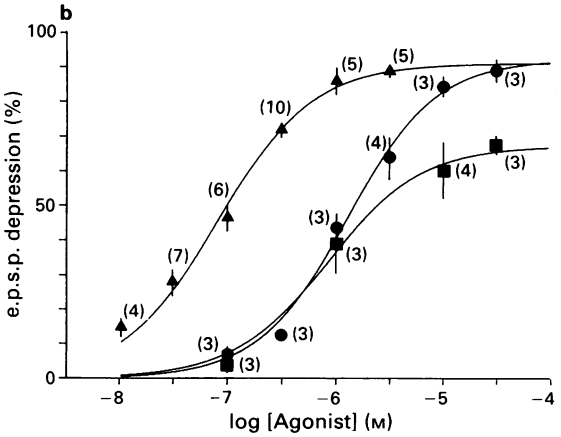


Figure 3 Striatal e.p.s.ps recorded in the presence of 30 μM bicuculline were depressed by $GABA_B$ agonists in a concentration-dependent manner. (a) Averaged (n = 10) records of e.p.s.ps from a single striatal neurone showing depression by SK&F 97541 (1 μM; upper records), 3-aminopropylphosphinic acid (3-APPA, 10 μM; middle records) and baclofen (10 μM; lower records). Drugs were applied in sequence shown following washout of preceding application, thus the effects of SK&F 97541 and 3-APPA can be seen to be reversible. Resting membrane potential −88 mV. (b) Concentration-effect curves for all three agonists constructed from data pooled from all experiments. Mean values are plotted, together with s.e.mean (bars). Data fitted by ANOVAR to a single-site logistic equation weighted according to the number of replicates for each point (numbers in parentheses) with the maximum effect unconstrained. The computed values of EC_{50} and maximum inhibition were respectively 0.092 μM and 92% for SK&F 97541 (▲), 0.83 μM and 67% (3-APPA; ■), and 1.25 μM and 92% (baclofen; ●).

reverse on washout than that of equieffective concentrations of either SK&F 97541 or 3-APPA (see Figure 6). Maximally effective concentrations of both baclofen (30 μM) and SK&F 97541 (3 μM) were unable to suppress the e.p.s.p. by more than 95% of its control amplitude. Data pooled from all experiments is represented graphically in Figure 3b. The EC_{50} values of the three agonists derived from logistic fits to the data were: SK&F 97541, 0.092 μM; 3-APPA, 0.83 μM; baclofen, 1.25 μM (Table 1). The estimated mean maximal depression of the e.p.s.p. by both SK&F 97541 and baclofen was 92%, whereas that of 3-APPA was 67% (Figure 3b).

There was no detectable effect of SK&F 97541, 3-APPA or baclofen at any of the concentrations examined on membrane potential or input resistance in any of the cells studied (see Figures 4, 6), indicating an absence of a postsynaptic action of these compounds (in contrast to that observed in substantia nigra compacta neurones; Figure 7a). In addition, depolarizations evoked by transient applications of L-glutamate were unaffected by SK&F 97541 (3 μM, 4 cells; Figure 4); SK&F 97541 therefore was not acting as an antagonist of postsynaptic glutamate receptors.

E.p.s.p. depression by SK&F 97541: effects of antagonists

Both CGP 35348 and 3-APHPA (30–300 μM; see Figure 1 for chemical structures) alleviated the e.p.s.p. depression caused by submaximal concentrations of SK&F 97541 (Figure 5a). The effects of both compounds were maximal within 5–10 min of their application and largely reversible within 10–30 min of washout. In 2 cells concentration-effect curves to SK&F 97541 were constructed and then repeated in the presence of first 30 and then 300 μM of one or the other of these antagonists. The antagonism observed was surmountable by application of higher concentrations of agonist (Figure 5b). Ratios of the EC_{50} values in the presence and in the absence of antagonist were used to construct Schild plots (Arunlakshana & Schild, 1959). For CGP 35348, the plot had a slope of 0.99 and gave an estimate of antagonist equilibrium dissociation constant (K_B) of 8.1 μM (Figure 5c). For 3-APHPA, the Schild plot had slope of 1.01 and gave a K_B estimate of 14.2 μM. Four additional experiments were performed where single dose-ratios were obtained with either 30 or 300 μM of antagonist. The K_B was estimated from the equation: K_B = antagonist concentration/(dose-ratio − 1). This assumes that antagonism was competitive, an assumption supported by the Schild plots for both antagonists having unity slope. Combining data from all these experiments yielded K_B values of 11.2 ± 1.7 μM (n = 4 cells) for CGP 35348 and 13.3 ± 0.4 μM for 3-APHPA (3 cells; Table 1).

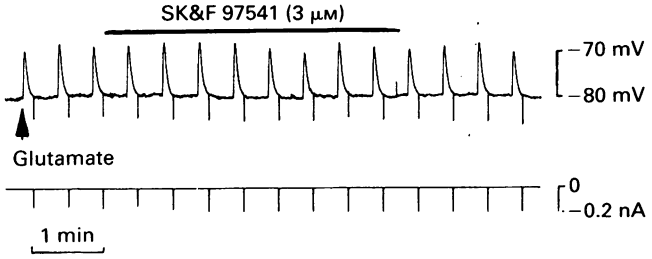


Figure 4 SK&F 97541 was without effect on membrane potential, input resistance or depolarizations evoked by exogenous glutamate in striatal neurones. Records of membrane potential (upper trace) and current passed by recording electrode (lower trace). Transient depolarizations due to repeated application of L-glutamate (100 mM) by pressure ejection (100 kPa, 20 ms) in vicinity of cell (first application indicated by arrow). Transient hyperpolarizations caused by passage of hyperpolarizing pulses of constant current (−0.2 nA, 200 ms) across membrane are proportional to input resistance. SK&F 97541 (3 μM), applied by superfusion for period indicated by the bar, was without effect on these variables.

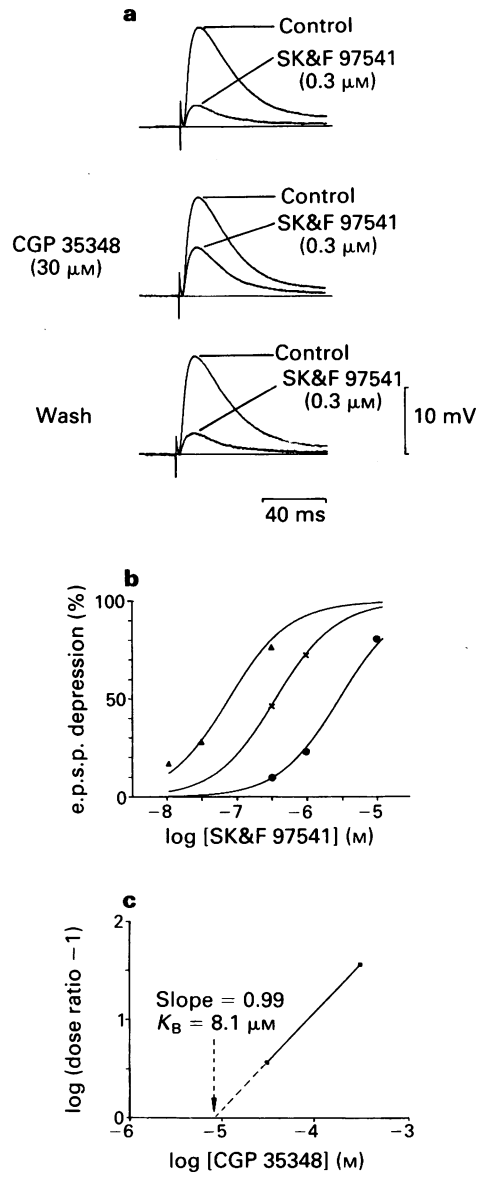


Figure 5 Depression of striatal e.p.s.ps by SK&F 97541 was competitively and reversibly antagonized by CGP 35348. (a) Three pairs of records from the same neurone each showing e.p.s.ps before and during three separate applications of SK&F 97541 (0.3 μM). The depression of e.p.s.p. amplitude by SK&F 97541 (upper pair of records) was reduced by CGP 35348 (30 μM; middle records), but was reestablished after 30 min wash of CGP 35348 (lower records). Bicuculline (30 μM) present throughout. (b) Concentration-effect curves from the cell depicted in (a) derived with SK&F 97541 in 0 (▲), 30 (×) and 300 μM CGP 35348 (●). Curves fitted by ANOVAR with maxima constrained to 100% depression of e.p.s.p. (c) Schild transform of data in (b) with dose-ratios calculated from EC_{50} values. Slope of 0.99 indicated antagonism by CGP 35348 was competitive with K_B (x-intercept) of 8.1 μM.

Phaclofen (1 mM) rapidly and reversibly antagonized the effect of SK&F 97541 (0.3 μM) by 58 ± 2.1% (3 cells; Figure 6a). If competitive antagonism is assumed, this indicates a K_B value for phaclofen of 405 ± 43 μM (n = 3; Table 1). In a single experiment phaclofen (1 mM) also reversibly antagonized the effect of baclofen (3 μM) by 52% (Figure 6b), similarly indicative of a K_B > 400 μM.

Substantia nigra pars compacta: cell properties

Intracellular recordings were made from 22 cells with properties comparable to those of the principal, presumed dopamine-

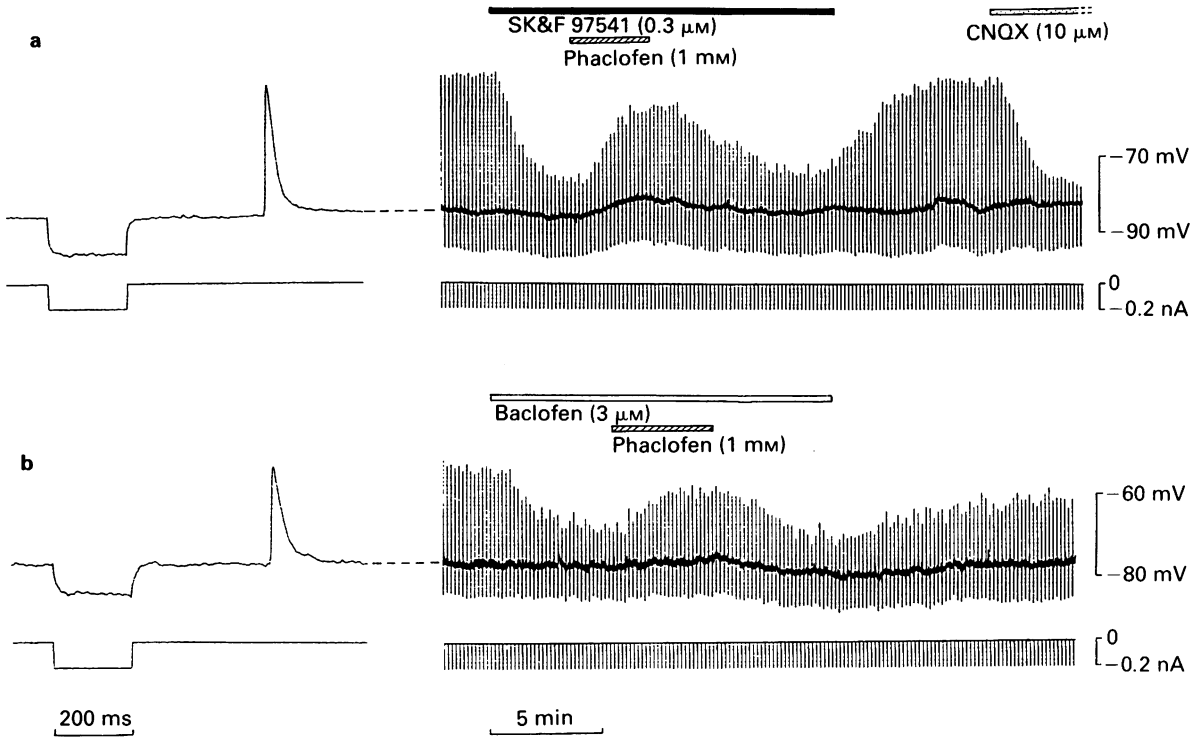


Figure 6 Depression of striatal e.p.s.ps by both SK&F 97541 (0.3 μM) and baclofen (3 μM) was reversibly antagonized by 1 mM phaclofen. (a) Continuous records of membrane potential (upper trace) and injected current (lower trace) showing (depolarizing) e.p.s.ps elicited at 0.1 Hz and (hyperpolarizing) electrotonic potentials caused by constant current pulses (−0.2 nA, 200 ms), as shown on expanded time scale in left hand panel. Depression of e.p.s.p. by SK&F 97541 (0.3 μM; solid bar) to 31% of control amplitude was reversibly antagonized by 58% with phaclofen (1 mM; hatched bar). Following washout of SK&F 97541, the recovered e.p.s.p. was subsequently depressed to 14% of control amplitude by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM). Bicuculline (30 μM) present throughout. (b) Records from another cell with the same experimental protocol as in (a), except that the depression of the e.p.s.p. to 39.5% of control amplitude was by baclofen (3 μM; open bar). This was reversibly antagonized by 52% by phaclofen (1 mM; hatched bar).

containing, cells of the substantia nigra zona compacta described previously (Lacey *et al.*, 1988; 1989). Of the 22 cells, 13 fired spontaneous action potentials at 2.3 ± 0.36 Hz and the other 9 were quiescent. Effects of $GABA_B$ agonists on membrane potential were quantified in the absence of spontaneous action potentials; the active cells were hyperpolarized with constant direct current injection to membrane potentials negative to firing threshold. The mean membrane potential at which compounds were tested was −56.6 ± 0.52 mV (n = 22).

Effect of $GABA_B$ agonists on membrane potential

SK&F 97541 (0.1–10 μM), baclofen, (1–30 μM) and 3-APPA (1–300 μM) hyperpolarized the membrane of substantia nigra pars compacta neurones by up to 25 mV in a concentration-dependent manner (Figure 7a), accompanied by a fall in input resistance, as has been reported previously in the case of baclofen (Lacey *et al.*, 1988). Spontaneous action potential firing where present was also inhibited (Figure 7a). The effects of all the agonists were maximal within 3 min of their application, but often failed to recover completely even following washout periods of 15 min (Figure 7a); this was more pronounced with applications of the higher drug concentrations (see also Lacey *et al.*, 1988). Pooled data from all cells examined is shown in Figure 7b. From the logistic curves fitted to the data, estimated EC_{50} values were: SK&F 97541, 0.15 μM; baclofen, 3.6 μM; 3-APPA, 9.0 μM (Table 1). The estimated mean maximum hyperpolarization with SK&F 97541 was 21.1 mV and 21.5 mV with baclofen, but only 17.8 mV with 3-APPA (Figure 7b).

Hyperpolarization by SK&F 97541: effects of antagonists

Membrane hyperpolarizations caused by SK&F 97541 were antagonized in a manner surmountable by higher concentrations of the agonist by 3-APHPA and CGP 35348 (30–300 μM; Figure 8a). Both antagonists were effective within 5–10 min of their application and showed reversibility within 10–30 min of washout. The estimated K_B for 3-APHPA, obtained from single dose-ratios as described above, was 14.0 ± 1.5 μM (n = 4; Table 1). A Schild plot constructed from one experiment with CGP 35348 had a slope of 0.97 and an x-intercept (K_B) of 18.3 μM (Figures 8b,c). This value was pooled with estimates from two other experiments in which single dose-ratios were obtained to give an estimated K_B for CGP 35348 of 17.6 ± 4.4 μM (n = 3; Table 1). In a single experiment, phaclofen (1 mM) reversibly antagonized the response to SK&F 97541 (0.3 μM) by 53%, indicative of a K_B > 400 μM (assuming competitive antagonism).

The ability of 3-APHPA and CGP 35348 to antagonize the effect of baclofen was also assessed. In two separate experiments, K_B estimates from single dose-ratios were 11.7 μM (3-APHPA) and 19.9 μM (CGP 35348).

Discussion

These experiments permit quantitative comparison of the effects of three agonists and three antagonists at $GABA_B$ receptors mediating two different functional responses in striatum (caudate-putamen) and substantia nigra compacta of the rat.

The hyperpolarization of substantia nigra neurones by baclofen has been demonstrated to be a direct postsynaptic action, mimicked by GABA itself, resulting from a GTP-

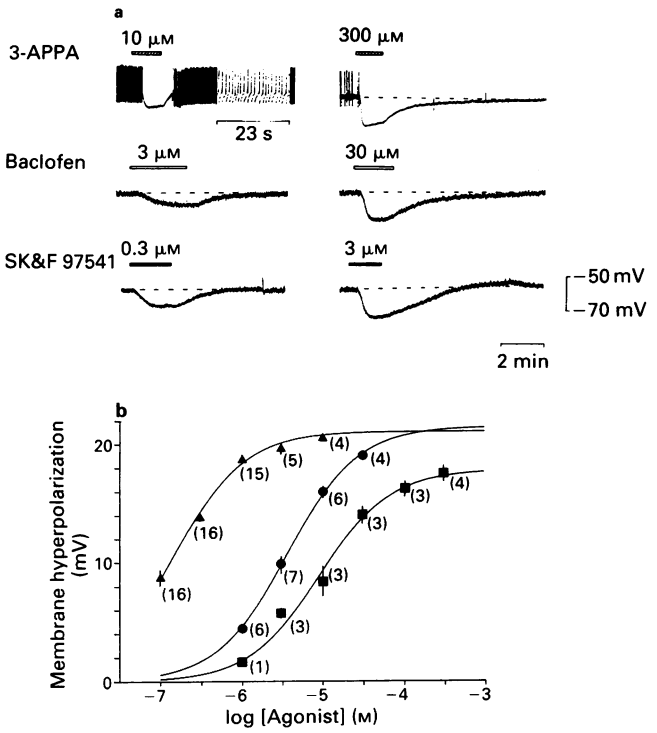


Figure 7 Substantia nigra compacta neurones were hyperpolarized in a concentration-dependent manner by $GABA_B$ agonists. (a) Records of membrane potential from a single substantia nigra compacta neurone showing reversible membrane hyperpolarization caused by sequential application of 3-aminopropylphosphinic acid (3-APPA, 10, 300 μM; hatched bars; top row), baclofen (3, 30 μM; open bars; middle row) and SK&F 97541 (0.3, 3 μM; solid bars; bottom row). The inhibition of action potential firing in top two traces did not recover fully on drug washout (note expanded time scale in top left trace; full action potential amplitude not reproduced). Dashed line indicates −57 mV. (b) Pooled data from all experiments with curves fitted by ANOVAR to a single-site logistic equation with the maximum effect unconstrained. Data from spontaneously-firing cells not included. Number of experiments in parentheses. Computed values of EC_{50} and maximum hyperpolarization were 0.15 μM and 21.1 mV for SK&F 97541 (▲), 3.6 μM and 21.5 mV for baclofen (●) and 9.0 μM and 17.8 mV for 3-APPA (■).

dependent potassium conductance increase (Lacey *et al.*, 1988). The striatal e.p.s.p., studied here in the presence of bicuculline to block the effects of GABA release, was reduced by 92% in DNQX (30 μM) and thus was mediated principally by glutamate release. Its inhibition by $GABA_B$ agonists may be considered due to an action on the presynaptic terminal for two reasons: firstly, no postsynaptic effect of $GABA_B$ agonists was observed in striatal neurones, even at maximal drug concentrations (Figures 4, 6). This makes the e.p.s.p. depression unlikely to be the result of an increase in postsynaptic membrane conductance (see Harrison, 1990; Uchimura & North, 1990). Secondly, depolarizations caused by exogenously applied glutamate were unaffected by $GABA_B$ agonists at concentrations that maximally depressed the e.p.s.p. (Figure 4). Additionally, striatal $GABA_B$ binding site density was unchanged following intrastriatal injections of kainic acid, which destroys intrinsic neurones but not afferent terminals (Kilpatrick *et al.*, 1983), further arguing against a postsynaptic location for striatal $GABA_B$ receptors.

The principal findings were that the three $GABA_B$ agonists exhibited a similar rank order of potency on both preparations, but each was more potent in depressing the striatal e.p.s.ps than in hyperpolarizing substantia nigra neurones. In addition, estimates of affinity for three antagonists showed no clear differences between the pre- and postsynaptic sites for each compound (see Table 1).

Table 1 Summary of agonist EC_{50} and antagonist K_B values for actions of $GABA_B$ ligands at both presynaptic (striatal) and postsynaptic (nigral) $GABA_B$ receptors

$GABA_B$ ligand	*Inhibition of striatal e.p.s.ps*	*Hyperpolarization of nigral neurones*
Agonists: EC_{50} (μM)		
SK&F 97541	0.092	0.15
3-APPA	0.83	9.0
Baclofen	1.25	3.6
Antagonists: K_B (μM)*		
CGP 35348	11.2 ± 1.7 (4)	17.6 ± 4.4 (3)
3-APHPA	13.3 ± 0.4 (3)	14.0 ± 1.5 (4)
Phaclofen	405 ± 43 (3)	>400 (1)

* With SK&K 97541 as agonist.
See text for abbreviations.

$GABA_B$ receptor agonists

The concentration-effect curves for the three agonists in both preparations were well fitted by the single-site logistic model, from which EC_{50} and maxima values were derived (Figures 3b, 7b). 3-APPA and baclofen were roughly equipotent, with SK&F 97541 at least tenfold more potent than either, in both preparations (Table 1). 3-APPA displayed a maximal effect of 67% and 84% that of baclofen and SK&F 97541 in striatum and substantia nigra respectively (Figures 3b, 7b), suggestive of a lower intrinsic efficacy or 'partial' agonism for this compound in both preparations. However, despite being about 11 fold less potent at the postsynaptic site, the lower efficacy of 3-APPA was no more pronounced here than at the presynaptic site. Thus the difference in absolute potency of each agonist between the preparations (lowest for SK&F 97541 and highest for 3-APPA) is not easily accounted for simply by a higher $GABA_B$ receptor 'reserve' at the presynaptic receptor.

Electrically stimulated contractions of several intestinal smooth muscle preparations are all inhibited by baclofen, 3-APPA and SK&F 97541 *in vitro* (Hills *et al.*, 1989; Hills & Howson, 1990; J.M. Hills, personal communication), presumably due to presynaptic inhibition of transmitter release. In these preparations the rank order of potency differed from that described here, with 3-APPA and SK&F 97541 being equieffective and baclofen at least five times less potent than either (Hills *et al.*, 1989; J.M. Hills, personal communication). In inhibiting the forskolin-stimulated production of adenosine 3':5-cyclic monophosphate (cyclic AMP) in slices of rat cerebral cortex, 3-APPA and (−)-baclofen were equipotent (Pratt *et al.*, 1989), as seen in the present study. However, in potentiating noradrenaline-stimulated cyclic AMP production, 3-APPA was both weaker and incapable of the same maximal effect as (−)-baclofen (Pratt *et al.*, 1989). Although there was no difference in the rank order of $GABA_B$ agonist potency between the two preparations studied here (Table 1), the differences in this respect from other preparations support the contention of Scherer *et al.* (1988) that there may be more than one type of $GABA_B$ receptor. In particular, there may be $GABA_B$ receptors of another type on the terminals of nerves innervating smooth muscle. Conclusive evidence for this will require selective antagonists.

$GABA_B$ receptor antagonists

CGP 35348 and 3-APHPA blocked the actions of SK&F 97541 at both pre- and postsynaptic sites. Unity slopes in a limited number of two-point Schild plots suggested that both compounds were competitive antagonists. Estimated K_B values, obtained mainly from single shifts of concentration-effect curves, were in the range 10–20 μM. Comparable results were obtained in substantia nigra when baclofen was used as the $GABA_B$ agonist, indicating that SK&F 97541 acted at the same site as baclofen. Baclofen hyperpolarizations and the slow i.p.s.p. in hippocampal pyramidal cells were inhibited by CGP 35348 in the range 30–100 μM (Bittiger *et al.*, 1990), in

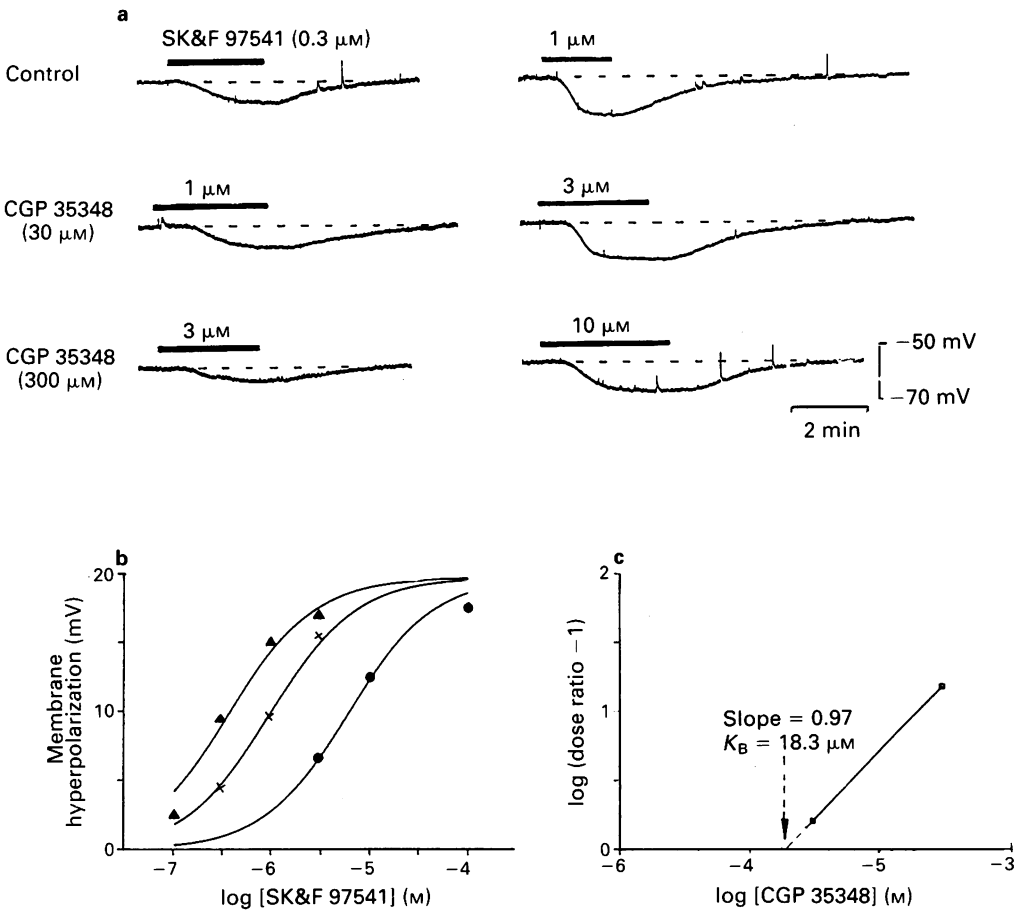


Figure 8 Hyperpolarization of substantia nigra neurones by SK&F 97541 was competitively antagonized by CGP 35348. (a) Records of membrane hyperpolarizations in a single neurone in response to SK&F 97541 (solid bars) in the absence (upper records), and presence of 30 (middle records) and 300 μM (lower records) CGP 35348. Equivalent responses to SK&F 97541 could be obtained in the presence of CGP 35348 by raising the agonist concentration. Dashed line indicates −56 mV. (b) Complete concentration-effect curves from the cell in (a). Points fitted to single-site logistic curves (× and ●, in CGP 35348 30 and 300 μM respectively) with maxima constrained to 19.8 mV, the value given by the unconstrained fit of points obtained in absence of CGP 35348 (▲). (c) Schild transform of data in (b) with dose-ratios calculated from computed EC_{50} values. Slope of 0.97 indicates antagonism by CGP 35348 was competitive with K_B (x-intercept) of 18.3 μM.

accord with the present estimates of its affinity. Similar estimates of $GABA_B$ antagonist affinity were obtained for CGP 35348 and 3-APHPA in peripheral tissue (J.M. Hills, personal communication). Thus if any of these actions of $GABA_B$ agonists are on different receptor subtypes, neither CGP 35348 nor 3-APHPA appear able to discriminate between them.

The antagonism of the striatal e.p.s.p. depression by SK&F 97541 or baclofen with phaclofen (Figure 6) is in contrast to earlier reports of insensitivity of presynaptic inhibition to this antagonist in other preparations (Dutar & Nicoll, 1988b; Wang & Dun, 1990; Harrison, 1990). However, presynaptic inhibition of hippocampal i.p.s.ps by low concentrations of baclofen has recently been reported to be alleviated by phaclofen (Davies *et al.*, 1990). Phaclofen is also weakly effective as an antagonist of the depression of electrically-evoked contractions of rat vas deferens by 3-APPA (J.M. Hills, personal communication). Moreover, 2-hydroxysaclofen, the sulphonic acid analogue of baclofen, which, like CGP 35348 and 3-APHPA, is also more potent than phaclofen, is effective at both pre- and postsynaptic sites of baclofen action in hippocampus (Davies *et al.*, 1990). It therefore seems likely that the previous failure to demonstrate antagonism of the presynaptic effects of baclofen by phaclofen may be attributable to its low potency and also to the use of excessive concentrations of the agonist which, according to the present findings, is likely to be more potent at pre- than post-synaptic sites.

The present estimates of affinity for phaclofen are approximate, but similar in both preparations ($K_B > 400\,\mu$M), and permit the conclusion that both CGP 35348 and 3-APHPA are at least 20 fold more potent than phaclofen, in agreement with Karlsson *et al.* (1990). Taken together, these results with $GABA_B$ antagonists do not support the generalisation that pre- and postsynaptic $GABA_B$ receptors represent different subtypes (Harrison, 1990), although it remains possible that some presynaptic $GABA_B$ receptors may be pharmacologically distinct from other types of $GABA_B$ receptors.

Functional implications

Fast excitatory transmission from thalamus and cortex to striatum has been demonstrated electrophysiologically (Buchwald *et al.*, 1973; Kawaguchi *et al.*, 1989); that originating from the cortex has been shown to be mediated by glutamate (Herrling, 1985; Malenka & Kocsis, 1988). Ligand binding studies of $GABA_B$ sites in rat striatal synaptosomes (Kilpatrick *et al.*, 1983) or in striatal tissue sections (Moratalla & Bowery, 1988) show marked decreases in density following decortication. Thus it is probable that a major component of the e.p.s.p. inhibited by $GABA_B$ agonists originated from the intrastriatal terminals of cortical neurones (but see Wilson & Wilson, 1985). Striatal neurones are well polarized *in vivo* as well as *in vitro*, firing only when significantly depolarized by excitatory synaptic input (Calabresi *et al.*, 1990). As the great majority of these neurones are projection neurones, the inhibition of fast excitatory input by $GABA_B$ agonists would be expected to have a considerable inhibitory influence on striatal output.

The component nuclei of the basal ganglia, through their interconnecting circuitry and net output, provide a powerful influence upon motor control and cognitive function, and may be viewed as a sensori-motor integration centre (Albin *et al.*, 1990). The striatum is the principal region of the basal ganglia for the receipt of input from all cortical regions; the dopamine-containing projection from the substantia nigra pars compacta constitutes a second major striatal input. Release of [^{3}H]-dopamine in striatal slices is reduced by baclofen (Bowery *et al.*, 1980), an effect which a systemically-administered $GABA_B$ agonist would reinforce through the inhibition of dopamine neurone firing at the level of the cell body. In addition to reducing release of glutamate and dopamine, a $GABA_B$ agonist *in vivo* would also inhibit GABA-mediated synaptic potentials (our unpublished observations; see also Uchimura & North, 1990, in nucleus accumbens). To predict the effect of $GABA_B$ agonists and antagonists on striatal output it will be necessary to consider the temporal relationship between cortical and nigral input to the striatum. Furthermore, there are many brain regions with higher $GABA_B$ binding site density than substantia nigra pars compacta or caudate putamen (Bowery *et al.*, 1987; Chu *et al.*, 1990). Thus the behavioural consequences of a perturbation of striatal function through drugs acting on $GABA_B$ receptors may be modified by additional effects elsewhere in the brain.

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Effect of clonidine, nimodipine and diltiazem on the *in vitro* opioid withdrawal response in the guinea-pig ileum

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1 The effects of clonidine, nimodipine and diltiazem, on the *in vitro* withdrawal contracture induced by naloxone in the guinea-pig ileum obtained from morphine-dependent animals, were evaluated.

2 The *in vitro* incubation with clonidine (0.01, 0.1 and 1 μM), diltiazem (0.25, 0.5 and 1 μM) or nimodipine (0.05, 0.1 and 1 μM) reduced significantly the force of the contracture induced by naloxone in the morphine-dependent guinea-pig ileum.

3 The intraperitoneal administration of clonidine (0.3 mg kg^{-1}), nimodipine (5 mg kg^{-1}) or diltiazem (20 mg kg^{-1}) reduced the contractile response induced by naloxone in the morphine-dependent guinea-pig ileum.

4 It is concluded that at least part of the effect of clonidine, nimodipine and diltiazem on withdrawal contractures is mediated via a peripheral, rather than a central site of action. Even though, the mechanism responsible for the effect of the calcium channel blockers differs from that of α_2-adrenoceptor agonists, all of the drugs tested prevented the contracture induced by naloxone in morphine-dependent guinea-pig ileum.

Introduction

After chronic morphine treatment, naloxone administration precipitates an abstinence syndrome in animals and man. During opioid withdrawal the release of numerous neurotransmitters is augmented, and specifically a direct relationship between noradrenergic hyperactivity and the intensity of the abstinence symptoms has been demonstrated (Swann *et al.*, 1983). The existence of a direct relationship between α_2-adrenoceptors in rat central nervous system and chronic morphine administration (Hamburg & Tallman, 1981) is well known, and clonidine, a drug widely used as an α_2-adrenoceptor agonist, has been used effectively to reduce withdrawal symptoms (Fielding *et al.*, 1978; Taylor *et al.*, 1988). Furthermore, peripheral mechanisms may be implicated in the anti-withdrawal effect of clonidine. It has been demonstrated that this drug suppresses the contracture of the opiate-dependent guinea-pig ileum elicited by administration of naloxone (Collier *et al.*, 1981). Nevertheless, the usefulness of clonidine in this regard is often limited by potential side effects such as hypotension and sedation (Washton & Resnick, 1980).

On the other hand, evidence suggesting an important role of calcium in the actions of opioid analgesics has accumulated. Chronic morphine administration is associated with a large increase in the content of calcium in the brain and during naloxone precipitated withdrawal these elevated calcium levels return toward control values (Yamamoto *et al.*, 1978). It has been shown that the number of calcium channel antagonist binding sites is greatly increased in the brain of morphine-tolerant mice (Ramkumar & El-Fakahany, 1988).

Recent studies have demonstrated the ability of the calcium entry blockers to inhibit the manifestation of the morphine abstinence syndrome in rats and mice (Bongianni *et al.*, 1986; Baeyens *et al.*, 1987) by a mechanism that differs from that of α_2-adrenoceptor agonists.

In order to compare the effect of clonidine on the abstinence signs with that of the calcium entry blockers we have evaluated the frequency of presentation of a contractile response after *in vitro* naloxone administration to the myenteric plexus-longitudinal muscle strips (MP-LM) obtained from guinea-pigs chronically treated with morphine. This contractile response represents a morphine withdrawal response (Ehrenpreis *et al.*, 1972; Schulz *et al.*, 1982).

Methods

Tissues were obtained from the small intestine caudal to the gastroduodenal junction of adult guinea-pigs weighing 250–400 g, fasted 24 h and killed by decapitation. MP-LM preparations were prepared from 5 cm segments (Ambache, 1954) and suspended in an organ bath containing 20 ml of Krebs solution (NaCl 118, KCl 4.75, K_2HPO_4 1.2, $CaCl_2$ 2.52, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 11.1 mM) at 34°C, aerated with 95% O_2 and 5% CO_2. A resting tension of 1 g was applied to each tissue. Contractions were elicited by electrical stimulation with a Grass SD9 stimulator (single square-wave pulses: 2 ms duration, 0.3 Hz and supramaximal voltage), or naloxone (1 μM) and detected with a Grass FT03 force displacement transducer and recorded on a Grass polygraph. Strips were allowed to equilibrate for 15 min before control measurements were taken.

Animals were made morphine-dependent by a single subcutaneous injection of a slow release suspension of morphine 200 mg kg^{-1} as described previously (Frederickson *et al.*, 1976). Four days after treatment the guinea-pigs were killed and the terminal ileum was removed for the *in vitro* study. Control animals were treated with the non-opiate containing vehicle. The *in vitro* withdrawal was precipitated by addition of naloxone (1 μM) to the organ bath. The naloxone effect was tested on the MP-LM strips obtained from the following groups of guinea-pigs: control animals; non-dependent animals, treated with clonidine, diltiazem or nimodipine; dependent animals; dependent animals treated with clonidine, diltiazem or nimodipine. Tissues obtained from dependent guinea-pigs were bathed in Krebs solution containing morphine (0.1 μM).

Intraperitoneal administration of clonidine (0.3 mg kg^{-1}), diltiazem (20 mg kg^{-1}) or nimodipine (5 mg kg^{-1}) were made 1 h before killing. The naloxone-induced contraction was also evaluated in morphine-tolerant preparations incubated for 15 min with clonidine (0.01, 0.1 and 1 μM), nimodipine (0.05, 0.1 and 1 μM) or diltiazem (0.25, 0.5 and 1 μM).

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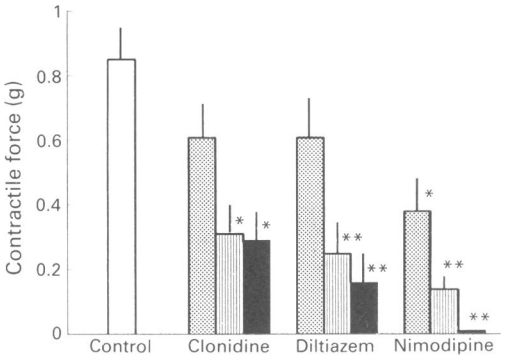

Figure 1 Effect of incubation with clonidine (0.01, 0.1 and 1 μM) diltiazem (0.25, 0.5 and 1 μM) and nimodipine (0.05, 0.1 and 1 μM) on the contraction induced by naloxone (1 μM) in MP-LM strips obtained from morphine-tolerant guinea-pigs. Open column-control (non-incubated), stippled columns lowest dose, lined columns middle dose, closed columns highest dose of each drug tested. Each column is the mean value of at least 9 preparations. Vertical bars represented s.e.mean. $*P < 0.05$; $**P < 0.01$ compared with control (non-incubated) preparations.

The drugs used were: clonidine (Boehringer Ingelheim, S.A. Barcelona, Spain), diltiazem (Esteve, S.A. Barcelona, Spain), morphine HCl (Alcaliber, S.A. Spain), naloxone (Sigma Chemical Co. St. Louis, MO, USA) and nimodipine (Bayer Leverkusen, Germany).

The force (g) of the naloxone-induced contractile response was measured in each group of guinea-pigs and compared with the observed responses in morphine-dependent preparations. Statistically significant differences were determined by Student's t test and by ANOVA test.

Results

The contractile response of the MP-LM strips induced by electrical stimulation was inhibited in a dose-dependent manner when morphine (0.1, 0.2, 0.4, 0.8 μM) was cumulatively administered to the organ bath. When tissues were obtained from guinea-pigs treated with morphine for 4 days the inhibitory effect of added morphine was greatly reduced. The % inhibition for each dose was: in control preparations ($n = 10$): 35.7 $\pm$ 3.4, 60.2 $\pm$ 3.9, 75.4 $\pm$ 4.3, 83.3 $\pm$ 2.3 and in morphine-tolerant preparations ($n = 10$): 2.8 $\pm$ 0.9, 5.8 $\pm$ 1.6, 16.1 $\pm$ 3.7, 30.1 $\pm$ 5.3, the difference being statistically significant ($P < 0.001$).

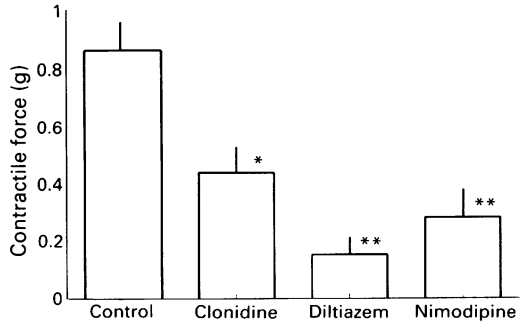

Figure 2 Contractile force of the naloxone-induced contraction in guinea-pig ileum obtained from morphine-dependent animals. Animals were pretreated with saline (control), clonidine (0.3 mg kg^{-1}), diltiazem (20 mg kg^{-1}) or nimodipine (5 mg kg^{-1}). Each column is the mean of at least 9 preparations. Vertical bars represent s.e.mean. $*P < 0.05$; $**P < 0.01$.

The amplitude of electrically-evoked twitches did not differ between control tissues and those chronically treated with morphine.

The addition of naloxone (1 μM) to the organ bath induced a contractile response in all MP-LM strips removed from morphine-treated guinea-pigs. Ileal segments from naive animals did not respond to naloxone.

When morphine-dependent preparations were incubated for 15 min before addition of naloxone, with clonidine (0.01, 0.1 and 1 μM), diltiazem (0.25, 0.5 and 1 μM) or nimodipine (0.05, 0.1 and 1 μM) the contractile response induced by naloxone was inhibited in a dose-dependent manner (Figure 1). Clonidine, diltiazem and nimodipine did not affect electrically-evoked twitches when added to the organ bath, either in control-tissues or in dependent-tissues at the doses tested.

A decreased sensitivity to naloxone was also found in the MP-LM strips obtained from morphine-dependent guinea-pigs pretreated, 1 h before killing, with clonidine (0.3 mg kg^{-1}), diltiazem (20 mg kg^{-1}) or nimodipine (5 mg kg^{-1}) (Figure 2). The amplitude of contraction induced by electrical stimulation did not differ between control tissues and those obtained from animals to which clonidine, diltiazem and nimodipine had been administered.

Discussion

It is well known that MP-LM preparations obtained from guinea-pigs chronically treated with morphine, develop tolerance to the inhibitory effect of morphine on the electrically-induced contractions. Our results agree with previous reports (Goldstein & Schultz, 1973). Moreover, in preparations of guinea-pig small intestine tolerant to morphine, naloxone induces a withdrawal sign consisting of a contracture of the longitudinal muscle. This abrupt contracture is produced by an increased release of acetylcholine (Frederickson *et al.*, 1976; Schulz *et al.*, 1985) and substance P (Chahl, 1983). Furthermore, withdrawal-induced electrophysiological events have been recorded in myenteric neurones. The experiments of Johnson *et al.* (1987) provided direct evidence for a naloxone-induced depolarization of the neuronal membrane. It is generally accepted that the typical symptoms of morphine withdrawal are caused by a rebound neuronal hyperactivity in the central and peripheral nervous systems.

The morphine abstinence symptoms can be effectively suppressed by administering clonidine. This drug has the ability to decrease noradrenaline (NA) release in the brain (Taylor *et al.*, 1988) and it has been used in the treatment of opioid withdrawal in man (Washton & Resnick, 1980). The main effect of clonidine on the withdrawal syndrome seems to be mediated by a decrease in NA release from locus coeruleus neurones. Nevertheless, clonidine also induces peripheral effects, like the inhibition of the neural activity in the guinea-pig ileum decreasing acetylcholine release (Malta *et al.*, 1981; Ilhan & Long, 1985), and the inhibition of the contractile response induced by naloxone in the morphine-dependent MP-LM preparation (Collier *et al.*, 1981).

On the other hand, after chronic morphine treatment the binding of $^{45}Ca^{2+}$ to synaptic vesicles was increased and a large increase in the content of calcium in the brain has been described. During the naloxone-precipitated withdrawal syndrome these elevated calcium levels return toward control values (Yamamoto *et al.*, 1978). The effects of calcium channel blockers on the behavioural signs of naloxone-induced abstinence in animals have been studied previously (Bongianni *et al.*, 1986; Baeyens *et al.*, 1987); however, the mechanism underlying this has not been elucidated and it is possible that central and peripheral sites of action could be implicated. In fact, in MP-LM strips, calcium entry blockers antagonize the contractile response induced by electrical stimulation and this effect has been attributed to a decrease of calcium entry into the smooth muscle through voltage-dependent channels (Martin *et al.*, 1990). The release of acetylcholine, induced by

electrical stimulation, was not affected by nifedipine in control preparations (Gilbert & Tucker, 1989); however, these authors found, in morphine-withdrawn tissues, that nifedipine significantly attenuated the electrically-induced release of acetylcholine, and suggested that this effect may be explained by changes in the number and/or characteristics of the voltage-operated calcium channels in the nerve endings of myenteric neurones.

The present work shows that when clonidine, diltiazem and nimodipine were administered *in vivo* to morphine-dependent guinea-pigs, or when morphine-dependent tissues were incubated with them, the *in vitro* withdrawal contracture was significantly decreased and this effect was dose-dependent.

These findings provide evidence to support the peripheral anti-withdrawal effects that have been suggested (Franz *et al.*, 1982; Baeyens *et al.*, 1987). All of the drugs studied are able to decrease the withdrawal contracture in morphine-dependent tissues, even though this decrease was associated with different mechanisms: clonidine activates α_2-presynaptic adrenoceptors and calcium antagonists reduce the entry of calcium into the neurones by blocking voltage-dependent calcium channels. Since calcium influx is an essential factor in neurotransmitter release, the calcium antagonist effect observed could be due to a blockade of the release of acetylcholine or substance P. Therefore, our results suggest that the effectiveness of the evaluated drugs in preventing *in vitro* naloxone withdrawal could be better attributed to an action at a neural site rather than a muscular site.

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Differential effects of calcium antagonists and Bay K 8644 on contractile responses to exogenous noradrenaline and adrenergic nerve stimulation in the rabbit ear artery

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1 The effects of three calcium antagonists (nifedipine, verapamil, diltiazem) and the calcium agonist Bay K 8644 were compared on contractile responses of similar amplitude elicited by noradrenaline (NA) and electrical nerve stimulation (ENS) in the rabbit isolated ear artery.

2 Contractions induced by both NA (3×10^{-7} M) and ENS (10 Hz, 10 s) were almost exclusively mediated by α_1-adrenoceptors, since 10^{-7} M prazosin abolished (NA) or almost abolished (ENS) the responses, and prazosin was more than three orders of magnitude more potent than rauwolscine on both types of response.

3 ENS-induced contractions were considerably less inhibited by nifedipine, verapamil and diltiazem than were those elicited by NA. Bay K 8644 enhanced responses to NA more than those to ENS.

4 The inhibitory effect of nifedipine and Ca^{2+} deprivation on NA-induced contractions decreased with increasing NA concentration. Reduction of the NA response by prazosin or phenoxybenzamine increased the nifedipine inhibition.

5 Reduction of the ENS-induced contractions by prazosin or phenoxybenzamine, or by use of a lower stimulation frequency did not increase the inhibitory effect of nifedipine.

6 In conclusion, the differential effects of the calcium antagonists on NA- and ENS-induced contractions were not related to differences in α-adrenoceptor subtype (α_1/α_2), receptor reserve or response amplitude, but may rather reflect temporal and spatial differences in α-adrenoceptor activation between the responses.

Introduction

Organic calcium antagonists and α-adrenoceptor blockers (non-selective and α_1-subtype selective) are effective antihypertensive agents. Orthostatic side effects are, however, more frequently encountered with α-adrenoceptor blockers than with calcium antagonists. A conceivable explanation may be that reflex sympathetic vasoconstriction is better preserved during treatment with the latter than with the former group of drugs.

In line with this notion, experimental studies on pithed rats have shown that calcium antagonists more effectively antagonize vasopressor responses to exogenous noradrenaline (NA) than those to sympathetic nerve stimulation (Pedrinelli & Tarazi, 1984). However, in studies on perfused tissues, calcium antagonists seem to affect vasoconstrictor responses to NA and electrical nerve stimulation (ENS) to a similar degree (Armstead *et al.*, 1987; Lippton *et al.*, 1987; Kadowitz *et al.*, 1988). In the rabbit isolated ear artery (REA), supplied with a dense adrenergic innervation, contractions elicited by ENS were even more sensitive to calcium antagonists than were those induced by NA (Kajiwara & Casteels, 1983). However, inhibitory effects were observed only at high calcium antagonist concentrations, and the relative amplitudes of the responses to NA and ENS were not defined.

A body of information suggests that intracellular Ca^{2+} release, in contrast to Ca^{2+} influx, contributes comparatively more to α_1- than α_2-adrenoceptor-mediated vasoconstriction in arteries (see Van Meel, 1982). It is also known that the α-adrenoceptor subtype located intra- and extrajunctionally vary considerably between different vascular preparations (Langer *et al.*, 1980; 1981; Yamaguchi & Kopin, 1980; Starke & Docherty, 1982; McGrath, 1982; Gardiner & Peters, 1982; Elsner *et al.*, 1984; Hicks *et al.*, 1984).

In the present study, the effects of three calcium antagonists (nifedipine, verapamil, diltiazem) and the calcium agonist Bay K 8644 were examined on responses elicited by NA and ENS in the isolated REA. The α-adrenoceptors stimulated by exogenous NA and neuronally released NA were characterized pharmacologically by use of selective α_1- and α_2-adrenoceptor agonists and antagonists. Since the inhibitory effects of CAs generally decrease with increasing NA concentration and consequently with the contraction amplitude (cf. van Breemen *et al.*, 1982b), drug effects were evaluated on contractions of similar amplitude induced by NA and ENS in the present study.

Methods

Female albino rabbits of the Danish Land strain were killed by air injection into an auricular vein. The middle third of the central ear artery with an inner diameter of 0.4–0.5 mm was rapidly dissected out under microscope. All dissections were performed in cold (8–10°C) Krebs solution of the following composition (in mM): NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 15, NaH_2PO_4 1.2, (+)-glucose 6.0. The arteries were divided into 2 mm long segments. Each segment was mounted on two L-shaped metal holders (0.2 mm diameter) in a temperature-controlled (37°C) organ bath containing 5 ml Krebs solution. The solution was continuously bubbled with 95% O_2 and 5% CO_2, resulting in a pH of approximately 7.4. One of the metal holders was fixed to a movable unit for adjustments of resting tension and the other was attached to a tension transducer (Grass FT03C). Detailed information on

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the experimental method has been given previously (Högestätt *et al.*, 1983; Skärby *et al.*, 1983).

During an equilibration period of 1 h, the resting tension was gradually increased to approximately 4 mN. Each preparation was then contracted by 10^{-4} M NA (giving a maximum response, see Figure 3) and the response was used as an internal reference. Agonist concentration-response relationships were obtained by exposure to increasing drug concentrations in a cumulative manner (i.e. the next drug concentration was introduced when the effect of the former reached a plateau). The effects of α-adrenoceptor antagonists, calcium antagonists and Bay K 8644 on NA-induced contractions were evaluated according to the following protocol. The preparations were repeatedly contracted by 3×10^{-7} M or 10^{-5} M NA with 30 min intervals, each NA exposure lasting for 5–10 min. When two reproducible contractions were obtained (difference <10%), the lowest drug concentration was introduced 15–20 min prior to the next NA application. This sequence was repeated with increasing drug concentrations. As shown in parallel control experiments with NA alone, the NA-induced contractions remained reproducible throughout the experimental period.

Electrical nerve stimulation was produced by a Grass S48 stimulator connected to two platinum wire electrodes placed immediately below and above each vessel segment. Square wave pulses with a duration of 0.3 ms were delivered with a frequency of 5 or 10 Hz in 10 s trains at 2.5 min intervals. The electrode polarity was changed after each pulse. The voltage was adjusted to give a maximum response in each experiment. When 6–10 reproducible contractions were obtained (difference <10%), the various drugs were added into the organ bath in a cumulative manner. Control experiments were always run in parallel. In a few experiments, preparations were stimulated continuously for several minutes with a pulse frequency of 10 Hz.

Drugs

(±)-Noradrenaline HCl, (−)-phenylephrine HCl (Sigma), rauwolscine HCl (Roth) and clonidine (Boehringer Ingelheim) were dissolved in 0.9% NaCl containing 1.0 mM ascorbic acid. Prazosin (Pfizer) was dissolved in 1% methanol and 1 mM HCl, whereas Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) (Bayer) and phenoxybenzamine were dissolved in ethanol, giving stock solutions of 1 mM. Verapamil HCl (Knoll) and diltiazem HCl (Tanabe) were dissolved in water. Stock solutions were stored at −70°C. Nifedipine (Bayer) was provided in ampoules containing 0.1 mg ml^{-1} nifedipine in 150 mg ml^{-1} ethanol, 150 mg ml^{-1} polyethylene glycol and water. Bay K 8644 and nifedipine were kept in the dark until use to avoid light-induced degradation. Drug dilutions were made with 0.9% NaCl containing 1 mM ascorbic acid.

Calculation and statistics

The maximum drug effect (E_{max}) and the drug concentration producing 50% of E_{max} (EC_{50}) were calculated for each drug when possible. pEC_{50} denotes the negative logarithm of EC_{50}. Results given in the text, tables and figures are expressed as mean ± s.e., followed by the number of experiments performed (n). Student's t test was used to determine differences between groups of data. Analysis of variance was performed when multiple comparisons were made. A probability level <0.05 was accepted as significant.

Results

Responses to noradrenaline

The NA-induced contraction was generally biphasic with an initial rapid component and an ensuing tonic component, reaching a maximum within 5–10 min. When related to the tonic component, the initial rapid response, where distinguishable, was considerably smaller at a concentration of 3×10^{-7} M NA than at 10^{-5} M or 10^{-4} M NA (Figure 1a). The maximum response to 3×10^{-7} M NA was 51 ± 3% (n = 38) of that to 10^{-4} M NA. Incubation in Ca^{2+}-depleted medium (with 10^{-4} M EGTA) for 10 min reduced the contractile responses to 3×10^{-7} M and 10^{-5} M NA by 87 ± 4% (n = 9) and 72 ± 6% (n = 8), respectively, leaving only a transient response. The difference in reduction between the two NA concentrations was statistically significant.

Responses to electrical nerve stimulation

Continuous ENS (10 Hz) also elicited a biphasic contraction similar to the exogenous NA response, although the second component gradually declined with time (Figure 1b). The initial rapid component was generally fully developed within 10 s. The second component reached a maximum after 1–3 min.

Electrical nerve stimulation (10 Hz) for 10 s periods every 2.5 min elicited reproducible phasic contractions (Figure 2). The contractions amounted to 37 ± 2% (n = 42) of the 10^{-4} M NA response. Tetrodotoxin (3×10^{-7} M) reduced the ENS-induced responses by 96 ± 1% (n = 27), and a few min incubation in Ca^{2+}-free medium (with 10^{-4} M EGTA) abolished them (n = 5).

Effects of α-adrenoceptor agonists

Noradrenaline, phenylephrine and clonidine elicited concentration-dependent contractions in the REA with pEC_{50} values of 6.26 ± 0.17 (n = 4), 6.54 ± 0.17 (n = 5) and

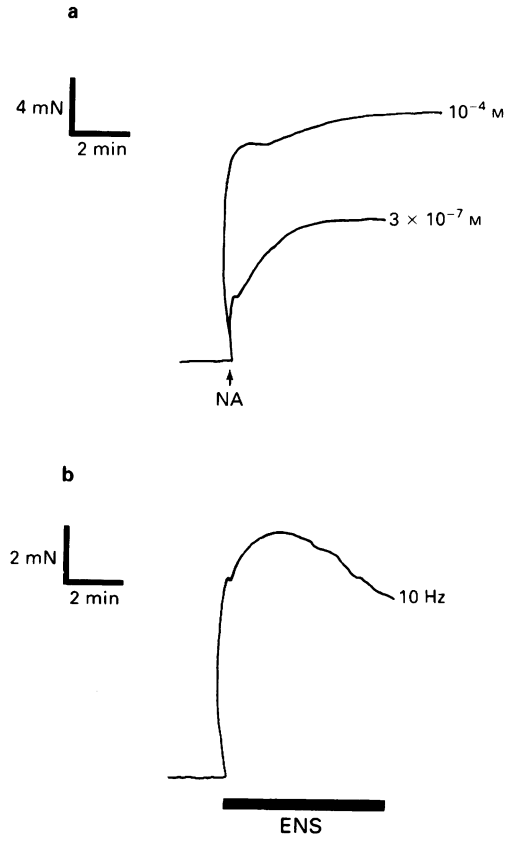

Figure 1 Contractile responses to noradrenaline (NA) and electrical nerve stimulation (ENS). (a) Superimposed tracings of contractions elicited by two NA concentrations in the same arterial segment; (b) shows the response to continuous ENS.

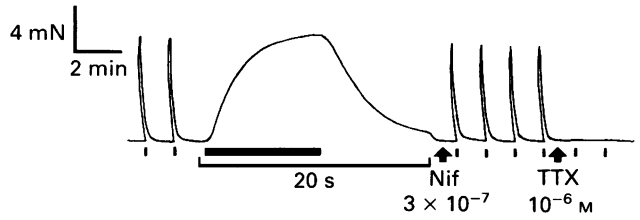

Figure 2 Tension tracing showing the effects of nifedipine (Nif) and tetrodotoxin (TTX) on responses induced by repeated electrical nerve stimulation (10 Hz, 10 s). Bars below tracings indicate the stimulation periods. The time scale was expanded during the third contraction to illustrate the time course of the response.

6.61 ± 0.11 (n = 6), respectively. The potencies of the agonists did not differ significantly. E_{max} for phenylephrine and clonidine were 108 ± 2.5% (n = 5) and 64 ± 10% (n = 6) of the 10^{-4} M NA-induced contraction, respectively (Figure 3).

Effects of α-adrenoceptor antagonists

Prazosin (10^{-6} M) reduced the contractile responses to repeated 10 s ENS (10 Hz) by 95 ± 1% (n = 6) and abolished those to 3×10^{-7} M NA. The ENS responses were enhanced by 10^{-8}–10^{-6} M rauwolscine, resulting in a biphasic concentration-response curve with an enhancement at low and inhibition at high ($\geq 10^{-5}$ M) concentrations (Figure 4). Prazosin was 3300 and 4500 times more potent than rauwolscine in inhibiting contractions elicited by NA and ENS, respectively (Figure 4, Table 1).

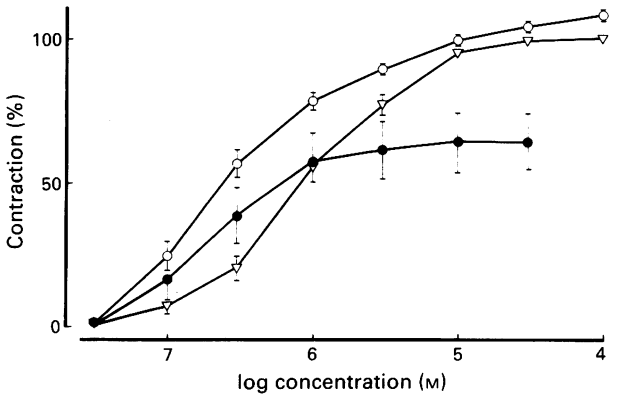

Figure 3 Concentration-response curves to noradrenaline (NA) (▽), clonidine (●) and phenylephrine (○). Contractions are expressed as a percentage of those induced by 10^{-4} M NA. Each point represents mean of 4–6 experiments; s.e.mean shown by vertical bars.

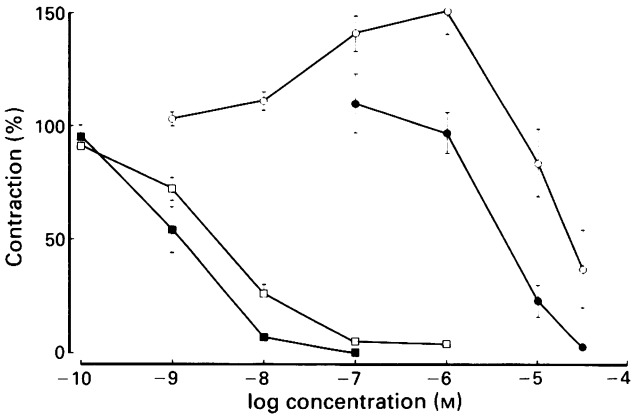

Figure 4 Concentration-inhibition curves showing the effects of prazosin (■, □) and rauwolscine (●, ○) on contractions elicited by 3×10^{-7} M noradrenaline (filled symbols) and 10 Hz electrical nerve stimulation (open symbols). Contractile responses are expressed as a percentage of those obtained before drug administration. Each point represents mean of 4–7 experiments; s.e.mean shown by vertical bars.

Effects of Bay K 8644 and calcium antagonists

None of the calcium antagonists nor Bay K 8644 *per se* affected the baseline tension of unstimulated preparations. However, during repeated 10 s ENS (10 Hz), Bay K 8644 induced a small but concentration-dependent tonic contraction, amounting to 13 ± 3% (n = 8) of the 10^{-4} M NA response. Bay K 8644 enhanced and nifedipine inhibited contractions induced by NA (3×10^{-7} M) significantly more than those induced by ENS (10 Hz, 10 s). The effects of nifedipine and Bay K 8644 on the ENS responses were too small to allow calculation of pEC_{50} (Figure 5a, Table 1).

Verapamil was 46 times and diltiazem 60 times more potent in inhibiting responses induced by NA than those elicited by ENS. At a concentration of 10^{-4} M, both verapamil and diltiazem abolished the ENS responses (Figure 5b and c, Table 1). Approximately 30% of the NA-induced contraction remained in the presence of 10^{-4} M diltiazem or 3×10^{-6} M nifedipine, whereas 10^{-4} M verapamil almost abolished the response.

Nifedipine (3×10^{-7} M) reduced the response to 3×10^{-7} M NA (64 ± 4%, n = 6) significantly more than that to 10^{-5} M NA (21 ± 4%, n = 5). The NA-induced contractions were sustained in the presence of nifedipine, whereas they were transient in Ca^{2+}-free medium (see above). Reduction of the

Table 1 Effects of α-adrenoceptor blockers, calcium antagonists and Bay K 8644 on contractile responses evoked by 3×10^{-7} M noradrenaline and electrical nerve stimulation (10 Hz, 10 s)

	Noradrenaline			*Electrical nerve stimulation*		
	pEC_{50}	E_{max}	n	pEC_{50}	E_{max}	n
Prazosin	8.96 ± 0.15	−100 ± 1	6	8.50 ± 0.09	−95 ± 1	6
Rauwolscine	5.44 ± 0.13	−97 ± 1	7	4.85 ± 0.11[a]		4
Verapamil	6.51 ± 0.25	−93 ± 1	6	4.85 ± 0.12	−100 ± 1	5
Diltiazem	6.45 ± 0.22	−72 ± 7[b]	5	4.67 ± 0.04	−99 ± 1	5
Nifedipine	8.23 ± 0.13	−67 ± 3	6		−10 ± 3	7
Bay K 8644	8.71 ± 0.28	+66 ± 24	6		+11 ± 4	8

The sign preceding each E_{max} value indicates inhibition (−) or enhancement (+) of contraction.

[a] Calculations based on a presumed E_{max} of 100%.

[b] The inhibition produced by 10^{-4} M diltiazem was accepted as E_{max}, although the concentration-response curve did not reach a clear plateau.

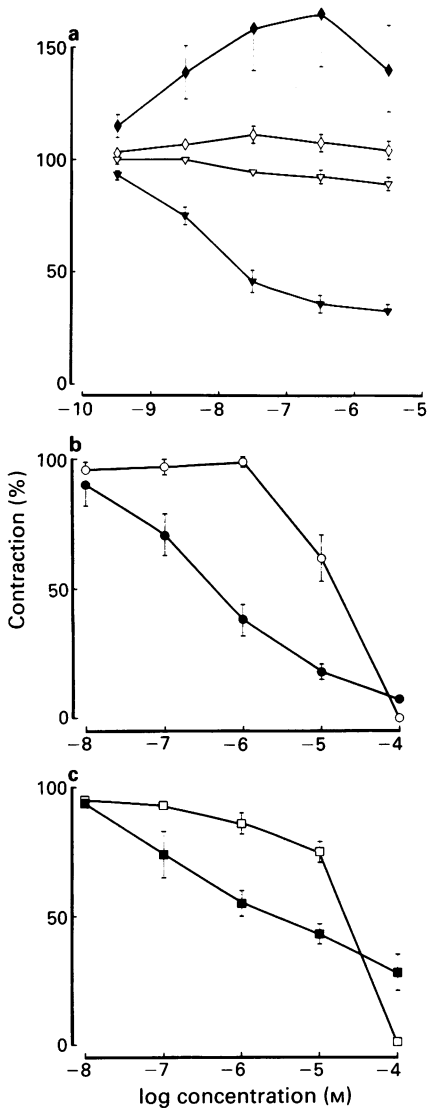

Figure 5 Effects of (a) nifedipine (▼, ▽) and Bay K 8644 (◆, ◇), (b) verapamil (●, ○) and (c) diltiazem (■, □) on contractions elicited by 3×10^{-7} M noradrenaline (filled symbols) and 10 Hz electrical nerve stimulation (open symbols). Contractile responses are expressed as a percentage of those obtained prior to drug administration. Each point represents mean of 4–8 experiments; s.e.mean shown by vertical bars.

10^{-5} M NA response by prazosin (1×10^{-7}–3×10^{-7} M) or phenoxybenzamine (Pbz) (1×10^{-8}–3×10^{-8} M, 5 min pretreatment) to a level similar to the 3×10^{-7} M NA response, significantly augmented the inhibitory effect of nifedipine (Figure 6). Furthermore, the inhibition produced by 3×10^{-7} M nifedipine did not differ between contractions induced by 3×10^{-7} M NA and those elicited by 10^{-5} M NA after α-adrenoceptor blockade by prazosin or Pbz. The contraction elicited by 5 Hz ENS was, however, not more affected by nifedipine than was that induced by 10 Hz, despite the fact that the response to 5 Hz was only 38 ± 4% ($n = 5$) of that to 10 Hz. Reduction of the 10 Hz ENS response to a level similar to the 5 Hz ENS response by prazosin (3×10^{-8}–10^{-7} M) or phenoxybenzamine (3×10^{-8}–10^{-7} M, 5 min pretreatment) did not enhance the effect of nifedipine. The results from these experiments are summarized in Figure 6.

Discussion

The REA is endowed with a dense adrenergic innervation and promptly constricts in response to perivascular nerve stimulation (Waterson & Smale, 1967; Steinsland *et al.*, 1973; 1985).

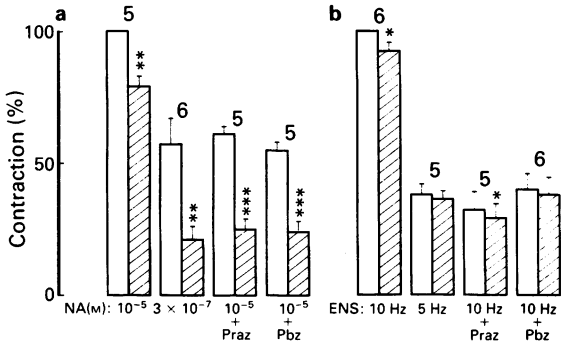

Figure 6 Effects of 3×10^{-7} M nifedipine (hatched columns) on contractions elicited by noradrenaline (NA) or repeated electrical nerve stimulation (ENS) in the presence and absence of partial α-adrenoceptor blockade by prazosin (Praz) or phenoxybenzamine (Pbz). Contractile responses are expressed as a percentage of those obtained by 10^{-5} M noradrenaline (a) or 10 Hz ENS (b) in the absence of inhibitors. Open columns, controls. Each column represents mean with s.e. shown by vertical bars. The numbers of experiments are given above each pair of columns. Asterisks indicate significant inhibition by nifedipine (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). For further explanation, see Results.

The contractile response was markedly reduced or abolished by superior cervical ganglionectomy or guanethidine (De la Lande & Rand, 1965). A significant phentolamine-resistant neurogenic contraction has, however, been demonstrated in small branches of the REA (Owen *et al.*, 1985). The ENS-induced contractions in the present study were Ca^{2+}-dependent and almost abolished by tetrodotoxin or α-adrenoceptor blockade, indicating their adrenergic origin.

Whether activated by exogenous NA or neuronally released NA, the α-adrenoceptor mediating the contractile response appeared to be mainly of the α_1-type. This conclusion, which agrees well with the results reported by others (Manzini *et al.*, 1983; Hieble & Woodward, 1984), was based on the following findings. Prazosin was more than three orders of magnitude more potent than rauwolscine in inhibiting contractions elicited by both NA and ENS. Prazosin at a concentration of 10^{-7} M, which in radioligand binding studies has virtually no effect on α_2-adrenoceptors (Hoffman & Lefkowitz, 1980; Andersson *et al.*, 1984), abolished (NA) or almost abolished (ENS) the responses. Inhibitory effects of rauwolscine were observed only at high concentrations ($>10^{-6}$ M) lacking α_2-adrenoceptor selectivity. The enhancement of the ENS-induced contractions by 10^{-8}–10^{-6} M rauwolscine rather reflects antagonism of prejunctional α_2-adrenoceptors (see Skärby & Larsson, 1987). The relative potencies of phenylephrine and clonidine are also consistent with an α_1-adrenoceptor (see Starke, 1981).

As clearly shown in the present study, contractile responses to exogenous NA were significantly more sensitive to nifedipine, verapamil and diltiazem than were those induced by ENS. Bay K 8644, which promotes Ca^{2+} influx by interacting with dihydropyridine receptors on the Ca^{2+} channels (Spedding, 1985), substantially augmented the NA-induced contraction, whereas those elicited by ENS were less affected. Obviously, these differential effects cannot be attributed to a heterogeneous distribution of α_1- and α_2-adrenoceptors on the vascular smooth muscle cells. It has, however, been suggested that the receptor reserve, rather than the α-adrenoceptor subtype, is a major determinant of intracellular Ca^{2+} release (see Ruffolo & Nichols, 1988). This concept is gaining wide support, and results obtained in several studies have shown that responses to α_1-adrenoceptor stimulation become sensitive to calcium antagonists only after reduction of the receptor reserve or by using partial agonists (Ruffolo *et al.*, 1984; Pedrinelli & Tarazi, 1985; Jim *et al.*, 1986; Wanstall & O'Donnell, 1988; Bognar & Enero, 1988). The REA contains a large α-adrenoceptor reserve, and half-maximum contraction is achieved at 1% receptor occupancy (Purdy *et al.*, 1983). As shown in the present study, reduction of the α-adrenoceptor

number by the irreversible receptor blocker Pbz did not increase the inhibitory effect of nifedipine on the ENS-induced contraction, whereas this pretreatment enhanced the inhibition of the 10^{-5} M NA-induced response. Thus, the differential effects of the calcium antagonists on contractions induced by exogenous and endogenous NA do not appear to be related to differences in receptor reserve. Interestingly, the response to 10^{-5} M NA after Pbz pretreatment was not more inhibited by nifedipine than was that obtained in the presence of prazosin, although the two α-adrenoceptor blockers reduced the NA response to the same extent (see Figure 6). This finding does not favour the view that the α-adrenoceptor reserve is of crucial importance for the calcium antagonist sensitivity in this preparation.

Nifedipine and Ca^{2+} depletion reduced the contractile response to 3×10^{-7} M NA significantly more than that to 10^{-5} M NA. This correlates well with previous studies showing that contractions induced by high NA concentrations are more dependent on intracellular Ca^{2+} release than are those elicited by low NA concentrations (Casteels *et al.*, 1977) and consequently less inhibited by calcium antagonists (van Breemen *et al.*, 1982b). Indeed, NA-induced intracellular Ca^{2+} release was shown to have a 10 fold higher threshold than stimulation of Ca^{2+} influx in the rabbit aorta (van Breemen *et al.*, 1982a). In the present study, reduction of the 10^{-5} M NA response by 50%, with prazosin or Pbz, considerably increased the inhibitory effect of nifedipine. Thus, it appears that the larger the NA-induced contraction, the smaller the calcium antagonist sensitivity. The ENS-induced contraction, however, turned out to be slightly smaller than the response to 3×10^{-7} M NA; still it was considerably less affected by the calcium antagonists.

Prazosin was approximately three times less potent in inhibiting contractions induced by ENS than those elicited by 3×10^{-7} M NA. Assuming equilibrium at the receptor level, this would predict an intrajunctional NA concentration of about 10^{-6} M according to receptor theory. However, since equilibrium conditions for NA are probably not obtained during a short period of ENS, leading to an overestimation of the prazosin-induced inhibition, the intrajunctional NA concentration may be even higher than 10^{-6} M (cf. Neild & Zelcer, 1982; Bevan, 1984). In contrast to the response to exogenous NA, a 70% reduction of the ENS-induced contraction by prazosin or Pbz, or by use of a lower stimulation frequency (5 Hz) did not enhance the inhibitory effect of nifedipine. This may indicate that contractions evoked by endogenous NA rely almost entirely on intracellular Ca^{2+} release irrespective of the response magnitude, although Ca^{2+} influx through calcium antagonist-insensitive membrane channels cannot be excluded.

As shown in a previous study on the perfused REA (Steinsland *et al.*, 1973), the contractile responses to NA and continuous ENS were found to be distinctly biphasic, composed of an initial rapid component, reaching a maximum within 10–15 s, and a second tonic component developing over several minutes. This largely agrees with the results obtained in the present study. Furthermore, the initial component of the NA response was rendered unaffected by several procedures interfering with Ca^{2+} influx in contrast to the tonic component (Steinsland *et al.*, 1973). Similar findings have been reported for several other arteries, suggesting that the initial rapid and the ensuing tonic component are due to intracellular Ca^{2+} release and Ca^{2+} influx, respectively (Bolton 1979; van Breemen *et al.*, 1982b; Skärby *et al.*, 1984). In a more recent study, the initial rapid response to ENS (3 Hz) was shown to be relatively resistant to verapamil and especially nitrendipine, whereas the tonic component was highly sensitive to these agents (Steinsland *et al.*, 1985). Since in the present study the preparations were stimulated for only 10 s, the effects of the calcium antagonists on the tonic ENS response were not evaluated. It would thus appear that the initial rapid component of the ENS response was compared with the tonic component of the NA response in the present study. The relevance of these components for α-adrenoceptor activation *in vivo* is, however, unclear. Based on microelectrode recordings from human sympathetic nerves *in vivo*, it was suggested that the sympathetic outflow to muscles is important for buffering temporary changes in blood pressure. The sympathetic impulses mostly appeared in high frequency bursts during 1–3 s with silent intervals of several seconds (see Wallin, 1981). In an attempt to reproduce this discharge pattern, the REA was stimulated with trains (2 s long) of pulses (25 Hz) every 5 s. This resulted in a sustained contraction, which was not appreciably affected by nifedipine (unpublished observations). It is conceivable that this type of intermittent ENS as well as short period ENS lead to a rapid and intense α-adrenoceptor stimulation restricted to membrane patches adjacent to the nerve terminals, and hence intracellular Ca^{2+} release, whereas exposure to exogenous NA activates α-adrenoceptors over almost the entire cell surface during a longer period of time, thus favouring Ca^{2+} influx. However, other possibilities such as, e.g., α_1-adrenoceptor subtype heterogeneity and release of other neurotransmitters along with NA (e.g. adenosine triphosphate, neuropeptide Y), influencing calcium antagonist sensitivity, cannot be ruled out at present.

The sensitivity of the 3×10^{-7} M NA-induced response to the calcium antagonists, Bay K 8644 and Ca^{2+} deprivation indicates that it was highly dependent on Ca^{2+} influx. In conjunction with the present study, we also examined the inhibitory effects of the calcium antagonists on the tonic component of the 124 mM K^+-induced contraction (unpublished observations). The mean amplitude of the tonic K^+ component and the 3×10^{-7} M NA response were almost identical (52% versus 51% of the 10^{-4} M NA response). Interestingly, neither pEC_{50} for nifedipine nor those for diltiazem and verapamil differed significantly between the two types of contraction. Although early electrophysiological studies on the REA were unable to record membrane potential changes in response to NA application (Droogmans *et al.*, 1977), later investigations have shown a close correlation between NA-induced contraction and depolarization in this artery (Trapani *et al.*, 1981; Suzuki & Kou, 1983). In the light of these findings it is tempting to suggest that NA, in concentrations that release only small amounts of intracellular Ca^{2+}, can promote Ca^{2+} influx through potential-operated channels.

In agreement with the present study, contractions induced by endogenous NA were fairly insensitive to calcium antagonists in the canine saphenous vein (Takata & Kato, 1984; 1987), rabbit pulmonary artery (Zelis *et al.*, 1985), rabbit ear artery (Kajiwara & Casteels, 1983) and rat vas deferens (Brown *et al.*, 1983; Amobi & Smith, 1985). Exposure to verapamil and diltiazem concentrations below 10^{-6} M had virtually no effect on the response to endogenous NA (present study; Kajiwara & Casteels, 1983; Takata & Kato, 1984; Amobi & Smith, 1985; Zelis *et al.*, 1985). When diltiazem concentrations above 10^{-6} M were used, a parallel inhibition of contraction and transmitter release was observed (Takata & Kato, 1984; Zelis *et al.*, 1985). Thus, a prejunctional action may well explain the complete inhibition of the ENS response produced by 10^{-4} M diltiazem in the present study. The ENS-induced contraction was also abolished by 10^{-4} M verapamil. Several studies have shown an enhancement of adrenergic transmitter release by verapamil concentrations above 10^{-6} M (Larsson *et al.*, 1984; Takata & Kato, 1984). In the nerve-stimulated canine saphenous vein, 10^{-5} M verapamil produced a 50% increase of NA release but still reduced the contractile responses by 30% (Takata & Kato, 1984). These findings agree with a simultaneous pre- and postjunctional α-adrenoceptor blocking effect of verapamil. Indeed, in radioligand binding studies, verapamil has been shown to interact with both α_1- and α_2-adrenoceptors in smooth muscle with dissociation constants ranging between 0.1 and 6.8 μM (Motulsky *et al.*, 1983; Larsson *et al.*, 1984; Descombes & Stoclet, 1985; Nishimura *et al.*, 1986). Bay K 8644, on the other hand, affected neither contraction nor NA release

evoked by ENS in the canine saphenous vein (Takata & Kato, 1987). Nifedipine appears also to have only minimal effects on adrenergic transmitter release (Högestätt *et al.*, 1982; Larsson *et al.*, 1984).

It is concluded that nifedipine, verapamil and diltiazem in concentrations $\leq 10^{-6}$ M affected only marginally ENS-induced contractions in the REA, whereas those elicited by exogenous NA were effectively reduced. In contrast, prazosin was an effective inhibitor of contractions elicited by both exogenous and endogenous NA. The differential effects of the calcium antagonists were not related to differences in α-adrenoceptor subtype (α_1/α_2), receptor reserve or response amplitude, but may rather reflect temporal and spatial differences in α-adrenoceptor activation between the two types of response.

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Philanthotoxin blocks quisqualate-, AMPA- and kainate-, but not NMDA-, induced excitation of rat brainstem neurones *in vivo*

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1 The effect of electrophoretic ejection of philanthotoxin (the polyamine toxin, from the Egyptian digger wasp) was tested on responses of brainstem and spinal neurones in the pentobarbitone-anaesthetized rat to excitatory amino acids.

2 Philanthotoxin caused a dose-dependent reduction of responses to quisqualate, α-amino-3-hydroxy-5-phenyl-4-isoxazolepropionate (AMPA) and kainate with little effect on those to N-methyl-D-aspartate (NMDA).

3 The time-course of this antagonist action was slow. In particular the rate of recovery was dependent on frequency of ejection of the agonist. This agonist-dependent recovery suggests that philanthotoxin has a channel blocking mode of action on mammalian central neurones.

Introduction

Transmission at the insect neuromuscular junction is mediated via postjunctional glutamate receptors of a type not dissimilar to those activated by quisqualate and α-amino-3-hydroxy-5-phenyl-4-isoxazolepropionate (AMPA), but quite distinct from N-methyl-D-aspartate (NMDA) receptors, in mammalian brain (Boden *et al.*, 1986). Venoms of orb-web spiders, containing low molecular weight polyamine-based toxins, paralyze their insect prey by a postsynaptic block at such neuromuscular junctions (see Jackson & Usherwood, 1988 for review). Philanthotoxin (PhTx), from the Egyptian digger wasp, *Philanthus triangulum*, is similar in structure and molecular weight to some of the above spider toxins, such as argiotoxin$_{636}$ (ATX) and Joro spider toxin (JSTX). Similarly, PhTx also causes neuromuscular paralysis when injected into honeybees on which the wasp preys (Eldefrawi *et al.*, 1988). Because of our interest in non-NMDA glutamate antagonists (Honore *et al.*, 1988) and because the actions of arthropod toxin have shown variable selectivity on mammalian glutamate receptors (Jackson & Usherwood, 1988), we decided to investigate the effects of PhTx on responses of rat central neurones *in vivo* to excitatory amino acids. A preliminary abstract has been published (Jones *et al.*, 1989).

Methods

Female Wistar rats (200–350 g), anaesthetized with 50 mg kg^{-1} sodium pentobarbitone i.p. were used in all experiments (see Honore *et al.*, 1988, for general methods). Briefly, the trachea, a major artery and vein were cannulated to maintain clear airways, record blood pressure and supplement anaesthesia, respectively. The rats were placed in a stereotaxic head frame and, after exposure of the dorsal medulla, recordings were made from single brainstem neurones with seven-barreled glass microelectrodes. Alternatively, the dorsal aspect of the spinal column was exposed and a partial lumbar laminectomy performed, allowing similar recordings to be made from single dorsal or ventral horn neurones in the lumbar cord. The microelectrode centre barrel, 4 M NaCl, was used to record action potentials; five of the six outer barrels were filled with solutions of: N-methyl-D-aspartate Na (NMDA, 200 mM, pH 8.1), quisqualate Na (5 mM in 200 mM NaCl, pH 7.8), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate Na (AMPA, 10 mM in 200 mM NaCl, pH 7.5), kainate Na (5 mM in 200 mM NaCl, pH 8.2), philanthotoxin (PhTx, 50 mM in 150 mM NaCl or 10 mM in 200 mM NaCl, pH 4.0), D-2-amino-5-phosphonovalerate Na (2-AP5, 25 mM in 175 mM NaCl, pH 7.6). The sixth barrel, 200 mM NaCl, was used for current balancing.

Action potentials from single brainstem neurones in the areas of the gracile, cuneate and trigeminal nuclei or lumbar spinal cord were monitored on an oscilloscope. Location of neurones was judged by the stereotaxic position of electrodes and from their responses to tactile stimuli of hindlimb, forelimb and oro-facial areas of skin. Firing rate of neurones was plotted continuously by a pen recorder and electrophoretic ejection of amino acids was adjusted to produce approximately equal and submaximal responses. After stable control responses had been obtained, PhTx or 2-AP5 was ejected and the size of the responses as a percentage of controls (mean ± s.d.) was taken to express the antagonist effect.

Results

Stable recordings were obtained from a total of 27 brainstem and 10 spinal neurones. Whether located in the gracile, cuneate or trigeminal nuclei, all brainstem neurones gave essentially similar results and so the data have been pooled. Although no differences with respect to pharmacology were observed between brainstem and spinal neurones in this study, data from these sets of neurones have not been pooled.

Administration of PhTx (−4 to +60 nA) selectively depressed quisqualate- and kainate-evoked firing of these neurones while having little or no effect on responses to NMDA. In the first experiments, very low ejecting currents of PhTx (50 mM in 150 mM NaCl) rapidly antagonized quisqualate responses. Subsequently, a 10 mM in 200 mM NaCl solution was preferred as this allowed more adequate control over the ejection of PhTx. Quisqualate and NMDA were tested on all 27 brainstem neurones. Responses to quisqualate were reduced to 25 ± 20% (mean ± s.d.) of control values, whereas those to NMDA showed an overall increase to 114 ± 40%. Responses to kainate, tested on 13 of the 27 neurones, were reduced to 36 ± 29%. On the same 13 neurones, quisqualate responses were reduced to 27 ± 21%. PhTx did not affect either the amplitude or the duration of the spike. At higher PhTx ejection currents, responses to NMDA were occasionally seen to diminish but, despite careful adjustments of the PhTx currents, there was no consistent difference in susceptibility of quisqualate and kainate responses to antagonism. Thus, in 29 tests of PhTx on 13 cells in which the antagonism of these two agonists by PhTx was compared, responses to quisqualate were reduced 20% more than those

Table 1 Selective effects of philanthotoxin (PhTx) on responses to quisqualate and kainate

Margin of difference	*Rank order* Quis > Kain	Kain > Quis
>10%	11	9
>20%	7	6
>30%	4	5

Frequency of occurrence of differences in sensitivity of quisqualate (Quis) and kainate (Kain) responses to PhTx, from 29 tests on 13 different neurones. On each neurone control responses to quisqualate and kainate were adjusted so as to give near equal responses before the administration of PhTx. The columns of numbers represent the times the responses to one agonist were reduced to a greater extent than the other. The rows show this analysis carried out at three separate levels of difference. Thus, in the middle row it can be seen that responses to quisqualate were reduced by 20% more than those to kainate on 7 occasions, whereas the reverse selectivity at this level was seen in 6 tests.

to kainate on 7 occasions and the reverse sensitivity was observed on 6 (see Table 1). 2-AP5 was tested on several of these neurones; its selective NMDA antagonist action was rapid in onset and recovered within 5 min of the end of its ejection. On 17 tests on a further 9 spinal neurones, PhTx reduced responses to AMPA by 87 ± 15%. On 5 of these cells, kainate responses were reduced by 96 ± 9% whilst responses to NMDA on all 9 cells were not affected significantly. Figure 1 illustrates the highly selective antagonist action of PhTx against non-NMDA responses on a spinal neurone. In several cases, after complete block of non-NMDA responses had been obtained, the PhTx ejecting current was increased several fold with no effect on responses to NMDA.

The full antagonist effect of PhTx at any given current was usually apparent within 5–10 min of the start of its ejection whereas, upon cessation of PhTx ejection, recovery was slow (Figure 1). In many cells with stable recording conditions full recovery often took up to 30 min. Because of this slow recovery and small changes in the cell-to-electrode tip relationship, full recovery from the effects of PhTx was only

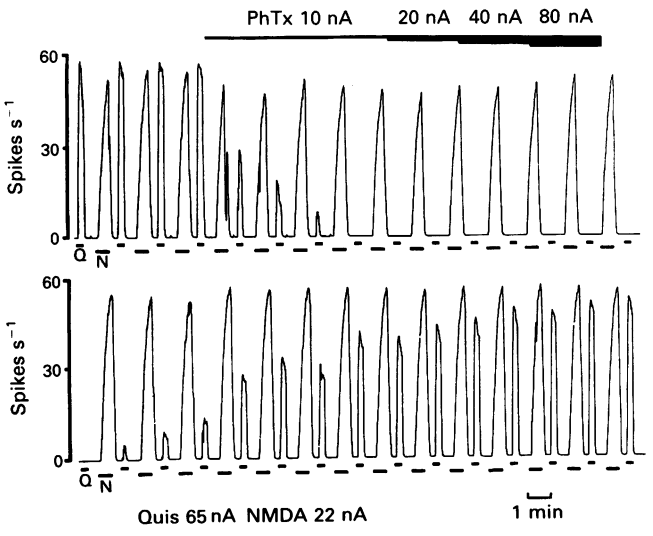

Figure 1 Ratemeter record from a single spinal neurone showing antagonism of quisqualate by philanthotoxin (PhTx) with no significant effect on responses to N-methyl-D-aspartate (NMDA). The uninterrupted upper record illustrates the gradual onset of action of PhTx (10 nA) to block the action of quisqualate (Quis, 65 nA). Further increases of ejecting current to 20, 40 and 80 nA were without effect on responses to NMDA (22 nA). The lower record shows the slow recovery from the effect of PhTx. There is a 15 min period of recording omitted between the upper and lower traces. Ordinate scale: firing rate in spikes s^{-1}.

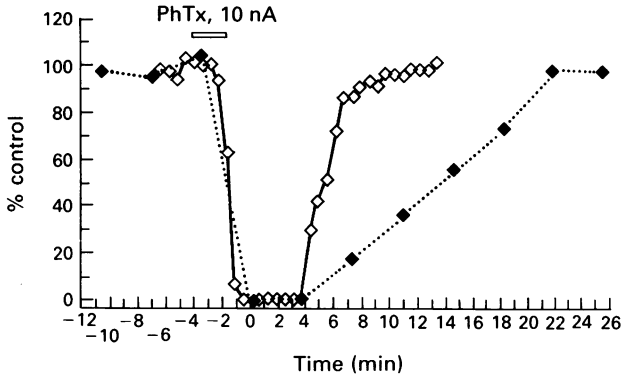

Figure 2 The difference in the time-course of recovery of responses to α-amino-3-hydroxy-5-phenyl-4-isoxazolepropionate (AMPA) 12 nA of a ventral horn spinal neurone, when the rate of agonist application is varied. In both cases AMPA responses were reduced to zero after 3–4 min application of philanthotoxin (PhTx). With fast application of AMPA (solid line; 20 s ejection, 15 s interval) the time taken for 50% recovery was approximately 4.5 min. With slower application (dotted line; 40 s ejection, 140 s interval), recovery to 50% of control took more than 13 min.

observed on about half the neurones tested. Nevertheless, the rate of recovery appeared to be dependent on the rate of agonist ejection, a result indicative of a channel blocking mode of action as suggested for its effects at the insect neuromuscular junction.

This was investigated in more detail by examining the rates of recovery with frequent or infrequent ejections of the agonist. On all cells tested, the rate of recovery from PhTx block of quisqualate or AMPA was slower for infrequent application (e.g. 20 s ejection at 3 min intervals) than for frequent application (e.g. 20 s every 20–40 s). An example of such a result, obtained from a ventral horn spinal neurone, is shown in Figure 2. This effect was not due to depolarization *per se* because frequent NMDA ejections did not hasten recovery of responses to AMPA or quisqualate.

Discussion

There are divergent views about the selectivity of the arthropod toxins for mammalian sub-types of glutamate receptors (Jackson & Usherwood, 1988), a situation which still exists in the more recent literature and to which must now be added the selectivity of PhTx toward non-NMDA subtypes of glutamate receptor observed here.

On the one hand, there is convincing evidence that they act as NMDA antagonists. Thus, the binding studies on rat brain membranes of Mena *et al.* (1989), the single cell voltage-clamp experiments (Kemp *et al.*, 1988) and whole animal experiments of Seymour & Mena (1989) all suggest that such toxins are NMDA blockers. PhTx has also been shown both to inhibit non-competitively NMDA-sensitive [^{3}H]-MK-801 binding to rat brain membranes and to reduce NMDA responses 10 times more effectively than non-NMDA responses in *Xenopus* oocytes (Ragsdale *et al.*, 1989). Furthermore, in support of an NMDA blocking action, PhTx reduces polysynaptic reflexes to a greater extent than monosynaptic reflexes in hemisected spinal cords of neonatal rats (N.A. Anis, unpublished observations).

On the other hand, other recent data provide contradictory evidence. Thus in electrophysiological studies on rat hippocampal neurones, Ashe *et al.* (1989) and Saito *et al.* (1989) demonstrated that ATX *in vitro* and JSTX *in vivo* respectively blocked glutamate-, but not aspartate-induced depolarizations of CA1 pyramidal cells. Ashe *et al.* (1989) also showed that ATX reduced Schaffer collateral-evoked field potentials, responses which are known to be mediated by non-NMDA receptors. All these effects were reversible on washing. The

present results with PhTx are in agreement with these latter studies.

In view of the structural similarity between PhTx, ATX and JSTX, we find it interesting to speculate upon whether small differences in structure may confer different selectivity for NMDA and non-NMDA receptor/channel complexes, or whether small differences in experimental protocol such as the extracellular environment may explain the apparent contradictory findings. It may be more instructive to compare several toxins on a given preparation.

With respect to the mode of action, our present results cannot provide a definitive answer, but the agonist-dependent nature of the recovery suggests that PhTx blocks and becomes trapped within the channels coupled to non-NMDA receptors. The failure of PhTx to distinguish between quisqualate and kainate is reminiscent of our results with the quinoxalinediones on spinal neurones, and supports our notion that quisqualate and kainate depolarize such central neurones by acting at similar or even common receptor-channel complexes (Honore *et al.*, 1988).

In conclusion, such toxins are potentially useful tools for investigating the pharmacology and physiology of glutamate receptor-channel complexes in the mammalian CNS and may have an important role to play in the design of useful therapeutic compounds.

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Actions of acetylcholine and GABA on spontaneous contractions of the filariid, *Dipetalonema viteae*

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1 Isotonic contractions were recorded from the filarial nematode, *Dipetalonema viteae* (*Acanthocheilonema viteae*), in an isolated tissue chamber.

2 Nicotine (10^{-6} M) and pilocarpine (10^{-5} M) increased the spontaneous contractions in the intact filariid, but acetylcholine (ACh, 10^{-4} M) and muscarine (10^{-5} M) were inactive.

3 When ACh was applied to an opened *D. viteae*, it was 10,000 times more potent. This indicates that the cuticle is an effective barrier to the penetration of ACh to the muscle cells.

4 The effects of ACh on the opened *D. viteae* were not affected by hexamethonium (10^{-3} M) or atropine (10^{-5} M) and were only partially reduced by (+)-tubocurarine (10^{-4} M).

5 γ-Aminobutyric acid (GABA, 10^{-3} M) reduced the spontaneous activity of the intact *D. viteae*; however, the effect of GABA had a slow onset and recovery. Muscimol (10^{-5} M) was more potent than GABA and had a more rapid onset and recovery.

6 GABA was 1,000 times more potent on the opened *D. viteae* than on the intact *D. viteae*. Baclofen (10^{-3} M) was inactive on both preparations.

7 The effect of GABA was not antagonized by bicuculline (10^{-4} M), picrotoxin (10^{-5} M) or penicillin G (10^{-3} M).

8 It is concluded that the filariid cuticle acts like a lipid structure and blocks the penetration of polar substances, such as ACh and GABA. Also, due to the lack of efficacy of the ACh and GABA antagonists, it was concluded that the nematode receptors are somewhat different from the mammalian ACh and GABA receptors.

Introduction

Filarial infections due to *Onchocerca*, *Wuchereria*, *Loa loa* and *Brugia* are world health problems, especially in the tropical climates (Dorozynski, 1976). However, the basic pharmacology of the filariids has not been adequately explored. There are only a few models of the human filariids. The most common pharmacological model is the intestinal nematode, *Ascaris*. Due to its intestinal localization, the clinical treatment of this nematode is quite different from the treatment of the filariids, which are localized subcutaneously or in the lymphatic system.

The body wall musculature of nematodes is involved in many essential processes, including locomotion, intestinal function, reproduction and structural support; thus disruption of muscle function would appear to be an important site of drug action. The muscle cells in the body wall of *Ascaris suum* are obliquely striated (Rosenbluth, 1965a); however, there are many physiological differences from mammalian skeletal muscle (DeBell *et al.*, 1963). Contractions appear to be initiated myogenically and the myogenic activity is probably modulated by an excitatory neurotransmitter, acetylcholine (ACh) and an inhibitory neurotransmitter, γ-aminobutyric acid (GABA) which are released by the neurones in the nerve cord (del Castillo *et al.*, 1963; 1964; Johnson & Stretton, 1985; 1987). In many ways, nematode muscle is similar, physiologically, to mammalian smooth muscle.

One filariid model is *Dipetalonema viteae* (*Acanthocheilonema viteae*). Rohrer *et al.* (1988) have described the physiological properties of single muscle cells in *D. viteae*, by use of intracellular recording methods. There were many similarities to *Ascaris*. ACh depolarized the muscle cells and increased the frequency of muscle action potentials and GABA reduced the frequency of the muscle action potentials. The depolarization by ACh, however, was not reduced by (+)-tubocurarine. Curare has been reported to reduce the responses to ACh in *Ascaris* (Norton & deBeer, 1957; del Castillo *et al.*, 1963).

Although ACh and GABA increase and decrease the frequency of muscle action potentials, respectively, the effects of these substances on the contractility of *D. viteae* have not been reported. Experiments on muscle contractility would be useful to characterize further the ACh and GABA receptors in the filariid to determine whether they are similar to the receptors in *Ascaris* and in mammalian tissues. This paper describes the effects of cholinoceptor and GABA-receptor agonists and antagonists on the spontaneous contractions of *D. viteae*. Preliminary accounts of these findings have been presented (Christ & Saz, 1986; Christ *et al.*, 1988).

Methods

Hamsters were infected with *D. viteae* larvae at the University of Georgia and shipped to the University of Notre Dame. The hamsters were killed by cervical dislocation and were kept warm and moist, while the adult *D. viteae* (4–12 months old) were removed from the subcutaneous tissues (Rohrer *et al.*, 1988). Intact worms were mounted in an isolated tissue bath (10 ml) by tying a thread to each end of the worm. One thread was attached to an isotonic transducer (Harvard Apparatus) and the other was attached to a mounting rod at the bottom of the bath. The length of the preparation was 4–5 cm, and the tension on the preparation was approximately 25 mg. The worm was bathed in a basic filarial medium (Bueding, 1949).

Other *D. viteae* were slit open, longitudinally. The reproductive and intestinal tracts were removed, before the worm was set up for the isotonic recording. The opened worms were bathed in a *Dipetalonema* body wall saline (DBWS) (Rohrer *et al.*, 1988).

The temperature was maintained at 37°C, and the chamber was bubbled with 95% N_2:5% CO_2. Agonists were added

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directly to the bath with microlitre syringes and mixing of the drugs was enhanced by the bubbling with N_2:CO_2. To remove the drugs, the chamber was flushed with prewarmed physiological solution. Each experiment was performed in three preparations. The magnitudes of the contractions from the cholinoceptor agonists were quantitated from the chart recording. Due to the rhythmic activity, it was necessary to estimate visually the peak of each response. These contractions were compared to the maximal response of each preparation, produced by either 10^{-4} M nicotine or 10^{-5} M ACh. Changes in the rhythmic contractions were not quantified. Since only small changes in length occurred with GABA and muscimol, these results were also not quantified. There were, however, marked changes in the rhythmic contractions with GABA and muscimol. Antagonists were usually added to the reservoir of solution that was used to flush the chamber.

D. viteae that were not used on the day of isolation were kept in an incubator at 37°C with 95% N_2:5% CO_2 for use in subsequent experiments. The incubation medium was composed of 3 ml NCTC 135, 3 ml Dulbecco's modified Eagles Medium, 1 ml horse serum, 0.7 mg penicillin G and 0.7 mg streptomycin. The worms could be maintained in this medium for 3 to 4 days with little loss of responsiveness to the drugs.

The drugs used were: acetylcholine chloride, muscarine chloride, nicotine, pilocarpine hydrochloride, atropine sulphate, (+)-tubocurarine chloride, hexamethonium chloride, GABA, muscimol, baclofen, picrotoxin, bicuculline and potassium penicillin G. All were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.), except baclofen (CIBA Pharmaceutical, Summit, NJ, U.S.A.) and hexamethonium chloride (U.S. Biochemical Corp., Cleveland, OH, U.S.A.).

Results

Cholinoceptor agonists on the intact D. viteae

The filariid contracted spontaneously and the magnitude of the contractions for each worm was consistent and stable for several hours in the tissue chamber. The worms that were used 1 to 2 days after isolation from the hamsters were much more active than those used 4 to 5 days after isolation.

When ACh was added to the solution bathing the intact filariid, it had no effect at concentrations up to 10^{-4} M (Figures 1 and 3). ACh, 10^{-3} M, usually induced a very small contraction. Muscarine, a muscarinic cholinoceptor agonist, was also inactive (Figure 1). ACh is considered to be the neurotransmitter that is liberated from the nerve cord neurones of nematodes and it increases the excitability of the muscle cells in *Ascaris* and *D. viteae* (del Castillo *et al.*, 1963; Rohrer *et al.*, 1988); thus it should have increased the contractility in this preparation. The weak response to ACh could be due to high cholinesterase activity (Bueding, 1952). Neostigmine (10^{-6} M) and physostigmine (10^{-6} M), however, did not enhance the actions of ACh, indicating that the lack of effect was not due to rapid metabolism of ACh by cholinesterases.

Norton & deBeer (1957) noted that the sensitivity to piperazine was much greater in the open *Ascaris* preparation than in the intact *Ascaris*. The cuticle apparently acted as a permeability barrier to piperazine and other polar substances in *Ascaris*. Although the filariid cuticle is quite different, morphologically, from the *Ascaris* cuticle (Fetterer, 1986); it may still function as a permeability barrier. In support of this concept, cholinoceptor agonists with high lipid solubilities were very active. Nicotine, a lipid-soluble nicotinic cholinoceptor agonist, induced an immediate contraction at concentrations greater than 10^{-7} M (Figure 1). This effect was rapidly reversed upon flushing the chamber with control solution. The effects of nicotine were concentration-dependent and appeared to reach a maximum at 10^{-4} M. Pilocarpine, a lipid soluble muscarinic cholinoceptor agonist, was also effective; although it had a lower intrinsic activity than nicotine (Figures 1 and 3). The maximal response was produced at 10^{-4} M pilocarpine. These results support the concept that ACh is inactive because it cannot penetrate to its site of action.

Cholinoceptor agonists on the opened D. viteae *preparation*

The opened filariid was surprisingly very active, spontaneously, although the contractions appeared to have a differ-

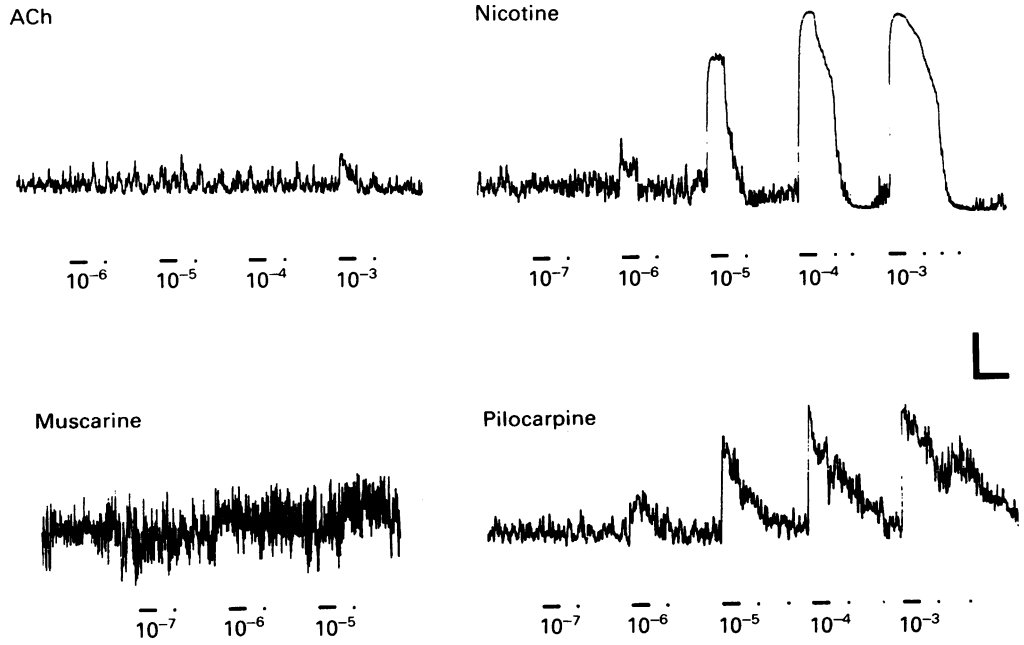

Figure 1 Effects of cholinoceptor agonists on the spontaneous isotonic contractions of the intact filariid, *Dipetalonema viteae*. Acetylcholine (ACh), nicotine, muscarine and pilocarpine were added to the bathing solution at the final molar concentrations indicated below each trace. The horizontal bar indicates the time of the exposure to the drug. At the end of the exposure the bath was flushed with approximately 15 ml of control solution. Additional flushes are indicated by dots. Note that nicotine and pilocarpine were very effective, however ACh and muscarine were ineffective. Calibrations: 2 mm and 2 min.

ent type of rhythmicity from those in the intact filariid. The activity was stable for several hours; in fact, the contractions often increased with time. It was not uncommon for a preparation to be totally inactive when it was first set up, and to become very active after 30 to 60 min.

ACh was much more effective on the opened preparation. Even 10^{-7} M ACh induced a rapid contraction, as well as an increase in the rhythmic activity of the preparation (Figure 2). The effect of ACh was readily reversible upon flushing the chamber. The magnitude of the contractions was reproducible and concentration-dependent. As can be seen in Figure 3, ACh was at least 10,000 times as potent on the open preparation as on the intact preparation. Muscarine was also much more potent on the open preparation. These differences probably occur because the cuticle blocks the diffusion of ACh and muscarine, both quaternary amines, to the cholinoceptors in the intact preparation. According to this hypothesis, the non-quaternary, highly lipid soluble agonists should have approximately the same potency on the intact and opened filariids. Nicotine and pilocarpine increased the contractions of the opened *D. viteae*, and they did have approximately the same potency on the opened as on the intact *D. viteae*, although the maximal response to pilocarpine of the opened *D. viteae* was somewhat less than that of the intact *D. viteae* (Figures 2 and 3).

GABA agonists on the intact D. viteae

GABA reduced the spontaneous activity of the intact filariid but high concentrations were necessary (Figure 4). GABA, 10^{-4} M, produced a weak suppression and complete blockade of the spontaneous contractions did not occur at concentrations less than 10^{-3} M. Also, the time course of the GABA action was very slow. The time to the maximal blockade was 1 to 2 min, and full recovery often required 10 to 15 min.

Muscimol, a $GABA_A$ receptor agonist, was much more potent than GABA on this preparation (Figure 4). Muscimol, 10^{-5} M, was very effective. Furthermore the blockade and recovery were very rapid with muscimol. Baclofen, a $GABA_B$ receptor agonist, was inactive at concentrations up to 10^{-3} M.

GABA agonists on the opened D. viteae *preparation*

GABA was more than 1000 times as active on the opened filariid, as on the intact filariid. Even 10^{-7} M GABA induced a depression of the spontaneous contractions, although it was not obvious in the record in Figure 5. The concentration-response relationship was very steep, as 10^{-6} M induced a complete paralysis in most preparations. The onset of action was rapid, as was the rate of recovery, even at the high concentrations. Thus the time-course of the GABA blockade was very different from that on the intact *D. viteae*. These differences were probably due to the low lipid solubility of GABA (Yunger & Cramer, 1981).

Muscimol was also active on the opened preparation; however it had about the same potency as on the intact preparation (compare Figures 4 and 5). Baclofen was inactive (Figure 5).

ACh antagonists on the opened D. viteae *preparation*

None of the ACh antagonists, hexamethonium, (+)-tubocurarine and atropine, had consistent effects on the spontaneous contractions at concentrations that would block mammalian preparations (Figure 6). Furthermore, in most experiments the antagonists had little effect on the contractions from the cholinoceptor agonists (Figure 7). Only (+)-tubocurarine reduced the contractions to ACh. This reduction was 69% in the three preparations at a concentration that would block mammalian cholinoceptor responses by 100%. Hexamethonium and atropine had insignificant effects on the ACh-induced contractions.

GABA antagonists on the opened D. viteae *preparation*

The GABA antagonists, picrotoxin, bicuculline and penicillin G, had few effects on the spontaneous contractions of the

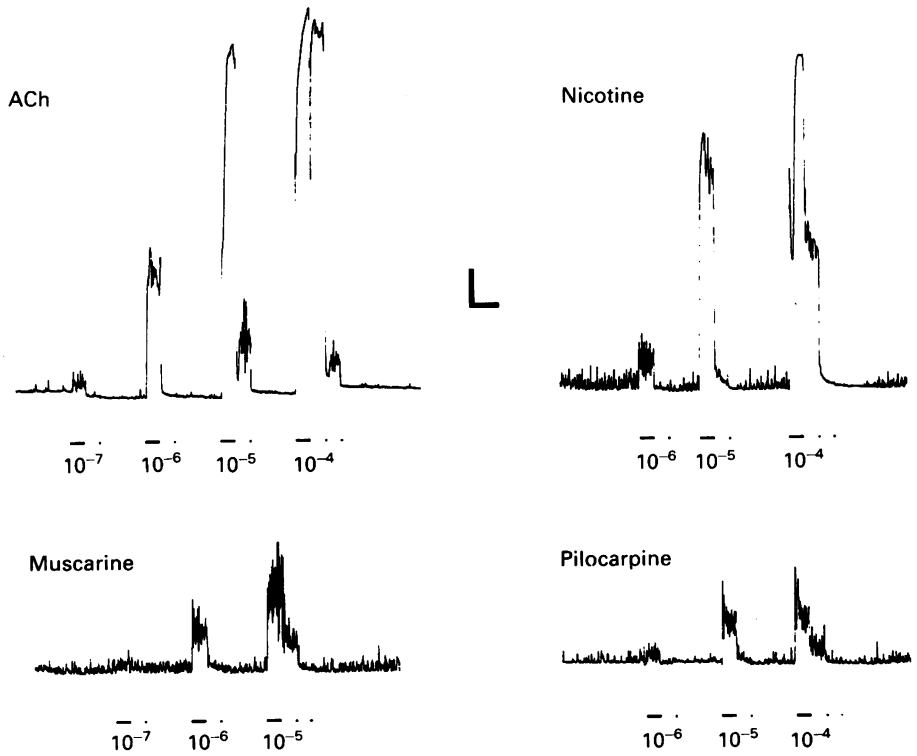

Figure 2 Effects of cholinoceptor agonists on the spontaneous isotonic contractions of the opened *D. viteae*. Each agonist was applied at the time indicated by the bar and at the concentration indicated below each bar. The bath was flushed at the end of the exposure (bar) and at each dot. Note that all the agonists were effective. Calibrations: 1 mm and 2 min (acetylcholine (ACh), nicotine and pilocarpine); 2 mm and 2 min (muscarine).

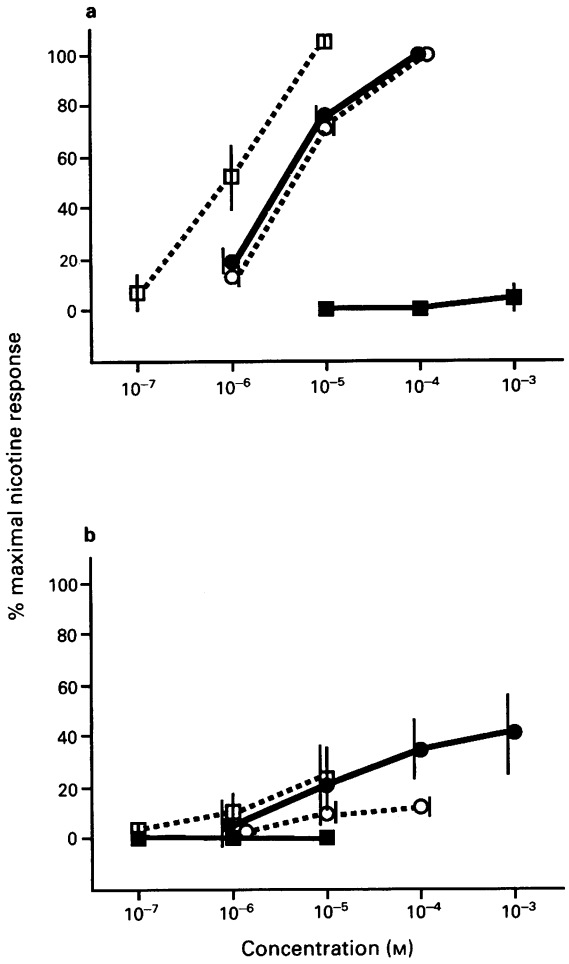

Figure 3 Concentration-response relationships for the cholinoceptor agonists. The contractions induced by acetylcholine (ACh, intact, ■; opened, □) and nicotine (intact, ●; opened, ○) are illustrated in (a). Each contraction was measured as a percentage of the contraction to 10^{-4} M nicotine, which was the maximal response in most preparations. In (b) the results from muscarine (intact, ■; opened, □) and pilocarpine (intact, ●; opened, ○) are shown. Each point is the mean of three experiments, and the vertical bar is the s.d.

opened preparation (Figure 8). Bicuculline (10^{-4} M) did tend to increase the spontaneous activity in the preparation illustrated in this figure. The antagonists did not affect the depression of the spontaneous contractions due to GABA (Figure 9).

Discussion

These experiments demonstrate that the recording of isotonic contractions from the filariid, *D. viteae*, is an easy and reliable method for observing the acute effects of drugs. Although the filariid is very small, it can easily contract against an isotonic transducer. The nerve ring is removed during the surgery, thus the preparation is essentially cuticle, muscle and nerve cord which makes it simple and homogeneous. The magnitude of the spontaneous activity is very constant for long periods of time. The intact filariid could be useful for drug screening of anthelmintics. The open filariid is very sensitive to cholinoceptor and GABA receptor agonists and it would be useful for the study of basic physiological and pharmacological mechanisms of drug actions, because substances can more readily reach their sites of action.

ACh and GABA were relatively inactive on the intact preparation but were very active on the open preparation. The major difference between these two preparations is that the cuticle does not act as a barrier in the open preparation. This indicates that the cuticle can reduce the penetration of ACh and GABA into intact filariids. Both of these compounds have low lipid solubilities and high water solubilities which indicates that the cuticle acts as a lipoidal barrier, in a similar fashion to the *Ascaris* cuticle (Fetterer, 1986). In support of this conclusion, the more lipid soluble substances, nicotine, pilocarpine and muscimol, had the same activities on the intact and opened preparations. Due to their high lipid solubilities, these drugs can readily penetrate the cuticle and quickly reach their sites of action, even in the intact preparation.

A drug with a high lipid solubility will have a rapid onset and high anti-filarial activity on the intact preparation. Drugs with low lipid solubilities will have low activities on the intact preparation, but they may still have anti-filarial efficacy. With long term exposure, polar drugs may eventually penetrate the cuticle and become trapped in the filariid or the filariid may ingest the drugs so they can be absorbed (Bone & Bottjer,

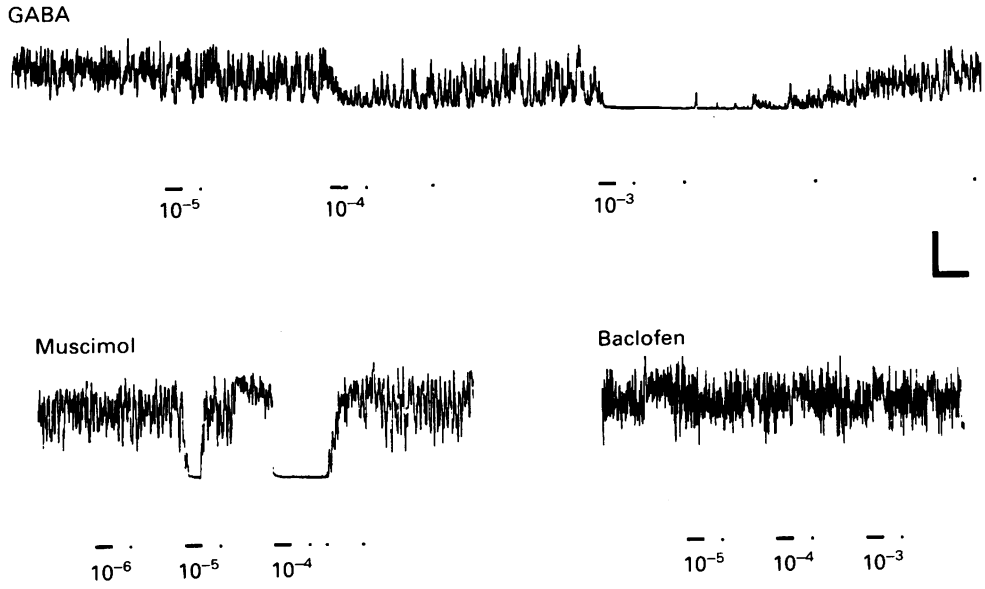

Figure 4 Effects of γ-aminobutyric acid (GABA) receptor agonists on the isotonic contractions of *D. viteae*. GABA, muscimol and baclofen were added to the bathing solution at the time indicated by the bar and at the concentration indicated below each bar. The bath was flushed at the end of the exposure (bar) and at each dot. Note that the onset and recovery of the GABA blockade were very slow. Calibrations: 2 mm and 2 min.

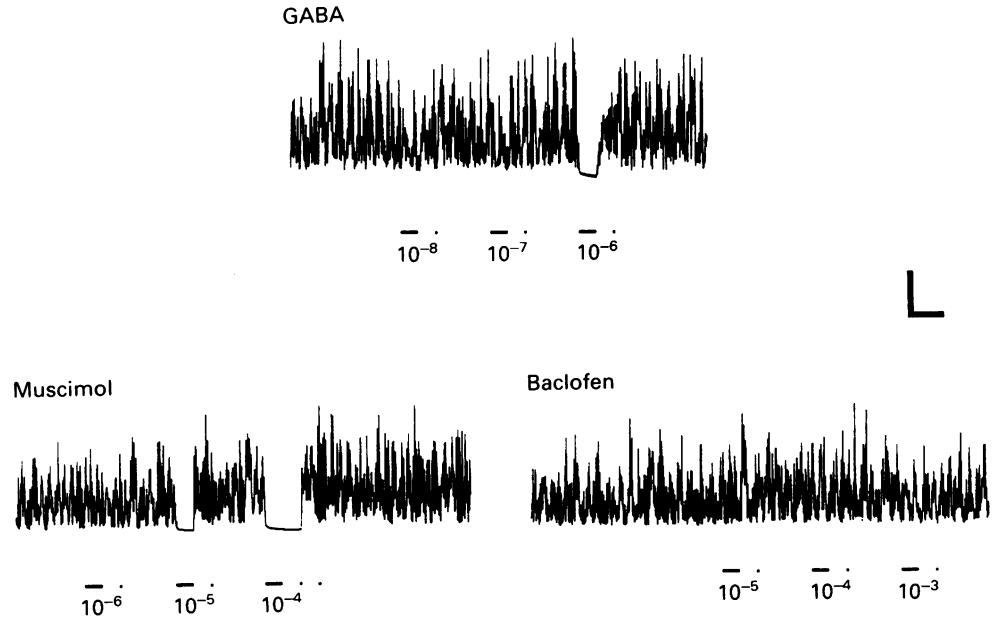

Figure 5 Effects of γ-aminobutyric acid (GABA) agonists on the isotonic contractions of the opened *D. viteae*. GABA, muscimol and baclofen were added to the bathing solution at the times indicated by the bars and at the concentration indicated below each bar. The bath was flushed at the end of the exposure (bar) and at each dot. Note that the onset and recovery of the GABA blockade were very fast in this preparation. Calibrations: 2 mm and 2 min.

1986). An advantage of drugs with low lipid solubilities is that they are less likely to penetrate the human blood brain barrier and induce CNS toxicity in the host.

The effectiveness of ACh on the opened *D. viteae* and the activity of the cholinoceptor agonists indicates that cholinoceptors are present in *D. viteae*, and that activation of the cholinoceptors can increase the muscle contractions. There are probably both nicotinic and muscarinic cholinoceptors in *D. viteae*, since the muscarinic agonists, pilocarpine and muscarine, and the nicotinic agonist, nicotine, increased the rhythmic contractions of the opened *D. viteae*. Since the antagonists, including (+)-tubocurarine, were much less effective in blocking the actions of ACh in the filariids, the cholinoceptors in filariids must have different properties from the cholinoceptors in mammals. Furthermore, the shift of the ACh concentration-response curve is not indicative of a competitive interaction, which is unlike the mammalian receptors. The lack of activity of the antagonists in reducing the actions of ACh also means that the antagonists are not ideal for testing whether ACh is a neurotransmitter in *D. viteae*. del Castillo *et al.* (1963) were able to demonstrate a blockade of the responses to exogenous ACh by tubocurarine in *Ascaris*; however, the antagonists were ineffective in reducing the spontaneous activity. On the basis of these results, they concluded that the spontaneous activity was myogenic in nature.

GABA was very effective as an agonist in *D. viteae* but the effects of GABA were not reduced by the GABA antagonists, bicuculline, picrotoxin and penicillin G. Thus the GABA receptors in this preparation are different from the classical $GABA_A$ receptors, although they are sensitive to muscimol, a $GABA_A$ receptor agonist. They could be classified as bicuculline-insensitive $GABA_A$ receptors, except that muscimol was less potent than GABA. Muscimol is usually more active than GABA at mammalian $GABA_A$ receptors. In this regard the GABA receptors in *D. viteae* are similar to those in *Ascaris* (Wann, 1987; Holden-Dye *et al.*, 1988; Holden-Dye & Walker, 1988). Of course, the antagonists had no consistent effects on the spontaneous contractions. Again, this result does not test the physiological role for GABA because the same antagonists did not reduce the effects of exogenous

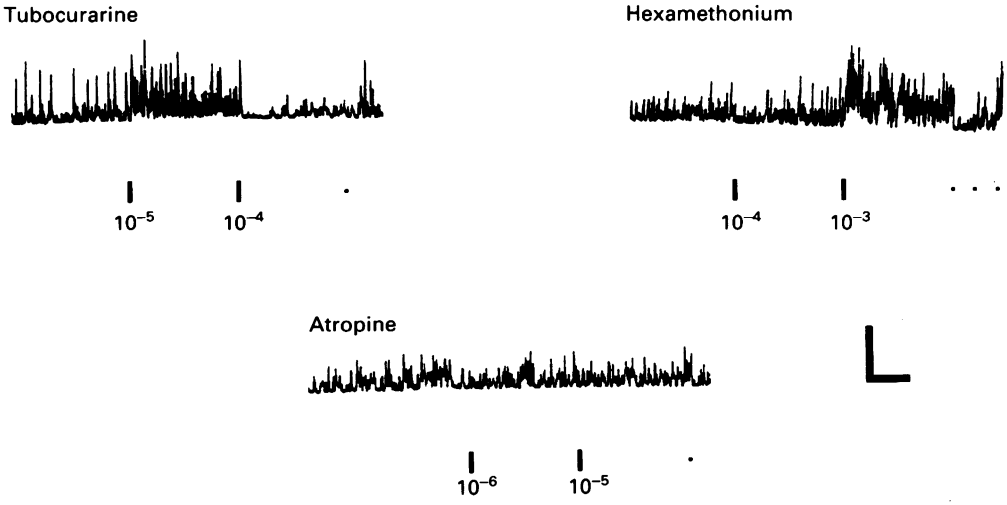

Figure 6 Effects of cholinoceptor antagonists on the spontaneous isotonic contractions of the opened *D. viteae*. Tubocurarine, hexamethonium and atropine were applied (cumulatively) at the vertical bars at the concentrations indicated below the bars. The bath was flushed at each dot. Calibrations: 2 mm and 2 min.

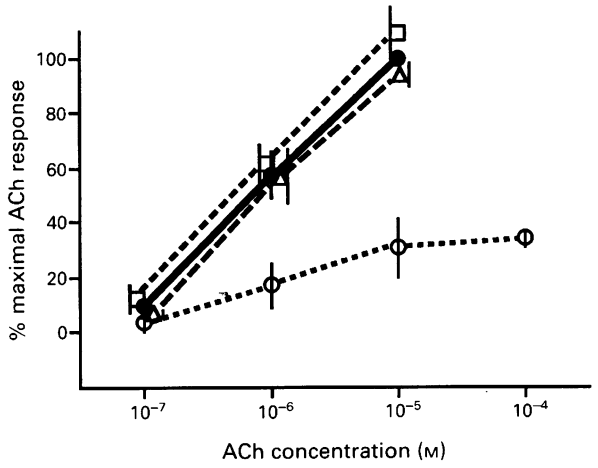

Figure 7 Effects of the cholinoceptor antagonists on the responses to acetylcholine (ACh) in the opened *D. viteae*. The contractions to ACh (●), ACh in the presence of 10^{-3} M hexamethonium (□), ACh in the presence of 10^{-5} M atropine (△), and ACh in the presence of 10^{-4} M tubocurarine (○) are illustrated. Each contraction was measured as a percentage of the contraction to 10^{-5} M ACh, which was the maximal response in most preparations. Each point is the mean of three experiments. The vertical line represents the s.d.

GABA and would not be expected to reduce the effects of endogenous GABA.

From these results, one can only speculate on the sites of action for ACh and GABA. The muscle cells in nematodes have an unusual anatomy with three components, a contractile portion, a cell belly and a muscle arm (Rosenbluth, 1965b). The muscle arms extend toward the nerve cord, where they make synaptic contact with the nerve cord neurones. Gap junctions exist between many muscle arms in the region of the nerve cord. The neurotransmitters (ACh and GABA) released by nerve cord neurones probably act at receptors on the muscle arms which alters the activity of the rest of the muscle cell. The receptors on the muscle arms should be

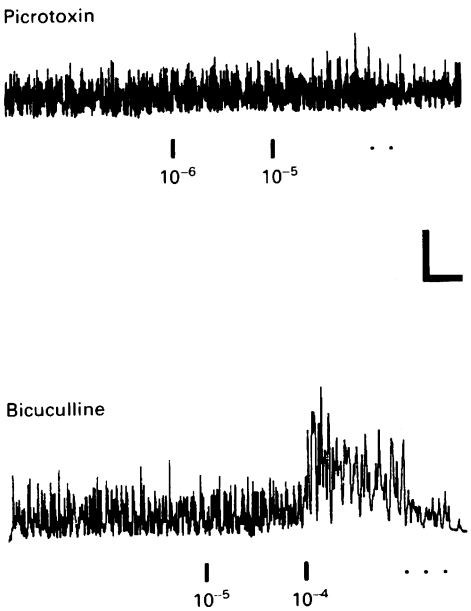

Figure 8 Effects of γ-aminobutyric acid (GABA) blocking drugs on the spontaneous isotonic contractions of the opened *D. viteae*. Picrotoxin and bicuculline were applied (cumulatively) at the vertical bars at the concentrations indicated below the bars. The bath was flushed at each dot. Calibrations: 2 mm and 2 min.

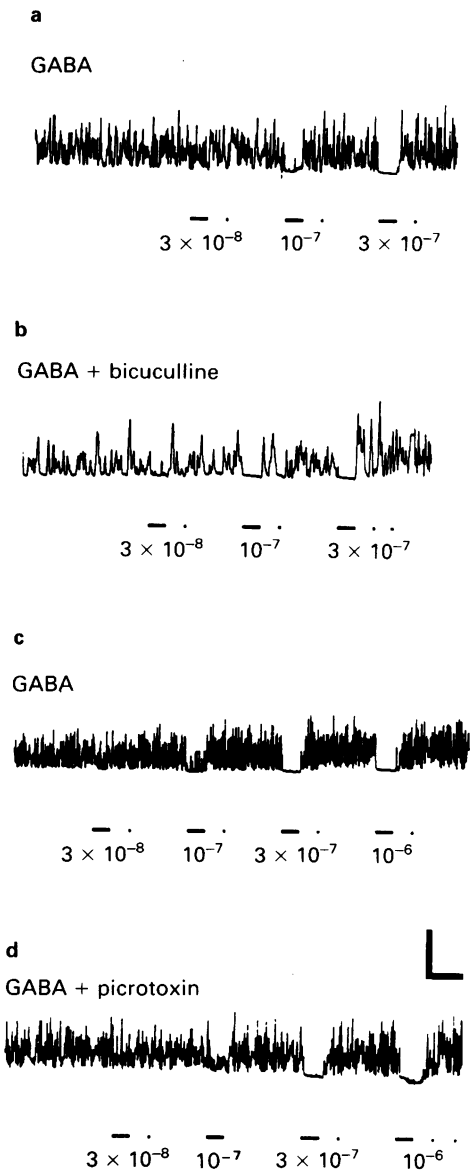

Figure 9 Effects of the γ-aminobutyric acid (GABA) blocking drugs on the responses of the opened *D. viteae* to GABA. GABA was applied at the time indicated by the bars and at the concentration indicated below each bar. Traces (a) and (c) are the control records and traces (b) and (d) are the responses in the presence of each blocking drug. The concentrations of the blocking drugs were 10^{-4} M bicuculline and 10^{-5} M picrotoxin. Calibrations: 2 mm and 2 min.

important sites of action for exogenously applied ACh and GABA. There is also evidence that ACh and GABA can act at the cell belly. If ACh or GABA is applied by superfusion after the nerve cord is removed, or if ACh or GABA is applied by iontophoresis to the muscle belly, changes in membrane potential and conductance can be observed in the *Ascaris* muscle belly (Brading & Caldwell, 1971; Martin, 1982; 1987). The physiological importance of these receptors is unclear; however, the changes in contractility from ACh and GABA in the present experiments probably involve receptors on both the muscle bellies and muscle arms.

D. viteae is a nematode that tends to localize in the subcutaneous tissues of the host. It appears to be very similar, physiologically, to the intestinal nematode, *Ascaris*. A major difference is that the antagonists are more effective in reducing the actions of ACh in *Ascaris* (Norton & DeBeer, 1957; del Castillo *et al.*, 1963; Martin, 1982) than in *D. viteae* (Rohrer *et al.*, 1988). Such a result may indicate a slight difference in the

cholinoceptors in these two species of nematodes. Also, ACh and GABA had a much more rapid onset of action and recovery in *D. viteae*. This is probably due to the fact that the opened *D. viteae* preparation is very thin and substances can quickly diffuse in and out of the muscle. The muscle wall of *Ascaris* is very thick; and even in the opened *Ascaris* preparation, it may take considerable time for ACh and GABA to diffuse to and from their sites of action.

In conclusion, the opened *D. viteae* preparation seems to be an excellent preparation on which to explore the effects of drugs on filariid contractility.

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Impaired induction of nerve ornithine decarboxylase activity in the streptozotocin-diabetic rat is prevented by the aldose reductase inhibitor ponalrestat

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1 The present study was designed to investigate if the aldose reductase inhibitor ponalrestat is capable of preventing the impairment of the response of ornithine decarboxylase (ODC) to nerve crush in streptozotocin (STZ)-diabetic rats.

2 ODC activity was measured in the dorsal root ganglia of crushed and uncrushed contralateral sciatic nerve of non-diabetic, ponalrestat-treated non-diabetic, STZ-diabetic and ponalrestat-treated STZ-diabetic rats.

3 Twenty four hours after crush, a significant ($P < 0.001$) increase in the ratio of ODC activity in ganglia of crushed relative to uncrushed nerves was found in non-diabetic but not in diabetic rats, as expected. In the ponalrestat-treated diabetic rats the ratio was significantly higher ($P < 0.001$) than that in the untreated diabetic rats and was not different from that in the non-diabetic group.

4 Ponalrestat also significantly decreased absolute levels of ODC activity in ganglia of uncrushed nerves from diabetic and non-diabetic animals. Despite the near-normal induction of ODC activity by nerve crush in the ponalrestat-treated diabetic animals, absolute ODC activity remained lower than that in ganglia of uncrushed nerves from non-diabetics.

5 We conclude that ponalrestat is able to prevent the impaired induction of ODC in experimental diabetes. The results, however, call into question the relationship between impaired ODC induction and diabetes-induced defects in nerve regeneration, which are insensitive to ponalrestat.

Introduction

One of the complications of streptozotocin (STZ)-induced diabetes is a delay in, or reduced rate of regeneration following a nerve crush injury (Longo *et al.*, 1986; Ekström & Tomlinson, 1989). After injury, the sensory neurones of rat sciatic nerve undergo a series of metabolic changes that lead to an increase in the activity of the enzyme ornithine decarboxylase (ODC) in the dorsal root ganglia (Tetzlaff & Kreutzberg, 1985; Wells, 1987). This increase is believed to be dependent on retrogradely transported signals from the site of injury (Kanje *et al.*, 1986). Recently, we have shown that the increase in the activity of ODC is absent or delayed during experimental diabetes (McLean *et al.*, 1987).

There is a close relationship between ODC activity and nerve regeneration (Kanje *et al.*, 1986; Kauppila & Sternberg, 1989). A number of studies have shown that ODC activity is related to RNA metabolism (Russell, 1983; Wells, 1984) and protein synthesis (Gilad & Gilad, 1983). It appears that an increase in ODC activity is an essential event for the regeneration and survival of the neurone.

It is well known that many of the defects in peripheral nerve in the experimentally diabetic rat can be prevented by administration of an aldose reductase inhibitor (Yue *et al.*, 1982; Dahlin *et al.*, 1987; Tomlinson *et al.*, 1982; 1985). However, recent evidence indicates that certain axonal defects, including the reduced rate of regeneration are resistant to aldose reductase inhibition (Ekström & Tomlinson, 1989; Mayer *et al.*, 1984; Willars *et al.*, 1987; Tomlinson *et al.*, 1987).

The purpose of this work was to determine if the impaired induction of ODC in diabetes was affected by the aldose reductase inhibitor ponalrestat.

Methods

Induction of diabetes

Diabetes was induced in female Wistar albino rats (weight 220–240 g) by intraperitoneal injection of STZ (50 mg kg^{-1}) in citrate buffer (pH 4.5). An equal number of non-diabetic rats received buffer alone. From 72 h after STZ injection, blood samples for the analysis of blood glucose were obtained from tail veins and measured with an Ames Glucocheck and glucose-sensitive sticks. One half of both the non-diabetic and diabetic groups was given 25 mg kg^{-1} ponalrestat (Statil; ICI 128436) suspension daily by gastric intubation during the experiment. The treatment was begun on the same day as injection of STZ.

Preparation of tissue

Three weeks later, rats were anaesthetized with ether and the right or left sciatic nerve randomly selected and crushed under aseptic conditions with 4/0 silk thread against a glass rod at mid-thigh level. The wound was closed and the animal allowed to recover. Twenty four hours later, the rats were killed and L4,5 dorsal root ganglia removed and placed on ice as quickly as possible. ODC activity was measured as the release of $^{14}CO_2$ from [^{14}C]-ornithine as described previously (McLean *et al.*, 1987).

The assay was performed as follows. The two dorsal root ganglia (DRG) from each nerve were pooled and homogenised in 200 μl cold 25 mM Tris buffer, pH 7.1, containing 0.1 mM pyridoxal-5'-phosphate, 5 mM dithiothreitol and 1 mM EDTA. The homogenates were centrifuged at 60000 *g* for 30 min at 4°C. One hundred μl of the ganglia supernatant were transferred to specially constructed tubes containing in their necks filter paper soaked in 40% KOH. All samples were incubated for 2 h at 37°C with 0.2 μCi D,L-[^{14}C]-ornithine (50 mCi mmol^{-1}; Amersham, Amersham, U.K.). The reaction was stopped by addition of 20 μl of 100% trichloroacetic acid and the tubes left to stand for a further 2 h. Filter papers were then removed and counted by liquid scintillation counting for their content of $^{14}CO_2$, liberated by conversion of the ornithine to putrescine.

Statistical analysis

Results were tested for statistical significance by the Mann-Whitney U test and Student's unpaired *t* test. Differences were considered to be statistically significant at the $P < 0.05$ level.

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Table 1 Blood glucose and body weight changes

Group	Number of rats	Body weight (g) Start	Body weight (g) End	Blood glucose (mM)
Non-diabetic	20	232 ± 6	240 ± 7	4.7 ± 1.8
Non-diabetic + ponalrestat	17	235 ± 6	242 ± 6	5.1 ± 1.3
Diabetic	15	235 ± 4	191 ± 9*	21.1 ± 2.9*
Diabetic + ponalrestat	20	238 ± 6	194 ± 9*	20.8 ± 2.2*

Values are mean ± s.d. of body weight and blood glucose 21 days after induction of diabetes.
* Significance of differences from non-diabetic group (Student's unpaired t test), $P < 0.001$.

Results

The mean blood glucose levels and body weights of all groups are shown in Table 1. All diabetic rats showed marked weight loss and general characteristics of diabetes, such as polyphagia, polydipsia, polyuria and significant hyperglycaemia. Ponalrestat had no effect on body weight or blood glucose.

ODC activity in DRG from crushed nerves was expressed as a ratio of that in uncrushed nerves from groups of non-diabetic, diabetic and ponalrestat-treated animals (Table 2). Twenty four hours after crush injury the mean ratio of ODC activity in ganglia of crushed nerve over that of uncrushed contralateral nerve was 1.87 ± 0.66 (s.d.) in non-diabetic rats. The mean ratio in diabetic rats was significantly ($P < 0.001$) less; the mean value of 1.01 implied that no induction of ODC had taken place at that time. There was no significant difference in this ratio between the non-diabetic and ponalrestat-treated non-diabetic rats; treatment of diabetic rats with ponalrestat led to the ratio of ODC activity being restored towards that in non-diabetic rats. The ratio in the ponalrestat-treated diabetic rats was significantly greater than that in the diabetic-only animals ($P < 0.001$).

It was necessary to determine if this effect of ponalrestat was a result of the drug affecting the activity of ODC in its own right. As seen in Table 3, in which the mean ODC activities are expressed as absolute values, ponalrestat significantly reduced ODC activity in ganglia of both crushed and uncrushed nerves of non-diabetic animals. In the diabetic rats, although ponalrestat restored the ratio of ODC activity (crushed/uncrushed) to a value similar to that in non-diabetic rats (Table 2), the absolute values of ODC in the crushed side after ponalrestat treatment did not reach that in ganglia of uncrushed nerves from untreated non-diabetic rats.

Table 2 The ratio of ornithine decarboxylase (ODC) activity in the dorsal root ganglia of crushed sciatic over that in the ganglia of contralateral control nerve, at 3 weeks of diabetes, with and without ponalrestat

Group	Number of rats	ODC ratio (mean ± s.d.)	P
Non-diabetic	20	1.87 ± 0.66	
Non-diabetic + ponalrestat	17	1.77 ± 0.86	*NS
Diabetic	15	1.01 ± 0.13	*<0.001
Diabetic + ponalrestat	20	1.69 ± 0.82	*NS †<0.001

Statistical comparison with *non-diabetic and †diabetic groups (Mann-Whitney U-test); NS: non-significant.

Table 3 The effect of ponalrestat on ornithine decarboxylase (ODC) activity in the dorsal root ganglia of crushed and uncrushed nerve

Group	Number of rats	ODC activity Crushed	ODC activity Uncrushed
Non-diabetic	20	$3.15 \pm 1.70^{a,b,c}$	$1.75 \pm 1.01^{d,e}$
Non-diabetic + ponalrestat	17	1.61 ± 1.03^{a}	0.91 ± 0.42^{d}
Diabetic	15	1.22 ± 0.60^{b}	1.22 ± 0.63^{f}
Diabetic + ponalrestat	20	1.36 ± 0.53^{c}	$0.86 \pm 0.36^{e,f}$

ODC activity is expressed in pmol CO_2 released per hour per pair of dorsal ganglia ± s.d. Significance of differences between groups with identical superscripts are ^{a,d} $P < 0.005$; ^{b,c,e} $P < 0.001$ and ^{f} $P < 0.05$ (Student's unpaired t test).

Discussion

Induction of ODC by nerve crush in 3 week diabetic rats was compared with that in non-diabetic rats. This finding is consistent with our previous data (McLean *et al.*, 1987). Recent work from our laboratory has also shown a similar effect in acrylamide- (Myall *et al.*, 1990b) and 2,5-hexanedione-treated rats (Myall *et al.*, 1990a). The neuropathies produced by these agents have in common with diabetes a defect in retrograde axonal transport (Jakobsen & Sidenius, 1983; Braendgaard & Sidenius, 1986; Logan & McLean, 1988). In the light of these results, we propose that the impaired induction of ODC is a consequence of a reduced signal from the site of nerve injury to the nerve cell bodies in diabetes. Because of variation in absolute ODC activity between animals, as reflected in the high s.d.s in Table 3, the effects of nerve crush are best expressed as a ratio of activity in ganglia of crushed nerves over that in ganglia of uncrushed nerves from the same animal (Table 2). The main finding from these data is the prevention of reduced ODC induction in sensory neurones of diabetic rats by ponalrestat therapy. Despite a reduction in ODC activity produced by ponalrestat itself, nerve crush was able to induce ODC activity even in diabetic animals. This is in keeping with the beneficial effect of the drug to prevent certain axonal transport defects (Tomlinson *et al.*, 1986; 1988), although its ability to prevent defects in retrograde transport has not been studied in any depth.

As revealed before by several authors, experimental diabetic neuropathy is characterized by a reduction in nerve conduction velocity, various biochemical changes, morphological abnormalities of axons and delayed nerve regeneration. Ponalrestat can prevent the diabetes-induced increase in sorbitol in rat sciatic nerves. Neither sorbitol nor myo-inositol levels were measured in this study, since the experimental conditions were identical to those in our previous work. In that case nerve sorbitol levels were significantly increased and nerve myo-inositol levels reduced; ponalrestat restored sorbitol to normal but not myo-inositol (Dahlin *et al.*, 1987). It may be that the prevention of elevated nerve polyols also prevents the deficits in retrograde axonal transport that lead to the impaired induction of ODC in the diabetic animals.

The relationship between the effects of ponalrestat on ODC induction and its action on regenerating nerve are less clear, since ponalrestat has been found to be ineffective at reversing the defects in nerve regeneration in STZ-diabetes (Ekström & Tomlinson, 1989). One interpretation of our results is that impairment of ODC induction and the deficits in nerve regeneration in diabetes are unrelated. In that case one may conclude that ODC induction is a necessary but not sufficient requirement for the regeneration process and that other aspects of nerve dysfunction in diabetes, e.g. defects in slow axonal transport, which are not prevented by ponalrestat, may be of more significance.

There is, however, an alternative interpretation, based on our finding that ponalrestat itself reduced ODC activity (Table 3). It may be that although ponalrestat treatment per-

mitted a near-normal induction of ODC in the diabetic animals, the absolute level to which the ODC was elevated in the presence of ponalrestat was not sufficient to permit the sequence of events that leads to normal nerve regeneration. This would be a further explanation of why the defects in nerve regeneration are not prevented by ponalrestat. However, if this were true one would expect to see impaired nerve regeneration in non-diabetic animals treated with ponalrestat, which is not the case (Ekström & Tomlinson, 1989). The correct interpretation will depend on the use of agents that restore ODC induction without affecting ODC activity.

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Synaptosomal tryptophan uptake and efflux following lesion of central 5-hydroxytryptaminergic neurones

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1 This study attempted to determine whether the activation of the tryptophan carrier in rat forebrain synaptosomes caused by depolarization or by extracellular sodium depletion occurred exclusively in 5-hydroxytryptaminergic nerve endings.

2 Ascending 5-hydroxytryptaminergic neurones were lesioned either electrolytically or by intraventricular administration of 5,7-dihydroxytryptamine. The extent of the lesion was assessed by comparing the uptake of [^{3}H]-5-hydroxytryptamine (5-HT) in lesioned animals and in sham-operated controls. [^{3}H]-5-HT uptake was reduced by 85.9 ± 1.63% (mean ± s.e.mean) in animals receiving electrolytic lesions, and by 87.4 ± 4.51% in those receiving 5,7-dihydroxytryptamine.

3 The uptake of [^{3}H]-tryptophan by synaptosomes from lesioned animals incubated in standard Na^+-rich media was slightly lower (278.8 ± 27.3 pmol mg^{-1} protein min^{-1}) than that observed in sham-operated controls (360.6 ± 30.3 pmol mg^{-1} protein min^{-1}). However, uptake in the absence of extracellular Na^+ was increased to a similar extent in both the sham-operated (539 ± 54.5 pmol mg^{-1} protein min^{-1}) and lesioned animals (507.2 ± 42.4 pmol mg^{-1} protein min^{-1}).

4 The efflux of [^{3}H]-tryptophan in response to extracellular Na^+ depletion was similar in sham-operated and lesioned animals. Release expressed as a percentage of tissue [^{3}H]-tryptophan released in response to the pulse of Na^+-free medium was 6.691 ± 0.585 ($n = 4$) in sham-operated controls and 8.195 ± 0.906 in lesioned animals.

5 The efflux of [^{3}H]-tryptophan in response to K^+ depolarization was also unchanged in lesioned animals when compared with sham-operated controls. Release, expressed as described above was, in sham-operated controls 3.76 ± 0.41 ($n = 4$) and 4.09 ± 0.30 in lesioned animals.

6 The results of this study show that the tryptophan carrier which is activated by depolarization or by extracellular Na^+ depletion is not located exclusively on 5-hydroxytryptaminergic nerve endings. Moreover the contribution made by 5-hydroxytryptaminergic neurones appears to be only minor.

Introduction

Recent studies have shown that tryptophan uptake into rat forebrain synaptosomes may be resolved into a single high affinity carrier-mediated process and diffusion (Wilkinson & Collard, 1985). The carrier may be activated by depolarization (Collard *et al.*, 1988), and by decreasing extracellular Na^+ concentration (Wilkinson & Collard, 1984). It has been suggested that a similar mechanism may operate within the nerve ending to activate the carrier in response to the two experimental procedures (Collard *et al.*, 1988). The identity of the neurones which possess this carrier, and the relationship between the carrier and the function of the neurones in which it is found have still to be elucidated. Because tryptophan is the precursor amino acid for 5-hydroxytryptamine (5-HT) synthesis, the possibility that the tryptophan carrier may be involved in the regulation of nerve terminal 5-HT synthesis has been suggested (Collard *et al.*, 1988). The synthesis of this transmitter in central 5-hydroxytryptaminergic neurones is known to be activated by a depolarization-induced increase in tryptophan hydroxylase activity (Boadle-Biber, 1978). Whether the supply of substrate to the activated enzyme is increased under these conditions is not known, but it would seem apppropriate for this to occur. A depolarization-induced activation of the plasma membrane tryptophan carrier in 5-hydroxytryptaminergic neurones could maintain an adequate supply of substrate for the activated enzyme. To determine whether this was likely to occur, this study examined the effect of electrolytic and chemical lesions of central 5-hydroxytryptaminergic neurones on the activation of the synaptosomal tryptophan carrier by depolarization or by extracellular Na^+ depletion.

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Methods

Lesions

Male Albino Wistar rats weighing between 200 and 250 g were used in all studies. Animals were anaesthetized with either sodium pentobarbitone (Sagatal, May and Baker, 60 mg kg^{-1} i.p.) or with halothane (0.9% in O_2). In order to introduce electrodes (for electrolytic lesions), or Hamilton syringe needles (for chemical lesions), animals were placed in a David Kopf No 900 stereotaxic frame. In the initial study on tryptophan uptake, 5-hydroxytryptaminergic neurones were lesioned electrolytically. In the later study on tryptophan efflux, it was decided to use the technically simpler procedure of intraventricular injection of 5,7-dihydroxytryptamine (5,7-DHT). This was considered a viable approach because although the method of inducing lesions was different in the two experiments, the extent of the lesions were monitored biochemically and shown to be very similar following both procedures (see Results and Table 1).

Electrolytic lesions of the dorsal and medial raphe nuclei were made with a concentric bipolar electrode (0.5 mm diameter, tip separation 1 mm, tip diameter 50 μm), stereotaxically positioned sequentially into both the dorsal and median raphe nuclei according to the following coordinates (Konig & Klippel, 1963): dorsal raphe nucleus – lateral 0; anterio-posterior +0.25 mm; vertical −1.2 mm; medial raphe nucleus – lateral 0; anterio-posterior +0.25 mm; vertical −3.6 mm. When the electrode was in place, a current 300 μA (d.c.) was passed for 10 s at each placement. Sham-operated animals

Table 1 The uptake of [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) into synaptosomes prepared from unoperated controls (control), sham-operated animals (sham) and animals lesioned electrolytically or with 5,7-dihydroxytryptamine (5,7-DHT) (lesioned)

5,7-DHT lesions ($n = 4$)		
Control	*Sham*	*Lesioned*
0.245 ± 0.010	0.255 ± 0.007	0.030 ± 0.010
Electrolytic lesions ($n = 6$)		
Control	*Sham*	*Lesioned*
0.251 ± 0.022	0.261 ± 0.016	0.033 ± 0.022

Uptake is expressed as the amount (pmol mg^{-1} protein) of [^{3}H]-5-HT actively accumulated over a 15 s incubation period at 37°C. The results are expressed as the mean ± s.e.mean. The numbers of observations are given in parentheses. No significant differences were observed between the unoperated controls and sham-operated animals in either group. The uptake in the lesioned animals was significantly lower than that of the sham-operated controls ($P < 0.001$, Student's t test).

were treated in the same way except that no current was passed. The animals were allowed to recover for 14 days before being used for transport studies.

The chemical lesions were administered by positioning the needle of a Hamilton syringe into the lateral ventricle according to the following coordinates: lateral 1.5 mm; anterio-posterior −1.3 mm from Bregma, vertical −1.2 mm from the intra-aural line (Paxinos & Watson, 1982). The fluid injected into the lateral ventricle consisted of 150 μg of 5,7-dihydroxytryptamine (5,7-DHT) free base dissolved in 20 μl of sterile saline containing 0.2% ascorbic acid. The 20 μl was injected in 2 μl aliquots over a period of 2.5 min. The needle was then left in place for 5 min before being slowly withdrawn. Sham-operated animals received injections of 20 μl of sterile saline containing 0.2% ascorbic acid but no 5,7-DHT. The animals were allowed to recover and used within 21–38 days following the injection. The extent of the lesions was assessed by comparing the uptake of [^{3}H]-5-HT into synaptosomes prepared from lesioned and sham-operated controls as described below. Histological investigation of the extent of the electrolytic lesions was also made. The section of mid and hindbrain remaining after the forebrain was removed for transport studies and stored in 4% formal saline. Seven days later, 50 μm serial sections were cut and stained with Neutral Red. The position and extent of the lesion was then determined with reference to the atlas of Konig & Klippel (1963).

Preparation of synaptosomes

Synaptosomes were prepared from whole forebrain either by the method of Gray & Whittaker (1962) as previously described (Collard *et al.*, 1981), or by the method of Dodd *et al.* (1981). The synaptosomal preparation obtained from either procedure showed almost identical properties with respect to the transport of 5-HT and tryptophan.

Measurement of [^{3}H]-5-HT and [^{3}H]-tryptophan uptake

Two different uptake studies were conducted. As mentioned above, the extent of the lesions to 5-hydroxytryptaminergic neurones was determined by examining 5-HT uptake. However, the major purpose of the study was to study the effect of the lesions on the stimulation of tryptophan uptake by decreasing extracellular Na^+. The experimental protocol used was essentially the same whether the uptake of [^{3}H]-5-HT or [^{3}H]-L-tryptophan was being examined, and was based on methods previously used in this laboratory (Wilkinson & Collard, 1984; Collard *et al.*, 1988). Each synaptosomal preparation was divided into two fractions before the final centrifugation. One of the synaptosomal pellets was resuspended in a standard Krebs solution of the following composition (mM): NaCl 124, KCl 5, KH_2PO_4 1.2, $MgSO_4$ 1.3, $CaCl_2$ 0.75, glucose 10, $NaHCO_3$ 26. The other pellet was resuspended in a Na^+-deficient Krebs solution in which NaCl and $NaHCO_3$ had been replaced isosmotically with sucrose and Tris/HCl (Wilkinson & Collard, 1984). The pH of both solutions was 7.4 at 37°C. Aliquots (1.0 ml) of synaptosomal suspension (containing about 0.5 mg synaptosomal protein ml^{-1}) were preincubated for 5 min at 37°C. [^{3}H]-L-tyryptophan (specific activity 0.08 Ci mmol^{-1}) or [^{3}H]-5-HT (specific activity 12.3–12.6 Ci mmol^{-1}) (Amersham International) was then added to give a final concentration of 10 μM or 10 nM, respectively. These concentrations were chosen on the basis of previous studies to ensure that the transport of the two substrates occurred by their respective high affinity carriers (Wilkinson & Collard, 1985; Evans & Collard, 1988). This was important for the 5-HT uptake studies as the high affinity carrier is believed to transport 5-HT predominantly into 5-hydroxytryptaminergic neurones (Ross, 1982) and, therefore, may be used as a monitor of 5-HT terminal function to determine the effectiveness of the lesions (Keller *et al.*, 1977). In the case of tryptophan, the concentration used was close to the K_m of the specific high affinity carrier, which had been identified by previous studies (Wilkinson & Collard, 1985) and which was being investigated further in this study. Incubation with [^{3}H]-tryptophan was continued for 1 min and that with [^{3}H]-5-HT for 15 s. Incubated synaptosomes were then separated from the incubation medium by Millipore filtration as described previously (Collard *et al.*, 1988). Membrane-bound and filter-bound radioactivity was subtracted from total radioactivity to provide a measure of transported [^{3}H]-tryptophan and [^{3}H]-5-HT in the presence and absence of extracellular Na^+ (Collard *et al.*, 1988). With respect to the transport of [^{3}H]-5-HT, the uptake in the absence of Na^+ was substracted from that in the presence of Na^+, to provide a value for the active transport of [^{3}H]-5-HT.

The protein concentration of the synaptosomal suspensions was measured by the method of Lowry *et al.* (1951). On the basis of the specific activity of the radiolabel added and the amount of protein present in each sample, the uptake of tryptophan and 5-HT was expressed as pmol accumulated mg^{-1} protein over the incubation time used.

Measurement of [^{3}H]-tryptophan efflux

The methods used to study the efflux of [^{3}H]-tryptophan were essentially as described previously (Collard *et al.*, 1988). Briefly, the synaptosomal pellet was resuspended in 20 ml of Krebs solution of the composition given in the previous section. Portions of the synaptosomal suspension (9.9 ml) were preincubated at 37°C for 10 min, after which time, 100 μl of [^{3}H]-L-tryptophan (specific activity 0.46 Ci mmol^{-1}) were added to give a final concentration of 4 μM. Incubation was continued for a further 10 min. Beds of synaptosomes were prepared from 5.0 ml portions of the incubated synaptosomes and set up in perfusion chambers (Collard *et al.*, 1981). The perfusion of the synaptosomes, the application of pulses of Na^+-deficient, or K^+-rich solutions and the separation and measurement of released [^{3}H]-tryptophan were carried out exactly as described previously (Collard *et al.*, 1988), except that the K^+ was applied for 60 s instead of 38 s as in the previous study.

Results

Histological and biochemical evaluation of the severity of the lesions to 5-hydroxytryptaminergic neurones

Histological examination of serial sections of the midbrain region of the lesioned animals confirmed that a relatively large

amount of tissue was destroyed, and that this included the dorsal and medial raphe nuclei in both the dorso-ventral and anterio-posterior directions. There was no evidence of misplaced electrodes in any of the animals, consequently, all lesioned animals were used for transport studies.

The measurement of the high-affinity uptake of [^{3}H]-5-HT is generally considered to be the most sensitive and unambiguous index of 5-hydroxytryptaminergic terminal degeneration available (Keller *et al.*, 1977). A comparison of the active uptake of [^{3}H]-5-HT in synaptosomes prepared from sham-operated and lesioned animals is shown in Table 1. It can be seen that in animals which had received electrolytic lesions, active uptake of 5-HT was reduced by 85.9 ± 1.63% ($n = 6$), and in those which had received 5,7-DHT lesions, uptake was reduced by 87.4 ± 4.51% ($n = 4$). Uptake in sham-operated animals was indistinguishable from that in non-operated controls.

The effect of electrolytic lesions of 5-hydroxytryptaminergic neurones on the synaptosomal uptake of [^{3}H]-tryptophan

The uptake of [^{3}H]-L-tryptophan into synaptosomes in the presence and absence of extracellular Na^+ is shown in Figure 1. It can be seen that the uptake of the amino acid in the presence of normal extracellular levels of Na^+ was slightly but not significantly lower in lesioned animals compared with sham-operated controls. Removal of extracellular Na^+ significantly increased the uptake of tryptophan in both the sham-operated and lesioned animals. Furthermore, the extent to which uptake was stimulated by Na^+ removal was similar in both sham-operated (179 ± 44 pmol mg^{-1} protein min^{-1}) and lesioned animals (229 ± 35 pmol mg^{-1} protein min^{-1}). Uptake in untreated control animals was also very similar to that observed in both experimental groups of animals both in the presence of extracellular Na^+ (316.05 ± 33.08 pmol mg^{-1} protein min^{-1}) and in its absence (493.92 ± 44.1 pmol mg^{-1} protein min^{-1}).

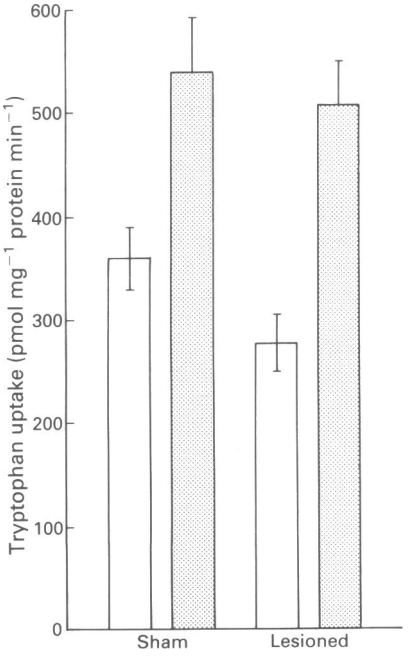

Figure 1 The uptake of [^{3}H]-tryptophan into synaptosomes prepared from sham-operated (sham) and lesioned animals in the presence (open columns) and absence (stippled columns) of extracellular Na^+. Uptake is expressed as the amount (pmol mg^{-1} protein) of [^{3}H]-tryptophan actively accumulated over a 1 min incubation period at 37°C. The results are expressed as the mean ($n = 6$); vertical lines indicate s.e.mean. Uptake in response to Na^+ depletion was significantly elevated in both the sham-operated ($P < 0.01$) and lesioned ($P < 0.001$) animals (Student's t test).

The effect of 5,7-DHT lesions on the efflux of [^{3}H]-tryptophan from preloaded and superfused synaptosomes

The results of the study are shown in Figures 2 and 3. The release of [^{3}H]-tryptophan in response to a 1 min pulse of Krebs solution containing an additional 15 mM K^+ is shown in Figure 2. It can be seen that the increased efflux of [^{3}H]-tryptophan in response to the application of the depolarizing pulse of K^+ was similar in both the sham-operated and lesioned animals. The efflux of [^{3}H]-tryptophan in response to a 2 min pulse of Na^+-free Krebs solution is shown in Figure 3. As with the previous study the increased efflux of [^{3}H]-tryp-

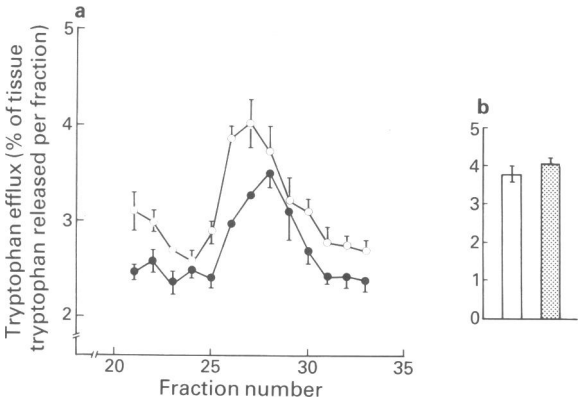

Figure 2 (a) The release of [^{3}H]-tryptophan in response to a 1 min pulse of Krebs solution containing an additional 15 mM K^+ in lesioned animals (○) and in sham-operated controls (●). Efflux is expressed as the percentage of tissue [^{3}H]-tryptophan released per fraction. Results are given as the mean ($n = 4$); vertical lines indicate s.e.mean. (b) The extra efflux above basal values caused by the application of the K^+ pulse in the lesioned animals (stippled column) and sham-operated controls (open column). Release is expressed as the mean percentage of tissue tryptophan released per pulse ($n = 4$); vertical lines indicate s.e.mean.

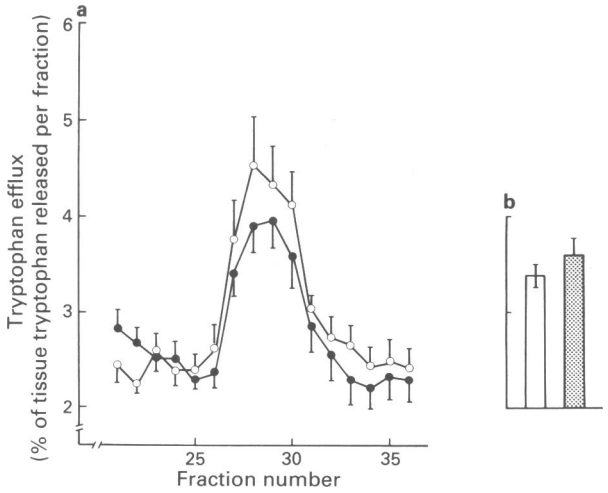

Figure 3 (a) The release of [^{3}H]-tryptophan in response to a 2 min pulse of Na^+-free Krebs solution in lesioned animals (○) and in sham-operated controls (●). Efflux is expressed as the percentage of tissue [^{3}H]-tryptophan released per fraction. Results are given as the mean ($n = 4$); vertical lines indicate s.e.mean. (b) The extra efflux above basal values caused by the application of the pulse of Na^+-free fluid in lesioned animals (stippled column) and in sham-operated controls (open column). Release is expressed as the mean percentage of tissue [^{3}H]-tryptophan released per pulse ($n = 4$); vertical lines indicate s.e.mean.

tophan can be seen to be similar in both the sham-operated and lesioned animals. Efflux is in fact slightly though not significantly elevated in the synaptosomes from the lesioned animals.

Discussion

This study attempted to determine more about the location and functional significance of the tryptophan carrier located in nerve ending fractions of rat brain. Previous studies had demonstrated that the carrier may be used to transfer tryptophan in either direction across the plasma membrane, depending on the experimental conditions employed and that the carrier could be activated by depolarization and by extracellular Na^+ depletion (Collard *et al.*, 1988). The identity of the neurones in which the carrier operated was not known, nor was it known whether activation of the carrier by extracellular Na^+ depletion or by depolarization occurred in the same cell, or whether the carrier present in some cell types was activated by one of the procedures and that in different cells by the other. The purpose of this study was to try to answer some of these questions by examining the involvement of 5-hydroxytryptaminergic neurones in the observed responses to these experimental manipulations.

The results clearly demonstrated that almost complete destruction of the ascending 5-HT nerve terminals (>85% loss of 5-HT uptake activity) had little effect on either the basal uptake of [^{3}H]-tryptophan or that stimulated by Na^+ depletion. No studies to date had examined the effect of lesions of 5-hydroxytryptaminergic neurones on the activation of [^{3}H]-tryptophan uptake caused by Na^+ depletion. However, uptake of [^{3}H]-tryptophan into hypothalamic synaptosomes incubated in normal Na^+ has been shown to be reduced by 5,7-DHT lesions (Denizeau & Sourkes, 1977). In this study, it was noted that [^{3}H]-tryptophan uptake into cerebellar synaptosomes was unaffected by lesions of 5-hydroxytryptaminergic neurones and that this reflected the paucity of 5-HT terminals in this brain region. The present study used synaptosomes prepared from whole forebrain and it is likely that the much smaller reduction in [^{3}H]-tryptophan uptake observed in this study resulted from the dilution of synaptosomes from 5-HT rich areas, with those derived from other areas of the forebrain containing a lower concentration of 5-HT terminals. However, the stimulation of [^{3}H]-tryptophan uptake by the removal of extracellular Na^+ was clearly not reduced by lesions of 5-hydroxytryptaminergic neurones.

In addition to these observations on the activation of the inward transport of the [^{3}H]-tryptophan, the stimulation of the outward transport of [^{3}H]-tryptophan caused by extracellular Na^+ depletion, or by depolarization was also similar in both lesioned and sham-operated animals. Thus, there appeared to be no obvious differences between the activation of the carrier by the two experimental procedures in either group of animals. Consequently it is not possible to determine from these data whether the carrier activated by the procedures used in this study resides in a single cell type, or whether the response to Na^+ depletion or depolarization occurs in different neurones.

The results of the study conclusively show that the tryptophan carrier activated by these experimental procedures is not located exclusively in 5-HT nerve terminals. Moreover, since there was very little change seen in the lesioned animals compared to controls, it can be predicted that the contribution made by 5-HT nerve endings to the overall response is only very minor. Therefore, these findings clearly do not support the suggestion that there may be a depolarization-induced coactivation of both nerve terminal tryptophan hydroxylase and the plasma membrane tryptophan carrier in 5-hydroxytryptaminergic neurones (Collard *et al.*, 1988).

There is little doubt, however, that the tryptophan carrier is located specifically in nerve endings (Wilkinson & Collard, 1984) and that it may be activated by depolarization. The selectivity of the carrier for tryptophan over its close relative phenylalanine (Collard *et al.*, 1988) would imply some functional significance other than that of a precursor for protein synthesis (Wedege *et al.*, 1977; Verity *et al.*, 1980). The route of tryptophan metabolism yet to be considered is that which gives rise to the kynurenines and quinolinic acid (Stone & Connick, 1985). This metabolic pathway may well be a significant route of metabolism of tryptophan in the CNS, although the cellular and subcellular sites of production of the various intermediates has yet to be established. Indirect evidence has indicated that quinolinic acid phosphoribosyltransferase, the enzyme responsible for the conversion of quinolinic acid to nicotinic acid, is concentrated in synaptosomal fractions (Schwarcz & Foster, 1984). There is little information available concerning the importance of the nerve ending fraction in the synthesis of kynurenine and its metabolites. However, it has recently been shown that quinolinic acid formation in the striatum may be driven by tryptophan availability (During *et al.*, 1989) and that treatment with parachlorophenylalanine, a compound which inhibits tryptophan transport by this carrier (Collard *et al.*, 1988), reduces the production of quinolinic acid in rat cortex (Moroni *et al.*, 1984). Thus increasing the transport of tryptophan across the nerve terminal plasma membrane could directly influence the production of these neuroactive compounds should it occur in nerve endings. However, until we have more information concerning this route of tryptophan metabolism in synaptosomal fractions, any relationship between the activation of the tryptophan carrier and the production of the kynurenines must remain speculative. The knowledge that some of these metabolites are at least neuroactive and probably neurotoxic (Stone & Connick, 1985) would make it worthwhile to investigate more fully the relationship between the precursor and its metabolites in the nerve ending fraction, and to determine any functional relationship which may exist between the activity of the neurone and the metabolism of tryptophan through this route.

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Pharmacological characterization of polycation-induced rat hind-paw oedema

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1 The inflammatory response induced by poly-L-arginine in the rat hind-paw was studied both by measuring paw oedema and histologically.

2 The paw volume was measured with a hydroplethysmometer at 0.5, 1, 2, 4, 6 and 18 h after the subplantar injection of the polycation. Protein extravasation was evaluated with Evans' blue and the histology studied by light microscopy.

3 Poly-L-arginine (12, 24, 43 and 115 kD) caused dose- and molecular weight-dependent oedema which had a rapid onset and long duration. Evans' blue extravasation paralleled the oedema induced by poly-L-arginine. Microscopic examination of the paws at early stages of oedema formation showed exuberant liquid exudate with no inflammatory cells. After 18 h, a cellular infiltrate was present, consisting mainly of mononuclear cells.

4 Indomethacin, dexamethasone, BW755c or the PAF-antagonist WEB 2086 caused no significant inhibition of the poly-L-arginine-induced oedema. Cyproheptadine had inhibitory effects only on the early stages of the polycation-induced oedema. Similar results were observed with rats depleted of histamine and 5-hydroxytryptamine.

5 Heparin, a polyanion, injected in the rat paw caused a marked inhibition of the polycation-induced oedema. N^G-monomethyl-L-arginine (LNMMA), an inhibitor of EDRF synthesis, injected locally also produced a marked inhibition, but this inhibition was reversed by iloprost.

6 These results suggest that the oedema induced by polycations was due to their cationic charge. The inhibitory effect of LNMMA is probably due to a decrease in vascular flow rather than a decrease in vascular permeability.

Introduction

Several basic and acidic (but not neutral) synthetic polyamino acids increase permeability and leucocytic infiltration when injected intradermally or intraperitoneally into rats (Stein *et al.*, 1956). Polycations such as poly-L-lysine induce charge- and size-dependent local oedema formation in the rabbit skin and also the release of prostacyclin and cytoplasmic purines from pig cultured aortic endothelial cells (Needham *et al.*, 1988). In addition, basic polyamino acids rich in arginine, lysine or ornithine stimulate the formation and/or release of the endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980) in perfused bovine segments of both artery and vein (Ignarro *et al.*, 1989).

In this paper we describe some pharmacological and histological characteristics of the inflammatory response induced by polycations (poly-L-arginine and poly-L-lysine) in the rat hind-paw. We have also assessed the involvement of EDRF as a possible mediator of polycation-induced oedema using an inhibitor of EDRF synthesis, N^G-monomethyl-L-arginine (LNMMA, Rees *et al.*, 1989).

Preliminary results have been communicated to the British Pharmacological Society (Antunes *et al.*, 1990).

Methods

Measurement of paw oedema

Male Wistar rats (150–200 g) were used. Oedema was induced by a single subplantar injection of the inflammatory agent (dissolved in saline) into the left hind-paw of the rat under light ether anaesthesia.

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The paw volume was measured immediately before the injection and at selected intervals thereafter with a hydroplethysmometer (model 7150, Ugo Basile, Italy). The final volume injected in the paw was always 0.1 ml. Results are expressed as increase in the paw volume (ml) calculated by subtracting the basal volume. In some cases, the area under the time-course curve was calculated (Lesser *et al.*, 1980) and the results expressed as percent of inhibition of the oedema total volume in comparison with control. The results are presented as mean ± s.e.mean.

Assessment of vascular response with Evans' blue

Evans' blue (25 mg kg^{-1} as a 2.5% solution in 0.45% NaCl) was injected intravenously immediately before subplantar injection of poly-L-arginine (24 kD, 1 mg per paw) into the rat hind-paw. At time intervals (0.5, 6 and 18 h) after poly-L-arginine injections, the animals were killed, the paws cut off and minced. The paws were then incubated with formamide (15 ml) for 72 h at 37°C. The solution was then filtered and the optical density of the filtrate assessed at 619 nm in a Uvikon 810 spectrophotometer (Lykke & Cummings, 1969; Garcia-Leme & Wilhelm, 1975).

Depletion of histamine and 5-hydroxytryptamine

Rats (Wistar, male, 180–220 g) were depleted of their stores of histamine and 5-hydroxytryptamine (5-HT) by repeated injections of compound 48/80 as previously described (Spector & Willoughby, 1959; Di Rosa *et al.*, 1971). Briefly, a 0.1% w/v solution of compound 48/80 in saline was given intraperitoneally morning and evening for eight doses, starting with an evening dose. The dose employed was 0.6 mg kg^{-1} for the first six injections and 1.2 mg kg^{-1} for the last two doses. Polycation was injected 5–6 h after the last injection of compound 48/80.

Histological studies

The foot pads were fixed in 10% formalin, embedded in paraffin and the histological sections stained by haematoxylin and eosin for light microscopical analysis.

Statistical analysis

The unpaired Student's t test was used for statistical evaluation of the results and $P < 0.05$ was taken as significant.

Drugs

Poly-L-arginine (12, 24, 43 and 115 kD), poly-L-lysine (85 kD), mepyramine, 5-HT, lambda-carrageenan, compound 48/80 and indomethacin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Cellulose sulphate and formamide were bought from Aldrich Chemical Co. (Dorset, UK) and Merck (FRG), respectively. Methysergide and BW 755C (3-amino-1-(*m*-(trifluoromethyl)-phenyl)-2-pyrazolone) were gifts from Sandoz Ltd (Switzerland) and the Wellcome Research Laboratories (Kent, UK), respectively. N^G-monomethyl-L(D)-arginine and 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-propane) were kindly provided by Italfarmaco (Milan, Italy) and Boehringer-Ingelheim (FRG), respectively. Iloprost and heparin sodium salt were gifts from Schering (FRG) and Roche (São Paulo, Brazil), respectively.

Results

Poly-L-arginine- and poly-L-lysine-induced dose-dependent rat hind-paw oedema

Figure 1 shows the dose-dependent relationships of the oedema produced by poly-L-arginine (43 kD) and poly-L-lysine (85 kD). At the higher dose (1.0 mg per paw), both poly-L-arginine and poly-L-lysine induced oedema which had rapid onset (at 30 min, 0.88 ± 0.03 ml and 0.62 ± 0.02 ml, respectively, $n > 35$) and long duration of action (at 6 h, 0.61 ± 0.03 ml and 0.57 ± 0.02 ml, respectively, $n > 35$). Increase in molecular weight of poly-L-arginine (12 kD to 115 kD, 1.0 mg per paw, $n > 5$) induced greater oedema (Figure 2).

Poly-L-arginine-induced Evans' blue leakage

Thirty min after the poly-L-arginine (24 kD, 1 mg per paw) injection, both the oedema and protein leakage were maximal whereas at 18 h the paw volume almost reached basal values and the protein leakage was decreased (Figure 3).

Histological analysis

Histological analysis of the hind-paw 30 min after the injection of poly-L-arginine (1 mg per paw; $n = 2$) showed an exuberant liquid exudate with no inflammatory cells. The intensive plasma leakage dissociated collagen fibres and a marked dilatation of lymphatic vessels was observed. The oedema and lymphatic vessel dilatation were still present 6 h after the polycation injection when a few polymorphonuclear as well as mononuclear cells were present; 18 h after the polycation injection the oedema decreased and an inflammatory cell infiltration mainly composed of mononucler cells was observed.

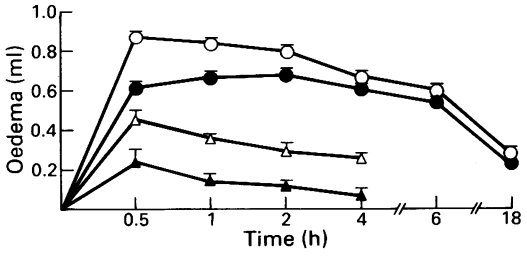

Figure 1 Poly-L-arginine 43 kD (△, 0.1 mg per paw; ○, 1 mg per paw; $n = 5$) or poly-L-lysine 85 kD (▲, 0.1 mg per paw; ●, 1 mg per paw; $n = 5$) injected into the rat hind-paw caused dose-dependent oedema. The oedema is expressed as the difference in volume (ml) of the paw compared to its basal volume. Each point represents the mean; s.e.mean shown by vertical bars.

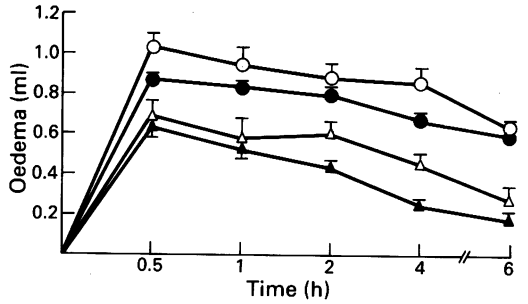

Figure 2 Molecular weight-dependent oedema formation in the rat hind-paw induced by subplantar injection of poly-L-arginine (▲, 12 kD; △, 24 kD; ●, 43 kD; ○, 115 kD; 1 mg per paw, $n > 5$). Each point represents the mean; s.e.mean shown by vertical bars.

Evaluation of histamine and 5-hydroxytryptamine participation

The involvement of 5-HT and/or histamine in the poly-L-arginine oedema was investigated in rats pre-treated with cyproheptadine ($0.5\ mg\ kg^{-1}$, i.p., 1 h), mepyramine ($0.25\ mg\ kg^{-1}$, i.v., 0.5 h) or with the mixture of the above antagonists (same doses). Rats treated with either cyproheptadine or the mixture of cyproheptadine and mepyramine showed an inhibition only 30 min later ($28.3 \pm 4.91\%$ and $34.2 \pm 6.4\%$, respectively; $n = 5$, $P < 0.05$, not shown). Mepyramine alone produced no significant inhibition of the poly-L-arginine oedema (not shown). At the doses used above, both cyproheptadine and the mixture of cyproheptadine and mepyramine (but not mepyramine alone) produced an inhibition of $45.1 \pm 8\%$ and $49.9 \pm 7\%$ of the 48/80 (10 μg per paw)-induced oedema ($n = 4$, $P < 0.05$, not shown).

In rats depleted of histamine and 5-HT stores, the oedema induced by poly-L-arginine (24 kD, 1 mg per paw) was inhib-

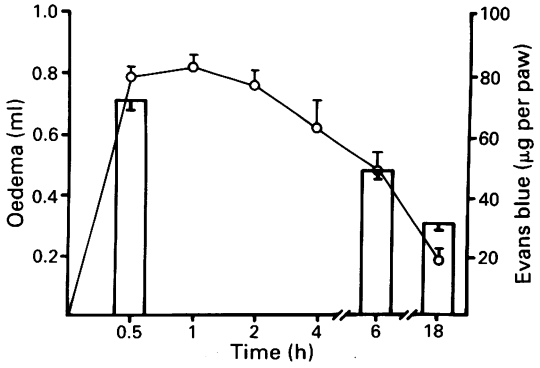

Figure 3 Protein leakage as measured by Evans' blue extravasation (columns). Poly-L-arginine (24 kD, 1 mg per paw) was injected into the hind-paw of rats pretreated with Evans' blue ($25\ mg\ kg^{-1}$, i.v.). At 30 min, 6 h and 18 h the animals were killed, the paws excised and the concentration of Evans' blue measured (see Methods). Oedema (ml) shown by (○).

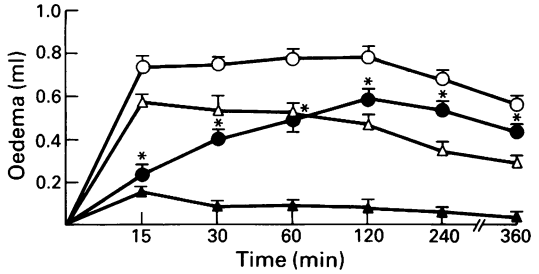

Figure 4 Effect of depletion of histamine and 5-hydroxytryptamine stores. Compound 48/80 (10 μg per paw) caused paw oedema in control rats (△, $n = 6$) but not in depleted rats (▲, $n = 8$). The poly-L-arginine (24 kD, 1 mg per paw)-induced oedema was reduced in depleted animals (●, $n = 8$) as compared to controls (○, $n = 6$). Each point represents the mean; s.e.mean shown by vertical bars.

ited by 41.6 ± 4.8% ($n = 8$, $P < 0.05$) whereas that induced by 48/80 was abolished (Figure 4).

Effect of WEB 2086, BW 755C, dexamethasone, indomethacin and heparin on poly-L-arginine-induced paw oedema

Rats treated 30 min beforehand with the PAF antagonist WEB 2086 (20 mg kg^{-1}, p.o., $n = 5$) or with the cyclo-oxygenase and lipoxygenase inhibitor BW 755 C (50 mg kg^{-1}, p.o., $n = 5$) exhibited no significant inhibition of the poly-L-arginine (43 kD, 1 mg per paw)-induced oedema (Figure 5). At the dose used above, BW 755C produced significant inhibition of the carrageenan (1 mg per paw)-induced paw oedema (49.9 ± 10.6%, $P < 0.05$, $n = 5$). Dexamethasone (1 mg kg^{-1}, s.c., 1 h before) or indomethacin (2 mg kg^{-1}, i.p., 30 min before) did not inhibit the poly-L-arginine-induced oedema ($n = 5$, not shown). Dexamethasone or indomethacin at these doses induced a marked inhibition of carrageenan (1 mg per paw)-induced oedema (67 ± 5.9%, $n = 8$ and 63.1 ± 4.1%, $n = 13$ respectively).

Heparin (50 u per paw) produced a marked inhibition of the oedema at all times (Figure 5). This compound had a dose-dependent inhibitory effect as measured by comparison of the area under the time-course curves (Table 1).

Heparin given systemically (5000 u kg^{-1}, 30 min beforehand) also caused an inhibition of the polycation-induced oedema (30.9 ± 6.7%, $P < 0.05$, not shown).

Poly-L-arginine-induced oedema is not abolished by a mixture of antagonists

Poly-L-arginine-induced oedema in rats treated 3 h before with cellulose sulphate (3 mg kg^{-1}, i.v.) and 30 min before with mepyramine (2 mg kg^{-1}, i.p.), methysergide (2 mg kg^{-1}, i.p.) and indomethacin (10 mg kg^{-1}, i.p.) was reduced by 39.9 ± 4.9% ($P < 0.001$, $n = 10$, not shown). Similar results were observed with poly-L-lysine (53.8 ± 3% inhibition, $P < 0.001$, $n = 5$, not shown).

Table 1 Inhibition by heparin of poly-L-arginine-induced oedema

Dose of heparin (u/paw)	*Inhibition* (%)	n	P
5	10.0 ± 7.41	10	NS
20	36.9 ± 7.60	10	0.01
50	62.9 ± 6.23	15	0.001

NS – not significant

N^G-monomethyl-L-arginine (LNMMA) inhibits poly-L-arginine-induced oedema

LNMMA (1 and 2 mg per paw) caused an inhibition of 27.6 ± 5.2% and 49.4 ± 5.3% ($n = 5$, $P < 0.05$, respectively) of the poly-L-arginine-induced oedema. However, LNMMA caused no reduction of the oedema when injected in the contralateral paw (2 mg per paw, $n = 5$, not shown). When injected systemically at a higher dose (100 mg kg^{-1}, 30 min before) LNMMA also exerted significant inhibition of the oedema (39.0 ± 5.8%, $n = 4$, $P < 0.05$). Inhibition of the poly-L-arginine-induced oedema was also observed with the D-form of N^G-monomethyl-arginine (DNMMA), although it was less potent than the L-isomer. At the dose of 2 mg per paw, DNMMA caused an inhibition of 25.1 ± 2.5% ($n = 5$, $P < 0.05$). In addition, iloprost (100 ng per paw) which did not induce oedema *per se*, partially prevented the inhibitory effect of the LNMMA (Figure 6).

LNMMA (4 mg per paw) produced no inhibition of carrageenan (1 mg per paw)-induced paw oedema (not shown, $n = 5$).

Discussion

Our results clearly demonstrate that the polycation poly-L-arginine injected into the rat hind-paw causes dose- and molecular weight-dependent oedema. The histological analysis of these lesions also showed a peculiar pattern of intense liquid exudation and lymphatic vessel dilatation, not charac-

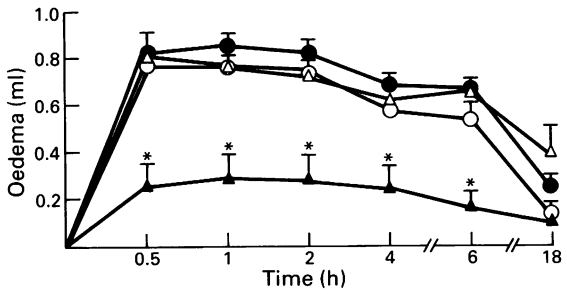

Figure 5 Effect of WEB 2086, BW 755C and heparin on oedema responses induced by subplantar injection of poly-L-arginine (43 kD, 1 mg per paw) in the rat hind paw. Rats were pretreated with WEB 2086 (●, 20 mg kg^{-1}, p.o. 30 min beforehand), BW 755C (△, 50 mg kg^{-1}, p.o., 30 min beforehand), heparin (▲, 50 U per paw) or saline (○). Each point represents the mean of $n = 5$; s.e.mean shown by vertical bars.

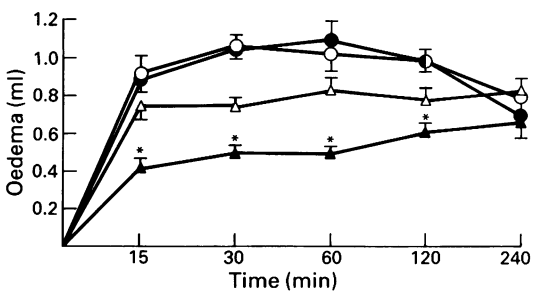

Figure 6 The inhibition caused by N^G-monomethyl-L-arginine (LNMMA) (▲, 4 mg per paw; $n = 5$) on poly-L-arginine-induced paw oedema (○, 1 mg per paw; $n = 5$) was partially reversed by iloprost (△, 100 ng per paw; $n = 5$). Iloprost (100 ng per paw) did not potentiate poly-L-arginine-induced oedema (●; $n = 5$). Each point represents the mean; s.e.mean shown by vertical bars.

teristic of other oedema producing substances, such as bradykinin, 5-HT or carrageenan (Spector & Willoughby, 1968). The increase in vascular permeability induced by the polycations was further confirmed by the extravasation of Evans' blue.

It is likely that the initial oedema was partially caused by the release of 5-HT since methysergide had an inhibitory action at this time and so did depletion of the 5-HT and histamine stores by 48/80. This is to be expected since irritant agents cause release of these mediators (Spector & Willoughby, 1968) and poly-DL-lysine (Padawer, 1970; Ennis *et al.*, 1980) and the polycation 48/80 (Kazimierczak & Diamant, 1978) degranulate mast cells. The lack of effect of mepyramine is not surprising, since histamine is not an important mediator of vascular permeability in rats (Rowley & Benditt, 1956; Wilhelm, 1962).

Although polycations induce release of prostaglandins (Shier *et al.*, 1984; Shier & DuBourdieu, 1986), it is unlikely that the persistence of the oedema was due to these mediators, since indomethacin, the dual inhibitor BW755C (Higgs *et al.*, 1979) and dexamethasone each failed to inhibit this oedema. It is interesting to note that the permeability increase induced by poly-L-lysine in the rabbit skin was sensitive to indomethacin (Needham *et al.*, 1988). The doses we used of the above antagonists were effective since they inhibited carrageenan-induced oedema.

Kinins are important modulators of vascular permeability (for review see Movat, 1985). We tried to evaluate the role of kinins by depleting the rats of kallikrein with cellulose sulphate (Di Rosa *et al.*, 1971). Our results indicate that it is unlikely that kinins play a major role in the polycation-induced oedema.

We have also investigated whether the increase of vascular permeability was due to PAF release. The failure of the PAF antagonist WEB 2086 (Casals-Stenzel *et al.*, 1986) to inhibit the poly-L-arginine-induced oedema indicates that PAF does not appear to be involved in the polycation-induced oedema.

Although the specific inhibitor of EDRF synthesis, LNMMA, caused a marked inhibition of the polycation-induced oedema, the finding that iloprost reversed the inhibition suggests that the inhibitory effect of LNMMA was due to a decrease in flow rather than a decrease in vascular permeability. It is interesting that LNMMA had no effect on carrageenan-induced paw oedema, indicating that in this oedema EDRF does not play an important role.

Polycations are naturally occurring polymers, released mainly from activated leukocytes and platelets (Peterson *et al.*, 1985; Tetta *et al.*, 1985; Camussi *et al.*, 1986). The vascular endothelium (Skutelsky *et al.*, 1975; Skutelsky & Danon, 1976; Simionescu *et al.*, 1981) and glomerular basement membrane (Barnes *et al.*, 1984) contain fixed anionic sites which confer an overall electronegative charge in these structures. The anionic sites are constituted primarily by sulphated glycosaminoglycans, most probably heparan sulphate (Simionescu *et al.*, 1981). It is likely that polycations increase vascular permeability due to electrostatic interactions with these anionic sites. Indeed, polycations of higher molecular weights have larger numbers of cationic sites, which increase their adsorption to surfaces (Hesselink, 1983). The finding that the ability of polycations to induce oedema was proportional to their molecular weights would support this conclusion. However, poly-L-arginine with lower molecular weight than poly-L-lysine was more potent in inducing paw oedema. This result indicates that other factors in addition to electrostatic forces, such as hydrophobic bonding and chain stiffness, modulate polycation-membrane interactions. Poly-L-lysine has lower hydrophobic bonding (Ichimura & Zama, 1977) and non-specific electrostatic binding ability (Ichimura *et al.*, 1978) than poly-L-arginine. There are also differences in the ability of these polycations to display helical conformation which increases chain stiffness, thus decreasing chain interaction with charged surfaces (Ichimura *et al.*, 1978).

Interestingly, heparin binds to vascular endothelium (Hiebert, 1981; Barzu *et al.*, 1985). Our results clearly demonstrated that heparin produced a marked inhibition of the polycation-induced oedema. The neutralization of the positively charged groups of the polycations could be explained if heparin after binding to the endothelial cell, competes with the anionic sites for the polycations. In this case, one might assume that polycations interact with specific endothelial anionic proteins to trigger the increase in the vascular permeability leading to oedema formation, since heparin inhibited the oedema. Alternatively, poly-L-arginine may be interacting with anionic sites on resident cells such as macrophages or mast cells.

Since there is some evidence that platelet polycations modulate glomerular vascular injury (Barnes & Venkatachalam, 1984), our results indicate that it may be worth developing heparin analogues devoid of anticoagulant activity as potential anti-inflammatory drugs.

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The use of oxyhaemoglobin to explore the events underlying inhibition of platelet aggregation induced by NO or NO-donors

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1 Full inhibition of thrombin-induced platelet aggregation was elicited by the least maximal platelet inhibitory concentrations of nitric oxide (NO; $7 \pm 1\,\mu$M) or NO-donors which included sodium nitroprusside (NaNp; $80 \pm 13\,\mu$M), 3-morpholinosydnonimine (SIN-1; $3 \pm 0.1\,\mu$M) or endothelial cells (EC; $2.36 \pm 0.12 \times 10^5$) added 1 min before thrombin. Oxyhaemoglobin (oxyHb; $10\,\mu$M) administered 30 s to 10 min after stimulation with thrombin caused a time-dependent reversal of the inhibition induced by these agents. OxyHb was ineffective when these agents were co-incubated with the non-selective phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 0.05 mM).

2 OxyHb did not reverse the platelet inhibition with IBMX (0.2 mM) or that caused by a selective guanosine 3': 5'-cyclic monophosphate (cyclic GMP) phosphodiesterase inhibitor 2-O-propoxyphenyl-8-azapurin-6-one, (M & B 22948; $20\,\mu$M). In addition, oxyHb did not reverse the inhibition with iloprost (1 nM) which inhibits platelet aggregation through stimulation of adenylate cyclase.

3 The inhibition of platelet aggregation by NO ($7 \pm 1\,\mu$M) or NaNp ($80 \pm 13\,\mu$M) was accompanied by a 13 fold increase in cyclic GMP levels occurring within 15 s of addition of these agents. In the continued presence of NO or NaNp, the reversing effect of oxyHb given 1 min after thrombin was associated with a pronounced decrease in cyclic GMP levels.

4 We conclude that the inhibition of platelet aggregation by activators of guanylate cyclase depends in the first few minutes on continuous stimulation of the enzyme in order to maintain intracellular concentrations of cyclic GMP, except when its breakdown is inhibited.

5 The addition of agents such as oxyHb after the inhibition of platelet aggregation offers another way of investigating the biochemical changes involved in maintaining platelets in an inactive state.

Introduction

Endothelium-derived relaxing factor (EDRF), which is released from the vascular endothelium in response to a number of stimuli, induces vasodilatation (Furchgott & Zawadzki, 1980) and inhibits platelet aggregation (Azuma *et al.*, 1986; Radomski *et al.*, 1987a,b) and platelet adhesion (Radomski *et al.*, 1987c; Sneddon & Vane, 1988) through stimulation of soluble guanylate cyclase. Nitric oxide (NO) accounts for the biological activity of EDRF (Palmer *et al.*, 1987; Ignarro *et al.*, 1987). Although there is still debate as to whether EDRF is NO or a closely related substance such as a nitrosothiol (Myers *et al.*, 1990), the inhibition of EDRF formation by L-arginine analogues such as N^G-monomethyl-L-arginine (Hibbs *et al.*, 1987), helps to substantiate the identity of EDRF as NO. Thus, we shall refer to EDRF as NO in this paper. Nitrovasodilators can be considered NO donors for they act through the release of NO. NO activates soluble GC by interacting with the ferrohaem centre of the enzyme resulting in increased concentrations of guanosine 3':5'-cyclic monophosphate (cyclic GMP; Ignarro *et al.*, 1986). The anti-aggregatory and anti-adhesive effects of NO or NO-donors on platelets and the elevation of cyclic GMP are reversed or decreased by the simultaneous addition of oxyhaemoglobin (oxyHb; Mellion *et al.*, 1980; 1983; Nishikawa *et al.*, 1982) which inactivates NO (Haussmann & Werringloer, 1985).

Little is known about the relationship between cyclic GMP concentrations and inhibition of platelet aggregation when NO is scavenged by oxyHb. We have studied this by inducing inhibition of aggregation by NO or NO-donors including cultured endothelial cells (EC) and adding oxyHb at different times after the inhibition of platelet aggregation. The platelet cyclic GMP levels were also measured.

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Methods

Preparation of washed platelets

Human washed platelets were prepared as described previously (Radomski & Moncada, 1983) and indomethacin ($10\,\mu$M) was added to the final platelet suspension to inhibit the formation of cyclo-oxygenase products. The platelet count was adjusted to 1×10^8 cells ml^{-1}.

Preparation of cultured endothelial cells

Endothelial cells (EC) from bovine aortae were grown as monolayer cultures in T75 tissue culture flasks. When confluent, the EC were treated briefly (10–20 s) with 0.05% (w/v) trypsin 1 h before use and spun at 1000 r.p.m. for 5 min to form a pellet. The supernatant was removed and the pellet containing the EC was then resuspended in Hanks balanced salt solution (without Ca^{2+} or Mg^{2+}) containing 10 mM HEPES buffer (HHBSS) and $10\,\mu$M indomethacin at pH 7.4. The final EC count was adjusted to 9×10^6 cells ml^{-1}.

Measurement of platelet aggregation

A suspension of washed platelets ($500\,\mu$l) was incubated at 37°C for 2 min with continuous stirring at 1000 r.p.m. in a Payton Dual channel aggregometer (Born & Cross, 1963) and then stimulated with submaximal concentrations of thrombin (40–80 mu ml^{-1}). The decrease in optical density was recorded for 5 min after addition of thrombin. Sodium nitroprusside (NaNp; $80 \pm 13\,\mu$M), nitric oxide (NO; $7 \pm 1\,\mu$M), 3-morpholinosydnonimine (SIN-1; $3 \pm 0.1\,\mu$M), iloprost (1 nM), 3-isobutyl-1-methylxanthine (IBMX; 0.2 mM), 2-O-propoxyphenyl-8-azurin-6-one (M & B 22948; $20\,\mu$M) or endothelial cells (EC; $2.36 \pm 0.12 \times 10^5$) at concentrations which just induced 100% inhibition of platelet aggregation were added to the platelet suspension 1 min before stimulation with

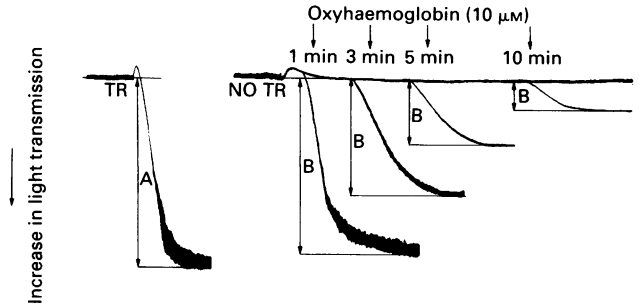

Figure 1 A typical aggregation trace of the reversal by oxyhaemoglobin of platelet inhibition by nitric oxide (NO). NO was preincubated with a suspension of washed platelets 1 min before thrombin (TR, 40–80 mu ml^{-1}) stimulation. Oxyhaemoglobin (oxyHb, 10 μM) or an equivalent volume of Krebs buffer (5 μl) was given at different times after the inhibition of platelet aggregation induced by NO (or NO-donors). The response was monitored for a further 5 min. Percentage aggregation obtained after oxyHb was calculated from the equation: % aggregation = B/A × 100, where A = change in light transmission obtained after administration of thrombin and B = change in light transmission obtained after administration of thrombin and oxyHb.

thrombin. Inhibition of platelet aggregation was expressed as % of maximal aggregation (Figure 1).

To assess whether the platelet inhibitory activity of the EC was related to the removal of thrombin by these cells, a suspension of EC (3×10^5) in Krebs buffer was incubated for 2 min with thrombin and then centrifuged at 10,000 g for 1 min. Platelet aggregatory activity of the supernatant was compared with the aggregatory activity of thrombin at the same concentration as that added to the suspension of EC.

Reversal of the inhibition of platelet aggregation by oxyhaemoglobin

Oxyhaemoglobin (10 μM) was added at different times after thrombin (from 30 s to 10 min) and the response monitored for a sufficient time (5 min) to achieve the plateau phase. The % aggregation obtained after oxyHb was calculated as shown in Figure 1 and was derived from the following equation: % aggregation = B/A × 100 where A = change in light transmission (mm) in control experiments obtained after administration of thrombin and B = change in light transmission (mm) obtained after the administration of thrombin and oxyHb.

Platelet guanosine 3':5'-cyclic monophosphate

Concentrations of cyclic GMP were measured with a radioimmunoassay kit using ^{125}I-labelled cyclic GMP. NO or NaNp were used in the same concentrations as in the aggregation experiments. A suspension of washed platelets (500 μl) was preincubated in the cuvette of an aggregometer for 1 min. The required concentration of NaNp or NO was then added to the platelet suspension and the incubation continued for different times both before (from 15 s to 10 min) and after stimulation with thrombin (from 1 to 15 min). The reaction was stopped by adding to the samples ice-cold trichloroacetic acid (TCA; 500 μl of a 10%, w/vol solution). In another series of experiments, NO or NaNp were added to the platelets 1 min before stimulation with thrombin. Oxyhaemoglobin (10 μM), an equivalent volume of Krebs buffer (5 μl) or a higher concentration of thrombin (200 mu ml^{-1}) was then added 1 min after thrombin (40–80 mu ml^{-1}) stimulation and the samples collected after 5 min. The samples were stored at −20°C, and the cyclic nucleotide extracted with 0.5 M tri-n-octylamine in 1,1,2-trichloro-trifluorethane. The addition of [^{3}H]-cyclic GMP to platelets showed a recovery of >99%, so the results have not been corrected for recovery. All experiments were performed in duplicate. The results are expressed as cyclic GMP pmol/0.5×10^8 platelets.

Statistics

Results are expressed as means ± s.e.mean of (n) experiments and each experiment was performed with blood obtained from different donors. The results were analysed by Student's unpaired t test to determine the significant difference between means, or by a two-way analysis of variance (ANOVA) followed by a least significance procedure to determine the nature of this response.

A P value of <0.05 was taken as significant.

Materials

The composition of the modified Krebs buffer was (mM): NaCl 137, KCl 2.7, $NaHCO_3$ 11.9, NaH_2PO_4 0.3, $MgSO_4$ 0.8, glucose 5.6 and $CaCl_2$ 1. The cell culture medium (CCM) consisted of Dulbecco's Modified Eagle's Medium with the addition of 10% foetal calf serum, L-glutamine (4 mM), penicillin (100 iu ml^{-1}), streptomycin (100 μg ml^{-1}) and gentamycin (0.1 mg ml^{-1}). Human thrombin, sodium nitroprusside and haemoglobin (from bovine blood) were obtained from Sigma (Poole, Dorset). 3'-Isobutyl-1-methylxanthine was obtained from Aldrich (Gillingham, Dorset). Kits for radioimmunoassay of cyclic GMP were purchased from Amersham (Little Chalfont, Buckinghamshire). Tri-n-octylamine and 1,1,2-trichloro-trifluoroethane and nitric oxide (NO) were obtained from British Drug Houses (Dagenham, Essex). HEPES buffer, penicillin, streptomycin and gentamycin were obtained from Flow Laboratories (Rickmansworth, Herts). Stock solutions of M & B 22948 or IBMX were dissolved in triethanolamine (20% v/v) and 0.1 N NaOH respectively and diluted with Krebs buffer as required. The concentrations of triethanolamine or NaOH in the platelet suspension did not exceed 0.05% or 0.01% respectively. Oxyhaemoglobin was prepared by reduction of bovine haemoglobin with sodium hydrosulphite as described previously (Salvemini *et al.*, 1989). Helium was obtained from B.O.C. Medical Gases (London). NO solutions were prepared by injecting 1 ml NO into 40 ml of helium-deoxygenated water kept in a Wheaton flask (de Nucci *et al.*, 1988). Prostacyclin was a gift from Wellcome Research Laboratories (Beckenham, Kent) and iloprost was a gift from Schering Ltd (Berlin, West Germany). M & B 22,948 was a gift from May & Baker (Dagenham, Essex) and SIN-1 a gift from Hoechst (Frankfurt, West Germany).

Results

Effects of oxyhaemoglobin on NO, NaNp, SIN-1 or EC-induced inhibition of thrombin-stimulated platelet aggregation

Thrombin (40–80 mu ml^{-1}) produced a submaximal (80–90% of maximum) aggregation within 3 min, and this response was abolished by NO (7 ± 1 μM, n = 7), NaNp (80 ± 13 μM, n = 6), SIN-1 (3 ± 0.1 μM, n = 3) or EC ($2.36 \pm 0.12 \times 10^5$, n = 4).

When oxyHb (10 μM) was added in the presence of NO or NaNp at different times after thrombin (from 30 s to 10 min), a time-dependent reversal of the anti-aggregatory effect was observed (Figures 1 and 2). Similar results were also obtained from SIN-1 or EC (Figure 3). When NaNp at the concentration tested was incubated for 5 min with a suspension of platelets and stimulated at this time with thrombin (40–80 mu ml^{-1}), no aggregation was observed (n = 3, not shown). This response was not altered if oxyHb (10 μM) was given together with thrombin 5 min after preincubation of the platelet suspension with NaNp. The reversal by oxyHb added 1 min after thrombin was not seen when NO (n = 4), NaNp (n = 4), SIN-1 (n = 3) or EC (n = 4) were preincubated with the platelets in the presence of IBMX (0.05 mM). This concentration of IBMX alone did not inhibit platelet aggregation. When given 30 s after thrombin, oxyHb did not reverse the

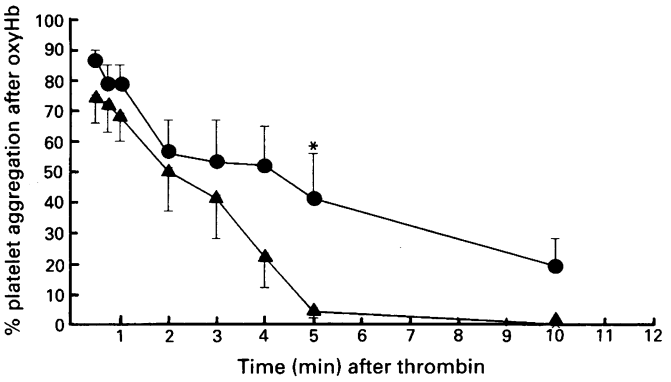

Figure 2 Reversal by oxyhaemoglobin (oxyHb, 10 μM) of the inhibition of thrombin (40–80 mu ml^{-1})-induced platelet aggregation elicited by nitric oxide (NO, 7 ± 1 μM, ●) (n = 7) or sodium nitroprusside (NaNp, 80 ± 13 μM, ▲) (n = 6). OxyHb when given at different times after thrombin promoted a gradual, time-dependent, reversal of the inhibitory effect of NO or NaNp. The points show mean and vertical bars represent s.e.mean for (n) experiments. * $P < 0.05$ when the values for NO were compared to those for NaNp by the ANOVA test followed by a least significant difference test.

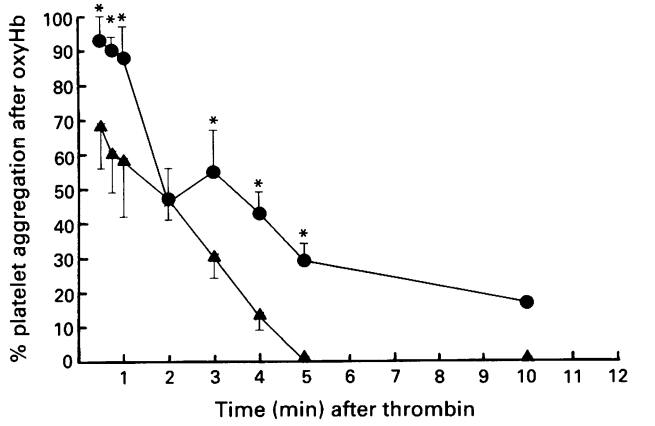

Figure 3 Reversal by oxyhaemoglobin (oxyHb, 10 μM) of the inhibition of thrombin (40–80 mu ml^{-1})-induced platelet aggregation by 3-morpholinosydnonimine (SIN-1, 3 ± 0.1 μM, ●) (n = 3) or endothelial cells (EC, 2.36 ± 0.12 × 10^5, ▲) (n = 4). OxyHb when given at different times after thrombin promoted a gradual, time-dependent, reversal of the inhibitory effect of SIN-1 or EC. The points represent the mean and vertical bars the s.e.mean for (n) experiments. * $P < 0.05$ when the values for SIN-1 were compared to those for EC by the ANOVA test followed by a least significant difference test.

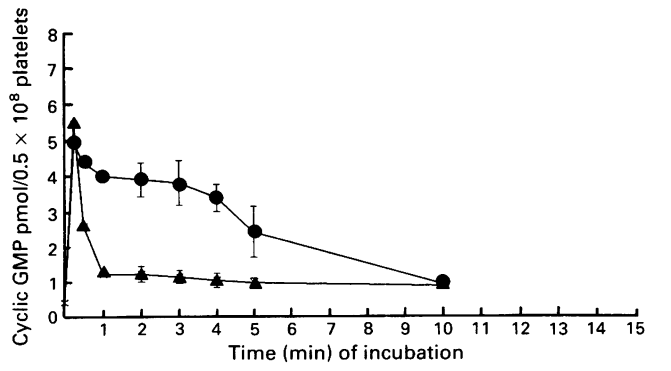

Figure 4 The effect of nitric oxide (NO, ●) or sodium nitroprusside (NaNp, ▲) on cyclic GMP concentrations. NO (7 ± 1 μM) (n = 7) or NaNp (80 ± 13 μM) (n = 6) were preincubated with a suspension of platelets for different times. NO or NaNp caused a sharp increase in the levels of cyclic GMP (13 fold in 15 s). In the presence of NO, the levels of cyclic GMP remained elevated for up to 4 min and then gradually declined, whereas with NaNp the levels of cyclic GMP fell within 1 min to approximately twice the basal levels. Each point is the mean with s.e.mean shown by vertical bars of (n) experiments.

anti-aggregatory activity of iloprost (1 nM, n = 4), IBMX (0.2 mM, n = 4) or of M & B 22948 (20 μM, n = 2). The concentration of oxyHb used was sufficient to reverse fully the inhibition of platelet aggregation by NO or NO-donors when given together with these agents before applying thrombin, and was, therefore, taken as being in sufficient amount to remove any NO present in the extracellular fluid. The inhibition of EC could not be explained by uptake of thrombin by these cells, for supernatants from suspensions of EC that had been incubated with thrombin for 2 min produced the same degree of aggregation as that obtained with thrombin solution.

Concentrations of cyclic GMP in platelets exposed to NaNp or NO

Nitric oxide (7 ± 1 μM, n = 7) caused a 12 fold increase in the platelet concentrations of cyclic GMP within 15 s. The cyclic GMP levels then gradually declined until after 10 min they were approximately double the basal levels (Figure 4).

NaNp (80 ± 13 μM, n = 6) also caused a rapid, 13 fold increase in platelet cyclic GMP concentrations within 15 s. Thereafter, unlike the changes recorded after addition of NO the levels fell sharply so that 1 min after addition of NaNp they were twice the basal levels (Figure 4). This concentration of cyclic GMP was maintained during the following 9 min of the experiment. These increases in cyclic GMP concentrations elicited by NaNp or NO were not altered by stimulation with thrombin when measured for up to 15 min after addition of the nitro compounds (not shown).

Effects of oxyHb on the levels of cyclic GMP in platelets exposed to NaNp or NO

During NO- (7 ± 1 μM, n = 7) or NaNp- (80 ± 13 μM, n = 6) induced inhibition of platelet aggregation the addition of oxyHb 1 min after stimulation with thrombin was associated with a fall in the platelet levels of cyclic GMP (as measured 5 min later) to values approximating basal levels (Figure 5a and 5b).

When platelets were preincubated with NO or NaNp together with IBMX (0.05 mM) for 1 min before stimulation with thrombin, oxyHb added 1 min later did not reverse the inhibitory activity of NO or NaNp. This dose of IBMX by itself did not significantly increase the levels of cyclic GMP (n = 6, $P < 0.1$) but markedly potentiated the increases induced by NO or NaNp (Figure 5a and 5b). Under these circumstances, that is, in the presence of IBMX (0.05 mM) oxyHb caused a 4 fold decrease in cyclic GMP levels with NO or a 2 fold decrease with NaNp (Figure 5a and 5b). However, these reduced levels were still twice as high as the basal levels recorded.

A high dose of thrombin (200 mu ml^{-1}) given 1 min after stimulation with the submaximal concentration of thrombin (40–80 mu ml^{-1}) fully reversed the platelet inhibitory activity of NaNp but did not decrease the levels of cyclic GMP (0.9 ± 0.1 after Krebs buffer and 0.8 ± 0.1 pmol/0.5 × 10^8 platelets after 200 mu ml^{-1} thrombin, n = 3, $P < 0.5$). Thus, the decrease in cyclic GMP observed after oxyHb cannot be due to a possible non-specific effect of this agent.

Discussion

Our results show that the inhibition of platelet aggregation by NO or NO-generating agents is maintained during the first few minutes by continuous stimulation of guanylate cyclase to compensate for a continuous breakdown of the cyclic GMP formed. Thus the inhibition of platelet aggregation by NO or NO-donors was readily reversed by the addition of oxyHb after the inhibition had occurred but not when the breakdown of cyclic GMP was prevented by a phosphodiesterase inhibitor.

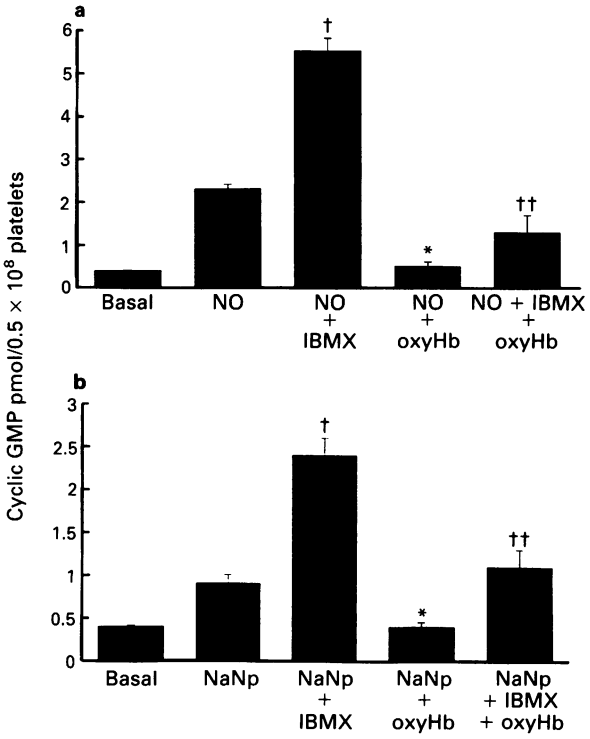

Figure 5 Changes in cyclic GMP concentrations in platelets in the presence of nitric oxide (NO) (a) or sodium nitroprusside (NaNp) (b) when oxyhaemoglobin (oxyHb, 10 μM) was added 1 min after stimulation with thrombin and the reaction stopped 5 min later. In the presence of NO ($7 \pm 1\,\mu$M, a) ($n = 7$) or NaNp ($80 \pm 13\,\mu$M, b) ($n = 6$), oxyHb decreased the platelet concentrations of cyclic GMP to values approximating basal levels. 3-Isobutyl-1-methylxanthine (IBMX, 0.05 mM) significantly increased the cyclic GMP levels in the presence of NO ($n = 7$) ($P < 0.005$, a) or NaNp ($n = 6$) ($P < 0.005$, b). Under these conditions, oxyHb caused a 4 fold (a) or 2 fold (b) decrease in cyclic GMP concentrations. The final levels reached were, however, at least twice the basal levels. Columns represent the mean and vertical bars show s.e.mean of (n) experiments. * $P < 0.01$ when compared to the values obtained with NO or NaNp in the absence of oxyHb, † $P < 0.005$ when compared to the value obtained with NO or NaNp alone and †† $P < 0.01$ when compared to basal cyclic GMP levels.

The effect of oxyHb was selective since it did not reverse the inhibition of platelet aggregation induced by the stable analogue of prostacyclin (PGI_2), iloprost. Iloprost like PGI_2, acts by stimulating the platelet adenylate cyclase leading to a rise in the levels of cyclic AMP (Tateson *et al.*, 1977; Gorman *et al.*, 1977). In addition, oxyHb failed to reverse the inhibition of platelet aggregation induced by either the non-selective phosphodiesterase inhibitor, IBMX, or that induced by the specific cyclic GMP phosphodiesterase inhibitor, M & B 22987. These agents inhibit platelet aggregation by preventing the destruction of cyclic GMP (Kramer *et al.*, 1977; Pavel & Coquil, 1978; Wells & Kramer, 1981).

A number of reports have shown that brief exposure of platelets to thrombin makes them less sensitive to subsequent activation by thrombin (Shuman *et al.*, 1978; McGowan *et al.*, 1983). In our experiments such a desensitization phenomena cannot explain the time-dependent reversal obtained from oxyHb. This is based on the finding that thrombin given 5 min after exposure of platelets to NaNp does not cause aggregation and this effect is not altered by administration of oxyHb together with thrombin.

Oxyhaemoglobin cannot penetrate platelets (Gruetter *et al.*, 1979) so the reversing effect observed with oxyHb must be due to removal of NO from the extracellular medium. By creating a concentration gradient, this may then lead to reduced NO concentrations inside the platelets.

Our results show that an increase of cyclic GMP to twice the basal levels after addition of NO or NaNp contributes towards keeping the platelets in an inhibited state for up to 15 min after stimulation with thrombin. In fact, inhibition of platelet aggregation by NO or NaNp was accompanied by a substantial initial increase in the platelet levels of cyclic GMP (12/13 fold after 15 s of contact) which decreased thereafter. However, levels of cyclic GMP decreased more rapidly after NaNp, in agreement with the data obtained by other groups (Mellion *et al.*, 1980; 1983; Nishikawa *et al.*, 1982). This suggests that the release of NO from NaNp was not maintained over the period of observation. Alternatively, the presence of cyanide in the NaNp molecule may have inhibited the guanylate cyclase (Gerzer *et al.*, 1988). Other possibilities such as stimulation of the cyclic GMP-dependent phosphodiesterase by NaNp cannot be ruled out.

The reversal by oxyHb of NO or NaNp-induced inhibition of thrombin-stimulated platelet aggregation was accompanied by a fall in the platelet cyclic GMP concentrations to basal levels. In the presence of IBMX, oxyHb also decreased cyclic GMP but the concentrations achieved were still twice as high as basal, and hence in a range sufficient to keep the platelets in an inhibited state. Clearly, the reversibility by oxyHb in the first few minutes after inhibition with NO or NO donors, demonstrates that stimulation of guanylate cyclase is the predominant mechanism at that time. Thereafter, there is a later process which does not involve NO since it is not reversed by oxyHb. As with NaNp, the inhibition by EC was not easily reversed by oxyHb after 4–5 min. This may reflect reduced NO release by these cells after a few minutes of stirring.

A number of hypotheses could explain the mechanism by which activators of soluble guanylate cyclase mediate their effects. These include: rapidly occurring changes such as phosphorylation of a 50 kD protein by the cyclic GMP-dependent protein kinase (Halbrugge *et al.*, 1990), cyclic GMP-dependent protein phosphorylation/dephosphorylation of myosin light chain (Kawahara *et al.*, 1984) or inhibition of phosphorylation of a 44 kD protein by protein kinase c (Waldmann & Walter, 1989) or long term changes such as activation of platelet ADP ribosyltransferase which seem to be independent of guanylate cyclase stimulation (Brune & Lapetina, 1989). At present however, there is no evidence for the involvement of a particular biochemical pathway in maintaining the inhibition of platelet aggregation by NO or NO-donors. This stems from the fact that it is difficult to monitor simultaneously both short and long term changes in platelets and reversal of their inhibition. Thus, the experimental model presented in this study may be of value in identifying some of the components involved in maintaining platelets in an inactive state.

Our results show clearly that the cyclic GMP concentrations needed to inhibit platelet aggregation can be maintained either by continuous formation of cyclic GMP or by preventing its breakdown. Thus, drugs which combine both NO-like activity and phosphodiesterase blocking activity could be more effective anti-platelet drugs than those possessing only NO-like properties.

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Tachykinin receptors in the circular muscle of the guinea-pig ileum

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1 We have studied the mechanical response of circular strips of the guinea-pig ileum to tachykinins and characterized the receptors involved by means of receptor-selective agonists.

2 The strips responded to both substance P (SP) and neurokinin A (NKA), as well as to [Pro^9]-SP sulphone (selective NK_1-receptor agonist), [βAla^8]-NKA(4–10) (selective NK_2-receptor agonist) and [MePhe^7]-neurokinin B (selective NK_3-receptor agonist). The ED_{50}s of the various peptides (calculated as the concentration of agonist which produced 50% of the response to 10 μM carbachol) were similar, in the range of 40–200 nM, i.e. no clearcut rank order of potency was evident.

3 The response to a submaximal (10 nM) concentration of SP or NKA was unaffected in the presence of peptidase inhibitors (thiorphan, captopril and bestatin, 1 μM each).

4 The response to the NK_1-agonist was totally atropine-resistant, but was reduced (about 30% inhibition) by tetrodotoxin. The response to the NK_3-receptor agonist was halved by atropine and abolished by tetrodotoxin. The response to the NK_2-agonist was unaffected by either atropine or tetrodotoxin.

5 The response to the selective NK_2-agonist was unchanged after desensitization of NK_1- or NK_3-receptors.

6 The response to the NK_2-selective agonist was strongly inhibited by [Tyr^5, D-Trp^{6,8,9}, Arg^{10}]-NKA(4–10) (MEN 10,207) a selective NK_2-receptor antagonist which did not modify the response to the NK_1-selective agonist.

7 Our findings indicate that all the three known types of tachykinin receptors mediate the contractile response of the circular muscle of the guinea-pig ileum to peptides of this family. The response to activation of NK_3-receptors is totally neurogenic and partially mediated by endogenous acetylcholine, the response to activation of NK_1-receptors is partly neurogenic and largely myogenic and the response to activation of NK_2-receptors is totally myogenic.

Introduction

Ample evidence, based on pharmacological, physiological, anatomical and neurochemical findings indicates that tachykinins (TKs) play a physiological role as excitatory transmitters in the guinea-pig ileum (Franco *et al.*, 1979; Costa *et al.*, 1981; 1985; Donnerer *et al.*, 1984; Smith & Furness, 1988; Llewellyn-Smith *et al.*, 1988; 1989; Bartho' *et al.*, 1989; Holzer, 1989; see also Bartho' & Holzer 1985 for review). In the guinea-pig intestine, substance P (SP) and other TKs, such as neurokinin A (NKA) (Too *et al.*, 1989) are stored in intrinsic neurones thought to play a role as final effectors of the atropine-resistant peristalsis (Bartho' & Holzer, 1985 for review).

The contractile response of the guinea-pig ileum to TKs is mediated by specific receptors (Lee *et al.*, 1982). In the longitudinal muscle, at least two TK receptors are present: one receptor (NK_1) mediates the direct response of muscle cells while the other (NK_3) activates intramural effector neurones which in turn release acetylcholine and possibly endogenous TKs (Kilbinger *et al.* 1986; Laufer *et al.*, 1986; 1988; Guard & Watson, 1987). Some studies have also suggested that a third type of TK receptor (NK_2) may mediate the contractile response of the guinea-pig ileal longitudinal muscle (Jacoby *et al.*, 1986; Dion *et al.*, 1987) while other investigations have excluded this possibility (Laufer *et al.*, 1988).

Also in the circular muscle of the guinea-pig ileum TKs are potent spasmogens (Holzer *et al.*, 1980; Costa *et al.*, 1985). However, the type of TK receptors mediating their contractile response at this level has not been determined. Complex mechanisms are likely to be involved, because the contractile response to SP has both a direct and an indirect component (Holzer *et al.*, 1980; Costa *et al.*, 1985).

The aim of this study was to characterize the TK receptors mediating the contractile response of the circular muscle of the guinea-pig ileum to these peptides. With this aim, the effect of SP and NKA as well as of receptor-selective synthetic TK agonists were investigated.

Methods

Male albino guinea-pigs (250–300 g) were stunned and bled. A segment of the ileum was excised and placed in oxygenated (96% O_2 and 4% CO_2) Krebs solution. The ileum was opened along the mesenteric border and pinned flat on a Petri dish. A small strip (< 3 mm wide) was then dissected along the circular axis as described by Costa *et al.* (1985) except that no attempt was made to remove the mucosal layer, in order to avoid any possible damage to the inner circular muscle. The strips were transferred to a 5 ml bath for isotonic recording (load 5 mN) of mechanical activity via a Basile 7050 Unirecord. In some experiments, the strips were electrically stimulated by means of two wire platinum electrodes placed at the top and bottom of the organ bath, connected to a Grass S11 stimulator.

All experiments started after a 90 min equilibration period. The contractile response to carbachol (10 μM) was assessed at 15–20 min intervals until reproducible responses were obtained. The response to carbachol was used as an internal standard to express contractile responses to peptides or field stimulation. Non-cumulative concentration-response curves to peptides were constructed at 15–20 min intervals with washing between doses. Some experiments were performed in presence of atropine (3 μM, contact time 15 min) or tetrodotoxin (1 μM, contact time 15 min). In these experiments the response to a 1 μM concentration of the three receptor-selective agonists was determined at 30 min intervals until reproducible responses were obtained before testing the effect of atropine or tetrodotoxin. In some experiments the response to a submaximal concentration of SP or NKA (10 nM) was determined before and after application of a mixture of peptidase inhibitors

(thiorphan, captopril and bestatin to block endopeptidase 24.11, angiotensin converting enzyme and aminopeptidases, respectively, 1 μM for each inhibitor).

In other experiments the response to a submaximal concentration of the peptides was studied before and after application (contact time 15 min) of [Tyr^5, D-Trp^{6,8,9}, Arg^{10}]-neurokinin A (4–10) (MEN 10,207) a newly developed selective NK_2-tachykinin receptor antagonist (Rovero *et al.*, 1990; Maggi *et al.*, 1990b). Attempts were made to construct cumulative concentration-response curves to the peptide in order to study the effect of the antagonist on the full concentration-response curves but marked desensitization was found in all cases tested ($n = 4$ from 4 animals).

All data in the text and figures are mean ± s.e.mean of 5–8 determinations. Statistical analysis was made by Student's t test for paired or unpaired data or by means of analysis of variance, when applicable. A P level < 0.05 was considered statistically significant.

EC_{50}s of SP, NKA and of the receptor-selective synthetic agonists were calculated, using linear regression analysis and the least square method, as the concentration of each peptide which produced 50% of the response to 10 μM carbachol.

Drugs used were: atropine HCl (Serva), tetrodotoxin (Sankyo), substance P and neurokinin A and bestatin (Peninsula), thiorphan (Sigma), captopril (Squibb). [Pro^9]-SP sulphone was a kind gift of Prof. D. Regoli, Department of Pharmacology, University of Sherbrooke, Canada. [β Ala^8]-NKA(4–10), [MePhe^7]-neurokinin B and [Tyr^5, D-Trp^{6,8,9}, Arg^{10}]-NKA(4–10) (MEN 10,207) were synthesized by Dr P. Rovero, Department of Chemistry, Menarini Pharmaceuticals by a conventional solid phase method. All peptides were dissolved in saline and kept frozen until use.

Results

About 50% of the circular strips showed an irregular, low-amplitude phasic activity (15% of the response to carbachol) which often waned and re-appeared during the course of the experiment. Electrical field stimulation at 20 Hz for 5 s (0.5 ms pulse width, 60 V) evoked phasic contractions which ranged between 72 and 94% of the response to carbachol. These responses were reduced by atropine (46 ± 7% inhibition, $n = 8$) and abolished by tetrodotoxin (1 μM). Atropine (3 μM) fully inhibited the contraction to 10 μM carbachol ($n = 4$).

Both SP and NKA produced a concentration (1 nM–1 μM)-dependent contraction of the circular muscle of the guinea-pig ileum (Figures 1 and 2). The curves to these two peptides were virtually superimposable. At 1 μM, the peak contractile response to SP and NKA averaged 86 ± 3 and 95 ± 2% of that to carbachol, respectively ($n = 5$ for each peptide). EC_{50}s and 95% confidence limits (in parentheses) were as follows: SP 42 nM (29–79), NKA 103 nM (62–129).

A consistent, concentration-dependent contractile response (Figures 1 and 2) was also obtained with each one of the three receptor selective agonists ($n = 6$–8). The responses at 1 μM averaged 61 ± 4, 68 ± 3 and 88 ± 4% of that to carbachol for [Pro^9]-SP sulphone ($n = 9$), [βAla^8]-NKA(4–10) ($n = 6$) and [MePhe^7]-neurokinin B ($n = 7$), respectively. EC_{50}s and 95% confidence limits (in parentheses) were as follows: [Pro^9]-SP sulphone 209 nM (115–402); [βAla^8]-NKA(4–10) 199 nM (152–279); [MePhe^7]-neurokinin B 68 nM (50–91).

The question was raised whether activity of TKs might have been underestimated because of local metabolism by tissue peptidases. To address this point the response to a submaximal concentration of SP and NKA (10 nM) was determined in the absence and the presence of a mixture of peptidase inhibitors (thiorphan, captopril and bestatin, 1 μM each, 15 min beforehand). The mixture of peptidase inhibitors did not change the response to carbachol ($n = 4$) nor affected the response to added peptides. In fact the responses to 10 nM SP averaged 18 ± 4 and 20 ± 5% and that to 10 nM NKA 25 ± 4 and 23 ± 3% of the response to carbachol before and after addition of the peptidase inhibitors, respectively ($n = 4$ for each peptide, NS).

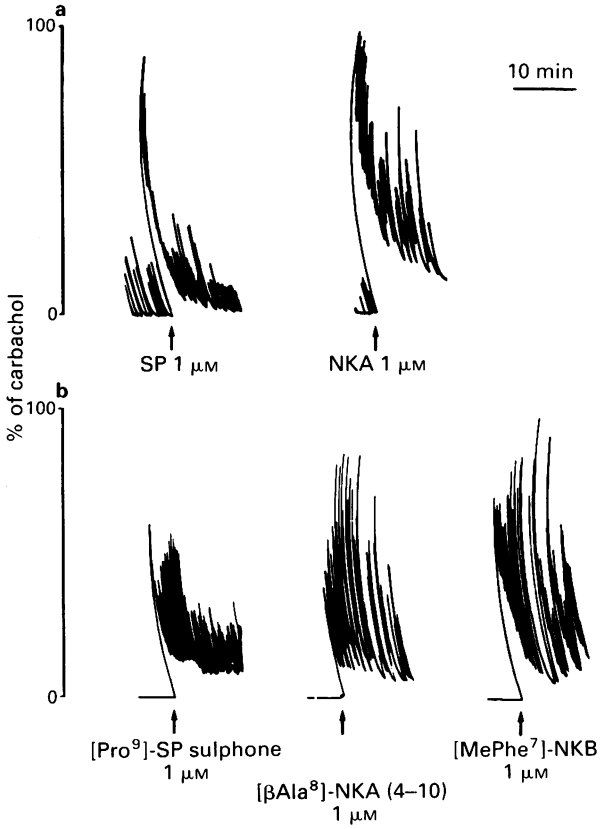

Figure 1 Typical tracings showing the contractile response of the circular muscle of the guinea-pig ileum to (a) 1 μM substance P (SP), neurokinin A (NKA) and (b) the receptor-selective agonists, [Pro^9]-SP sulphone (NK_1-receptor), [βAla^8]-NKA(4–10) (NK_2-receptor) and [MePhe^7]-neurokinin B (NKB). Vertical bars indicate the maximal contractile response to carbachol (10 μM).

Figure 3 shows the effect of atropine (3 μM) and tetrodotoxin (1 μM) on the contractile responses produced by the synthetic receptor-selective agonists. The response to [Pro^9]-SP sulphone was not significantly changed by atropine, although in individual experiments either an enhancement or a reduction was observed ($n = 11$). Conversely, tetrodotoxin produced a statistically significant reduction (about 30%) of the response to the NK_1-receptor agonist ($n = 8$, Figure 3). The response to [MePhe^7]-neurokinin B was significantly reduced (about 50%) in the presence of atropine and was vir-

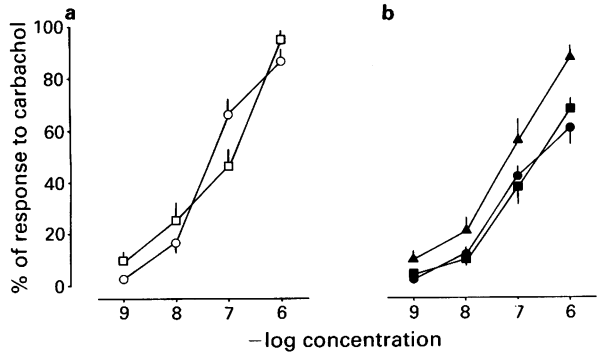

Figure 2 Concentration-response curves showing the contractile effect of (a) substance P (SP, ○, $n = 5$), neurokinin A (□, $n = 5$) and (b) the receptor-selective agonists [Pro^9]-SP sulphone (●, NK_1-receptor, $n = 8$), [βAla^8]-NKA(4–10) (■, NK_2-receptor, $n = 7$) and [MePhe^7]-neurokinin B (▲, NK_3-receptor, $n = 6$). Each point is mean with s.e.mean shown by vertical lines.

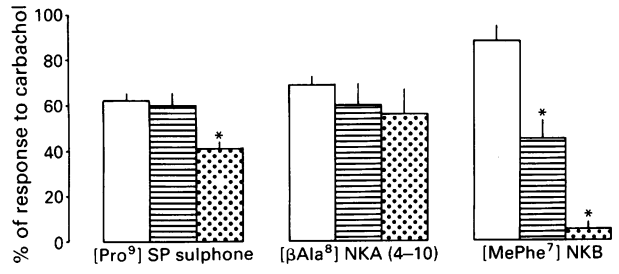

Figure 3 Effect of atropine (3 μM horizontally lined columns) or tetrodotoxin (1 μM, stippled columns) on the contractile response of the circular muscle of the guinea-pig ileum to 1 μM application of [Pro^9]-SP sulphone (NK_1-receptor, $n = 8$–11), [βAla^8]-NKA(4–10) (NK_2-receptor, $n = 6$) and [MePhe^7]-neurokinin B (NK_3-receptor, $n = 6$). Control: open columns. Significantly different from the control response, $^*P < 0.05$.

tually abolished by tetrodotoxin ($n = 6$ each). By contrast the response to [βAla^8]-NKA(4–10) was unaffected by either atropine or tetrodotoxin ($n = 6$ each, Figure 3).

Cross-desensitization experiments were also performed in order to assess whether NK_2-receptors might contribute to the contractile response to TKs. As shown in Figure 4, a second application of either [Pro^9]-SP sulphone or [MePhe^7]-neurokinin B (1 μM for each peptide) failed to reproduce the contractile response observed at the first challenge, indicating desensitization of NK_1- and NK_3-receptors, respectively. In the presence of each of the two desensitizing peptides, 1 μM [βAla^8]-NKA(4–10) produced a contractile response not different from the control, indicating the lack of cross-desensitization. Similar results were obtained for each desensitizing peptide in 4 preparations from different animals.

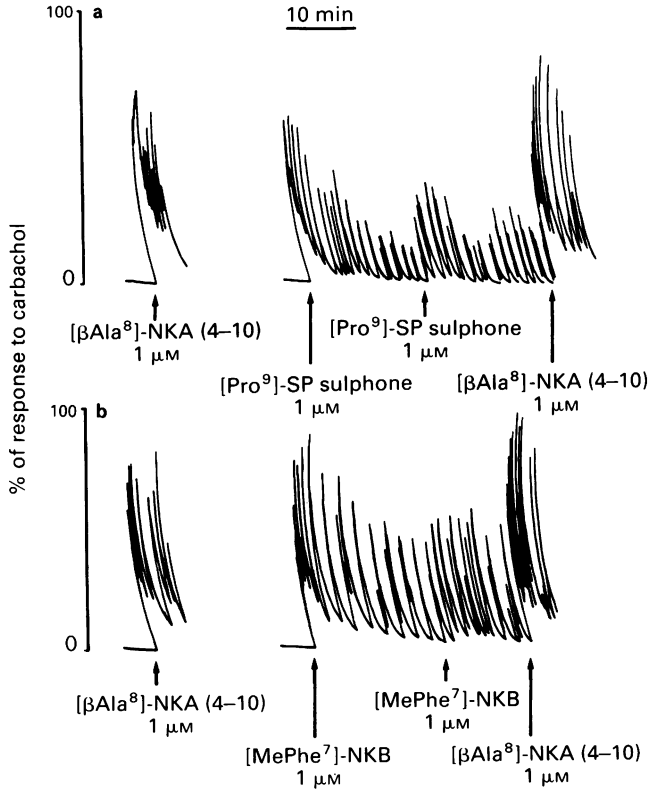

Figure 4 Typical tracings showing the lack of cross desensitization between the response to [Pro^9]-SP sulphone (NK_1-receptor selective) and [βAla^8]-NKA(4–10) (NK_2-receptor selective) (a) and [MePhe^7]-neurokinin B (NKB, NK_3-receptor selective) and [βAla^8]-NKA (4–10) (b). In each panel the control response to the NK_2-receptor agonist, obtained 30 min before application of the first dose of the NK_1- or NK_3-receptor selective agonist is shown. Experiments were performed on paired strips from the same animal.

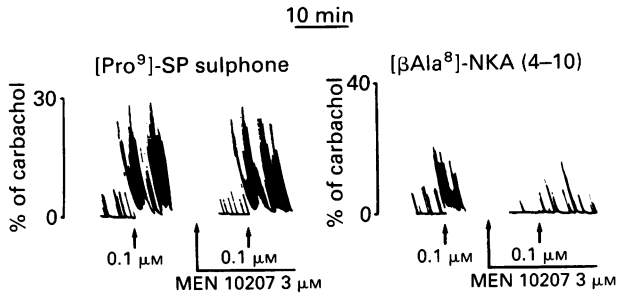

Figure 5 Typical tracings showing the effect of MEN 10,207 ([Tyr^5, D-Trp^{6,8,9}, Arg^{10}]-neurokinin A (4–10)) on the contractile response produced by a submaximal concentration (0.1 μM) of the NK_1-receptor selective agonist, [Pro^9]-SP sulphone or the NK_2-receptor selective agonist [βAla^8]-NKA(4–10).

Data in Figures 5 and 6 show the effect of MEN 10,207 (3 μM, contact time 15 min), a newly developed NK_2-receptor selective antagonist (Rovero *et al.*, 1990; Maggi *et al.*, 1990b) on the responses produced by a submaximal concentration (0.1 μM) of [Pro^9]-SP sulphone or [βAla^8]-NKA(4–10). MEN 10,207 did not affect the response to the NK_1-selective agonist, while strongly reduced the contraction produced by the NK_2-selective agonist ($n = 5$ in each case).

Discussion

This study confirms previous reports describing the powerful contractile effects exerted by TKs on the circular muscle of the guinea-pig ileum (Holzer *et al.*, 1980; Costa *et al.*, 1985). As compared to the earlier study of Costa *et al.* (1985) we noted a lower sensitivity of the circular strip to SP, a finding possibly related to the presence, in our preparation, of the mucosal layer which was not removed in order to prevent any possible damage to the inner circular muscle. To be noted is that Bauer & Kuriyama (1982) observed a similar low sensitivity to SP in mucosa-free preparations of the circular muscle of the guinea-pig ileum.

Recently, evidence has been obtained indicating a role for certain tissue peptidases in degrading TKs in the periphery (Turner, 1987; Devillier *et al.*, 1988; Patacchini *et al.*, 1989).

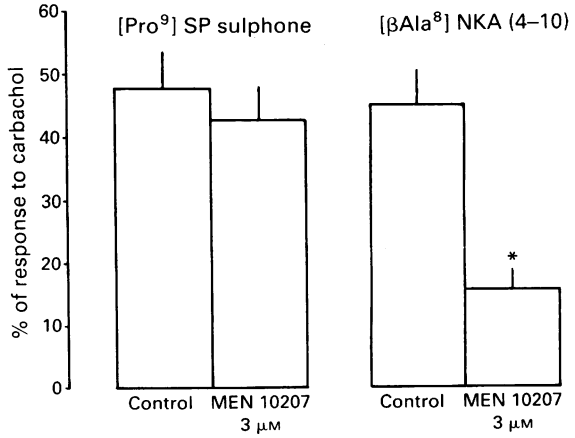

Figure 6 Effect of MEN 10,207 ([Tyr^5, D-Trp^{6,8,9}, Arg^{10}]-neurokinin A (4–10)) on the contractile response produced by application of a submaximal concentration (0.1 μM) of the NK_1-receptor selective agonist [Pro^9]-SP sulphone or the NK_2-receptor selective agonist [βAla^8]-NKA(4–10). Each column is mean of 5 experiments; s.e.mean shown by vertical lines. Statistically different from the control response $^*P < 0.05$.

The present findings do not provide evidence for a significant degradation of TKs by either endopeptidase 24.11 (thiorphan as inhibitor), angiotensin converting enzyme (captopril as inhibitor) or aminopeptidases (bestatin as inhibitor) in the guinea-pig ileum. However, the possibility cannot be ruled out that other peptidases are important for TK degradation at this level.

The contractile response of the circular strip of the ileum to TKs application is likely to involve activation of the specific TK receptors detected on circular muscle cells by autoradiography (Burcher *et al.*, 1986). However the possibility that the contractile responses to TKs and synthetic peptides might have been influenced by other mediators released from the mucosal layer cannot be ruled out.

The results obtained with the receptor-selective synthetic agonists are of interest because they delineate differentiated functions and discrete localizations of the TK receptor subtypes in the guinea-pig ileum. It is evident that the conclusions which can be drawn from the present experiments are critically dependent by the specificity of the selective agonists and antagonists used to identify TK receptors. The available evidence, obtained in selected, monoreceptorial bioassays for NK_1-, NK_2- and NK_3-receptors, indicates that [Pro^9]-SP sulphone is as potent as SP at NK_1-sites while being virtually inactive at NK_2- or NK_3-receptors (Drapeau *et al.*, 1987). For both the NK_2- ([βAla^8]-NKA(4–10)) and NK_3-([$MePhe^7$]-neurokinin B) agonists used in this study, the available evidence indicates that they possess similar or stronger affinity as compared to natural TKs at the respective preferred receptor while their activity at NK_1/NK_3 sites for the NK_2-agonist or at NK_1/NK_2-sites for the NK_3-agonist is markedly reduced, by about two orders of magnitude (Drapeau *et al.*, 1987; Rovero *et al.*, 1988). To improve the possibility of discriminating between the responses produced by the synthetic receptor-selective agonists we also investigated the effect of atropine and tetrodotoxin.

The response to [$MePhe^7$]-neurokinin B, a selective NK_3-receptor agonist (Drapeau *et al.*, 1987; Dion *et al.*, 1987) was totally neurogenic, being abolished by tetrodotoxin. A consistent fraction of this response was also atropine-resistant indicating that endogenous acetylcholine is not the only mediator involved. In fact the possibility that the endogenous TKs account for the atropine-resistant responses to the NK_3-agonist (cf. Guard & Watson, 1989) and to electrical field stimulation (Costa *et al.*, 1985 and present findings) deserves consideration.

The response to [Pro^9]-SP sulphone, a selective NK_1-receptor agonist (Drapeau *et al.*, 1987; Dion *et al.*, 1987) was partially neurogenic as indicated by the effect of tetrodotoxin. Thus both a direct and indirect components might participate in circular muscle contraction produced by NK_1-receptors activation. The lack of a significant inhibitory action by atropine indicates that the cholinergic contribution to the NK_1-mediated response was quantitatively very small, if any. The ability of NK_1-receptors to activate neurogenic mechanisms affecting motility was also shown in canine small intestine (Fox & Daniel, 1986; Fox *et al.*, 1986). In view of the extremely high selectivity of [Pro^9]-SP sulphone for NK_1- vs. NK_2-/NK_3-receptors, the possibility of a cross-talk of this peptide with the neurogenic response activated by the NK_3-agonist seems very unlikely.

[βAla^8]-NKA(4–10) is a newly developed NK_2-receptor selective agonist (Rovero *et al.*, 1989). The use of this peptide has enabled us to obtain evidence that NK_2-receptors mediate the contractile response to TKs in both the longitudinal and circular muscle of the human ileum (Maggi *et al.*, 1989; 1990a). The possibility that [βAla^8]-NKA(4–10) was acting on NK_1- or NK_3-receptors in the circular muscle of the ileum can be excluded because: (a) the response mediated by NK_3-receptor activation is totally indirect while the response to [βAla^8]-NKA(4–10) is atropine- and tetrodotoxin-resistant and (b) the action of [βAla^8]-NKA(4–10) is not modified after desensitization of NK_1- or NK_3-receptors achieved by repeated exposure to [Pro^9]-SP sulphone or [$MePhe^7$]-neurokinin B.

Further differentiation of the response to [Pro^9]-SP sulphone and [βAla^8]-NKA(4–10) comes from the use of MEN 10,207, a newly developed highly selective NK_2-receptor antagonist (Rovero *et al.*, 1990; Maggi *et al.*, 1990b).

In conclusion, NK_1-, NK_2- and NK_3-receptors appear to mediate, with different mechanisms, the contractile response of the circular muscle of the guinea-pig ileum to TKs. In this respect, a marked species-related difference exists in comparison to the results of similar experiments performed on the circular muscle of the human ileum. In this latter tissue we found that the contractile response is entirely sustained by activation of NK_1- and NK_2-receptors (Maggi *et al.*, 1990a).

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Pharmacological characterization of tachykinin-stimulated inositol phospholipid hydrolysis in peripheral tissues

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1 Tachykinin-stimulated inositol phospholipid hydrolysis was examined in slices of rat parotid gland, hamster urinary bladder and guinea-pig ileum longitudinal muscle.

2 In the presence of lithium, substance P and other naturally-occurring and synthetic tachykinins induced large, dose-dependent increases in [^{3}H]-inositol monophosphate accumulation.

3 In slices of rat parotid gland, [pGlu^6,L-Pro^9]SP(6–11) was considerably more potent in stimulating inositol phospholipid hydrolysis than [pGlu^6,D-Pro^9]SP(6–11).

4 In contrast, in slices of hamster urinary bladder, [pGlu^6,D-Pro^9]SP(6–11) exhibited greater potency in evoking inositol phospholipid breakdown than [pGlu^6,L-Pro^9]SP(6–11).

5 The differential selectivity of these C-terminal fragments of substance P suggests that they may be useful tools for distinguishing between NK_1 and NK_2 receptors.

6 L-659,837 and L-659,874 antagonized eledoisin-stimulated inositol phospholipid hydrolysis in slices of hamster urinary bladder. Neither compound significantly reduced substance-P evoked inositol phospholipid breakdown in slices of rat parotid gland, or senktide-induced inositol phospholipid hydrolysis in slices of guinea-pig ileum.

7 L-659,837 and L-659,874 had no effect on the atropine-sensitive, carbachol-stimulated inositol phospholipid hydrolysis in slices of rat parotid gland.

8 These data further support the notion that L-659,837 and L-659,874 are potent and selective NK_2 receptor antagonists.

Introduction

There is now considerable evidence for the existence of multiple tachykinin receptors (for reviews see Quirion & Dam, 1988; Guard & Watson, 1991). The current classification (Henry *et al.*, 1987) recognises three main types (NK_1, NK_2 and NK_3) characterized according to the rank order of potency of a variety of tachykinin agonists in various bioassay systems.

The tachykinin agonist profile of NK_1 receptors in both the central nervous system (CNS) and periphery is similar, with the preferred endogenous tachykinin, substance P, showing activity at nanomolar concentrations. The non-mammalian tachykinins eledoisin, physalaemin and kassinin are approximately equipotent with substance P, but the mammalian tachykinins neurokinin A (NKA) and neurokinin B (NKB) are slightly less potent than substance P.

In contrast, at NK_2 receptors in rat vas deferens and hamster urinary bladder, NKA, NKB, eledoisin and kassinin are all active in the nanomolar range, whereas substance P and physalaemin are approximately two orders of magnitude less potent. In addition, substance P methyl ester, a selective NK_1 receptor agonist, which is approximately equipotent with substance P at NK_1 receptors, is 100–1000 fold less active than substance P at NK_2 receptors (Watson *et al.*, 1983).

At NK_3 receptors, the preferred tachykinin, NKB (Laufer *et al.*, 1985) is 100 fold more potent than NKA or substance P (Mastrengelo *et al.*, 1986; McKnight & Maguire, 1987). The demonstration of NK_3 receptors has been much facilitated by the availability of the potent and highly selective NK_3 receptor agonist, senktide (Wormser *et al.*, 1986).

A putative fourth type of tachykinin receptor has recently been described in guinea-pig tracheal tissue (McKnight *et al.*, 1988a). The rank order of potency of naturally occurring tachykinins at this site, appears to be similar to that of NK_2 sites, although distinct differences are apparent in potencies of certain synthetic tachykinin analogues. Further studies are required to confirm the existence of these receptors.

NK_1, NK_2 and more recently NK_3 receptors have all been shown to be associated with inositol phospholipid hydrolysis in various tissues. Thus, Hanley *et al.* (1980) reported that substance P and related tachykinins induce inositol phospholipid hydrolysis in rat salivary glands. The rank order of potencies in eliciting this response was similar to the rank order of affinities at the NK_1 binding site, suggesting that inositol phospholipid hydrolysis in this tissue is coupled to NK_1 receptors. Similarly, we have previously reported that the same response in hamster urinary bladder is mediated via NK_2 receptors (Bristow *et al.*, 1987). Finally, tachykinin-induced inositol phospholipid hydrolysis in guinea-pig ileum myenteric plexus has been characterized by Guard *et al.* (1988) and shown to be linked to NK_3 receptors.

In the present study, we describe the effects of a pair of synthetic tachykinin agonists, [pGlu^6,L-Pro^9]SP(6–11) and [pGlu^6,D-Pro^9]SP(6–11), on NK_1 and NK_2 receptor-mediated inositol phospholipid hydrolysis. Radioligand binding studies suggest that [pGlu^6,L-Pro^9]SP(6–11) and [pGlu^6,D-Pro^9]SP(6–11) have selectivity for NK_1 and NK_2 receptors respectively (Lee *et al.*, 1986). It was therefore of interest to assess these compounds with respect to their ability to elicit a biochemical response in tissues previously characterized as possessing NK_1 and NK_2 receptors. We have also examined the effects of two synthetic tachykinin antagonists L-659,837 and L-659,874, on NK_1, NK_2 and NK_3 receptor-mediated inositol phospholipid hydrolysis. Both compounds show selectivity for NK_2 sites in radioligand binding studies (Williams *et al.*, 1988) and antagonize responses mediated by NK_2 receptors in *in vitro* pharmacological models of tachykinin receptors (McKnight *et al.*, 1988b). Thus, the present studies were undertaken to examine further the selectivity of these compounds for NK_2 receptors. Some of these data have

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been published in preliminary form (Suman-Chauhan et al., 1988).

Methods

Tachykinin-induced inositol phospholipid hydrolysis was examined in slices of rat parotid gland (NK_1), hamster urinary bladder (NK_2) and guinea-pig ileum (NK_3) by previously described methodology (Hanley et al., 1980; Bristow et al., 1987; Guard et al., 1988). Tissues were rapidly removed, cross-chopped ($350 \times 350 \times 350\,\mu m$) with a McIlwain tissue chopper, and dispersed in 10 ml Krebs Ringer bicarbonate (KRB) of the following composition (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 1.25, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11, (pH 7.4) with a Pasteur pipette. Slices were pre-incubated in 250 ml oxygenated KRB at 37°C for 30 min with two changes of buffer. Twenty-five microlitre aliquots (approximately 0.5 mg protein) of gravity-packed slices were then placed in polypropylene tubes containing 2 μCi [^{3}H]-inositol, 10 mM LiCl, bovine serum albumin (1 mg ml^{-1}) and bacitracin (40 μg ml^{-1}) in freshly gassed KRB. Tubes were gassed, capped and incubated for 30 min to allow incorporation of [^{3}H]-inositol into tissue phospholipids. Agonists (10 μl) were added (to give a final assay volume of 250 μl) and tubes incubated at 37°C for a further 45 min. Antagonists (10 μl) were added, where appropriate, 15 min before exposure to agonists.

Reactions were terminated by addition of 1 ml chloroform/methanol (1:2; vol/vol), followed by 300 μl chloroform and 300 μl deionized water. Tubes were vortexed and aqueous and organic phases allowed to separate by standing at room temperature for 30–60 min. Water soluble [^{3}H]-inositol monophosphates were separated by anion exchange chromatography (Berridge et al., 1982). Aliquots (750 μl) of the upper aqueous phase were removed and diluted with 2 ml deionized water before loading onto glass columns containing 1 ml of a 1:1 (vol/vol) suspension of Dowex AG 1-X8 formate resin in deionized water. Free [^{3}H]-inositol and [^{3}H]-glycerophosphoinositol were removed by eluting columns with 10 ml water followed by 15 ml 25 mM ammonium formate solution, respectively. [^{3}H]-inositol monophosphates were eluted into scintillation vials with 10 ml 150 mM ammonium formate solution containing 5 mM sodium tetraborate. Total radioactivity present in this 10 ml fraction was determined by addition of 10 ml Hydrofluor and counting in the gel phase.

Analysis of results

All data points represent the mean of triplicate determinations. Results were plotted as the mean [^{3}H]-inositol monophosphate accumulation (d.p.m.) versus log tachykinin concentration. EC_{50} values (concentration of tachykinin inducing 50% of the maximum response, as defined by 30 μM substance P) were determined from individual dose-response curves composed of at least four different tachykinin concentrations (range 0.001 μM to 100 μM). The geometric means of EC_{50} values ($n = 4$–6 separate experiments) were obtained by calculating the results in logarithmic form and expressing as the antilog of the mean (−s.e.mean; +s.e.mean).

IC_{50} values (concentration of antagonist resulting in 50% inhibition of the response to a given concentration of agonist) were determined from individual inhibition curves employing at least four different antagonist concentrations (range 0.01 μM to 100 μM). Geometric means of IC_{50} values (−s.e.mean; +s.e.mean) were calculated from three separate experiments as described for EC_{50} values.

Materials

[^{3}H]-inositol, specific activity 16.3 Ci mmol^{-1}, was purchased from Amersham International plc. Substance P methyl ester was obtained from Cambridge Research Biochemicals. All other tachykinins were purchased from Bachem Biochemicals. Senktide (succ-[Asp^6,MePhe^8]SP(6–11)), [pGlu^6,D-Pro^9]-SP(6–11), [pGlu^6,L-Pro^9]SP(6–11), L-659,837 and L-659,874 were synthesized and characterized in the Medicinal Chemistry Department of the MSDRL Neuroscience Research Centre. Carbachol, atropine, bovine serum albumin and bacitracin were obtained from Sigma Chemical Co, Ltd.

Results

Effects of agonists

Basal accumulation of [^{3}H]-inositol monophosphates was 679 ± 72 d.p.m. ($n = 38$) and 769 ± 69 d.p.m. ($n = 34$) per 25 μl slices in rat parotid gland and hamster urinary bladder, respectively. In the presence of lithium, the addition of substance P and other naturally occurring or synthetic tachykinins (0.001–500 μM) resulted in large, concentration-dependent increases in accumulation of [^{3}H]-inositol monophosphates, giving rise to 4–7 fold maximal increases above basal in slices of rat parotid gland and 10–15 fold maximal increases above basal in hamster urinary bladder. These data reflect the ability of lithium to inhibit inositol monophosphatase activity in these tissues, thus preventing hydrolysis of [^{3}H]-inositol monophosphates to free [^{3}H]-inositol.

A comparison of the EC_{50} values obtained for a series of tachykinins in these tissues is shown in Table 1. The rank order of potency of tachykinins for evoking inositol phospholipid hydrolysis in rat parotid gland is typical of an NK_1 receptor-mediated response. Thus, substance P and physalae-

Table 1 Comparison of EC_{50} values (μM) for tachykinin-induced [^{3}H]-inositol monophosphate production in slices of rat parotid gland and hamster urinary bladder

	EC_{50} (μM) (−s.e.mean, +s.e.mean)	
Peptide	NK_1 (rat parotid gland)	NK_2 (hamster urinary bladder)†
Substance P	0.007 (0.005, 0.008)	5.09 (3.95, 6.56)
Physalaemin	0.005 (0.004, 0.007)	3.97 (3.16, 5.00)
Eledoisin	0.031 (0.021, 0.046)	0.045 (0.033, 0.060)
Kassinin	0.045 (0.04, 0.051)	0.007 (0.005, 0.009)
Neurokinin A	0.383 (0.308, 0.475)	0.013 (0.010, 0.018)
Neurokinin B	0.667 (0.430, 1.035)	0.036 (0.030, 0.044)
Substance P methyl ester	1.56 (0.72, 3.37)	106.6 (66.7, 170.6)
[pGlu^6,D-Pro^9]SP(6–11)	40.60 (29.10, 56.70)	0.216 (0.164, 0.285)
[pGlu^6,L-Pro^9]SP(6–11)	1.04 (0.800, 1.340)	29.00 (21.00, 40.00)

Mean EC_{50} values (concentration required to induce 50% of the maximum response, as defined by 30 μM substance P) were determined from 4–6 separate dose-response curves each composed of at least four different tachykinin concentrations. Results were calculated in logarithmic form and are expressed as the antilog of the mean (−s.e.mean, +s.e.mean).

† Data taken from Bristow et al. (1987).

min, with EC_{50} values of 0.007 μM and 0.005 μM respectively, were considerably more potent than neurokinin A and neurokinin B (EC_{50} values 0.383 μM and 0.667 μM, respectively), but approximately equipotent to eledoisin and kassinin (EC_{50} values 0.031 μM and 0.045 μM, respectively). In contrast, we have previously shown that tachykinin-induced inositol phospholipid hydrolysis in hamster urinary bladder involves activation of NK_2 receptors (Bristow *et al.*, 1987). Thus, eledoisin, kassinin, neurokinin A and neurokinin B were all active in the nanomolar range (EC_{50} values 0.045 μM, 0.007 μM, 0.013 μM and 0.036 μM respectively), whereas substance P and physalaemin were 100–1000 times less potent (see Table 1). In addition, the synthetic NK_1 receptor selective tachykinin analogue, substance P methyl ester, was 100 fold less active at stimulating inositol phospholipid hydrolysis in hamster urinary bladder (EC_{50} value 106.6 μM) than in rat parotid gland.

We have also compared the effects of two synthetic C-terminal fragments of substance P, [pGlu^6,D-Pro^9]SP(6–11) and [pGlu^6,L-Pro^9]SP(6–11) (Figure 1), on inositol phospholipid hydrolysis in rat parotid gland and hamster urinary bladder (Table 1). These C-terminal hexapeptides, originally synthesized by Piercey *et al.* (1985), have been shown to exhibit differential selectivity for NK_1 and NK_2 binding sites with [pGlu^6,D-Pro^9]SP(6–11) being selective for NK_2 sites whilst [pGlu^6,L-Pro^9]SP(6–11) is selective for NK_1 sites (Lee *et al.*, 1986). In the present study, [pGlu^6,D-Pro^9]SP(6–11) was considerably more potent in stimulating inositol phospholipid hydrolysis in slices of hamster urinary bladder (EC_{50} value 0.216 μM) than in slices of rat parotid gland (EC_{50} value 40.6 μM). In contrast, [pGlu^6,L-Pro^9]SP(6–11) showed greater potency in evoking inositol phospholipid breakdown in slices of rat parotid gland as compared to hamster urinary bladder (EC_{50} values 1.04 μM and 29.00 μM respectively). Data relating to NK_2 receptor-mediated inositol phospholipid hydrolysis in hamster urinary bladder (Table 1) has been previously published (Bristow *et al.*, 1987).

Effects of antagonists

The effects of two synthetic hexapeptides, L-659,837 and L-659,874 (Figure 1) on tachykinin-induced inositol phospholipid hydrolysis mediated via NK_1, NK_2 and NK_3 receptors have also been studied. Concentrations of substance P, eledoisin, and the NK_3 receptor selective agonist senktide (Wormser *et al.*, 1986) resulting in submaximal accumulation of [^{3}H]-inositol monophosphates were used to stimulate inositol phospholipid hydrolysis in rat parotid gland, hamster urinary bladder and guinea-pig ileum, respectively. In slices of hamster urinary bladder, eledoisin (0.1 μM) produced a 7–10 fold increase above basal in accumulation of [^{3}H]-inositol monophosphates. Both L-659,837 and L-659,874 inhibited the response to eledoisin in a concentration-dependent manner (Figure 2) with IC_{50} values of 0.89 μM (0.65, 1.24; −s.e.mean; +s.e.mean) and 0.49 μM (0.32, 0.76; −s.e.mean; +s.e.mean), respectively. Complete inhibition of eledoisin-evoked inositol phospholipid hydrolysis was effected by 10 μM L-659,837 or 10 μM L-659,874. In contrast, in slices of rat parotid gland, neither compound significantly reduced the 3–5 fold increase in the accumulation of [^{3}H]-inositol monophosphates induced by 0.01 μM substance P, at concentrations up to 100 μM. Similarly, no significant effects were observed on the 2–3 fold increase in [^{3}H]-inositol monophosphate accumulation produced by 1 nM senktide in guinea-pig ileum, at concentrations up to 10 μM. In addition, L-659,837 and L-659,874 failed to stimulate basal activity in all three tissues studied, at concentrations up to 100 μM.

In order to investigate further the selectivity of L-659,837 and L-659,874, their effects on carbachol-induced inositol phospholipid hydrolysis were examined in slices of rat parotid gland. Carbachol (10 μM) induced a 10 fold increase in the accumulation of [^{3}H]-inositol monophosphates over basal levels (Figure 3), a response which was almost completely

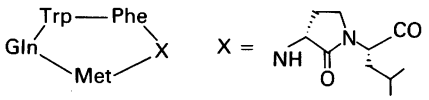
Agonists

[pGlu^6,D-Pro^9]SP(6–11) pGlu-Phe-Phe-D-Pro-Leu-Met-NH_2

[pGlu^6,L-Pro^9]SP(6–11) pGlu-Phe-Phe-L-Pro-Leu-Met-NH_2

Antagonists

L-659,837



L-659,874

Ac-Leu-Met-Gln-Trp-Phe-Gly-NH_2

Figure 1 Structures of synthetic tachykinin agonists and antagonists. pGlu = pyroglutamic acid.

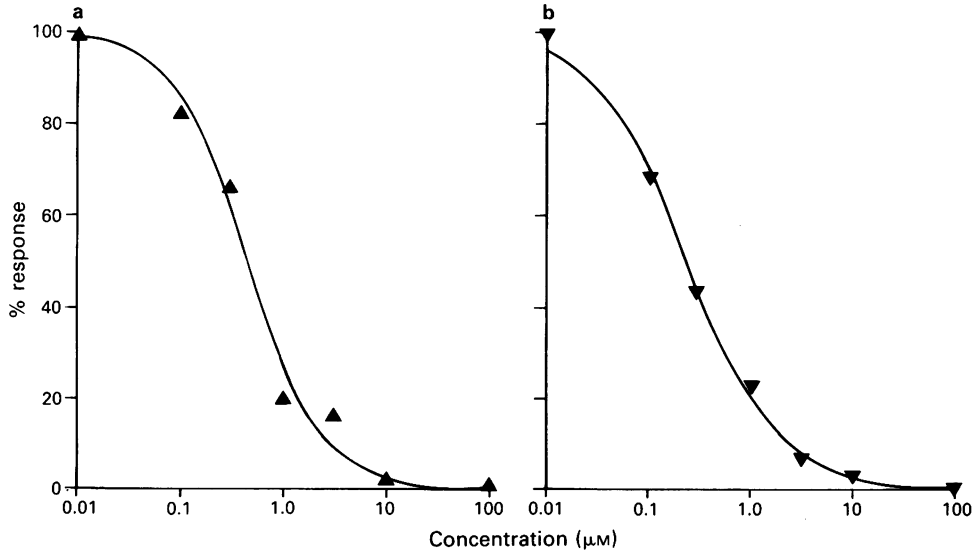


Figure 2 Effects of L-659,837 (a) and L-659,874 (b) on 0.1 μM eledoisin-stimulated inositol phospholipid hydrolysis in slices of hamster urinary bladder. Results are expressed as the percentage response to eledoisin remaining in the presence of increasing concentrations of the antagonist. The data shown are from a single representative experiment, where eledoisin (0.1 μM) stimulated [^{3}H]-inositol monophosphate production approximately 10 fold from 313 to 3044 d.p.m. per 25 μl slices. Each data point represents the mean of three determinations. Mean IC_{50} values were calculated from three such experiments.

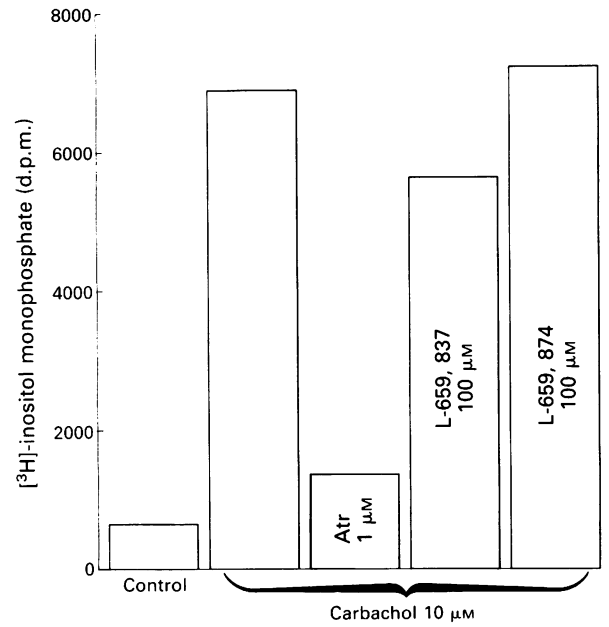


Figure 3 Effects of atropine (Atr), L-659,837 and L-659,874 on 10 μM carbachol-stimulated inositol phospholipid hydrolysis in slices of rat parotid gland. Results are expressed as d.p.m. [^{3}H]-inositol monophosphate accumulation above basal levels per 25 μl slices. The histogram shows data from a single representative experiment performed in triplicate.

abolished by the muscarinic antagonist, atropine (1 μM). In contrast, concentrations of up to 100 μM L-659,837 and L-659,874 had little or no effect on carbachol-induced inositol phospholipid hydrolysis (Figure 3).

Discussion

The current classification of tachykinin receptors depends largely on agonist potency profiles in various assay systems. Definitive classification requires the development of potent, highly selective agonists and antagonists. In the present study, we have compared the ability of the two synthetic C-terminal substance P fragments, reported to have differential selectivity for NK_1 and NK_2 receptor binding sites, to elicit a functional response, i.e. inositol phospholipid hydrolysis. Lee *et al.* (1986) reported that [pGlu^6,L-Pro^9]SP(6–11) was considerably more potent in displacing [^{125}I]-BH-substance P binding to NK_1 sites (e.g. rat parotid gland) than [pGlu^6,D-Pro^9]SP(6–11), whereas the latter showed greater potency in displacing [^{125}I]-BH-eledoisin binding to NK_2 sites (e.g. hamster urinary bladder). Thus, D-Pro/L-Pro ratios (IC_{50} value for [pGlu^6,D-Pro^9]SP(6–11)/IC_{50} value for [pGlu^6,L-Pro^9]SP(6–11)) for NK_1 tissues fell within the range 87–124, whereas NK_2 tissues yielded a D-Pro/L-Pro ratio of 0.003. The D-Pro/L-Pro ratios (EC_{50} value for [pGlu^6,D-Pro^9]SP(6–11)/EC_{50} value for [pGlu^6,L-Pro^9]SP(6–11)) calculated for induction of inositol phospholipid hydrolysis in slices of rat parotid gland and hamster urinary bladder were 39 and 0.007 respectively. These results are in close agreement with the data of Lee *et al.* (1986). We have not examined the effects of the synthetic agonists [pGlu^6,D-Pro^9]SP(6–11) and [pGlu^6,L-Pro^9]SP(6–11) on NK_3 receptor-mediated inositol phospholipid hydrolysis because of the inherent problems which would be encountered as a result of the mixed population of NK_1 and NK_3 receptors present in the guinea-pig ileum (Guard *et al.*, 1988). Such studies would need to be performed either in tissues containing predominantly NK_3 receptors, or in the presence of selective antagonists to block NK_1 receptors. McKnight *et al.* (1988a) have reported that [pGlu^6,D-Pro^9]SP(6–11) and [pGlu^6,L-Pro^9]SP(6–11) are equipotent at the putative NK_4 receptor-mediated contractions of guinea-pig trachea. There is to date no published evidence for coupling of this receptor to inositol phospholipid hydrolysis, therefore we were unable to investigate the biochemical correlates of this observation. Nevertheless, it is clear that comparison of D-Pro/L-Pro ratios can provide a useful means of distinguishing between tachykinin receptor types.

L-659,837 and L-659,874 are both synthetic hexapeptides derived from the parent compound L-363,851 (Cascieri *et al.*, 1985), which was originally developed within our laboratories as an NK_2 agonist. L-659,837 possesses a cyclic structure, which retains the constraining lactam unit present in the parent compound. L-659,874 is a linear structure with a primary sequence closely related to that of L-659,837. Both compounds have been evaluated in radioligand binding assays for NK_1, NK_2 and NK_3 receptor sites, and shown to be NK_2 selective (Williams *et al.*, 1988). L-659,837 and L-659,874 both potently displaced [^{125}I]-BH-eledoisin binding from membranes prepared from hamster urinary bladder with IC_{50} values of 0.15 μM and 0.04 μM respectively. L-659,837 and L-659,874 were essentially inactive in displacing [^{125}I]-BH-substance P binding from NK_1 sites and [^{125}I]-BH-eledoisin binding from NK_3 sites in membranes prepared from rat cerebral cortex (IC_{50} values greater than 100 μM). This selectivity for NK_2 receptors was also reflected in *in vitro* pharmacological models, with L-659,837 and L-659,874 having no significant effects on NK_1-mediated substance P-induced contractions of guinea-pig ileum (pA_2 values less than 4.5) or on NK_3-mediated, eledoisin-induced contractions of rat portal vein (pA_2 values 4.9 and 4.4 respectively), but potently antagonizing the NK_2-mediated eledoisin-induced contractions of rat vas deferens (pA_2 values 6.7 and 6.8 respectively; McKnight *et al.*, 1988b). Our own studies on inositol phospholipid hydrolysis further substantiate these findings that L-659,837 and L-659,874 are potent and selective antagonists at NK_2 receptors, with essentially no activity in antagonizing NK_1 and NK_3 receptor-mediated inositol phospholipid hydrolysis. The selectivity of L-659,837 and L-659,874 for NK_2 receptors is further emphasized by the observation that neither compound showed significant activity against carbachol-stimulated inositol phospholipid hydrolysis. Preliminary studies suggest a competitive mode of action for the effects of L-659,837 and L-659,874 against eledoisin-stimulated phospholipid hydrolysis in slices of hamster urinary bladder. Thus, the presence of 1 μM L-659,837 or L-659,874 resulted in a rightward shift of the eledoisin dose-response curve in this tissue (unpublished). If competitive antagonism is assumed, pKi values of 6.6 and 6.8 can be calculated for L-659,837 and L-659,874, respectively, using the Cheng-Prusoff equation (Cheng & Prusoff, 1973). These values are in good agreement with the previously published pA_2 values for these antagonists versus NK_2 receptor-mediated responses to eledoisin in the rat vas deferens preparation (McKnight *et al.*, 1988b).

Multiple tachykinin receptors appear to be involved in nociception (Dourish *et al.*, 1988b), and therefore tachykinin antagonists may possess antinociceptive properties. Intracisternal injection of L-659,837 potently blocked eledoisin-induced reciprocal hindlimb scratching in mice, but was without effect on substance P-induced scratching, again suggesting NK_2 selectivity (Dourish *et al.*, 1988a). L-659,837 also induced significant latency in the mouse hot plate test suggesting analgesic properties in thermal pain (Dourish *et al.*, 1988a).

In conclusion, we have demonstrated that the tachykinin analogues [pGlu^6,D-Pro^9]SP(6–11) and [pGlu^6,L-Pro^9]SP(6–11) are useful tools for the characterization of tachykinin receptors. We also provide further evidence for the notion that L-659,837 and L-659,874 are novel, potent and selective NK_2 antagonists. The availability of such selective, high affinity compounds should facilitate research into the physiological roles of multiple tachykinin receptors.

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Effects of hypomagnesia on transmitter actions in neocortical slices

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1 The effects of hypomagnesia on the neuronal responses induced by iontophoretically applied acetylcholine, glutamate, N-methylaspartate (NMDA) and γ-aminobutyric acid (GABA) were investigated using intracellular recording techniques in *in vitro* slices of sensorimotor cortex (guinea-pigs).

2 Perfusion with Mg-free media, with or without tetrodotoxin (TTX), induced a small hyperpolarization (~4 mV) and a small decrease (~10%) in the input resistance of neurones. During TTX-blockade of Na-spike genesis, spontaneous depolarizing waves of low frequencies were observed in neurones of slices under Mg-free conditions.

3 The effects of acetylcholine and to a lesser extent, GABA actions, were depressed in a dose-dependent, reversible manner by decreases in the $[Mg^{2+}]$ of the perfusing media. In neurones of slices that had been incubated in Mg-free artificial cerebrospinal fluid to ensure a maximal depletion, the responses to these transmitters were potentiated by each sequentially administered increase in extracellular $[Mg^{2+}]$. The actions of NMDA were potentiated during perfusion of Mg-free media. However, the responses to glutamate, which may activate receptors for NMDA, were either depressed or unchanged under these conditions.

4 A regulatory role for external Mg cations in the responses of neocortical neurones to the transmitter substances, acetylcholine and GABA, can be inferred from these investigations which simulate hypomagnesemia. The dose-dependent depression of GABA actions by low extracellular $[Mg^{2+}]$ additionally provides a plausible mechanism that may contribute to the neuronal hyperexcitability that is observed during conditions of hypomagnesemia.

Introduction

In man and experimental animals, hypomagnesemia is associated with manifestations of central nervous system (CNS) dysfunction (Martindale & Heaton, 1964; Agus *et al.*, 1982). These can be attributed to the following mechanisms, based on *in vitro* investigations of the extracellular effects of Mg on CNS neurones: (1) removal of the well-known antagonistic actions of Mg on Ca-entry at both pre- and postsynaptic sites (Katz & Miledi, 1969; Lambert & Heinemann, 1986; Czèh & Somjen, 1989), (2) reduced surface charge screening on neuronal membranes and an associated facilitation of inward ionic currents and action potentials (Frankenhauser & Hodgkin, 1957; Llinás & Walton, 1980), and (3) removal of a voltage-dependent Mg-blockade of excitatory amino acid actions at N-methyl-D-aspartate (NMDA)-receptors (Thomson, 1986; Stanton *et al.*, 1987; MacDonald *et al.*, 1987; Mayer *et al.*, 1988; Collingridge & Lester, 1989). Another obvious possibility is that the hypomagnesemia may interfere with the role of Mg in regulating a wide variety of enzymatic reactions and phosphorylation-dependent processes, including second messenger systems (Gurwitz & Sokolovsky, 1980; Grubbs & Maguire, 1987; Stelzer *et al.*, 1988), that are essential for neuronal excitability in the CNS. An explanation for this effect may require that the decrease in extracellular $[Mg^{2+}]$ ($[Mg^{2+}]_o$) would result in a corresponding drop in intracellular $[Mg^{2+}]$ (Baker & Crawford, 1972; Heinonen & Akerman, 1986) which would compromise certain receptor sensitivities to transmitter actions.

Here, we investigated the possibility that hypomagnesia in *in vitro* slice preparations may modify neuronal responses to known transmitter substances such as acetylcholine, glutamate and γ-aminobutyrate (GABA) in neocortical neurones (Krnjević, 1974). Some of these results have been published in preliminary form (El-Beheiry & Puil, 1989a).

Methods

Slice preparations and recording

Sensorimotor cortex was identified in halothane-anaesthetized guinea-pigs, excised, and cut into ~500 μm thick slices according to previously described procedures (El-Beheiry & Puil, 1989b). The slices were submerged in artificial cerebrospinal fluid (ACSF) oxygenated with a 95% O_2/5% CO_2 mixture and kept at room temperature until required for recording (bath temperature, 32–34°C). The constituents of the ACSF were (in mM): NaCl 124, KCl 3.75, KH_2PO_4 1.25, $MgSO_4 \cdot 7H_2O$ 2, $CaCl_2 \cdot 2H_2O$ 2, dextrose 10 and $NaHCO_3$ 26. When changes were made in the $[Mg^{2+}]_o$ by altering the ACSF composition, the total concentration of divalent cations was maintained constant by appropriately increasing or decreasing the $[Ca^{2+}]$. In 4 experiments, Ca-deficient ACSF was made by equimolar substitution of $CoCl_2$ for $CaCl_2$. The techniques for intracellular recording from neocortical neurones in *in vitro* slice preparations with K_2SO_4 (0.6 M) filled electrodes (cf. Eccles, 1964) have been described; these descriptions included measurements of input resistance by intracellular injections of hyperpolarizing current pulses (100–150 ms duration) and current-voltage relationships (El-Beheiry & Puil, 1989b). In cases where a change in resting membrane potential was observed during an imposed alteration in $[Mg^{2+}]_o$, d.c.-injection was used to return the resting potential to its initial level.

Drugs and their applications

Acetylcholine (0.5 M, pH 4.5), glutamate (0.5 M, pH 8.5), N-methyl-D-aspartate (NMDA; 100 mM, pH 9) and γ-aminobutyrate (GABA; 100 mM, pH 4.5) (all obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.), or NaCl for control applications, were administered by iontophoresis from a three- or five-barrelled microelectrode which was inserted into the dendritic region at a distance of ~50 μm from the location of neuronal impalement. The substances were applied consistently at recurring intervals (10–60 s) for observations of their interactions with imposed changes in $[Mg^{2+}]_o$. The durations of the applications were 4 to 7 s for GABA, 6 s for glutamate and 7 to 20 s for acetylcholine. In 20 neurones, tetrodotoxin (TTX; 1–1.5 μM) (Sigma Chemical Co.) was applied in the ACSF to block action potential-dependent transmitter release.

Results

Successful penetrations of neurones were obtained mostly in regions corresponding to layers IV–V of typical cortex. The effects of a reduction in the $[Mg^{2+}]_0$ were investigated in 32 sensorimotor neurones.

Membrane properties and spikes

Before hypomagnesia, the mean resting membrane potential and mean input resistance were -70.4 ± 7.3 (mean $\pm$ s.d.) mV and 48.8 ± 18.5 MΩ, respectively ($n = 24$). Perfusion of the slices with Mg-free media, with or without the application of TTX, induced a slight hyperpolarization (3–5 mV) in 8 neurones (cf. Table 1). After 30–45 min in Mg-free and TTX perfusion, the resting membrane potentials in 4 neurones exhibited abruptly appearing episodes of depolarizing oscillations (4–7 mV), each lasting 10–20 s. The input resistances were unchanged in 11 of the neurones, and decreased by ~10% in 5 neurones during the hyperpolarization. The current-voltage relationships in the hyperpolarizing direction displayed an inward rectification in ~85% of the neurones ($n = 24$) which was not significantly affected by the Mg-depletion. Intracellular injections of depolarizing current pulses (~100 ms duration) evoked spikes of >80 mV amplitude and <2 ms duration, with overshoots of ~20 mV. The spikes also were not apparently affected by the Mg-deficient conditions.

Responses to transmitter substances

Applications (~ED_{50} s) of acetylcholine, glutamate and GABA produced mean peak depolarizations of 15.8 ± 4.6, 20.5 ± 7.7 and 21.5 ± 7.8 mV respectively in the 24 neurones. The input resistances of these neurones were either increased (~15%) or unaffected near the peak of the depolarizing responses induced by acetylcholine. In contrast, the input resistances were decreased (35–80%) by glutamate or GABA applications. These responses were obtained either in the absence or presence of TTX (1.5 μM).

Perfusion of the Mg-free ACSF was commenced shortly after (3–5 min) 3 or more applications of each transmitter substance had produced responses of relatively constant amplitudes. Application of acetylcholine or GABA induced depolarizations which were sharply reduced during Mg-free perfusion (Figure 1a; Table 1). The responses to glutamate were not consistently affected during perfusion with Mg-free media ($n = 9$). The amplitudes of the depolarizations evoked by glutamate application were either depressed by <20%, unchanged, or enhanced by ≤20%. Depolarizing responses to NMDA application that were comparable in amplitude to those elicited by glutamate could not be obtained during the perfusion of ACSF containing 2 mM Mg^{2+} ($n = 10$). However, during 5–10 min perfusion of Mg-free media, the application of NMDA evoked a depolarization of ~20 mV amplitude in all 10 neurones. All of the above effects of Mg-free media were fully reversible; generally, recoveries were observed at 12–18 min after reperfusion with Mg (perfusion flow rates, 1–3 ml min^{-1}).

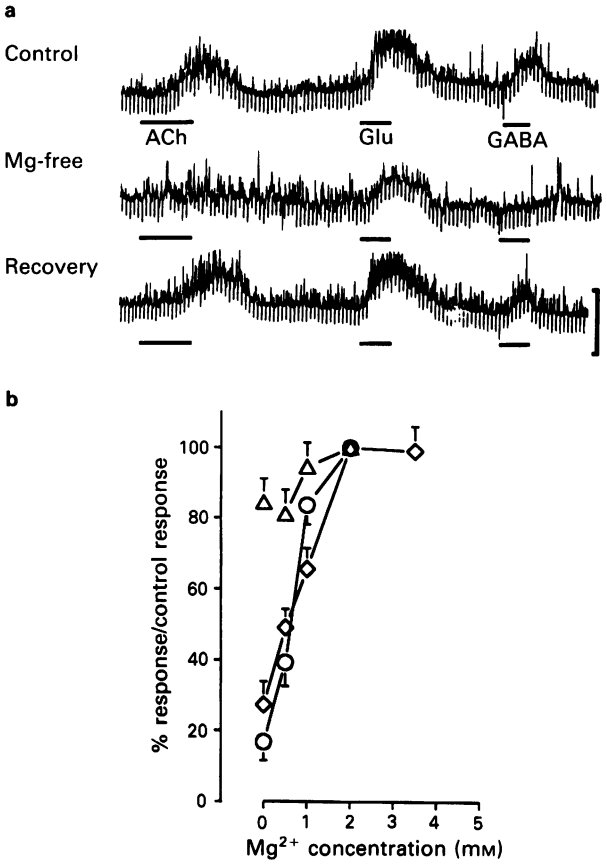

Figure 1 (a) Blockade of acetylcholine (ACh, 90 nA for 8 s) and GABA (50 nA) actions under conditions of Mg-free perfusion for 10 min. ACh and GABA responses were completely attenuated in a neurone ($V_m = -75$ mV) whereas the effects of glutamate (Glu) application (90 nA) were still evident, although reduced. The hyperpolarization (~4 mV) induced by Mg-free perfusion was compensated by d.c.-injection. Complete recovery of potential and transmitter responses was observed at ~15 min of reperfusion with Mg. Vertical bar, 15 mV. Time calibration, 8 s (ACh application bar). (b) Dose-response relationships obtained from 18 neurones indicate dependencies of the depolarizations evoked by ACh (○), GABA (◇) and Glu (△) on $[Mg^{2+}]_0$. Each of the points represents the mean data pooled from at least 4 neurones and vertical lines show s.e.mean.

Dose-response relationships

The depolarizations evoked by acetylcholine, glutamate and GABA were averaged in 18 neurones bathed in conditions containing different concentrations of Mg^{2+} (Figure 1b). The responses to acetylcholine and GABA approached their respective maxima sooner and were larger in peak amplitude, with increasing $[Mg^{2+}]_0$ (not shown). However, changes in the rate of rise and the magnitude of the glutamate responses were very inconsistent and seemed to be largely independent of the $[Mg^{2+}]_0$.

Table 1 Effects of Mg-free media on transmitter responses in sensorimotor neurones

Resting membrane properties							
	Decrease				*Glutamate response*		
Hyperpolarization	*in input resistance*	*Depression* of ACh response*	*Depression of GABA response*	*Potentiation of NMDA response*	*Depression*	*No change*	*Potentiation***
8/16	5/16	7/7	13/13	10/10	3/9	4/9	2/9

* Defined as >20% attenuation of control amplitudes of ACh-induced depolarization.
** Defined as >20% increase of control amplitudes of glutamate-induced depolarization.

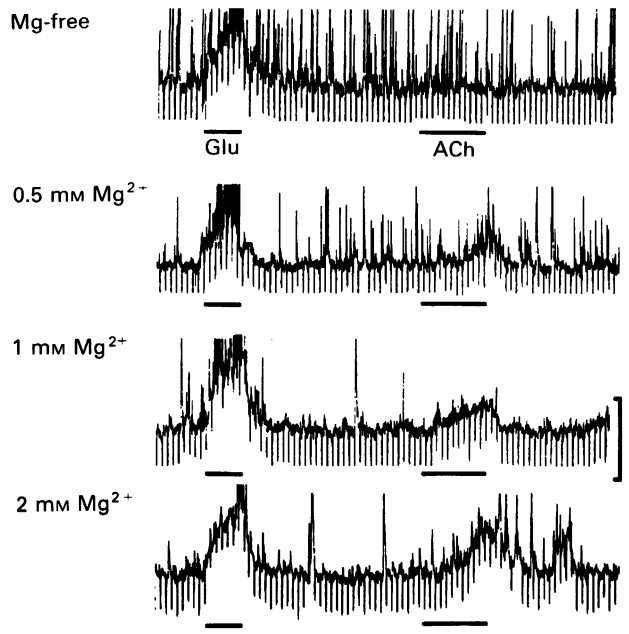

Figure 2 Gradual potentiation of the effects of acetylcholine (ACh) application (85 nA) in a neurone ($V_m = -70$ mV) that corresponded to increases in $[Mg^{2+}]_0$. The slice had been previously incubated in Mg-free media for ~2 h. Glutamate (Glu) was applied with a 100 nA current for 5 s. The responses to ACh and Glu were obtained at ~7 min of perfusion at each concentration of Mg^{2+}. Vertical bar, 15 mV. Time calibration, 9 s (ACh application bar).

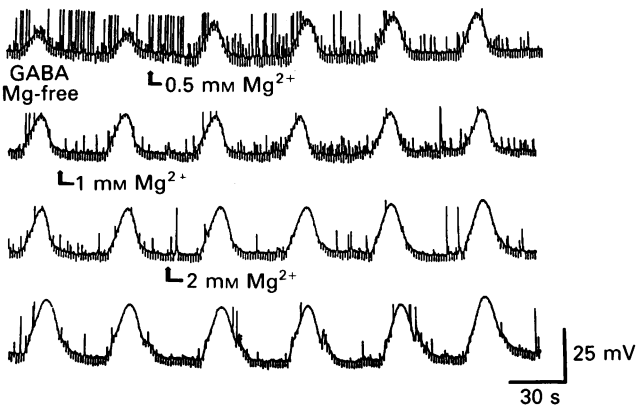

Figure 3 Continuous record shows that γ-aminobutyric acid (GABA) application (80 nA) produced responses which were gradually potentiated by sequential increases in $[Mg^{2+}]_0$. The slice had been previously incubated in Mg-free artificial cerebrospinal fluid for ~3 h. ($V_m = -72$ mV).

Effects of sequentially increasing $[Mg^{2+}]_0$

In a separate series of experiments, the neuronal responses to the transmitter substances were investigated under conditions where the slices had been first incubated for 3–7 h in Mg-free ACSF to ensure a maximal depletion of $[Mg^{2+}]_0$ (cf. Sutor *et al.*, 1987). Neurones ($n = 8$) in slices that had been incubated in Mg-free ACSF since cortical excision showed pronounced spontaneous (presumably synaptic) activities. During perfusion of increasing levels of $[Mg^{2+}]_0$ the spontaneous firing gradually subsided, although synaptic transients and spiking were still evident at the highest concentration (cf. Figures 2 and 3). This activity was associated with a small depolarization (~4 mV), although input resistance remained unchanged from that observed in Mg-free conditions in 6 out of the 8 neurones.

Under the Mg-free conditions none of the neurones responded to the applications of acetylcholine. However, during perfusion with ACSF containing 0.5 mM Mg, depolarizations could be evoked with acetylcholine. These increased in amplitude with perfusion of ACSF containing 1.0 and 2.0 mM [Mg] (5 out of 8 neurones; Figure 2). In contrast to the absence of acetylcholine sensitivity, the neurones responded to GABA and glutamate applications during Mg-free conditions. However, the GABA-induced depolarizations were potentiated in a dose-dependent manner by the administration of additional Mg^{2+} (Figure 3). During Mg-free perfusion, application of glutamate depolarized all 8 neurones and a build-up in $[Mg^{2+}]_0$ to 0.5, 1.0 and 2.0 mM did not consistently produce much change in these responses.

In 2 of the above neurones, the responses to acetylcholine were entirely blocked, and the GABA responses were depressed, when the slices were reperfused with Mg-free ACSF, demonstrating a reproducibility in the reversible effects of hypomagnesia. In general, the results obtained from neurones under Mg-free conditions after cortical excision, and the effects of the cumulative applications of Mg, were entirely consistent with the results obtained from neurones under conditions where the $[Mg^{2+}]_0$ had been 'acutely' lowered by perfusion of Mg-free media.

Effects of changes in divalent cations in the external media on the transmitter responses

In order to investigate the possibility that the increase in the external $[Ca^{2+}]$ used to maintain the total cationic concentration constant contributed to the observed effects, slices were perfused with ACSF solutions containing 4 mM Ca^{2+}. No significant changes were observed in the depolarizations evoked by acetylcholine, glutamate and GABA under these Ca-rich conditions. Two other neurones were bathed in Mg-free ACSF which contained only 2 mM Ca^{2+}. In this medium, the depressions of the acetylcholine and GABA responses were very similar in amplitude and duration to those obtained in neurones bathed in hypomagnesic conditions, i.e., with 4 mM Ca^{2+}.

Because of the variability in the effects of a reduction or a build-up in $[Mg^{2+}]_0$ on the responses to glutamate, we also investigated the effects when systematic changes were made in the divalent cation concentration of the perfusing media. Most of these experiments were carried out without changing the total cationic concentration and during concomitant application of TTX. Exclusion of both Ca and Mg from the perfusing media together with their substitution by 4 mM Co^{2+}, attenuated the peak depolarization produced by glutamate application by 38.8 ± 7.5% in 3 out of the 4 neurones (Figure 4a). Substitution of Ca alone by Co^{2+} in the external media suppressed the glutamate responses by 15 ± 5.7% in 4 neurones (Figure 4b). Recovery was complete after 15–20 min at a perfusion flow rate of 2–3 ml min^{-1}.

Discussion

In the present investigations, hypomagnesia was produced in *in vitro* slice preparations by substituting Ca for Mg in the perfusing media, such that the total divalent cation concentration was constant at 4 mM. Any interference with the surface charge screening of the membrane that would have facilitated an activation of inward currents was thereby avoided (Frankenhauser & Hodgkin, 1957; Llinás & Walton, 1980). The effects induced by the simulated hypomagnesemia could not be attributed to changes in the extracellular $[Ca^{2+}]$ (cf. Results) and one can be reasonably confident that they are mostly due to changes in the extracellular concentration of Mg (Frankenhauser & Hodgkin, 1957; McLaughlin *et al.*, 1971).

Unlike hippocampal neurones (Mody *et al.*, 1987), an exposure to Mg-free media hyperpolarized neocortical neurones and decreased their input resistances. Conversely, a depolar-

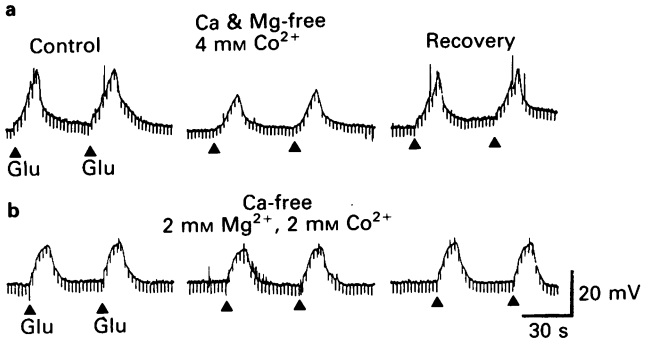

Figure 4 Effects of changing the external $[Ca^{2+}]$ and $[Mg^{2+}]$ on responses to glutamate applications (100 nA) in two neurones (V_m = −68 mV in a, −78 mV in b). Responses to glutamate (Glu) were depressed in Ca- and Mg-free artificial cerebrospinal fluid (ACSF) (a) more than in the absence of Ca alone (b). Recovery was observed at ~15 min in (a), and at ~20 min in (b), after reperfusion with control ACSF.

ization with a magnitude comparable to the peak hyperpolarization was observed on several occasions during the buildup of $[Mg^{2+}]_0$ from hypomagnesia. The hyperpolarization during hypomagnesia may be attributed to an interruption of tonic, blocking actions of Mg on resting Ca-activated K-channels, (cf. Llinás & Walton, 1980; Begenisich, 1988). Such blocking actions have been observed in spinal motoneurones where intracellular injections of Mg^{2+} produce membrane depolarization by reducing a Ca-activated K-conductance (Krnjević *et al.*, 1976). To explain the neocortical results (see below) this mechanism would probably require a dependence of the internal $[Mg^{+}]$ on $[Mg^{2+}]_0$, which has been suggested to be a general feature of Mg regulation in nerve cells (Heinonen & Akerman, 1986; Baker & Crawford, 1972).

Suppression of the acetylcholine-induced responses

A dose-dependent suppression of acetylcholine-induced depolarizations in neurones by removal of Mg from the extracellular medium has not been described previously. External applications of Mg by iontophoresis have been found to depress acetylcholine- and carbachol-evoked spike firing in the spinal cord and dorsal root ganglion (Davies & Watkins, 1977), but not to antagonize nicotinic actions on cerebellar neurones (Garza *et al.*, 1987). Although the K-conductance mechanism is different, a blockade of the muscarinic actions by a reduced $[Mg^{2+}]_0$ has been observed in cardiac muscle (Kurachi *et al.*, 1986). In the neocortex, the interactions of acetylcholine with muscarinic receptors depolarize pyramidal neurones by reducing their membrane conductances for K (Krnjević *et al.*, 1971). In sympathetic ganglion neurones the muscarinic actions involve a reduction in the Ca-activation of K-channels (Belluzzi *et al.*, 1985; Wanke *et al.*, 1987).

The depression of muscarinic responses in neocortical neurones can be attributed to one or more of the following mechanisms: (1) a decrease in $[Mg^{2+}]_0$ may remove the tonic inhibition of a Ca-conductance, thereby increasing Ca-dependent K-efflux (cf. Belluzzi *et al.*, 1985; Wanke *et al.*, 1987); this would effectively counteract the depolarizing effects of the acetylcholine. (2) Decreasing $[Mg^{2+}]_0$ may cause corresponding decrements in intraneuronal $[Mg^{2+}]$ (Heinonen & Akerman, 1986; Baker & Crawford, 1972) that would change the internal Ca^{2+}/Mg^{2+} ratio; this could interfere with the actions of acetylcholine (Krnjević *et al.*, 1976). (3) A decrease in internal $[Mg^{2+}]$ might also lead to a reduction in guanosine 5'-triphosphate (GTP) binding to the agonist/receptor complex, thereby producing the depression of acetylcholine responses (Gurwitz & Sokolovsky, 1980; Aronstam *et al.*, 1985). (4) Low $[Mg^{2+}]_0$ favours a conversion of the muscarinic receptor with high affinity binding sites to low affinity conformational states in the cerebral cortex, whenever Mg is added to the incubating medium (Birdsall *et al.*, 1984); this is consistent with the observed reduction in the responses to acetylcholine.

$[Mg^{2+}]_0$ does not significantly affect glutamate-induced responses

As in previous intracellular investigations on rat and human neocortical slices (Thomson *et al.*, 1985; Sutor *et al.*, 1987; Avoli *et al.*, 1987), no substantial changes in the neuronal responses evoked by glutamate were observed in guinea-pig neocortical slices under Mg-free conditions. In the frog and rat spinal cords, large reductions or increases in $[Mg^{2+}]_0$ do not produce appreciable effects on the extracellularly recorded responses to glutamate (Ault *et al.*, 1980; cf. Lacey & Nistri, 1988). The absence of effects in neocortex could be due to a failure of Mg^{2+} to influence significantly the binding of glutamate to its receptors (Baudry & Lynch, 1979), or may result from the interactions of glutamate predominantly with non-NMDA-sensitive sites in layers IV and V (Monaghan & Cotman, 1985). The observed trend towards a depression of the glutamate responses in the absence of external Ca (cf. Figures 1 and 4) may be significant in view of the possible charge contribution of Mg^{2+} to the depolarization evoked by glutamate actions (Pumain *et al.*, 1988).

Attenuation of GABA-depolarizations by Mg-free ACSF

The effects of $[Mg^{2+}]_0$ on the activation of Cl-currents by GABA-receptor interactions have not been observed previously in mammalian CNS neurones. However, extracellular applications of Zn^{2+}, Co^{2+}, Ni^{2+} or Cd^{2+} antagonize the responses to GABA in various vertebrate neurones (Kaneko & Tachibana, 1986). Intracellular Mg^{2+} has been suggested to be essential for preventing a 'rundown' of $GABA_A$-receptor function (Stelzer *et al.*, 1988; Gyenes *et al.*, 1988). Without clear biochemical evidence that the $[Mg^{2+}]_0$ affects the binding characteristics of $GABA_A$-receptors, one may assume that the Mg-dose-dependent inhibition of the depolarizations produced by GABA applications in our investigations is due to a decrease in the intracellular $[Mg^{2+}]$ subsequent to the removal of $[Mg^{2+}]_0$ (Baker & Crawford, 1972; Heinonen & Akerman, 1986); this could lead to a 'rundown' of $GABA_A$ receptor function or to a reduced affinity of the receptors for GABA (cf. Inoue *et al.*, 1986).

Enhancement of neuronal excitability in Mg-free ACSF

A reduction of $[Mg^{2+}]_0$ is known to enhance neuronal excitability in cortical systems including the hippocampus and entorhinal (Walter *et al.*, 1986; Stanton *et al.*, 1987; Mody *et al.*, 1987; Hamon *et al.*, 1987) as well as sensorimotor cortex (Thomson, 1986). Several mechanisms have been proposed for the presumed regulating role of $[Mg^{2+}]_0$ (cf. Introduction). The dose-dependent depression of the responses to GABA, a cortical inhibitory transmitter (Krnjević, 1974), which was observed in the present investigations, provides an additional mechanism that may contribute to the neuronal hyperexcitability observed during conditions of hypomagnesemia.

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Differences in control of descending inhibition in the proximal and distal regions of rat colon

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1 Descending inhibition in the proximal and distal portions of rat colon was studied separately, *in vitro*.

2 In the proximal colon, localized distension with a small balloon caused three types of response (contraction; relaxation; relaxation, then contraction) of the circular muscle on the anal side of the distended region.

3 Distension caused descending relaxation of circular muscle in all segments of the proximal colon, although for this prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was necessary in some segments to increase muscle tone.

4 Atropine and guanethidine did not inhibit this descending relaxation, but tetrodotoxin did.

5 Hexamethonium inhibited the descending relaxation in 14 of 17 preparations of proximal colon tested, but not in the others.

6 In the distal colon, distension consistently caused an increase in the tone of the circular muscles. Descending relaxation was observed only after development of higher tone. Atropine and guanethidine did not inhibit the relaxation, but tetrodotoxin did.

7 Hexamethonium did not inhibit the descending relaxation in most of the preparations of distal colon examined.

8 AF64A, an inhibitor of choline uptake, inhibited the response mediated by cholinergic neurones *in vitro* to electrical transmural stimulation of the longitudinal muscle of proximal colon.

9 Treatment of colonic preparations with AF64A *in vitro* resulted in inhibition of descending relaxation in those of proximal, but not those of distal, colon.

10 The participation of intrinsic cholinergic neurones in the descending neuronal pathway is strongly suggested by the results in the proximal colon, but less so in the distal colon.

11 The tone and spontaneous contractile activity of colonic circular muscles are discussed in relation to their neuronal control.

Introduction

Stimuli applied to the intestinal wall produce contraction on the oral side (ascending contraction) and relaxation on the anal side (descending relaxation) of the stimulated region, resulting in peristaltic movements ('The law of the intestine', Bayliss & Starling, 1899). Descending relaxation has been observed in cat colon (Crema, 1970), dog intestine (Bayliss & Starling, 1899), rabbit colon (Julé, 1980) and guinea-pig colon (Costa & Furness, 1976). Myenteric neurones are known to contain neural pathways responsible for peristalsis of the intestine (reviewed by Kosterlitz & Lees, 1964; North, 1982). These neuronal pathways have been studied by inflation of a small balloon in the intestine (Frigo & Lecchini, 1970) or by mechanical distension (Costa & Furness, 1976) to produce a localized stimulus. Hirst & McKirdy (1974) demonstrated the presence of a descending neural pathway in the guinea-pig small intestine excited by distension of the intestinal wall or transmural electrical stimulation. They also suggested the involvement of cholinergic interneurones in the pathway, because the electrical response was inhibited by (+)-tubocurarine. The neural pathway of the descending reflex contains afferent sensory neurones, interneurones and inhibitory motor neurones. The inhibitory neurones have been suggested to be non-adrenergic, non-cholinergic (NANC; Costa & Furness, 1976). Grider & Makhlouf (1986) showed that the NANC inhibitory neurones in rat colon receive an input from cholinergic interneurones and release vasoactive intestinal peptide. Other studies have demonstrated cholinergic myenteric neurones whose depolarizing effect is reversibly blocked by hexamethonium (Sato *et al.*, 1973; Nishi & North, 1973; North *et al.*, 1980; Tokimasa *et al.*, 1981).

In the present work, we studied the responses of the circular muscles of the proximal and distal portions of rat colon to inflation of a balloon in the lumen. We also examined the participation of cholinergic interneurones in the descending inhibitory pathway by use of ethylcholine mustard aziridinium ion (AF64A), which has been shown to cause selective injury of cholinergic neurones in the peripheral nervous system (Mantione *et al.*, 1983a; McArdle & Hanin, 1986; Hoyle *et al.*, 1986).

Methods

Male Wistar rats (250–350 g) were used. They were stunned by a blow on the head and bled via the carotid artery. Segments of the proximal and distal colon were removed and placed in Tyrode solution consisting of (in $g l^{-1}$) NaCl 8, KCl 0.2, $CaCl_2$ 0.2, $MgCl_2$ 0.1, NaH_2PO_4 0.05, $NaHCO_3$ 1.0 and glucose 1.0. The faeces in the excised segments were gently flushed out with Tyrode solution. Segments of 4 to 6 cm of the proximal colon and distal colon were used. In all experiments the preparation was kept in 30 ml of Tyrode solution at 37°C aerated with 95% O_2 and 5% CO_2. The preparation was equilibrated for at least 30 min before the experiment was started. Drugs were added to the organ bath in volumes of less than 1% of the bathing solution.

Recording of responses of colonic circular muscle to the stimulus of distension

Colonic segments were held horizontally with the side adherent to the mesentery at the bottom in a specially designed organ bath (Figure 1). The middle of the segment was connected by a stainless-steel hook at the joint of the mesentery to an anchor fixed to the bottom of the bath. A rubber balloon, connected to a syringe by thin polyethylene tubing, was introduced into the lumen and positioned at the middle of the segment. The balloon was inflated with 0.1 to 0.2 ml of

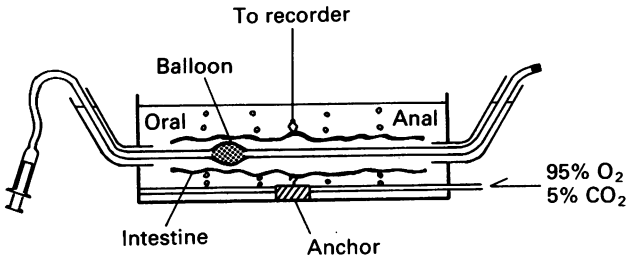

Figure 1 Diagram of the system used for recording the responses of isolated colonic segments to balloon inflation.

warm water from the syringe to produce slightly greater local distension than that produced by a faecal bolus. The duration of distension was 30 or 60 s. The mechanical response of the circular muscle about 1.0 cm anal to the balloon was recorded, by connecting a frog heart clip to a small area of the wall opposite to the anchor and then connecting the clip via a thread to an isotonic transducer (TD-112A, Nihonkohden, Tokyo, Japan). Both ends of the segment were free. This arrangement allowed preferential recording of the response of the circular muscle, since the balloon was deflated immediately after the development of the responses to avoid an effect on the spontaneous activity. The circular muscle was subjected to a resting load of 0.5 g.

Recording of responses of longitudinal muscle to electrical transmural stimulation

Colonic segments were suspended in an organ bath filled with aerated Tyrode solution, maintained at 37°C. The oral end of the segment was attached to a transducer and the anal end mounted on an anodal electrode placed at the bottom of the bath. Responses of the longitudinal muscle to transmural stimulation, with trains of 50 pulses of 0.1 ms width and supramaximal voltage (usually 30 V) at a frequency of 10 Hz, were recorded isotonically and successively with a 10 min interval between tests and described in the preceding paragraph.

Preparation of AF64A

AF64A was prepared from ethylcholine mustard (AF64) picrate as described by Fisher *et al.* (1982). Briefly, AF64 picrate was dissolved in saline and picrate was removed with anion exchange resin. The solution was then adjusted to pH 7.4 by addition of solid $NaHCO_3$, stood for 1 h at room temperature and then used as AF64A. AF64A was prepared just before each experiment. The conversion of AF64 to AF64A measured by the iodine-thiosulphate method (Sandberg *et al.*, 1984) was 91.5%, so the concentration of AF64A was corrected on the basis of this value.

Treatment of colon segments with AF64A

Colonic segments *in vitro* were treated with AF64A prepared as described above by adding the agent into the bath at a final concentration of 62.5 μM.

Drugs

Ethylcholine mustard picrate (AF64) was a gift from Mitsubishi Kasei Kogyo Co., Yokohama, Japan. Tetrodotoxin was a gift from Sankyo Co., Osaka, Japan, and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) a gift from Ono Pharmaceutical Co., Osaka, Japan. $PGF_{2\alpha}$ was dissolved in ethanol and added to the organ bath at a final concentration of 10 ng ml^{-1} in 0.1% ethanol. This concentration of ethanol did not affect the responses. All other chemicals were of analytical grade and dissolved in distilled water.

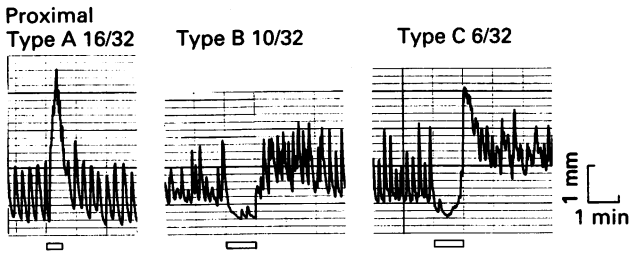

Figure 2 Responses of proximal segments of rat colon to balloon inflation. The segments were radially distended with a small balloon and the responses of the circular muscle 1 cm anal to the balloon were recorded. Small rectangles indicate 30 s distension and large ones 60 s distension. Ratios indicate the number of preparations that showed the indicated response to the total numbers of preparations studied. Details of experimental conditions are described in the Methods.

Results

Descending responses of the proximal colon to local distension

At rest, circular muscle of the proximal colon exhibited spontaneous rhythmic contractions. On stimulation, three types of response were observed on the anal side of an inflated balloon (Figure 2). Of 32 preparations, 16 showed only contraction (designated as type A), 10 showed only relaxation (type B) and 6 showed relaxation followed by contraction (type C). The type B response occasionally changed to a type C response with spontaneous decrease in the resting tone of the preparation during the experiment. The type of response, especially development of relaxation, appeared to be closely related to the resting tone of the preparation, because the type B response was elicited in preparations that initially exhibited a type A response to local distension when their resting tone was increased by treatment with $PGF_{2\alpha}$ (Figure 3).

Hexamethonium inhibited the descending relaxation in 14 of 17 preparations of the proximal colon, but did not affect relaxation in the other 3 preparations (Figure 4). Guanethidine (4 μM) did not have any significant effect on the descending relaxation, but tetrodotoxin (1 μM) completely blocked the relaxation of all preparations tested. Tetrodotoxin slightly stimulated the spontaneous contractile activity of circular muscle, as has been found previously (Manzini *et al.*, 1986). Atropine (1 μM) did not diminish the inhibitory phase of the response. These results indicate that NANC neurones mediate the descending relaxation of the proximal colon.

Descending responses of distal colon to local distension

Preparations of the distal colon exhibited either no spontaneous contraction of circular muscle or slight contraction at low frequency, even after a long period of equilibration in the

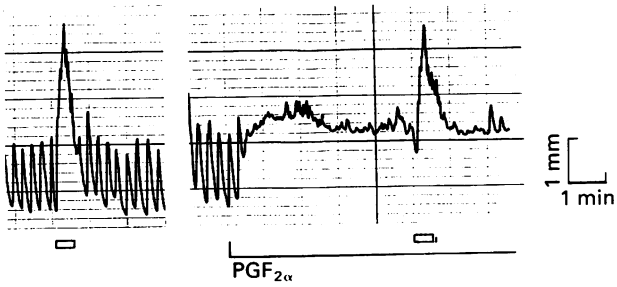

Figure 3 Effect of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on proximal colonic segments that exhibited the type A response. $PGF_{2\alpha}$ (10 ng ml^{-1}) was added to the organ bath. For further details see legend of Figure 2 and Methods.

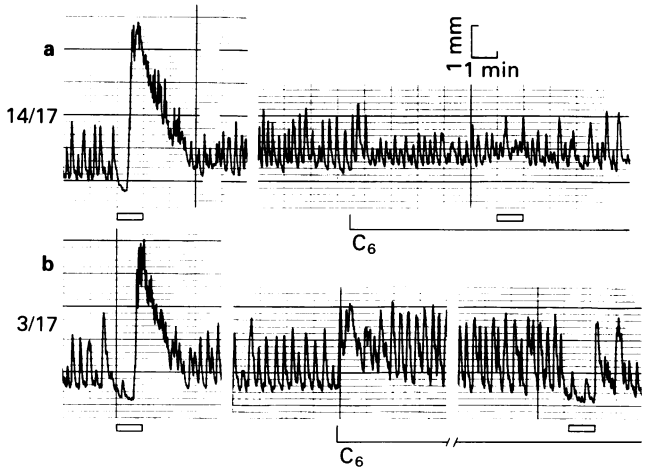

Figure 4 Effect of hexamethonium (C_6, 500 μM) on descending response of a proximal colon to balloon inflation. The descending response was either abolished (a) or not affected (b). For further details see legend of Figure 2 and Methods.

organ bath. Almost all preparations showed only contraction of circular muscle anal to the distended region in the initial 3 or 4 tests with local distension. However, the resting tone of the circular muscle gradually increased during successive distensions and never returned to the pre-stimulus level, even after repeated changes of the bathing solution. When a higher resting tone was acquired in this way, the preparation began to exhibit descending relaxation followed by contraction instead of contraction only (Figure 5). Neither atropine nor guanethidine affected the inhibitory response. Tetrodotoxin increased the resting tone and abolished the response to local distension, as in the proximal portion of the colon. In 17 experiments in which the effect of hexamethonium treatment was investigated, descending relaxation was abolished in only one case, partially inhibited in 6 and unaffected in the other 10. This agent increased the tone in all preparations (Figure 6).

Effect of AF64A treatment on the cholinergic response of rat proximal colon

Electrical transmural stimulation induced a small phasic contraction followed by a transient rapid relaxation and a large rebound contraction. These responses were consistently observed after each stimulus during a period of 2 h. The initial small phasic contraction seemed to be cholinergic because atropine abolished it without affecting the following two phases of the response (Figure 7). Treatment of the colonic segment with AF64A *in vitro* resulted in selective inhibition of the small phasic contraction in all 4 preparations tested within 2 h, with no other changes (Figure 8).

Effect of AF64A on descending relaxation

The descending relaxation of the proximal colon in response to local distension was inhibited by pretreatment with AF64A

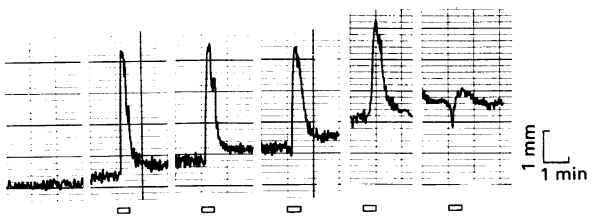

Figure 5 Change in response of the distal colon to balloon distension with time. The preparation was distended repeatedly with a small balloon. For further details see legend of Figure 2 and Methods.

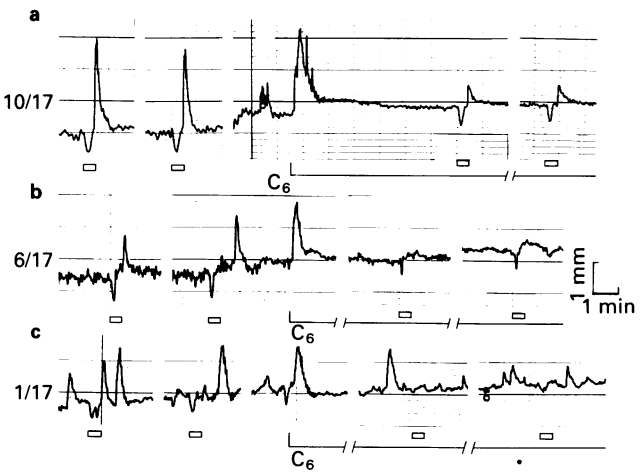

Figure 6 Effect of hexamethonium (C_6, 500 μM) on descending responses of distal colon to balloon inflation. (a) Results from a preparation in which hexamethonium did not affect relaxation. (b) Results from a preparation showing partial inhibition by C_6. (c) Results from a preparation showing complete inhibition by C_6. Ratios are numbers of preparations showing the response to total numbers of preparations tested. For further details, see legends of Figures 2 and 4.

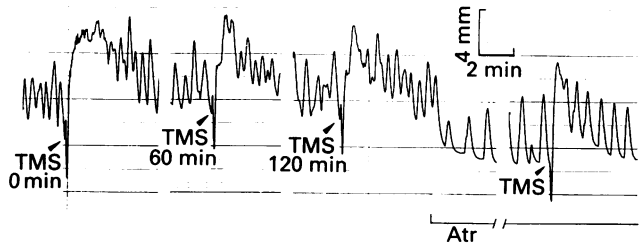

Figure 7 Response of longitudinal muscle of colonic segments to electrical transmural stimulation and effect of atropine (Atr, 1 μM) on the response. A preparation of proximal colon was stimulated transmurally by trains of electrical pulses (TMS) at the point marked by an arrowhead. The time after the beginning of the experiment is shown. For further details see Methods.

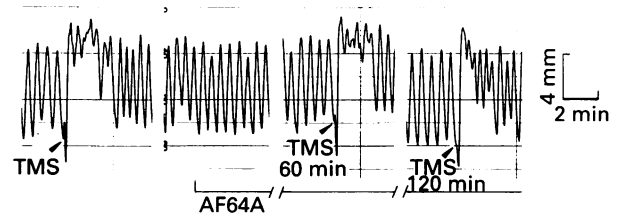

Figure 8 Effect of AF64A treatment on the response to transmural electrical stimulation. A preparation of proximal colon was treated with AF64A (62.5 μM) *in vitro*. The time indicates that after the beginning of the treatment. For further details see legend of Figure 7 and Methods.

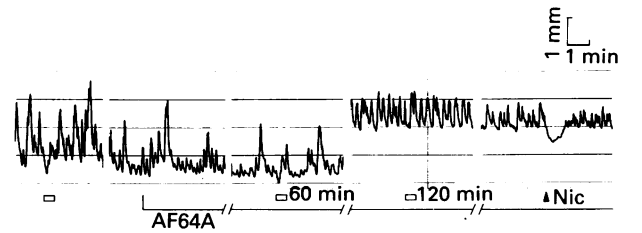

Figure 9 Effect of AF64A treatment on descending relaxation of the proximal colon on balloon distension. A preparation of proximal colon was distended with a balloon before and after AF64A treatment. Nicotine (Nic, 10 μg ml^{-1}) was added in the presence of AF64A. For further details see legend of Figure 7 and Methods.

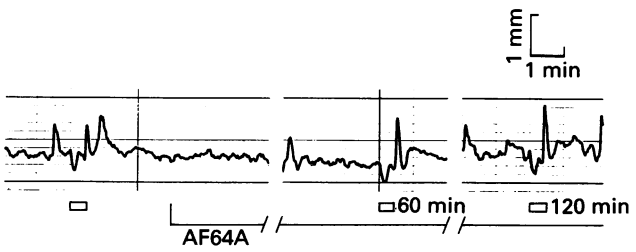

Figure 10 Descending relaxation of a distal colonic segment on balloon distension before and after AF64A treatment. For further details see legends of Figures 7 and 9 and Methods.

within 2 h in all 4 preparations studied. The preparations were still responsive to nicotine when the descending relaxation had been abolished (Figure 9). AF64A had no effect on any of the 4 preparations of distal colon studied (Figure 10).

Discussion

There are differences in the morphology (Christensen *et al.*, 1984) and density (Hukuhara & Neya, 1968) of the myenteric plexus and in its sensitivities to drugs (Fink & Friedman, 1960) in the proximal and distal portions of rat colon. The contents of the intestinal lumen are dehydrated in the proximal colon and pellets of faeces are mainly formed in the distal colon (Ferré & Ruckebusch, 1985). Thus, the two portions of the colon differ greatly in motility and physiological functions.

Rubbing the mucosa with an uninflated balloon did not induce any descending responses. Inflation of the balloon with a volume over 0.1 ml was effective in inducing descending responses in almost all of the segments. By increasing the volume up to 0.2 ml the responses became more distinct without any changes in their shape. With volumes over 0.25 ml, mechanical artifacts were often observed on the record. Hence, we assumed that the inflation of the balloon induced the responses via the mechanoreceptors within the intestinal wall. Paintal (1954a,b) have alluded to the fact that mucosal afferents tend to respond in an 'all or none' manner to a local stimulus while the in-series mechanoreceptors in muscle show a graded response.

Some preparations of proximal colon showed only descending contraction (type A) in response to local distension, whereas others showed descending relaxation (type B, C; Figure 2). However, segments that exhibited the type A response also showed descending relaxation when their tone was increased by treatment with $PGF_{2\alpha}$ (Figure 3). $PGF_{2\alpha}$ is known to induce contraction of the circular muscle of rat colon (Flesher & Bennett, 1969) and increase the resting tone of the colon (Eckenfels & Vane, 1972). Thus the reason why preparations exhibiting the type A response did not show an inhibitory phase in response to local distension was probably that the initial tension of their circular muscle was low. In general, we supposed that inflation of the balloon caused a relaxation followed by contraction. The finding that distal segments showed descending relaxation only after some increase in tone induced by repetitive distensions (Figure 5) supports this hypothesis. Tetrodotoxin increased the spontaneous contractile activity in both the proximal and distal colonic segments. It also significantly increased the tone of circular muscle in the distal segment, but not in the proximal segment. These findings indicate the existence of a tonic inhibition of the spontaneous contractile activity of the colon by inhibitory neurones. Similarly, tonic sympathetic nerve activity exerts an inhibitory effect on proximal and midcolonic motility of the cat (Gillis *et al.*, 1987) and tonic NANC-like activity suppresses the proximal rat colon (Maggi *et al.*, 1987). The above findings also indicate the existence of some neural inhibitory control of the tone of the circular muscle in the distal colon.

Bayliss and Starling (1899) proposed that a local stimulus to the intestine causes contraction on the oral side and relaxation on the anal side of the stimulated region, thereby resulting in transport of the intestinal contents from the oral region to the anus. Some exceptions to descending relaxation have been obtained, such as in the small intestine of the guinea-pig (Yanagiya & Ohkubo, 1958), domestic fowl (Hodgkiss, 1986) and rabbit (Ozaki, 1979). However, there is also an interesting finding that in guinea-pig small intestine local distension produces excitation of descending inhibitory neurones (Hirst & McKirdy, 1974). These and the present findings indicate that excitation of descending inhibitory neurones occurs in the intestine of many species, but that the degree of tone of the tissue may determine whether relaxation occurs.

The inhibitory effect of tetrodotoxin and the absence of effects of atropine and guanethidine on the descending relaxation in the proximal and distal colon of the rat indicate that NANC neurones mediate the relaxation observed in the present study.

Hexamethonium, an antagonist of nicotinic cholinoceptors, inhibited the descending relaxation to local distension in 14 of 17 preparations of the proximal colon, but in only one preparation of the distal colon. The results suggest that there are many cholinergic interneurones of the descending inhibitory pathway in the proximal colon, but few in the distal colon. Crowcroft *et al.* (1971) have shown that cholinergic neurones within the wall of the distal colon extend their axons to the inferior mesenteric ganglion and terminate on the noradrenergic neurones which could depress the activity of excitatory neurones of the colon in guinea-pig. In the present study, the experiments were carried out with the colonic segments of rat. Therefore, the contribution of the nerve pathway proposed by Crowcroft *et al.* (1971) is not relevant to the present experiments on descending relaxation.

AF64A has been shown to cause selective degeneration of cholinergic neurones in the central nervous system (Fisher & Hanin, 1980; Fisher *et al.*, 1982; Mantione *et al.*, 1983b; Sandberg *et al.*, 1984; Walsh *et al.*, 1984). One of its main effects is thought to be inhibition of the high affinity choline uptake system at the nerve terminals (Rylett & Colhoun, 1980; 1984; Mantione *et al.*, 1981; Fisher *et al.*, 1982; Curti & Marchbanks, 1984). There are few studies on the effect of AF64A on the peripheral nervous system (Mantione *et al.*, 1983a; Allen, 1983; Hoyle *et al.*, 1986). We found that AF64A selectively inhibited the small phasic contraction (Figure 8) that could be inhibited by atropine. Therefore, AF64A probably caused dysfunction of cholinergic neurones in the colon *in vitro* as has previously been suggested (Rylett & Colhoun, 1978; Mantione *et al.*, 1983a; Potter *et al.*, 1985). Our finding that AF64A inhibited the descending relaxation in all 4 segments of the proximal colon examined, but not in any of the 4 segments of the distal colon examined, are consistent with findings that hexamethonium abolished the input of signals from cholinergic interneurones by blocking nicotinic cholinoceptors on NANC neurones. The present results, therefore suggest a significant participation of cholinergic interneurones in the proximal, but not the distal portion of the rat colon in the descending neural pathway.

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Dr G. M. Lees' term of office as Secretary to the Editorial Board of the *British Journal of Pharmacology* will end later this year. Dr Lees is to be succeeded by Dr R. W. Horton and Dr W. A. Large, as Joint Secretaries to the Editorial Board. Consequently, the Editorial Office is to be moved from Aberdeen to London. The transfer will occur in stages.

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A. T. Birmingham
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