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Streptozotocin-induced diabetes reduces the density of [^{125}I]-endothelin-binding sites in rat cardiac membranes

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The effect of acute, streptozotocin-induced diabetes on the affinity (K_D), density (B_{max}) and selectivity of specific, high affinity binding sites for [^{125}I]-endothelin ([^{125}I]-ET) in rat cardiac membrane fragments was determined. Three days after a single i.v. bolus dose of streptozotocin (60 mg kg^{-1}), the density of [^{125}I]-ET binding sites was reduced ($P < 0.01$) without changes in affinity or selectivity.

Introduction Endothelin (ET), a vasoconstrictor polypeptide secreted by vascular endothelial cells (Yanagisawa *et al.*, 1988), has positive inotropic (Ishikawa *et al.*, 1988a; Hu *et al.*, 1988) and chronotropic (Ishikawa *et al.*, 1988b) effects on the heart, increases the amplitude and duration of the cardiac action potential (Ishikawa *et al.*, 1988a), stimulates phospholipase C (Resink *et al.*, 1988) and phosphatidylinositol metabolism (Sugiura *et al.*, 1989) and causes bronchoconstriction (Uchida *et al.*, 1988).

High affinity ET (as [^{125}I]-ET) binding sites have been identified in the heart (Ambar *et al.*, 1989; Gu *et al.*, 1989a,b), where the bound [^{125}I]-ET is displaceable by cold ET and sarafotoxin S6b (Gu *et al.*, 1989b; Ambar *et al.*, 1989) but not by Ca^{2+}- or α-adrenoceptor antagonists (Gu *et al.*, 1989a). Pretreatment with ET causes 'down-regulation' of [^{125}I]-ET binding sites (Hirata *et al.*, 1988). Little else is known about the factors which modify their density, affinity or selectivity. Our results show that streptozotocin-induced acute diabetes, a model often used to study the cardiac effects of diabetes (Tani & Neely, 1988), alters the density of cardiac [^{125}I]-ET binding sites.

Methods Hearts from adult (200–250 g) Sprague Dawley rats which had been fasted overnight were used for these experiments. Half the rats were injected via the tail vein with a bolus dose of streptozotocin (60 mg kg^{-1}) dissolved in sodium-citrate buffer (pH 4.5), to produce non-ketoacidotic diabetes (Mansford & Opie, 1968). The others received an equal volume of citrate buffer i.v. All rats received rat chow and water *ad libitum*.

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On the third day the rats were anaesthetized with a diethylether-O_2 mixture and heparinized. Blood was taken for non-fasting plasma glucose determinations, measured by a glucose oxidase technique (Beckman Astra autoanalyser, U.S.A.).

Cardiac membranes were harvested (Gu *et al.*, 1989a), in a homogenizing medium containing 20 mM $NaHCO_3$ and 0.1 mM phenylmethylsulphonylfluoride (PMSF) pH 7.4. Protein was assayed by the Lowry method (Lowry *et al.*, 1951) with bovine serum albumin as standard. [^{125}I]-ET binding was monitored as previously described (Gu *et al.*, 1989a) with a final protein concentration of 0.16–0.24 mg protein ml^{-1} in 0.25 ml. Non-specific binding was defined in the presence of 2×10^{-7} M ET. The reaction mixture contained 50 mM Tris and 0.1 mM PMSF, pH 7.4, with 2×10^{-11} M–1×10^{-9} M [^{125}I]-ET. Incubation was at 37°C for 60 min. Bound and free [^{125}I]-ET were separated by rapid vacuum filtration across GF/C Whatman filters after dilution with 3.5 ml of ice-cold 10 mM Tris buffer containing 6.6% polyethyleneglycol 6000 (PEG), pH 7.4. After two additional washes with Tris-PEG buffer the radioactivity of the filters was counted in a LKB multiwell γ counter (80% efficiency).

Binding selectivity was established with 10^{-13}–10^{-8} M ET, 10^{-12}–10^{-7} M (+)-PN200-110 (isopropyl-4 (2,1,3-benzoxadeazol-4-γ-1)-1,4 dihydro-2,6-dimethyl-5-methoxycarbonyl-pyridine-3-carboxylate), (10^{-13}–10^{-8} M) sarafotoxin S6b, and [^{125}I]-ET (10^{-10}–2×10^{-10} M).

Data analysis was as previously described (Gu *et al.*, 1988). K_D is the concentration of ligand required to occupy 50% of binding sites, B_{max} the density of binding sites, K_i the inhibition constant of the competing ligand, and IC_{50} the concentration of ligand which displaces 50% of the specifically bound ligand ([^{125}I]-ET). Results are presented as mean ± s.e.mean of 6 experiments, unless otherwise stated. Tests of significance were calculated by Student's t test, with $P = 0.05$ as the limit of significance.

Porcine ET, (Protein Research Foundation, Osaka, Japan) was iodinated (Gu *et al.*, 1989a) to a specific activity of approximately 1600 Ci mmol^{-1}. Sarafotoxin S6b, (+)-PN200-110 and streptozotocin came from Peninsula Research Laboratories, Cali-

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fornia, U.S.A., Sandoz Ltd, Basle, Switzerland, and Boehringer Mannheim, Mannheim, Germany, respectively.

Results Plasma from control and streptozotocin-treated diabetic rats contained 10.22 ± 0.35 and 28.93 ± 1.07 mmol glucose l^{-1} respectively. The membrane yields were similar for both groups (9.01 ± 0.32, and 9.12 ± 0.42 mg g^{-1} for control and diabetic hearts respectively). [^{125}I]-ET binding reached asymptote within 60 min, and was linear over a protein range of 0.16–0.24 mg ml^{-1}. Binding to reaction tubes and filters was negligible.

Specific [^{125}I]-ET binding for non-diabetic rat membranes was to a single population of sites (Hill coefficient, 0.999 ± 0.004), with a K_D of 0.098 ± 0.007 nM, and a B_{max} of 100.6 ± 5.3 fmol mg^{-1} protein (Figure 1a). [^{125}I]-ET binding for diabetic rat membranes was also to a single population of sites (Hill coefficient, 0.999 ± 0.003), with a K_D (0.090 ± 0.05 nM) not significantly different from the controls. Non specific binding was unchanged. However, (Figure 1a) the B_{max} was reduced ($P < 0.01$) to 70.9 ± 1.4 fmol mg^{-1} protein. Despite this reduction in B_{max}, the selectivity was maintained, with cold ET and sarafotoxin S6b, but not (+)-PN200-110, displacing bound [^{125}I]-ET (Figure 1b and c). The K_i and IC_{50} values for ET and sarafotoxin S6b displacement of [^{125}I]-ET for control membranes were:- K_i 0.04 nM for ET and 0.12 nM for sarafotoxin S6b; $IC_{50} = 0.08$ nM for ET and 0.23 nM for sarafotoxin S6b. For diabetic rat membranes the K_i was 0.04 nM for ET and 0.17 nM for sarafotoxin S6b, with IC_{50} values of 0.08 nM for ET and 0.33 nM for sarafotoxin S6b.

Adding streptozotocin to isolated membranes, to provide a concentration equivalent to the maximum plasma levels achievable after the bolus injection, altered neither the B_{max} nor the affinity of the [^{125}I]-ET binding.

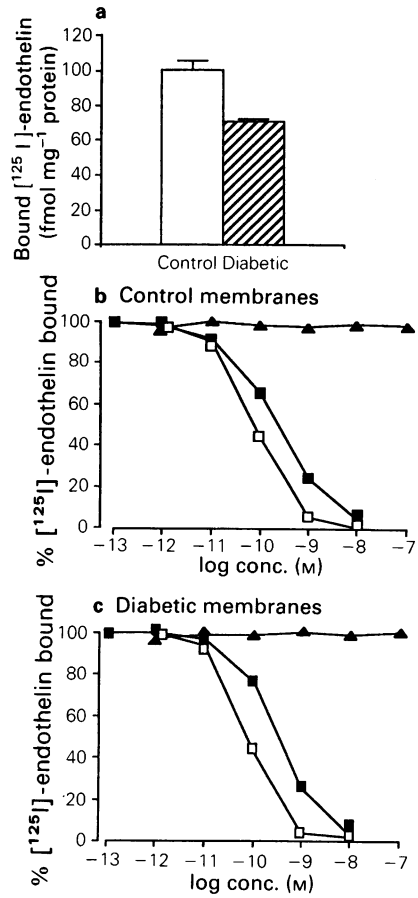

Figure 1 Effect of stretozotocin-induced acute diabetes on [^{125}I]-endothelin ([^{125}I]-ET) binding to cardiac membranes. (a) [^{125}I]-endothelin bound to cardiac membranes from control (open column) and diabetic (hatched column) rats. Each column is mean of 6 experiments (with s.e.mean shown by vertical bars), the estimates for which were performed in duplicate. * = $P < 0.01$. Control (b) + diabetic (c) displacement curves for the effect of cold endothelin (10^{-12}–10^{-8} M) (□), sarafotoxin S6b (10^{-12}–10^{-8} M) (■)and (+)-PN200-110 (10^{-13}–10^{-8} M) (▲) on specifically bound [^{125}I]-endothelin from cardiac membranes. Similar results were obtained in 3 other experiments.

Discussion These results confirm the ability of [^{125}I]-ET to bind to a single population of sites in rat cardiac membranes (Gu *et al.*, 1989a), and extend them by showing that the density of these sites is reduced in membranes harvested from streptozotocin-treated rats, without changes in affinity or selectivity.

This effect of acute diabetes on the density of cardiac high affinity [^{125}I]-ET binding sites will have to be considered in future studies in which hearts from acutely diabetic rats are used as experimental models. For example, Tani & Neely (1988) used hearts from acutely diabetic rats to study the effect of diabetes on ischaemia- and reperfusion-induced injury. They described a protective effect of diabetes. The reduction in [^{125}I]-ET binding site density described here could contribute to this protection, since ET promotes Ca^{2+} influx (Ishikawa *et al.*, 1988a) and increases energy utilization by way of its positive inotropic and chronotropic activity (Hu *et al.*, 1988). These effects, together with the stimulant effect of ET on phosphoinositol metabolism (Sugiura *et al.*, 1989) and Na^+/H^+ exchange (Wann

et al., 1989) could implicate ET in the loss of Ca^{2+} homeostasis observed during ischaemia and reperfusion.

Our results do not indicate whether the diabetes-induced reduction in [^{125}I]-ET binding site density is due to receptor 'down regulation'. However, they rule out a direct effect of the streptozotocin.

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Effects of α- and β-calcitonin gene-related peptides upon ion transport in rat descending colon

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Both α- and β-calcitonin gene-related peptide (CGRP) exhibit concentration-dependent dual actions upon rat colonic epithelia. At low concentrations (i.e. 0.03–10 nM) both CGRPs produce antisecretory effects that are abolished by pretreating tissues with tetrodotoxin (TTX). At higher concentrations (30–200 nM) secretory responses predominate and these effects are not abolished by TTX. It is suggested that the mediator of the antisecretory actions of CGRPs is somatostatin.

Introduction Two calcitonin gene-related peptides (CGRPs) exist in the enteric nervous system (Mulderry *et al.*, 1988). The first was described in rat brain (Rosenfeld *et al.*, 1983) and designated α-CGRP while the second, β-CGRP which differs from α-CGRP by only one of its 37 amino acids was predicted following the discovery of a novel mRNA from rat thyroid carcinoma (Amara *et al.*, 1985). The rat gene coding for α-CGRP also encodes the precursor of the calcium-regulating hormone, calcitonin (Rosenfeld *et al.*, 1983). In contrast there is no evidence for a second mature transcript from the gene encoding β-CGRP (Amara *et al.*, 1985). Both peptides appear to be coexpressed in the CNS, while in the enteric nervous system it is α-CGRP which coexists with substance P (SP) in sensory neurones (Ekblad *et al.*, 1988; Mulderry *et al.*, 1988) and β-CGRP is thought to be located in intrinsic submucous and myenteric neurones (Mulderry *et al.*, 1988).

Detailed investigations of CGRP containing neurones in the rat colon (Ekblad *et al.*, 1988) have identified several populations of CGRP containing nerves. Some perivascular CGRP fibres contain SP and are extrinsic in origin and probably sensory, whilst other perivascular fibres contain both CGRP and vasoactive intestinal peptide. Another more extensive CGRP-containing population of neurones is present in both intramural ganglia, the cell bodies present in the submucous plexi projecting varicose fibres towards the mucosa. These cells do not appear to contain any other neuropeptides. In contrast a further subpopulation of CGRP positive cells present only in the submucous plexi also contain somatostatin (SOM) and these neurones issue fibres which are located in the submucosa and around the basal areas of crypts. The presence of CGRP containing nerves so close to the mucosa is suggestive of a role for this peptide in the regulation of ion transport. Rat colon epithelium responds to a variety of endogenous substances which cause either anion secretion and increase the short circuit current Y (SCC) e.g. kinins (Cuthbert & Margolius, 1982) or they exhibit antisecretory effects resulting in a fall in SCC, e.g. neuropeptide Y (NPY) (Cox *et al.*, 1988). This study describes the effects of both α- and β-CGRP upon rat colonic epithelial ion transport.

Methods Preparations of descending colon were removed from male Sprague-Dawley rats (200–250 g) and the overlying smooth muscle layers were removed (together with the accompanying myenteric plexi). Mucosal sheets were placed between two halves of perspex Ussing chambers with a window size of 0.6 cm^2 and bathed in oxygenated Krebs-Henseleit solution at 37°C as described previously (Cox *et al.*, 1988). Four adjacent preparations of colon mucosa were routinely obtained, all exhibiting similar basal SCC when automatically voltage clamped (WP Instruments, New Haven, CT, U.S.A.). The resultant SCC was continuously recorded. EC_{50} values are expressed as geometric means with 95% confidence limits.

Both α- and β-CGRP were purchased from Peninsula Laboratories Europe Ltd (St Helens, Merseyside). Tetrodotoxin (TTX) was obtained from Sigma (Poole).

Results Complex concentration-response profiles were obtained following addition of either α- or β-CGRP to the basolateral bathing fluid of untreated colonic preparations. Between concentrations of 0.03 nM and 10 nM α- and β-CGRP, reductions in SCC were recorded (Figure 1a) from

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the original stable baseline. Addition of higher concentrations (30–200 nM) resulted in increases in SCC, reversing the effects of the lower doses of both peptides. Following application of β-CGRP at the highest concentration, the elevated SCC returned, not to the original baseline but to the lower SCC achieved after 10 nM β-CGRP. Similar profiles were obtained with α-CGRP (data not shown). Application of 100 nM TTX to colon preparations reduced the baseline SCC by $-12.0 \pm 3.2\,\mu A/0.6\,cm^2$ ($n = 6$, from $20.5 \pm 4.1\,\mu A/0.6\,cm^2$ to $8.5 \pm 1.1\,\mu A/0.6\,cm^2$). Subsequent addition of either α- or β-CGRP (using the same concentration range) resulted in a loss of the antisecretory effects previously observed with low concentrations (Figure 1b) and only the secretory responses remained. These increases in SCC were observed with threshold concentrations of 1 nM for α-CGRP and 3 nM for β-CGRP. EC_{50} values of 13.9 nM (10.7, 18.2; $n = 3$) and 65.0 nM (25.6, 165.3; $n = 3$) were obtained for α- and β-CGRP respectively in TTX pretreated tissues. Taking the antisecretory responses from control tissues in isolation, threshold concentrations were 0.1 nM for both α- and β-CGRP. EC_{50} values of 0.3 nM (0.1, 1.1; $n = 3$) and 2.0 nM (0.4, 10.9; $n = 3$) were obtained for α- and β-CGRP respectively. It does appear that α-CGRP is the more potent secretory and antisecretory agent of these two peptides, although the antisecretory effects of β-CGRP were greater.

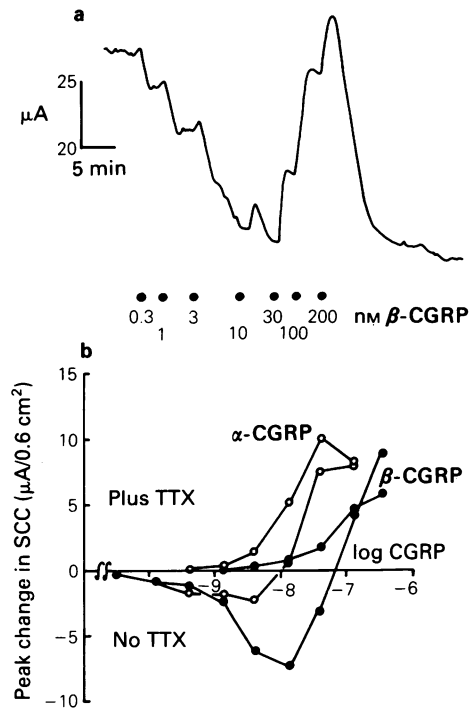

Figure 1 The effect of α-calcitonin gene-related peptide (α-CGRP) (○) and β-CGRP (●) upon electrogenic ion transport in mucosal preparations of rat colon. (a) A representative record of changes in SCC following application of increasing concentrations of β-CGRP (added to basolateral surface only) in the absence of tetrodotoxin (TTX). Note that the direction of changes in SCC is reversed between 10 and 30 nM β-CGRP. (b) Pooled data from 3–8 separate experiments showing the differences in concentration-response curves to α- and β-CGRP either in the presence or absence of TTX. Peak changes in SCC from the original baseline with or without TTX were recorded as $\mu A/0.6\,cm^2$. Each point represents the mean of 3–8 observations. Standard errors (which are not shown for reasons of clarity) ranged from 5–25% of the means.

Discussion Both α- and β-CGRP produce biphasic effects upon colonic epithelial ion transport. At low concentrations, the antisecretory effects were found to be sensitive to TTX while at higher concentrations, secretory responses were observed which were insensitive to TTX. The antisecretory responses could be mediated by one or more of three known antisecretory agents (all of which are located in neurones innervating the mucosa), either noradrenaline, NPY or SOM. The last of these three is perhaps the most likely candidate as it is by far the most potent antisecretory agent in the rat colon. In addition, preliminary data indicate that preincubation of tissues with 30 nM SOM results in loss of the antisecretory effects of CGRPs, while in the presence of 50 μM yohimbine these effects are still observed. Interestingly in two other gastrointestinal systems, CGRP has been found to stimulate SOM release, namely rat stomach (Yamatani *et al.*, 1986) and canine stomach and intestine (Dunning & Taborsky, 1987). The TTX sensitivity of low-concentration CGRP responses in the rat colon, therefore, indicate the presence of CGRP receptors on submucous neurones, which are retained in our preparations. If SOM is the antisecretory mediator, then it is important to note that the only SOM-containing neurones innervating the mucosa also contain CGRP (Ekblad *et al.*, 1988). Only one other study has so far investigated the effects of α-CGRP upon intestinal ion transport (McCulloch & Cooke, 1989). Using guinea-pig distal colon these authors observed only TTX-sensitive secretory responses in full thickness preparations. They concluded from their observations that α-CGRP activated myenteric neurones which then by stimulation of submucous neurones released a secretory neurotransmitter. Their findings together with ours, highlight the significant differences now being observed between species in neuropeptide control of mucosal ion transport.

We conclude that α- and β-CGRP elicit both indirect, antisecretory and direct, secretory effects upon colonic epithelia. The antisecretory agent mediating the low concentration effects of CGRPs has yet to be positively identified; nevertheless SOM is a likely candidate.

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Comparison of the sodium currents in normal Purkinje fibres and Purkinje fibres surviving infarction—a pharmacological study

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1 Purkinje fibres surviving infarction showed a lower maximum upstroke velocity ($\dot{V}_{max}$) and a longer action potential duration when compared to normal Purkinje fibres. A reduction in the fast sodium current and an increase in the sodium 'window' current may be responsible for the observed alterations in $\dot{V}_{max}$ and action potential duration respectively.

2 Since voltage clamp studies were not feasible, a pharmacological approach was used. The responses to tetrodotoxin (TTX) and lignocaine in normal Purkinje fibres and Purkinje fibres surviving infarction were used to examine the sodium currents in these fibres.

3 $\dot{V}_{max}$, an indirect measure of the fast sodium current, was more sensitive to lignocaine in Purkinje fibres surviving infarction than in normal Purkinje fibres. The reduction in $\dot{V}_{max}$ by lignocaine was more prominent at the shorter stimulation cycle length. Significant reduction of $\dot{V}_{max}$ was observed with the higher concentration of TTX and no differential effect on $\dot{V}_{max}$ between normal Purkinje fibres and Purkinje fibres surviving infarction was detected.

4 Reduction of action potential duration in the presence of TTX or lignocaine was used as a measure of the sodium 'window' current. A greater reduction of action potential duration by TTX and lignocaine was observed in normal Purkinje fibres than in Purkinje fibres surviving infarction.

5 The results suggested that the fast sodium current in Purkinje fibres surviving infarction is more sensitive to pharmacological agents with local anaesthetic properties and the prolonged action potential duration in these Purkinje fibres cannot be due to an increase in the sodium 'window' current. The results are compatible with an enhanced effect of antiarrhythmic drugs on $\dot{V}_{max}$ and conduction in the ischaemic heart.

Introduction

It has been shown that subendocardial Purkinje fibres can survive in the infarcted area after coronary artery occlusion. These Purkinje fibres display a lower maximum diastolic potential, reduced action potential amplitude, depressed maximum rate of depolarisation and prolonged action potential duration when compared to normal Purkinje fibres (Friedman *et al.*, 1973; Lazzara *et al.*, 1973). In addition, enhanced automaticity and triggered activity have been observed in surviving Purkinje fibres in the infarcted area (El-Sherif *et al.*, 1983). It has therefore been suggested that ventricular arrhythmias seen after 24 h of coronary artery occlusion may originate from these Purkinje fibres that survive infarction (Horowitz *et al.*, 1976).

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The rapid upstroke of the cardiac action potential ($\dot{V}_{max}$) is produced by the entry of sodium ions. This fast sodium current can be reduced by tetrodotoxin (TTX) and Class 1 antiarrhythmic drugs (Vaughan Williams, 1984; Bigger & Hoffman, 1985). A reduction of the fast sodium current will decrease $\dot{V}_{max}$ and slow cardiac conduction. Recently, another sodium current has been observed during the plateau phase of the action potential in Purkinje fibres (Attwell *et al.*, 1979; Coraboeuf *et al.*, 1979). This current is termed the sodium 'window' current (Attwell *et al.*, 1979) and can be blocked by a low concentration of tetrodotoxin (TTX) and by lignocaine (Colatsky, 1982). At present, little information is available concerning the possible changes in the fast sodium current and the TTX-sensitive sodium 'window' current in the ischaemic heart. Since it is

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not possible to obtain small free running Purkinje fibres from the infarcted areas for voltage clamp study, a direct assessment of these currents is not feasible. Hence, a pharmacological approach was used in the present study to investigate the possible alterations in the sodium currents in Purkinje fibres surviving infarction, as compared to normal fibres. The effects of TTX and lignocaine on $\dot{V}_{max}$ and the action potential duration in these Purkinje fibres were used as measurements of the fast sodium current and the sodium 'window' current respectively.

Methods

Normal tissue

Mongrel dogs of either sex were anaesthetized by intravenous injection of pentobarbitone ($30\,mg\,kg^{-1}$). After a left lateral thoracotomy, the heart was rapidly excised and placed immediately in oxygenated Tyrode solution. Free running Purkinje fibres were removed from the left ventricle. The preparation was superfused with oxygenated Tyrode solution maintained at $37 \pm 1°C$.

Infarcted tissue

Infarcted tissue was obtained 20–24 h after ligation of the left anterior descending coronary artery. Dogs were anaesthetized with an intravenous injection of pentobarbitone ($30\,mg\,kg^{-1}$) and ventilated with room air by a Harvard pump through an endotracheal tube. By use of sterile surgical techniques, the heart was accessed through the fourth left intercostal space. The left anterior descending coronary artery was isolated at a point proximal to the first diagonal branch and a two-stage ligation was performed according to the method of Harris (1950). The chest was closed in layers to provide an airtight seal. Occasionally, the second stage of ligation resulted in frequent premature ventricular contractions necessitating the use of $3\,mg\,kg^{-1}$ lignocaine (i.v.). This administration of lignocaine did not alter the action potential parameters observed 24 h later. All animals received 7.5 mg morphine sulphate (i.m.) and 5 mg diazepam (i.m.) at the first sign of them emerging from anaesthesia and again about 4–8 h later. After 20–24 h of coronary artery ligation, the animals were reanaesthetized with pentobarbitone ($20–30\,mg\,kg^{-1}$). The heart was rapidly removed and placed in oxygenated Tyrode solution. An endocardial preparation of $1.5–2.5\,cm^2$ was carefully removed from the infarcted region with a small corner of normal tissue. External stimuli could be delivered by bipolar electrodes in contact with surviving Purkinje fibres in the infarcted region or in the normal region. Since reduced excitability was observed in the infarcted region, the stimulus was usually applied to the normal region. Preparations were superfused with oxygenated Tyrode solution maintained at $37 \pm 1°C$.

Solutions

The modified Tyrode solution contained the following (mM): NaCl 125, $NaHCO_3$ 24, $MgCl_2$ 0.5, $CaCl_2$ 1.8, KCl 4.0, NaH_2PO_4 0.9 and glucose 5.5. The solution was equilibrated with a gas mixture of 95% O_2 – 5% CO_2. Tyrode solutions containing lignocaine, $2\,mg\,l^{-1}$ (7.4×10^{-6} M) or $5\,mg\,l^{-1}$ (1.8×10^{-5} M), or TTX (Sigma), 5×10^{-7} M or 2×10^{-6} M were also used. Recordings were made after 20 min equilibration. The lignocaine concentrations used are within the therapeutic range.

Intracellular recordings

After a stabilisation period (at least 1 h), intracellular action potentials were recorded using the standard microelectrode technique. External stimuli were delivered to the preparations with bipolar platinum electrodes. The pulse duration was 2 ms and the amplitude was 2 times threshold. The basic cycle lengths were 1000 and 400 ms. The signals were recorded on a Tandberg 4 channel FM tape recorder.

Measurements were made of the maximum diastolic potential, activation voltage, overshoot, action potential amplitude and action potential durations at 50 and 95% repolarization (APD_{50} and APD_{95} respectively). The maximum upstroke velocity ($\dot{V}_{max}$) was obtained by electronic differentiation. Only recordings in which the same impalement was maintained throughout the entire experiment were used for statistical analysis.

Statistical analysis

All values are expressed as mean ± s.d. and the statistical significance was calculated by use of analysis of variance, Student's paired and unpaired t tests and Tukey tests. A P value of <0.05 was considered statistically significant.

Results

Action potential characteristics of normal Purkinje fibres and Purkinje fibres surviving infarction

The action potential characteristics recorded from normal preparations (normal Purkinje fibres) and infarcted preparations, from hearts after 24 h of cor-

Table 1 Action potential characteristics of normal Purkinje fibres and Purkinje fibres surviving infarction

	Normal Purkinje fibres ($n = 25$)	*Purkinje fibres surviving infarction* ($n = 25$)
Maximum diastolic potential (mV)	88.5 ± 5.3	84.9 ± 4.4*
Activation potential (mV)	87.4 ± 5.3	84.2 ± 4.2*
Overshoot (mV)	38.1 ± 8.3	40.3 ± 5.8^{NS}
Amplitude (mV)	126.7 ± 5.2	125.2 ± 5.5^{NS}
APD_{50} (ms)	223 ± 40	226 ± 42^{NS}
APD_{95} (ms)	320 ± 45	362 ± 51**
$\dot{V}_{max}$ ($V s^{-1}$)	387 ± 71	343 ± 63**

Values represent mean ± s.d. n = number of experiments. Statistical comparisons were made by Student's t test for unpaired data. *$P < 0.05$; **$P < 0.01$; NS, $P > 0.05$.

onary artery ligation (Purkinje fibres surviving infarction), are summarized in Table 1. Normal Purkinje fibres had a more negative maximum diastolic potential and activation potential than Purkinje fibres surviving infarction. A higher $\dot{V}_{max}$ was observed in normal Purkinje fibres when compared with Purkinje fibres surviving infarction. Action potential duration (APD_{95}) was in general longer in Purkinje fibres surviving infarction. However, APD_{50} was similar in both types of fibres.

Effects of TTX and lignocaine on $\dot{V}_{max}$

The effects of a lower and higher concentration of TTX (5×10^{-7} and 2×10^{-6} M) and lignocaine (7.4×10^{-6} and 1.8×1.0^{-5} M) were used to compare

Table 2 Effects of tetrodotoxin (TTX) and lignocaine on the maximum upstroke velocity in normal Purkinje fibres and Purkinje fibres surviving infarction

	Maximum upstroke velocity ($V s^{-1}$)			
Drug	*Normal Purkinje fibres*	n	*Purkinje fibres surviving infarction*	n
(A) Cycle length 1000 ms				
Control	366 ± 44	7	297 ± 45	11
TTX 5×10^{-7} M	361 ± 43		289 ± 60	
Control	385 ± 57	10	326 ± 60	9
TTX 2×10^{-6} M	309 ± 73***		276 ± 69**	
Control	368 ± 76	9	317 ± 60	9
Lignocaine 7.4×10^{-6} M	371 ± 90		300 ± 50	
Control	374 ± 50	7	298 ± 48	8
Lignocaine 1.8×10^{-5} M	368 ± 48		250 ± 64	
(B) Cycle length 400 ms				
Control	366 ± 37	7	294 ± 50	11
TTX 5×10^{-7} M	345 ± 41		281 ± 57	
Control	370 ± 53	10	315 ± 57	9
TTX 2×10^{-6} M	280 ± 69***		249 ± 59***	
Control	362 ± 81	9	310 ± 54	9
Lignocaine 7.4×10^{-6} M	359 ± 87		276 ± 45**	
Control	376 ± 43	7	288 ± 51	8
Lignocaine 1.8×10^{-5} M	362 ± 52		235 ± 70*	

Values represent mean ± s.d. n = number of experiments. Comparisons were made between control and the results in the presence of TTX or lignocaine by Student's t test for paired data. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

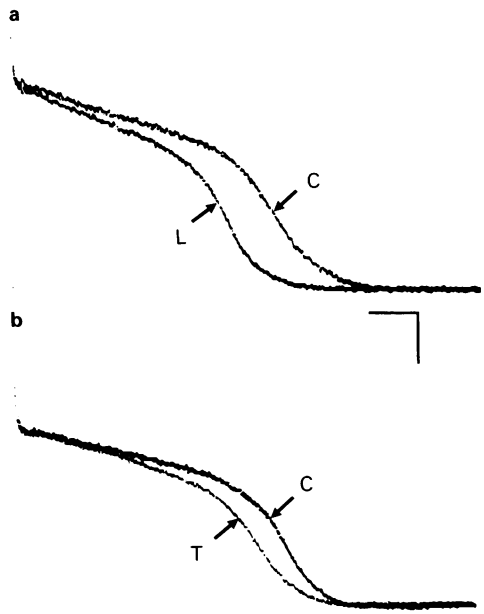

Figure 1 Effects of lignocaine (a) and tetrodotoxin (b) on the contour of the action potential of a normal Purkinje fibre. The records shown are obtained from the same fibre stimulated at a basic cycle length of 1000 ms. C represents control; T represents the presence of tetrodotoxin (5×10^{-7} M) and L represents the presence of lignocaine (1.8×10^{-5} M). Calibration: horizontal line = 50 ms, vertical line = 25 mV.

their effects on $\dot{V}_{max}$ of normal Purkinje fibres and Purkinje fibres surviving infarction. The results are summarized in Table 2. The lower concentration of TTX had no significant effect on $\dot{V}_{max}$ while the higher concentration of TTX reduced $\dot{V}_{max}$ in normal Purkinje fibres and Purkinje fibres surviving infarction. The reductions in $\dot{V}_{max}$ by the higher concentration of TTX were similar in both fibres and at the two cycle lengths tested (15–24%, Table 2). In contrast, neither concentration of lignocaine affected $\dot{V}_{max}$ in normal Purkinje fibres. In Purkinje fibres surviving infarction, the effect of lignocaine on $\dot{V}_{max}$ was more prominent than in normal Purkinje fibres. At the stimulation cycle length of 1000 ms, the reduction in $\dot{V}_{max}$ by the higher concentration of lignocaine was still not statistically significant ($P = 0.06$). Significant reduction in $\dot{V}_{max}$ by both concentrations of lignocaine was observed in Purkinje fibres surviving infarction at the shorter cycle length of 400 ms (11 and 18% by 7.4×10^{-6} and 1.8×10^{-5} M, respectively, Table 2).

Effects of TTX and lignocaine on action potential durations

Both TTX and lignocaine reduced action potential duration in a dose-dependent manner in both normal Purkinje fibres and Purkinje fibres surviving infarction. In order to compare the effects of reducing the sodium 'window' current and thus the

Table 3 Effects of tetrodotoxin (TTX) and lignocaine on action potential durations in normal Purkinje fibres and Purkinje fibres surviving infarction

Cycle length (ms)	*Drug*	*Action potential duration* (ms) *Normal Purkinje fibres* ($n = 7$)	*Purkinje fibres surviving infarction* ($n = 7$)
(A) APD_{50}			
1000	Control	201 ± 32	199 ± 23
	TTX	164 ± 33**	193 ± 27
	Lignocaine	148 ± 22**	164 ± 18**
400	Control	148 ± 17	153 ± 15
	TTX	127 ± 20**	151 ± 16
	Lignocaine	116 ± 17**	135 ± 18**
(B) APD_{95}			
1000	Control	296 ± 35	339 ± 39
	TTX	254 ± 39**	324 ± 47**
	Lignocaine	236 ± 32**	291 ± 33**
400	Control	226 ± 15	277 ± 29
	TTX	212 ± 26**	269 ± 29*
	Lignocaine	199 ± 26**	256 ± 24**

Values represent mean ± s.d. n = number of experiments. Analysis of variance followed by Tukey test was used to determine statistical significance between control and data after TTX or lignocaine. *$P < 0.05$, **$P < 0.01$ when compared to the corresponding control.

action potential duration in these fibres, concentrations of TTX (5×10^{-7} M) and lignocaine (1.8×10^{-5} M) that had been shown to reduce this current were used (Coraboeuf *et al.*, 1979; Colatsky, 1982). Since the effects of TTX and lignocaine were completely eliminated after a washout period, it is possible to examine both drug effects in the same fibre and to increase the sensitivity of the comparison. The order of exposure to TTX and lignocaine was reversed in different experiments.

The effects of TTX (5×10^{-7} M) and lignocaine (1.8×10^{-5} M) on the contour of the action potential in a normal Purkinje fibre are illustrated in Figure 1. The changes in APD_{50} and APD_{95} are summarized in Table 3. The action potential duration was shortened by lignocaine and TTX, as indicated by a significant decrease in APD_{50} and APD_{95} (Table 3). The reduction of APD_{50} and APD_{95} in the presence of TTX and lignocaine, although attenuated at the shorter cycle length of 400 ms (rate effect, $P < 0.001$), was still statistically significant.

The effects of TTX (5×10^{-7} M) and lignocaine (1.8×10^{-5} M) on the contour of the action potential in a Purkinje fibre surviving infarction are shown in Figure 2. TTX had a minimal effect on action potential durations at both cycle lengths of 1000 and 400 ms. The reduction of APD_{95}, though small, was still statistically significant (Table 3). The reduction of action potential durations by lignocaine in Purkinje fibres surviving infarction was less than that observed in normal Purkinje fibres but it was also statistically significant (Table 3).

Stability of recordings from Purkinje fibres surviving infarction

In order to assess the time effect on the action potential parameters in Purkinje fibres surviving infarction, the results obtained during the control period (after at least 60 min of stabilisation) were compared to values at the end of the experiment, after a 40–60 min washout period with normal Tyrode solution. Only activation potential was significantly different (-83.5 ± 4.0 to -84.5 ± 4.1 mV, $P < 0.05$, $n = 21$). All other action potential characteristics returned to within 3% of the measurements taken before exposure to the drugs.

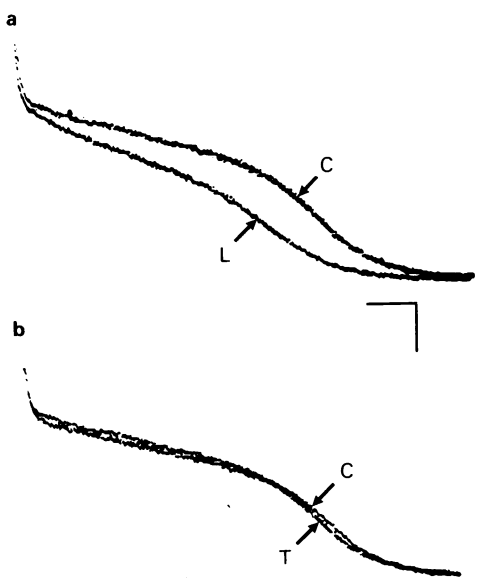

Figure 2 Effects of lignocaine (a) and tetrodotoxin (b) on the contour of the action potential of a Purkinje fibre surviving infarction. The records shown are obtained from the same fibre stimulated at a basic cycle length of 1000 ms. C represents control; T represents the presence of tetrodotoxin (5×10^{-7} M) and L represents the presence of lignocaine (1.8×10^{-5} M). Calibration: horizontal line = 50 ms, vertical line = 25 mV.

Discussion

In the present study, a pharmacological approach was used to examine the possible alterations of the sodium currents in subendocardial Purkinje fibres that survived myocardial ischaemia. Electrical activities from ventricular muscle fibres were rarely observed in the infarcted region similar to that observed by Friedman *et al.* (1973). The lack of strong muscle contraction enabled us to maintain the stable recordings required for this study from surviving subendocardial Purkinje fibres. However, the difficulty in maintaining stable recordings due to active contractions limited the use of a similar preparation obtained from the normal heart for comparison. Hence, recordings from normal free running Purkinje fibres were chosen for comparison. In addition, most of our knowledge on cardiac action potential characteristics was obtained from these free running Purkinje fibres.

In cardiac Purkinje fibres, the ionic current crossing the cell membrane at the time of $\dot{V}_{max}$ is mainly due to the fast sodium current and therefore $\dot{V}_{max}$ has been used as an indirect indicator of the fast sodium current (Weidmann, 1955). However, there is considerable controversy as to whether $\dot{V}_{max}$ is linearly related to peak sodium conductance measured in voltage clamp studies (Hondeghem, 1978; Cohen *et al.*, 1984; Fozzard *et al.*, 1985). In spite of this limitation, changes in $\dot{V}_{max}$ by pharmacological agents

observed in the present study would still reflect relative changes in the fast sodium current. The changes in $\dot{V}_{max}$ in normal Purkinje fibres and Purkinje fibres surviving infarction would still provide us with a valid comparison of the fast sodium current, although quantitative assessment of the fast sodium current may be difficult to achieve.

It is clear from the present study that lignocaine was more effective in reducing $\dot{V}_{max}$ in Purkinje fibres surviving infarction than in normal Purkinje fibres. Reduction of $\dot{V}_{max}$ by lignocaine has been described in infarcted preparations at a slow rate of stimulation (Allen *et al.*, 1978; Wang *et al.*, 1979). In the present study, we observed that the effect of lignocaine was more prominent at a faster stimulation rate than at a slower rate. This is compatible with previous studies showing that the effect of lignocaine on $\dot{V}_{max}$ in normal tissue was rate-dependent and lignocaine bound predominantly to the inactivated sodium channels (Chen *et al.*, 1975; Hondeghem & Katzung, 1977). The enhanced depressant effect of lignocaine in Purkinje fibres surviving infarction may be due to a slowing of the recovery kinetics of the sodium channels. Since the recovery kinetics are voltage-dependent, they can be affected by the significant membrane depolarisation observed in Purkinje fibres surviving infarction. This would result in more sodium channels remaining in the inactive state with a higher affinity for lignocaine. An enhanced depressant effect of lignocaine on conduction *in vivo* in the ischaemic myocardium has been demonstrated (Kupersmith *et al.*, 1975; El-Sherif *et al.*, 1977; Man & Dresel, 1979). However, the quantitative changes in the recovery kinetics of the sodium channels in Purkinje fibres surviving infarction remain to be determined.

The effects of TTX on $\dot{V}_{max}$, unlike lignocaine, were similar in normal Purkinje fibres and Purkinje fibres surviving infarction. TTX has been shown to slow the recovery kinetics of the sodium channels and affect the channel gating in a manner different from local anaesthetics (Cohen *et al.*, 1982). Due to the long time constant of recovery for the sodium channels in the presence of TTX, a significant rate-dependent effect of TTX might not be observed at the stimulation rates used in the present study.

A low concentration of TTX ($3.3–6.6 \times 10^{-7}$ M) shortened the action potential duration without any significant effect on $\dot{V}_{max}$ (Coraboeuf *et al.*, 1979). Our results also showed that TTX (5×10^{-7} M) shortened action potential duration and had no significant effect on $\dot{V}_{max}$ in both normal Purkinje fibres and Purkinje fibres surviving infarction. However, the reduction of action potential duration by TTX at this concentration in Purkinje fibres surviving infarction was less than that observed in normal Purkinje fibres. These results suggest that the longer duration seen in Purkinje fibres surviving infarction is not due to an increase in the TTX-sensitive sodium 'window' current. At the concentration used in our study (1.8×10^{-5} M), lignocaine has been shown to shorten action potential duration in Purkinje fibres also by an effect on the TTX-sensitive sodium 'window' current (Colatsky, 1982). The effect of lignocaine on action potential duration in the present study is more complex since the concentration of lignocaine used was accompanied by a reduction in $\dot{V}_{max}$. Although the role of the fast sodium current on action potential duration is not known, we cannot assume lignocaine shortened action potential duration only through its effect on the TTX-sensitive window current. In addition, lignocaine may reduce action potential duration by increasing potassium conductance (Weld & Bigger, 1976), although this effect is not supported by the recent study of Colatsky (1982).

Changes in intracellular ion activities in Purkinje fibres surviving infarction may be responsible for some of the observed electrophysiological abnormalities. However, it has been shown that only a portion of the alteration in the diastolic potential can be explained by a reduction of the intracellular potassium activity and the potassium equilibrium potential during myocardial ischaemia (Dresdner *et al.*, 1987). Furthermore, cells in the infarcted zone of cat hearts showed normal steady-state potassium activity and Na^+-K^+ ATPase activity (Kimura *et al.*, 1986).

Two major possibilities remain to be investigated that can explain the longer action potential duration seen in Purkinje fibres surviving infarction. A reduction of potassium conductance can induce diastolic depolarisation (Hiraoka *et al.*, 1980; Malecot *et al.*, 1984), spontaneous activity and a lengthening of the action potential duration (Mugelli *et al.*, 1983). This alteration could be responsible for some of the electrophysiological derangements seen in Purkinje fibres surviving infarction. Another possible mechanism for prolongation of the action potential duration could be an increase in sodium-calcium exchange (Noble, 1986). Indeed it has been shown that the inhibition of sodium-calcium exchange decreased the action potential duration (Mitchell *et al.*, 1984). However, the exchange current is thought to be less important in Purkinje fibres than in ventricular muscle fibres (Mitchell *et al.*, 1984).

In conclusion, our results showed that the fast sodium current in Purkinje fibres surviving infarction was more sensitive to antiarrhythmic agents than normal Purkinje fibres. This suggested a slowing of the recovery kinetics of the fast sodium current. The prolonged action potential duration in Purkinje fibres surviving infarction was not due to an increase in the TTX-sensitive sodium 'window'

current. These results are compatible with an enhanced effect of antiarrhythmic drugs on $\dot{V}_{max}$ and conduction in the ischaemic heart, especially at increased heart rates.

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Effects of experimental diabetes on the responsiveness of rat aorta

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1 Vascular responsiveness was examined in aortic ring preparations, with or without endothelium, from rats with experimental diabetes induced by streptozotocin and from vehicle-treated (control) rats.

2 There were no significant differences between diabetic tissues and control tissues in the responsiveness to the vasoconstrictors noradrenaline, 5-hydroxytryptamine and KCl, and to the vasodilators sodium nitroprusside, isoprenaline and acetylcholine.

3 When maximum contractions to vasoconstrictors was expressed relative to tissue weight, maximum contractions were significantly greater in diabetic tissues.

4 When expressed in terms of the KCl contraction, there were no significant differences between diabetic and control tissues in the maximum contraction to vasoconstrictors.

5 These results demonstrate that diabetic-induced changes in vascular responsiveness, if any, do not occur at the receptor level.

Introduction

The rat has been widely studied as a model for the effects of experimental diabetes on vascular responsiveness, often with conflicting results. Recently, Head *et al.* (1987) reported that the maximum contractile response to noradrenaline (NA) is significantly reduced in aortae with intact endothelium from diabetic rats, and suggested that results such as those of Scarborough & Carrier (1983), who reported an increased maximum contractile response to NA in diabetic aorta, may have been due to the lack of an endothelium. Since Head *et al.* (1987) did not examine aortae without endothelium, the object of the present study was to examine whether the endothelium does influence the effects of diabetes on the responsiveness of the rat aorta.

Methods

General

Male Sprague Dawley rats (150–200 g, 6–8 weeks) were fasted for 24 h, then injected intraperitoneally with streptozotocin (65 mg kg^{-1}) in a citric acid/sodium phosphate buffer (pH 4) or with the vehicle alone. Glucose in urine was measured 3 days after injection and on the day of the experiment by use of Ames Multistix reagent strips. All rats injected with streptozotocin developed severe diabetes as indicated by urine levels of glucose of 55 mmol l^{-1} or above. Rats injected with vehicle gave negative glucose tests indicating levels of less than 5.5 mmol l^{-1} in urine. No evidence was found for ketones in the urine of either group of animals. Of 17 rats injected with streptozotocin, 2 died during the period before the experiment.

Aortic rings

Rings of approximately 3–5 mm in length were prepared from a section of the thoracic aorta between the aortic arch and the diaphragm following stunning and exsanguination, 14–21 days after injection of streptozotocin or vehicle. Three types of ring preparation were obtained: the first in which rings were cleared of connective tissue and no attempt was made to preserve the endothelium (group 1 aorta: without endothelium), the second in which the endothelium was preserved (group II aorta: with endothelium) and the third in which the endothelium was destroyed by vigorous rubbing with a glass rod (group III aorta: without endothelium). Rings were placed in organ baths and attached between a fixed rod and an isometric tension transducer under 1 g tension. Tissues were bathed in Krebs-Henseleit solution of the following composition (mM): NaCl 119,

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Table 1 Potency of noradrenaline (NA) and 5-hydroxytryptamine (5-HT) in rat aorta with (group II) or without (groups I and III) a functional endothelium

	NA EC_{50} (nM)	*5-HT* EC_{50} (nM)
Group I		
Diabetic	26.3	346
	(9.1–75.8)	(115–1050)
	$n = 6$	$n = 7$
Vehicle (control)	23.4	251
	(9.3–58.9)	(138–457)
	$n = 5$	$n = 7$
Group II		
Diabetic	49.0	
	(24.0–100)	
	$n = 5$	
Vehicle (control)	50.1	
	(22.9–110)	
	$n = 5$	
Group III		
Diabetic	38.0	
	(11.2–129)	
	$n = 5$	
Vehicle (control)	40.7	
	(5.4–309)	
	$n = 5$	

EC_{50} values (mean and 95% confidence limits, nM) are shown together with n, the number of experiments.

$NaHCO_3$ 25, (+)-glucose 11.1, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.0. Cocaine (3 μM) and corticosterone (40 μM) were present to block neuronal and extraneuronal monoamine uptake and additionally propranolol (3 μM) was present to block β-adrenoceptor-mediated actions in experiments in which noradrenaline was used and prazosin (0.1 μM) was present to block α-adrenoceptor-mediated actions in experiments in which isoprenaline was used.

In another series of experiments, segments of aorta were accurately measured and cut to 3 mm lengths and the water content of the tissue was obtained by subtracting dry weight from wet weight.

Agonist-induced contractions

Tissues were exposed to KCl 40 mM for 5 min to test the viability of the preparation. Tissues were then washed, and thereafter bathing fluid was changed every 15 min except during cumulative agonist administration. After 30 min, agonists were added cumulatively in 0.5 log unit increments beginning with 1 nM (noradrenaline) or 0.1 μM (5-hydroxytryptamine: 5-HT). Agonist potency was expressed as an EC_{50} (concentration producing 50% of maximum contraction). In some experiments, acetylcholine (ACh) (1–100 μM) was added once a maximum contractile response to NA had been obtained.

Agonist-induced relaxations

Tissues were exposed to KCl 40 mM for 5 min and washed. After 30 min, additional KCl 20 mM (isoprenaline) or 40 mM (NA or ACh) was administered and, when the contraction had reached a maximum, the agonist was added cumulatively in 0.5 log unit increments beginning with 1 nM (isoprenaline and sodium nitroprusside) or in 1 log unit increments beginning with 1 μM (ACh). In control experiments (one tissue from each animal), equivalent volumes of distilled water vehicle were administered. Where necessary, responses were corrected for changes occurring in vehicle experiments.

Drugs

The following were used: acetylcholine chloride (Sigma, Poole, UK); cocaine hydrochloride (Sigma); corticosterone (Sigma); 5-hydroxytryptamine hydrochloride (Sigma); (−)-isoprenaline hydrochloride (Sigma), (−)-noradrenaline bitartrate (Sigma), prazosin hydrochloride (gift: Pfizer, Sandwich, U.K.); sodium nitroprusside (Sigma), streptozotocin (Sigma).

Drugs were dissolved in distilled water except for corticosterone (100% ethanol) and streptozotocin (citric acid/sodium phosphate buffer pH 4), and dilutions were made up in distilled water. All stock solutions were prepared just prior to use and sodium nitroprusside solutions were additionally stored in the dark.

Statistics

Values are expressed either as arithmetic means (with s.e.mean) or as geometric means (with 95% confidence limits). Potency of agonists was expressed as an EC_{50} value (concentration producing 50% of maximum response). Differences between groups were assessed by analysis of variance (Anovar) and Student's t test, where appropriate.

Results

Fourteen to twenty one days after injection, diabetic rats had body weights significantly less than those of vehicle rats with values of 222 ± 14 g ($n = 5$) and 309 ± 40 g ($n = 5$), respectively ($P < 0.01$). Tissue wet weight was significantly less in aorta from diabetic than from vehicle animals with weights of

Table 2 Maximum contraction obtained to noradrenaline (NA) and 5-hydroxytryptamine (5-HT) and contraction to KCl (40 mM) in rat aorta with (group II) or without (groups I and III) a functional endothelium

	NA max T (g)	*5-HT* max T (g)	*KCl* (40 mM) (g)
Group I			
Diabetic	1.01 ± 0.15 (7)	0.87 ± 0.21 (7)	0.36 ± 0.08 (6)
Vehicle (control)	0.89 ± 0.20 (5)	0.92 ± 0.18 (7)	0.32 ± 0.07 (6)
Group II			
Diabetic	1.17 ± 0.22 (5)		0.48 ± 0.10 (8)
Vehicle (control)	0.73 ± 0.18 (5)		0.41 ± 0.08 (8)
Group III			
Diabetic	0.58 ± 0.11 (5)		
Vehicle (control)	0.45 ± 0.06 (5)		

Maximum contraction (mean ± s.e., absolute tension in g) is shown together with *n*, the number of experiments, in parentheses.

4.14 ± 0.41 mg ($n = 10$) and 7.93 ± 0.62 mg ($n = 10$), respectively ($P < 0.01$).

Agonist-induced contractions

Tables 1 and 2 show the potencies of NA and 5-HT and maximum responses (absolute tension in g) obtained to NA and 5-HT, as well as the contraction obtained to KCl (40 mM) in aortic rings from diabetic and control rats. Aortae from groups I and III did not relax to ACh (100 μM), whereas aortae from group II (with endothelium) relaxed to ACh. Hence only aortae from group II (with preserved endothelium) demonstrated endothelium-dependent relaxations. There were no significant differences between diabetic and control tissues for any variable. However, when maximum contractions obtained were expressed per mg tissue wet weight, maximum tension response to NA was significantly greater in tissues from diabetic animals (e.g. group II aorta: 308 ± 38 mg and 132 ± 27 mg per mg tissue wet weight in diabetic and vehicle tissues, respectively, $n = 5$ each, $P < 0.01$). Similarly, the maximum contractions to 5-HT and KCl were significantly larger in tissues from diabetic animals, when expressed per mg tissues wet weight (data not shown, cf. Table 2). However, when maximum tension to NA or 5-HT was expressed in terms of the contraction to KCl 40 mM, there were no significant differences between diabetic and control (data not shown, cf. Table 2).

Agonist-induced relaxations

In experiments examining relaxations to agonists in tissues precontracted with KCl 40 mM, there were no significant differences between aortae from control or diabetic animals in the potency of maximum relaxation to sodium nitroprusside or in the maximum relaxation to acetylcholine (Table 3). In tissues contracted with noradrenaline (100 μM) there were no differences in the maximum relaxation to acetylcholine. In tissues precontracted with KCl 20 mM, isoprenaline produced relaxations in most tissues, but there were no significant differences between control and diabetic aortae in the maximum relaxation to isoprenaline (Table 3). Due to the relatively small magnitude of the relaxations to isoprenaline in some tissues, isoprenaline potency was not calculated.

Water content of tissues

In another series of experiments, segments of aorta 3 mm in length, were obtained from control and diabetic animals. The water content was found to be 59.2 ± 2.4% ($n = 5$) and 53.2 ± 8.4% ($n = 4$) in vehicle and diabetic tissues, respectively (no significant difference).

Discussion

Although the effects of diabetes on the vascular responsiveness of the rat aorta have been widely studied, with conflicting results, there are several variables which should perhaps be considered when comparing results: strain of rat employed, drug employed to induce diabetes, duration of diabetes, age of the animal, presence or absence of endothelium, method used to calculate maximum response.

Table 3 Maximum relaxations to sodium nitroprusside (SNP) and isoprenaline (Isop) in group I aorta (without endothelium) and maximum relaxation to acetylcholine (ACh) in group II aorta (with functional endothelium)

		SNP max relax (%)	*SNP* EC_{50} (nM)	*Isop* max relax (%)	*ACh* max relax (%)
Group I					
Diabetic	KCl 40 mM	121.8 ± 20.0 ($n = 5$)	5.89 (1.95–17.8) ($n = 5$)		
	KCl 20 mM			50.6 ± 8.3 ($n = 6$)	
Vehicle (control)	KCl 40 mM	107.5 ± 18.8 ($n = 5$)	7.76 (3.2–19.0) ($n = 5$)		
	KCl 20 mM			30.9 ± 7.0 ($n = 7$)	
Group II					
Diabetic	KCl 40 mM				41.8 ± 6.4 ($n = 8$)
	NA 100 μM				15.7 ± 4.9 ($n = 4$)
Vehicle	KCl 40 mM				38.1 ± 5.3 ($n = 8$)
	NA 100 μM				17.3 ± 1.7 ($n = 4$)

Relaxations are expressed as a percentage of the contraction to the contractile agent (KCl or NA). Values are mean ± s.e., with n, the number of experiments, in parentheses. Also shown is the potency (EC_{50}) of sodium nitroprusside and 95% confidence limits (nM).

Since streptozotocin has been employed in most studies and since most authors employed Sprague-Dawley or Wistar rats with no obvious differences, it is intended to discuss the remaining points in relation to the present results.

The present results demonstrate that diabetes of 14–21 days duration fails to affect the potency or maximum contraction (absolute tension in g) to NA, 5-HT or KCl, or the potency and maximum relaxation to SNP, isoprenaline or ACh. However maximum contractions to NA, 5-HT and KCl were significantly larger in diabetic tissues when expressed per mg wet weight of tissue. Maximum contractions to NA and 5-HT were unaltered in diabetic tissues when expressed relative to the maximum contraction to KCl. Hence the calculation used to express maximum response can affect interpretation of the data.

There is no particular reason to assume that expressing contractions per mg tissue weight confers more validity on the results, simply because aortae from diabetic rats are found to be markedly lighter than aortae from control animals. Admittedly, the difference in aortic weight is not due to differences in water content of the tissues since water content is reported to be unchanged (present results; Turlapaty *et al.*, 1980) or slightly increased (Fiol de Cuneo *et al.*, 1988) in blood vessels from diabetic rats, suggesting that the loss of tissue weight does include loss of muscle mass. An analogous problem occurs in ageing studies where aortic weights (and indeed body weights) are significantly less in immature (1–2 month) as compared to aged (18–24 month) rats, yet the maximum response to contractile agents in absolute terms can be unchanged (O'Donnell & Wanstall, 1986) or increased (Hyland *et al.*, 1987; Emmick & Cohen, 1986) by increasing age. Returning to diabetic studies, there are reports of no change (Fortes *et al.*, 1983 [with endothelium]; present results), an increase (Ramanadham *et al.*, 1984; Fortes *et al.*, 1983 [without endothelium]) or a decrease (Turlapaty *et al.*, 1980; Pfaffman *et al.*, 1982; Head *et al.*, 1987) in the maximum response in absolute terms to NA or phenylephrine in aortae from diabetic rats, but of those observing a decrease, only Head *et al.* (1987) reported a decreased maximum contraction when responses were expressed per mg tissue. In the present study, we can at least say that diabetes did not decrease the maximum contractile response to the agents examined and that the response to each agonist relative to KCl was unchanged by diabetes.

In the present study animal tissues were examined 14–21 days after inducing diabetes. While some

studies examined tissues 8 weeks after induction of diabetes, there is no evidence that this longer duration of diabetes produces additional effects. For instance, Pfaffman *et al.* (1982) examined durations of diabetes for 2–12 weeks but found no evidence for increased effects with a duration longer than 2 weeks, and Owen & Carrier (1980) report effects of diabetes of duration 14–20 days. However, a duration of diabetes of 7 days failed to produce changes in rat aorta (McLeod & McNeil, 1985).

The role of the vascular endothelium in diabetes-induced changes in vascular responses has been examined in some studies. Head *et al.* (1988) and Fortes *et al.* (1983) found that the endothelium-dependent relaxation to ACh in rat aorta was unchanged by diabetes, but Oyama *et al.* (1986) reported a decreased maximum relaxation to both ACh and histamine in diabetic tissues. The present results agree with those of Head *et al.* (1987) in that we also fail to observe altered relaxation to ACh, but although Head *et al.* (1987) suggested that differences between their own results and those of Scarborough & Carrier (1983) may have been due to the presence or absence of endothelium respectively, our present data suggest that this is not so. We obtained similar results whether or not endothelium was present. However, some authors, although finding no change in the relaxation to ACh, found that the presence of endothelium influenced the contractile response in diabetic tissues, but with conflicting results. In rat aorta, Fortes *et al.* (1983) found that there was an increased potency or maximum response to NA in diabetic tissues with endothelium, but not without endothelium. In contrast, Gebremedhin *et al.* (1987), employing dog femoral artery, found an increased maximum response to phenylephrine in diabetic tissues, but only without endothelium.

It is perhaps now worth considering the effects of the diabetic state, not on the maximum response, but on the potency of NA or phenylephrine in rat aorta. Most studies report no change in potency of NA (Owen & Carrier, 1980; Pfaffman *et al.*, 1982; Ramanadhan *et al.*, 1984; Scarborough & Carrier, 1985; Head *et al.*, 1987; present results), but an increased potency of NA has been reported in 100 and 360 days, but not in 7 day or 180 day, diabetic tissues (McLeod & McNeil, 1985) and an increased potency of NA in alloxan diabetic tissues at 10 days but not 6 months (without endothelium (Fortes *et al.*, 1983)). Hence, despite the controversy concerning the effects of diabetes on the maximal response to the α-adrenoceptor agonist, most studies agree that agonist potency is not altered by diabetes.

There is one major difference between the present study and the majority of previous studies of the effect of diabetes on the responsiveness of the rat aorta: the use of adjuvant drugs for optimal characterization of individual receptor types. The use of corticosterone to block extraneuronal uptake of catecholamines, propranolol to block β-adrenoceptor actions of NA, and prazosin to block α-adrenoceptor actions of isoprenaline, and perhaps the use of cocaine (although it is thought there are few, if any, sympathetic nerves in rat aorta), should optimise each receptor-mediated response. For instance, in the absence of β-adrenoceptor blockade, both NA and phenylephrine may have significant effects at β-adrenoceptors which may prevent attainment of the maximum α_1-adrenoceptor-mediated vasoconstriction. The problem is further complicated by the fact that this β-adrenoceptor-mediated relaxation is greatly attenuated, and α-adrenoceptor-mediated vasoconstriction increases, during maturation (see Hyland *et al.*, 1987; Sawyer & Docherty, 1987). Although no significant diabetes-induced alterations in β-adrenoceptor-mediated relaxation were found in the present study, failure to prevent such actions of NA is an added complication to studies of rat aorta.

In conclusion, the present results demonstrate only one diabetes-induced change in the responsiveness of rat aorta: an increased maximum response to vasoconstrictor agonists, but even then, only when expressed in terms of tissue wet weight. Considering that the maximum tension obtained to α-adrenoceptor agonists in rat aorta is altered during maturation and there is a lack of consistency between studies both in the magnitude of the maximum tension and in the effects of diabetes, the conclusion must be that there is no clear evidence that experimental diabetes alters the responsiveness of the rat aorta.

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Classification of tachykinin receptors in muscularis mucosae of opossum oesophagus

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1 Muscularis mucosae of the distal oesophagus of the opossum contracts in response to substance P and to a variety of tachykinins. To delineate the nature of the receptors present in this tissue, we evaluated contractile responses to substance P, neurokinin A, neurokinin B, eledoisin and analogues believed to be highly selective for NK-1, NK-2 and NK-3 receptors. In addition, the effects of prolonged exposure to each of these agents (10^{-6} M or 10^{-5} M) on contractile responses to substance P and to itself were evaluated. Similarly effects of prolonged exposure to the various tachykinins and their analogues on the field-stimulated responses of this muscle were studied.

2 All naturally occurring tachykinins were full agonists and differed in potency (comparing ED_{50} values) by less than ten fold. In nearly all cases there was cross tachyphylaxis between substance P and the other tachykinins and each reduced tonic responses to field stimulation, a response previously shown to be mediated by a substance P like agent. Eledoisin failed to cause tachyphylaxis under the conditions of these experiments.

3 When highly selective tachykinin analogues were used, only that believed to activate NK-1 receptors was a full agonist. [β-Ala^4,Sar^9,Met$O_2{}^{11}$]$SP_{(4-11)}$ was also only slightly less potent than substance P. In contrast, an agonist selective for NK-2 (NK-A) receptors, [Nle^{10}]$NKA_{(4-10)}$, and one selective for NK-3 (NK-B) receptors, [β-Asp^4,MePhe^7]$NKB_{(4-10)}$ were unable to produce a response equal to 50% of the maximum even at 10^{-5} M. However, all three selective tachykinin analogues reduced responses to substance P but not to carbachol. They usually reduced both phasic and tonic responses to field stimulation.

4 We conclude, based on this and earlier study, that the tachykinin receptors of opossum oesophagus muscularis mucosae recognize all naturally occurring tachykinins but may represent only NK-1 receptors. The ability of analogues selective for other types of tachykinin receptors to reduce responses to substance P raises the possibility that their selectivity depends in part on diminished efficacy rather than totally on diminished affinity at some classes of receptor.

Introduction

The muscularis mucosae of opossum oesophagus has numerous nerves containing substance P-immunoreactive material (Domoto *et al.*, 1983; Daniel *et al.*, 1987) and responds to field stimulation of its nerves by a tonic (following phasic) contraction which appears to be mediated by substance P (SP) or a related tachykinin (Domoto *et al.*, 1983; Robotham *et al.*, 1985). The tachykinin or tachykinins present in this tissue or released on nerve stimulation have not been identified but resemble substance P (Daniel *et al.*, 1987). An initial attempt to identify the tachykinin receptor present gave results interpreted as showing a non-selective receptor (Daniel *et al.*, 1986). This conclusion was reached because eledoisin, physalaemin and kassinin, all full agonists, were similar in potency to SP. Eledoisin and physalaemin were originally used to differentiate SP-E and SP-P receptors (Lee *et al.*, 1982).

This classification of receptors is no longer accepted (Henry, 1987). Recently, three classes of tachykinin receptors have been recognized, corresponding to three naturally occurring members of the tachykinin family: a receptor for substance P (SP), for neurokinin A (NKA), and for neurokinin B (NKB) (Henry, 1987). It is recognized that these naturally occurring tachykinins are not highly selective for their own receptors (Iversen *et al.*, 1987). Furthermore eledoisin, physalaemin and kassinin all

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interact with all three receptors with varying but disputed potencies (for example, see Iversen et al., 1987). Thus identification of receptor type with the natural agonists may not be straight-forward. However, recently synthetic tachykinins have been synthesized which are more selective for each of these receptor classes (Drapeau et al., 1987a,b) based upon studies in tissues thought to contain only or predominantly one class of receptor.

The objective of this study was to utilize the naturally occurring tachykinins (SP, NKA, NKB and eledoisin) as well as the synthetic, more selective tachykinins [β-Ala^4, Sar^9, MetO$_2^{11}$]SP$_{(4-11)}$ for SP, [Nle^{10}]NKA$_{(4-10)}$ for NKA, and [β-Asp^4,MePhe^7]-NKB$_{(4-10)}$ for NKB receptors, to characterize tachykinin receptor types present in opossum oesophagus muscularis mucosae.

Methods

Opossums were killed by an overdose (65 mg kg^{-1}) of pentobarbitone, the oesophagus was rapidly removed and the mucosa including the muscularis mucosae, separated. The mucosal folds were trimmed off and the thickened distal region of the mucosa used to prepare eight 2–3 mm wide longitudinal strips. These consisted mostly of muscularis (see Domoto et al., 1983). These strips were mounted under 1 g tension in 3 ml muscle baths containing Krebs Ringer solution (composition in mM: NaCl 115.5, KCl 4.16, $CaCl_2$ 2.5, $MgSO_4$ 1.16, $NaHCO_3$ 21.9, NaH_2PO_3 1.16, glucose 11.1). The bath solution was bubbled with 95% O_2 : 5% CO_2 and maintained at a temperature of 37°C. The tissues were mounted on glass rods to which concentric Pt electrodes were attached. Tissues were tied by a silk thread to a hook at one end of the glass rod and pulled through the concentric electrodes and attached at the other end by a silk thread to a Grass FT .03 strain gauge transducer. Tension records were made on an eight channel Beckman R 611 oscillograph.

After 1 h for recovery from dissection, the viability of tissues was assessed by field stimulation at 5 and at 40 Hz (0.5 ms square pulses at 40 V cm^{-1}). Only tissues which showed typical phasic and tonic contractions were used (Domoto et al., 1983). Then 10^{-6} M carbachol was administered to determine the maximum response. This was washed out and cumulative tachykinin concentration-response curves obtained (concentration range 10^{-10} to 10^{-6} or 10^{-5} M). Usually 2 or 4 strips were exposed to each peptide.

The protocol for obtaining concentration-response curves usually involved 10 fold increases in concentration with each step, adding the next highest concentration as soon as the response to the preceding one had reached a plateau. This protocol avoided tachyphylaxis (demonstrated by repeating the exposure to concentrations of peptide producing about half maximal and maximal responses 60 min after washout of peptide), reduced the time for completion of experiments (since both contraction and relaxation were slow and since the range of effective concentrations is wide) and provided reproducible results. In some experiments, attempts were made to produce selective tachyphylaxis to various peptides. In one protocol using naturally occurring tachykinins to generate the concentration-response curves, the maximum concentration of each peptide was left in the bath until the response had faded. Then the bath was rapidly washed, responses to field stimulation were tested and another concentration-response curve was generated. The same peptide or another one was used in some of the muscle strips in each experiment. This procedure did not affect responses to the subsequent addition of carbachol. Another protocol involved desensitization to 10^{-6} M SP by the selective agonists or SP itself. Tissues were first exposed to the selective agonist (10^{-6} M), SP (10^{-6} M) or the equivalent solution without tachykinin. After the maximum response was achieved, the bath was rinsed rapidly with Ringer solution and SP added. After the maximum response was achieved the bath was again rapidly rinsed and carbachol 10^{-6} M added.

Contractile responses were expressed as % of the maximum response to carbachol. Concentration-response curves were plotted as logarithm of concentration versus response. The ED_{50} values for each strip were determined for each peptide. Means of ED_{50} values and their standard errors were calculated after transformation of the value to a logarithmic scale. In addition the maximum response (usually occurring at 10^{-6} M) was determined for each peptide as well. Mean values for various tachykinins were compared using unpaired comparisons and two tailed t tests of their significance; P values less than 0.05 were considered significant.

Drugs used were: substance P, neurokinin A, neurokinin B, eledoisin and carbachol from Sigma and the synthetic analogues from Dr D. Regoli.

Results

In the first series of experiments, potencies and maximum responses were compared when different peptides were used. Table 1 summarizes the findings. Substance P was slightly but consistently more potent than NKA and NKB, and roughly equipotent with eledoisin. All of these peptides were full agon-

Table 1 ED_{50} values and % maximum responses for tachykinins in opossum muscularis mucosae

	n	ED_{50} (M)	Max
SP vs NKA			
Exp. 1			
SP	6	$4.0 \pm 0.2 \times 10^{-9}$	95.7 ± 3.3
NKA	6	$2.1 \pm 0.1 \times 10^{-8}$*	100.8 ± 3.8
Exp. 2	6	$1.5 \pm 0.2 \times 10^{-9}$	92.2 ± 3.1
	6	$1.2 \pm 0.1 \times 10^{-8}$*	99.3 ± 4.4
Exp. 3	6	$4.7 \pm 1.3 \times 10^{-9}$	99.8 ± 4.7
	6	$3.0 \pm 1.3 \times 10^{-8}$*	99.5 ± 5.3
Exp. 4	6	$5.1 \pm 1.5 \times 10^{-9}$	94.5 ± 8.2
	6	$1.1 \pm 0.2 \times 10^{-8}$*	105.7 ± 3.4
Exp. 5	4	$6.8 \pm 14.8 \times 10^{-9}$	89.8 ± 2.5†
	4	$1.2 \pm 1.4 \times 10^{-8}$	89.3 ± 4.9
Exp. 6	4	$10 \pm 15 \times 10^{-9}$	88.0 ± 2.6†
	4	$3.1 \pm 1.2 \times 10^{-8}$	92.0 ± 6.2
Overall mean			
SP	32	3.8×10^{-9}	93.9
NKA	32	1.73×10^{-8}	98.3
SP vs Eledoisin			
Exp. 1			
SP	6	$9.4 \pm 1.5 \times 10^{-9}$	94.0 ± 7.8
El	6	$1.1 \pm 1.5 \times 10^{-8}$	98.7 ± 4.3
Exp. 2	4	$4.5 \pm 1.5 \times 10^{-9}$	93.5 ± 5.3
	6	$4.7 \pm 1.1 \times 10^{-9}$	110.0 ± 3.2
Overall mean			
SP	10	6.7×10^{-9}	93.9
El	12	7.2×10^{-9}	104.4
SP vs NKB			
Exp. 1			
SP	4	$3.0 \pm 1.3 \times 10^{-8}$	102.5 ± 5.6
NKB	4	$1.5 \pm 2.1 \times 10^{-7}$*	99.8 ± 6.9
Exp. 2			
SP	4	$3.5 \pm 1.6 \times 10^{-9}$	112.8 ± 3.7
NKB	4	$4.0 \pm 1.4 \times 10^{-8}$*	103.3 ± 6.4
Overall mean			
SP	8	1.0×10^{-8}	107.7
NKB	8	6.0×10^{-8}	101.6

*Significantly different ($P < 0.05$) from corresponding value for SP.
†Significantly different from 100%.
SP, substance P; NKA, neurokinin A; NKB, neurokinin B.

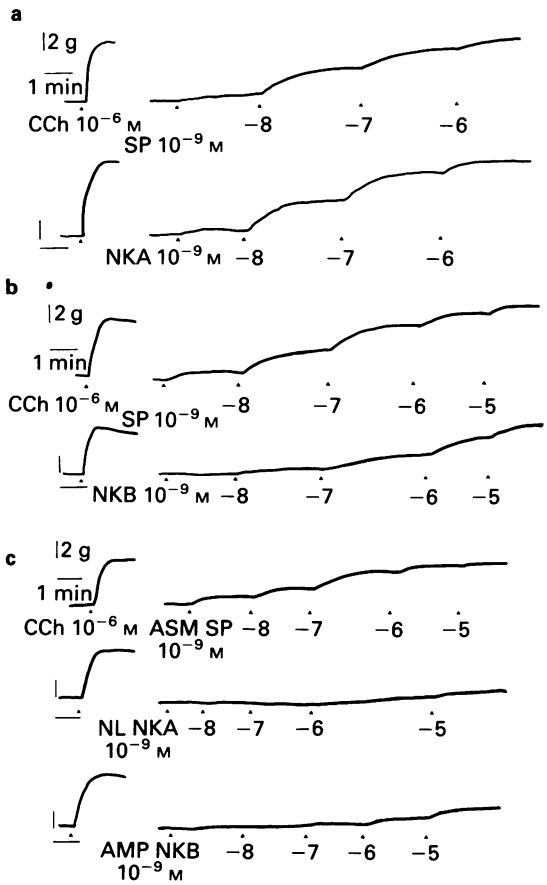

Figure 1 Typical responses to several agonists used in this study. In each case, an initial response to 10^{-6} M carbachol (CCh) is shown to define the maximum contraction of the strip and later a cumulative concentration-effect curve is shown. Tension is in g and time in minutes. (a) Compares cumulative responses to substance P (SP) and neurokinin A (NKA), 10^{-9} to 10^{-6} M. (b) Compares cumulative responses to SP and NKB, 10^{-9} to 10^{-5} M. (c) Compares cumulative responses to [β-Ala^4, Sai^9, Met(O$_2$)^{11}]-SP$_{(4-11)}$ (ASMSP$_{4-10}$), (NLNKA) and (AMPNKB) - [β-As^4, MePhe^7]-NKB$_{(4-10)}$ 10^{-9} to 10^{-5} M.

ists. There was thus no clear basis for distinguishing the class or classes of receptors present from use of the natural agonists. From these data one might conclude that there was a mixture of receptors selective for NKA and substance P or a non-selective receptor.

Therefore, more selective agonists were tested. Table 2 compares these results. The results of this study were clear cut. Only the agonist selective for NK-1 receptors (ASM-SP$_{4-10}$) was a full agonist. Its potency was slightly less than that of substance P. However, agonists selective for NK-2 or NK-3 receptors seemed to be partial agonists; i.e., at 10^{-5} M they produced less than 50% of the maximum response. Figure 1 illustrates these findings.

In several experiments, after completion of the concentration-response curve the maximum concentration (10^{-5} M) of each peptide was left in the bath for 2 to 2.5 h while the contractions gradually faded.

Table 2 A Actions of selective tachykinin peptides on opossum muscularis mucosae

Peptide	n	ED_{50} (M)	*Max response*
SP	6	$8.3 \pm 3.3 \times 10^{-9}$	92.5 ± 6.0
[β-Ala^4,-Sar^9,MetO$_2^{11}$]SP$_{4-11}$	5	$2.5 \pm 1.7 \times 10^{-8}$	81.5 ± 5.2
[Nle^{10}]NKA$_{4-10}$	6	$> 10^{-5}$	19.8 ± 4.4†
[β-Asp^4,MePhe^7]NKB$_{4-10}$	6	$> 10^{-5}$	35.5 ± 5.0†

B Effect of exposure to 10^{-6} M tachykinin on response to 10^{-6} M substance P

Agent	n	% *CCh maximum*
None	4	78.6 ± 4.3
SP	4	0 ± 0††
[β-Ala^4,-Sar^9,MetO$_2^{11}$]SP$_{4-11}$	3	26.0 ± 0.6††
[Nle^{10}]NKA$_{4-10}$	3	42.3 ± 11.4††
[β-Asp^4,MePhe^7]NKB$_{4-10}$	3	38.3 ± 13.5††

Values are mean ± s.e.mean.
† Significantly different ($P < 0.05$) from 100%.
†† Significantly different ($P < 0.05$) from no treatment.
CCh, carbachol.

Then responses to field stimulation at 5 and 40 Hz were tested before and just after washing of the bath. Afterwards, the concentration-effect curves for various peptides were repeated. In all cases, some strips were exposed to the same peptide as was used for producing tachyphylaxis while other strips were exposed to other peptides to determine if cross-tachyphylaxis had occurred. Table 3 summarizes these results. It shows that there was cross tachyphylaxis between SP and NKA. SP produced tachyphylaxis to both SP and eledoisin, but the latter was relatively ineffective against both SP and itself. NKB produced tachyphylaxis against both itself and SP and SP also caused tachyphylaxis to NKB. Thus there was no evidence that the naturally occurring tachykinins caused selective desensitization to NK-2 or NK-3 receptors compared to NK-1 receptors.

Desensitization by more selective peptides was tested and compared to that by SP. Exposure to 10^{-6} M of each caused reduction of the responses to 10^{-6} M substance P. The data are summarized in Table 2B. Addition of SP itself abolished the responses to a subsequent exposure. None of these agonists affected the response to 10^{-6} M carbachol added immediately afterwards.

When effects of prolonged exposure to each peptide on tonic responses to 40 Hz field stimulation were compared (Table 4), it was evident that all of

Table 3 Effects of prolonged exposure to tachykinins (10^{-6} M) on responses

Test peptide	*Exposed to*	n	ED_{50}(M)	*Max*(%)
SP	NKA	4	$4.5 \pm 2.2 \times 10^{-6}$†	34.8 ± 10.9†
	SP	4	$1.2 \pm 2.5 \times 10^{-7}$†	52.3 ± 7.5†
NKA	NKA	4	$1.3 \pm 2.3 \times 10^{-6}$†	40.8 ± 12.2†
	SP	4	$1.0 \pm 9.2 \times 10^{-6}$†	50.8 ± 8.65†
SP	Eledoisin	2	8.4×10^{-7}	54††
	SP	2	5.0×10^{-7}††	61††
Eledoisin	Eledoisin	2	1.5×10^{-8}	98.5
	SP	2	5.6×10^{-9}	115
SP	NKB	4	$6.3 \pm 1.2 \times 10^{-7}$†	83.5 ± 14.3
	SP	4	$1.6 \pm 0.17 \times 10^{-7}$†	92.0 ± 6.3
NKB	NKB	4	$9.4 \pm 2.0 \times 10^{-6}$†	50.8 ± 10.5†
	SP	4	$4.7 \pm 1.5 \times 10^{-7}$†	76.8 ± 2.4†

† Significantly different from control values in Table 1.
†† Outside the range of values observed in experiments summarized in Table 1.

Table 4 Effect of prolonged exposure to tachykinins on responses to field stimulation (40 Hz)

Experiment	n	*Exposed to*	*Decrease in response* (%)
1	4	SP 10^{-6} M	28.3 ± 5.7
	4	NKA 10^{-6} M	37.5 ± 6.0
2	4	SP 10^{-5} M	50.5 ± 8.1
	4	NKA 10^{-5} M	24.0 ± 10.8
3	4	SP 10^{-6} M	15.0 ± 3.2
	4	Eledoisin 10^{-6} M	14.3 ± 3.3

them were capable of reducing the response. In the case of the highly selective peptides, none was capable of selectively inhibiting the tonic responses (compared to the phasic response) to field stimulation. In most cases, non-selective peptides inhibited both phasic and tonic contractions even though they did not reduce the response to 10^{-6} M carbachol. The mechanism of this effect has not been investigated further but may reflect a presynaptic action of these substances.

Discussion

These results confirm and extend our previous finding (Daniel *et al.*, 1986) that tachykinin receptors in muscularis mucosae of opossum oesophagus appear to be non-selective in the sense that they do not discriminate between the putative naturally occurring agonists for three postulated classes of receptors; NK-1, NK-2 and NK-3. Thus in this tissue, substance P, NKA, NKB eledoisin and physalaemin are all full agonists and differ in potency by less than 1 log unit. However, when agonists designed to be more selective than the natural agonists were used (Drapeau *et al.*, 1987a,b), it was found that only the one highly selective for NK-1 receptors was a full agonist. Furthermore, the naturally occurring agonists produced tachyphylaxis of responses to substance P and to tonic responses to high frequency (40 Hz) field stimulation, but the agonists highly selective for various NK receptors reduced both phasic and tonic responses to field stimulation.

There are two possible explanations for these findings: (1) these receptors are one class of receptors which recognize several naturally occurring tachykinins with roughly similar potencies; or (2) these receptors are a mixture of all three classes of receptor, present in sufficient densities to permit maximal contractions when each class was occupied by naturally occurring tachykinins. If the second explanation was correct and some of these agonists were truly selective, then selective desensitization to receptors for each class should be possible; i.e., there should be no or minimal cross desensitization between agonists acting on different classes of receptors. In fact there was cross desensitization between most natural tachykinin peptides (Table 3) and even selective peptides for NK-2 and NK-3 receptors caused some desensitization to substance P, but did not depress responses to carbachol. Therefore it is possible that these substances achieve selectivity by low efficacy at some receptors but still recognize all receptors. Thus the present findings support that neurokinin receptors in this tissue may represent a predominant NK-1 population which retains the ability to recognize several synthetic and naturally occurring tachykinins. However, these findings do not rule out the existence of some NK-2 and NK-3 receptors in this tissue which are insufficient in number or efficacy to cause a maximum response when selectively activated but which do contribute to the maximum response. Ligand binding studies and ultimately receptor isolation and biochemical characterization are required to confirm whether only one receptor is present and if this receptor is identical to that found in other tissues.

However, comparison of these results to those reported by Regoli *et al.* (1987) suggest that in fact this tissue contains a single type of receptor which resembles other NK-1 receptors. Regoli *et al.* reported (1987) that this receptor type in canine carotid artery was characterized by potency ratios such that substance P was more potent than NKA and even more potent compared to NKB (i.e., SP > NKA > NKB). The ED_{50} values were NKA four times higher and NKB twelve times higher than that for substance P; very similar to those found in this study (four and eight times higher). However, in carotid artery, eledoisin was about 1/3 as potent as substance P and physalaemin equipotent while kassinin was 1/6 as potent as substance P. Our previous data (Daniel *et al.*, 1986) suggested that physalaemin was equipotent to substance P in muscularis mucosae but that eledoisin and kassinin were slightly less potent than substance P. In the present study which followed a different protocol, eledoisin, was equipotent with substance P. Previous studies of the C terminal fragments of substance P on putative NK-1 receptors in the carotid artery (Regoli *et al.*, 1987) and in our previous study of muscularis mucosae (Daniel *et al.*, 1986) also revealed some quantitative differences, but all were full agonists in both studies. These discrepancies may reflect minor differences in the receptor or in the experimental conditions, including susceptibility to degradation.

The responses of the tissue to all agonists studied were slow in onset and in offset compared to those to muscarinic agonists (Daniel *et al.*, 1986). There appeared to be no basis for distinguishing their

effects based on duration of action in contrast to observations in the rabbit iris sphincter (Too *et al.*, 1988). Similarities in the onset and offset of actions could depend on the kinetics of interaction of each agonist with the receptor. If this is the case and there is only one receptor subtype with which most agonists interact with similar affinity then all might be expected to have similar durations of action. However, potencies of the selective agonists varied considerably. If their potencies were determined by their affinities for the receptor, then their common time course of action is surprising. The duration of agonist actions can, of course, be determined by variation in rates of agonist degradation, receptor down-regulation or the time course of the system coupling receptor occupation to response. We propose that a common coupling mechanism determines the time course of these agonist since degradation rates of the agonists are unlikely to be similar and the extent of desensitization also varied.

Overall, the findings of this and the earlier study (Daniel *et al.*, 1986) are consistent with the conclusion that opossum muscularis mucosae contains only one major class of tachykinin receptor, NK-1. Its interaction with tachykinin antagonists has not been studied quantitatively. The basis for persistence in evolution of an NK-1 receptor which still recognizes all naturally occurring tachykinins is unclear. This preparation may be useful for binding studies to NK-1 receptors and such studies will help clarify the small differences between this preparation and dog carotid artery.

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Effects of pinacidil on the sympatho-adrenal system in dogs

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1 The aim of the present work was to study the antihypertensive effect of pinacidil, a potassium channel opener, in sinoaortic denervated (SAD) conscious dogs and to investigate whether the involvement of the sympathetic nervous system induced by this vasodilator compound is only of baroceptor reflex origin.

2 Pinacidil (0.1, 0.2, 0.4 mg kg^{-1} i.v.) induced a dose-dependent decrease in blood pressure in normal as well as in SAD dogs. In contrast, the induced-tachycardia observed in normal dogs was not found in SAD animals.

3 Since pinacidil induced an increase in plasma catecholamines, free fatty acids, glucose, plasma renin activity and aldosterone in SAD dogs, it is suggested that this sympathetic activation is independent of the baroreceptor reflex pathways.

4 The sympathetic activation is mainly of peripheral origin, since pinacidil (0.7 mg kg^{-1} i.v.) induced an increase in adrenaline release from the adrenal gland after section of the great splanchnic nerve in anaesthetized dogs. This increase is probably due to an effect that does not involve K^+ channel opening. However, this effect of pinacidil was not observed during splanchnic nerve stimulation (in this case, the involvement of K^+ channel opening is suggested).

Introduction

Pinacidil, a substituted guanidine, is a new antihypertensive agent which produces haemodynamic changes typical of a vasodilator compound acting directly on vascular smooth muscle (Carlsen *et al.*, 1981; Kawashima & Liang, 1985). This drug is one of a new pharmacological class of drugs thought to open apamin-insensitive potassium channels (Weston & Abbot, 1987; Cook, 1988). Kawashima & Liang (1985) suggested that the pinacidil-induced dose-dependent decrease in total peripheral vascular resistance could elicit a secondary sympathetic activation of the baroreceptor reflex and hence explain its positive inotropic and chronotropic effects. The aim of the present work was to study the antihypertensive effect of pinacidil in dogs and to investigate whether the pinacidil-induced involvement of the sympathetic nervous system is only of baroreceptor reflex origin. In fact, would pinacidil-induced opening of potassium channels elicit a decrease in sympathetic tone, thus partly explaining its antihypertensive properties? In the present study, two different *in vivo* protocols were used: firstly, chronic neurogenic hypertension in conscious sinoaortic denervated (SAD) dogs, a model deprived of baroreceptor reflex pathways (Damase-Michel *et al.*, 1987), and, secondly, catecholamine release from the adrenal medulla in anaesthetized dogs. For this purpose, the effects of pinacidil on plasma and adrenal catecholamine levels were investigated.

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Methods

Protocol 1: Study of pinacidil in conscious SAD dogs

Sinoaortic denervation Eight mongrel dogs of either sex, weighing 11 to 20 kg, were made hypertensive by SAD as previously described (Damase-Michel *et al.*, 1987). Briefly, carotid and aortic nerves were cut under chloralose anaesthesia (80 mg kg^{-1} i.v.) during two successive procedures, one at time 0 and the

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second one seven weeks later. During the surgery, care was taken to keep intact both vagal and sympathetic fibres in the vagus.

Experimental protocol Three successive doses of pinacidil (0.1, 0.2, 0.4 mg kg^{-1}) (dissolved in appropriate solvent: NaCl) or vehicle alone were administered at 15 min intervals to two groups of conscious dogs: seven normal (10 to 19 kg body weight) and eight chronic SAD dogs after a resting period of at least 30 min. This protocol was selected according to the data of Kawashima & Liang (1984): cumulative doses were thus 0.1, 0.3, 0.7 mg kg^{-1} of pinacidil in each dog. For several reasons, it was assumed that the doses used in this study are true cumulative ones. First, the half-life of pinacidil is 2 to 4 h (Word *et al.*, 1984). Moreover, Nicholls *et al.* (1986) and Ward (1984) found that the maximum pinacidil-induced changes were seen a few minutes after injection and lasted for 70 to 180 min. Cardiovascular parameters were measured before and every 5 min for 45 min, plasma catecholamines and free fatty acids at time 0, 15, 30 and 45 min, plasma glucose, aldosterone and renin activity just before (time 0) and at the end of the experiment (time 45 min).

Protocol 2: Study of pinacidil on adrenal medulla in anaesthetized dogs

General procedure Fourteen mongrel dogs of either sex, weighing 15 to 25 kg, were anaesthetized with α-chloralose (80 mg kg^{-1} i.v.), curarized by gallamine (2 mg kg^{-1} i.v.) and artificially ventilated with a Ideal Palmer pump (insufflated air volume: 15 ml kg^{-1} with frequency of 16 min^{-1}). The vagus nerves were cut at the cervical level (C_4). Fully adequate anaesthesia was maintained by an injection of 15–20 mg kg^{-1} of chloralose each hour. Body temperature of the animals was maintained at a constant level around 38°C and arterial pH monitored using a Metrohm pH meter.

Arterial venous blood sampling Adrenal venous blood sampling was done according to our previously described technique (Valet *et al.*, 1988a). Briefly, the right adrenal vein was dissected and its collaterals occluded. The animal was heparinized (500 u kg^{-1} every 2 h). The adrenal vein was occluded at the level of its junction with the inferior vena cava, and the other end was cannulated for blood sampling. When not being sampled, adrenal venous blood was returned directly to the femoral vein via a cannula. Blood sampling started 30 min after adrenal vein cannulation. Each blood sample (2 ml) was collected in a tube containing anticoagulant heparin (0.1%) and immediately frozen. Plasma was immediately separated by centrifugation and stored below −80°C. Adrenal plasma flow rate (ml kg^{-1} min^{-1}) was measured and catecholamine output (adrenaline and noradrenaline) expressed as ng kg^{-1} min^{-1}.

The right great and lesser splanchnic nerves were dissected, cut below the diaphragm and the surrounding tissue containing sympathetic fibres carefully destroyed in order to obtain a fairly complete denervation. For that reason, we use the term 'adrenal medulla with sectioned great splanchnic nerve' rather than 'denervated'. Section of the splanchnic nerve was made one hour before blood collection was started. The peripheral end of the great splanchnic nerve was stimulated via a bipolar stainless steel electrode. The stimuli consisted of 3 min trains of rectangular pulses of supramaximal intensity (5 V), 0.5 ms duration and frequency 1 Hz. Stimulation was repeated twice for periods of 3 min each separated by 30 min. In the control group, 0.9% NaCl solution (saline) (the vehicle of pinacidil) was given 10 min before the two periods of stimulation and in the pinacidil-treated group, saline or pinacidil was given before the first and the second stimulation periods, respectively. Adrenal venous blood was collected (3 min) before stimulation and 90 s after the beginning of the stimulation.

Drug administration Pinacidil (0.7 mg kg^{-1}) or saline was injected intravenously 10 min before electrical stimulation.

Cardiovascular parameters

Systolic and diastolic blood pressures were recorded by means of a catheter introduced into the abdominal aorta via the left femoral artery and connected to a Gould P23ID transducer on one channel of a Honeywell recorder. Heart rate was obtained by use of a heart period (pulse interval) meter triggered by the electrocardiogram (lead II). Protocol 1 was performed in conscious dogs whereas in protocol 2 the dogs were under chloralose anaesthesia.

Biochemical assays

Plasma or adrenal catecholamines were measured by high performance liquid chromatography using electrochemical detection. Blood samples were obtained from femoral artery for protocol 1 and adrenal vein for protocol 2. Plasma free fatty acids were analysed by the method of Dole & Meinertz (1960). Plasma glucose was determined by an enzymatic method (Biotrol kit, Paris). Plasma renin activity and plasma aldosterone concentrations were measured with commercial radioimmunoassays kits.

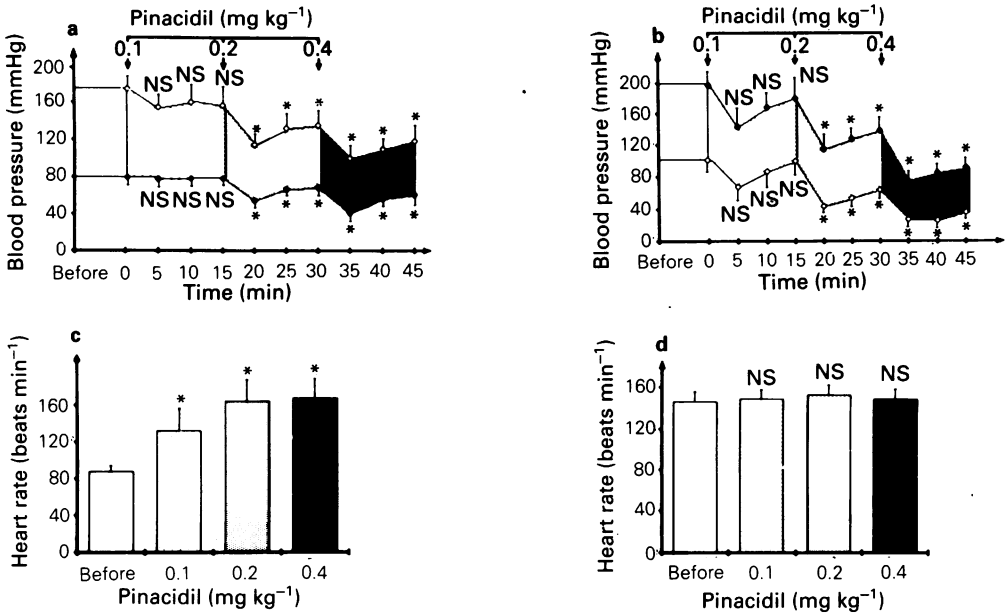

Figure 1 Effects of three serial doses of pinacidil (0.1, 0.2, 0.4 mg kg^{-1} i.v.) on systolic (○), diastolic (●) blood pressures (mmHg), each measured 5 min after drug injection (a, b) and heart rate (beats min^{-1}) measured 15 min after drug injection (c, d) in 7 normal (a, c) and 8 sinoaortic denervated (SAD, b, d) conscious dogs. Statistical analysis was made by Student's t test for paired comparisons. Mean values are given; vertical lines show s.e.mean. *$P < 0.05$ when compared with pretreatment values. NS = not significant.

Statistical analysis

All data are presented as mean values ± s.e.mean. Statistical analysis was performed by use of Student's t test for paired or unpaired data. The level of significance was $P < 0.05$.

Results

Protocol 1: Study of pinacidil in conscious SAD dogs

Only the results with pinacidil are presented since sham experiments (not shown), using saline as a vehicle, failed to show any significant changes in blood pressure, heart rate, plasma catecholamines and other studied parameters in normal as well as SAD conscious dogs.

Blood pressure and heart rate (Figure 1)

Normal dogs: Pinacidil produced a dose-dependent decrease in systolic and diastolic blood pressures and a simultaneous increase in heart rate. Its peak effects occurred shortly (5 min) after each administration. These acute changes lessened gradually and reached a steady state within 10 to 15 min after each administration.

SAD dogs: Acute injection of pinacidil (0.1, 0.2, 0.4 mg kg^{-1}) induced a dose-dependent decrease in blood pressure. The time course of this change was similar to that observed in normal dogs. By contrast, pinacidil did not elicit any change in heart rate in conscious SAD dogs during the whole experiment.

Plasma catecholamine levels (Figure 2a and b)

Normal dogs: Under resting conditions (before treatment), plasma catecholamine levels were 289 ± 63 and 187 ± 52 pg ml^{-1} for noradrenaline and adrenaline respectively. After 0.4 mg kg^{-1} pinacidil (i.e. cumulative dose of 0.7 mg kg^{-1}) a significant increase was observed.

SAD dogs: Resting values were significantly ($P < 0.05$) higher in SAD than in normal dogs: 418 ± 101 pg ml^{-1} and 333 ± 107 pg ml^{-1} for noradrenaline and adrenaline respectively. Pinacidil (0.2 and 0.4 mg kg^{-1}; i.e. cumulative doses of 0.3 and 0.7 mg kg^{-1}) induced a significant increase in plasma noradrenaline whereas 0.1 mg kg^{-1} pinacidil was

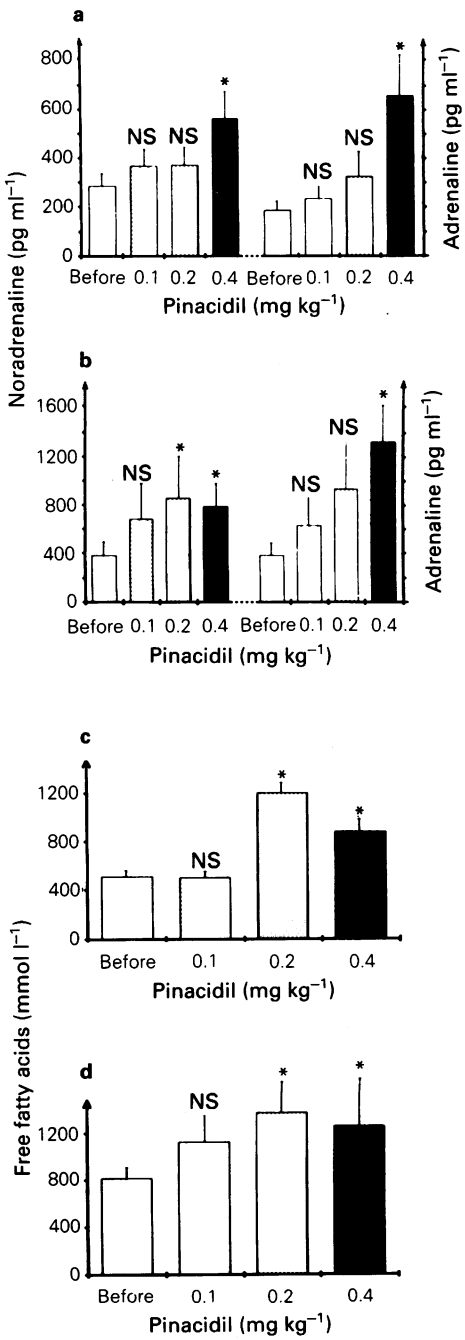

Figure 2 Effects of three serial doses of pinacidil (0.1, 0.2, 0.4 mg kg^{-1} i.v.) on plasma catecholamines (pg ml^{-1}; a, b) and free fatty acids (mmol l^{-1}; c, d) measured 15 min after drug injection in 7 normal (a, c) and 8 SAD (b, d) conscious dogs. For explanation of analysis of results see Figure 1 legend.

ineffective. The changes in adrenaline levels were only significant after the cumulative dose of 0.7 mg kg^{-1}.

Plasma free fatty acids (Figure 2c and d) As previously found (Valet *et al.*, 1988b), plasma free fatty acid levels were higher in SAD dogs than in normal dogs (821 ± 122 versus 517 ± 57 mmol l^{-1}, $P < 0.05$). In the two groups of animals, pinacidil elicited a significant increase in plasma free fatty acids: these changes were significant after 0.2 and 0.4 mg kg^{-1} pinacidil (i.e. for cumulative doses of 0.3 and 0.7 mg kg^{-1}).

Plasma glucose, aldosterone and renin activity (Table 1) These values were only measured at time 0 (before the drug) and 45 min after a cumulative dose 0.7 mg kg^{-1}. Pinacidil induced a significant increase in all these parameters in normal as well as in SAD dogs.

Protocol 2: Study of pinacidil on adrenal medulla in anaesthetized dogs

Fourteen mongrel dogs (6 controls and 8 treated animals) were used. Under our experimental conditions (chloralose anaesthesia and gallamine), blood pH (7.42), body temperature (38°C) and haematocrit did not vary. Gallamine was used since it prevented the variations in arterial pressure elicited by respiratory irregularities without inducing ganglioplegy. Sumikawa *et al.* (1979) found that it does not alter the secretion rate of adrenaline from the gland.

Splanchnic nerve stimulation (at a frequency of 1 Hz) significantly increased adrenal catecholamine release in the two groups (controls and pinacidil-treated dogs) (Table 2). Thus, adrenaline secretion was ten to fifteen fold more than the basal value: 10.50 ± 1.66 and 11.87 ± 1.54 ng kg^{-1} min^{-1} in controls and experimental groups respectively. A frequency of 1 Hz was chosen since the output (mainly adrenaline) obtained with this frequency was almost identical to the basal value measured when the adrenal nerve was intact: in 18 dogs, the mean basal value of adrenaline secretion was 12.81 ± 1.29 ng kg^{-1} min^{-1} (Valet *et al.*, 1988a). Electrical stimulation induced a slight increase in plasma flow rate in the two groups which was only significant in the control group (9.80 ± 3.89% of the basal value; $P < 0.05$). This effect could be due to vasoactive intestinal peptide release (Edwards, 1982).

In the control group, the values obtained, before and during the second stimulation, for adrenaline, noradrenaline and adrenal plasma flow rate were not significantly different from the corresponding values observed before and during the first stimulation. Figure 3 shows the ratio $R_{2/1}$ between the values for

Table 1 Effect of pinacidil (cumulative dose: 0.7 mg kg^{-1} i.v.) on plasma glucose, aldosterone and renin activity in normal (n = 7) and sinoaortic denervated (SAD) (n = 8) conscious dogs

		Normal	*SAD*
Plasma glucose (mmol l^{-1})	Before	5.2 ± 0.4	4.5 ± 0.3
	After	6.8 ± 0.6*	6.4 ± 0.5*
Plasma aldosterone (ng 100 ml^{-1})	Before	14.0 ± 2.8	34.8 ± 12.6
	After	29.3 ± 3.7**	65.3 ± 18.6**
Plasma renin activity (ng ml^{-1} h^{-1})	Before	1.50 ± 0.61	4.44 ± 2.58
	After	13.49 ± 3.12***	15.51 ± 3.66**

Statistical analysis was made by means of Student's t test for paired comparisons. Mean values ± s.e.mean are given.
*P < 0.05, **P < 0.01, ***P < 0.001.

the second stimulation and the corresponding values for the first period of electrical stimulation. For each parameter, the ratio was around 1, the highest being for noradrenaline (1.17). In this case, the basal values before the second stimulation were not significantly different from the basal values before the first stimulation.

In the treated group, pinacidil was given 10 min before the second period of electrical stimulation. A dose of 0.7 mg kg^{-1} was selected because this dose was found to increase plasma adrenaline levels in conscious SAD. Pinacidil induced a significant increase in the basal secretion (before stimulation) of noradrenaline and adrenaline when compared with control groups (Figure 3). Thus, the ratios $R_{2/1}$ for adrenaline and noradrenaline were 2.16 and 3.96, respectively. In addition, pinacidil significantly raised adrenal plasma flow rate when compared with the control group: the ratio $R_{2/1}$ was 1.51. By contrast during stimulation, pinacidil failed to modify adrenaline and noradrenaline release when compared with the control group (Figure 3). It is important to note that the ratio $R_{2/1}$ for plasma flow rate (1.48) was similar to the basal value obtained before stimulation.

Discussion

In the present study, the effect of pinacidil, a new potassium channel opener, was studied on sympathetic tone in normal and SAD dogs. This antihypertensive drug is known to have a direct arterial vasodilator effect (Arrigoni Martelli *et al.*, 1980). However, an effect on venous return cannot be excluded as recently described by Grant *et al.* (1989) on the canine saphenous vein using another potassium channel opener (BRL 34915). Moreover, in the model of chronic neurogenic hypertension (SAD), the effectiveness of baroreceptor denervation was checked by the failure of phenylephrine (0.1, 1 and 10 μg kg^{-1} i.v.) to induce bradycardia and nitroglycerin (1, 3, 10 and 30 μg kg^{-1} i.v.) to induce tachycardia. Thus, the involvement of baroreceptor reflex pathways in the antihypertensive action of drugs in

Table 2 Adrenal catecholamine levels and adrenal venous plasma flow rate before and during the first splanchnic nerve stimulation of the denervated adrenal gland (i.e. before any injection) in control (sham) and pinacidil experiments

	First stimulation			
	Controls		*Pinacidil*	
	Before	*During*	*Before*	*During*
Adrenaline (ng kg^{-1} min^{-1})	1.02 ± 0.36	10.50 ± 1.66***	0.82 ± 0.37	11.87 ± 1.54***
Noradrenaline (ng kg^{-1} min^{-1})	0.08 ± 0.03	1.86 ± 0.37***	0.06 ± 0.01	1.57 ± 0.30***
Adrenal plasma flow rate (ml kg^{-1} min^{-1})	0.181 ± 0.019	0.196 ± 0.018*	0.178 ± 0.01	0.189 ± 0.01

A frequency of stimulation of 1 Hz was applied. Adrenaline and noradrenaline were measured 3 min before and during nerve stimulation. Blood sampling during stimulation was started 90 s after initiation of the stimulus. Each value represents the mean ± s.e.mean (n = 6 for control group, n = 8 for pinacidil group). Catecholamine release and adrenal plasma flow rate during stimulation were compared with values obtained before stimulation by means of Student's t test for paired comparisons. *P < 0.05, ***P < 0.001.

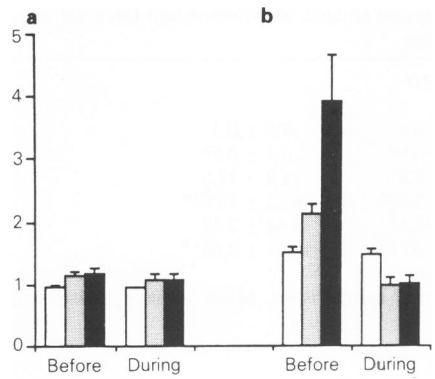

Figure 3 Adrenal catecholamine levels (adrenaline: stippled (middle) columns, noradrenaline: solid columns) and adrenal plasma flow rate (open columns) in the control (a) and pinacidil (b) group before and during splanchnic nerve stimulation. Ordinate scale: each value is expressed as the ratio $R_{2/1}$ between the values of the second stimulation and the corresponding values of the first period of electrical stimulation. Mean values in the pinacidil group were compared with the corresponding value from the control group by means of unpaired Student's t test. $^{**}P < 0.01$, $^{***}P < 0.001$.

conscious dogs can be investigated in this SAD preparation. The main finding in the present study is that, besides the classical involvement of baroreceptor pathways (triggered by the decrease in peripheral resistances, as shown in normal dogs by Kawashima & Liang, 1985), pinacidil induced an unexpected increase in sympathetic tone totally independent of the baroreceptor reflex (as shown by the experiments on SAD dogs). As far as we know, such results have never been described previously.

In fact, the importance of baroreceptor pathways in the chronotropic responses to potassium channel openers has been emphasized. Conflicting results have been obtained: Kawashima & Liang (1985) showed that propranolol only reduced but did not totally suppress the increase in heart rate produced by pinacidil in conscious dogs. In contrast, Cook & Hof (1988) found that the increase in heart rate elicited by BRL 34915 (another vasodilatator thought to act by opening potassium channels) was completely abolished by prior administration of bopindolol in normotensive rats. The incomplete abolition of the response by propranolol might be compatible with a chemoreceptor response which has, in dog, a sympathetic component. However, these chemoreceptor reflex pathways are known to be activated when blood pressure dramatically decreases below 80 mmHg or when Po_2 is greatly reduced (for example 15 to 5 kPa), but that was not the case under our experimental conditions.

In order to study the pinacidil-induced variations in sympathetic tone, plasma levels of catecholamines, glucose and free fatty acid were determined. It is well known that catecholamines are powerful lipolytic and hyperglycaemic stimulating agents both *in vivo* and *in vitro* (Berlan, 1976; Milavec-Krisman & Wagner, 1978). These parameters were increased as expected after pinacidil in normal animals (via baroreceptor reflex) but they also rose in SAD dogs (i.e. animals deprived of baroreceptor reflex pathways). The rise in plasma noradrenaline in normal dogs is in agreement with the data of Kawashima & Liang (1985) who found a 66% increase in plasma noradrenaline levels (for a cumulative dose of $0.4\,mg\,kg^{-1}$). Finally, an increase in sympathetic tone is also indicated by the rise in plasma renin activity and aldosterone observed in the two groups of dogs.

These results in SAD conscious dogs question the mechanism of the sympathetic activation. The action of pinacidil can be mainly explained by peripheral mechanisms since the drug hardly crosses the blood brain barrier (I. Ahnfelt-Ronne, personal communication). In order to confirm the peripheral origin of this activation, we investigated the effect of pinacidil on splanchnic nerve stimulation-induced catecholamine release, since cells from the adrenal medulla are embryologically analogous to sympathetic autonomic ganglia. First, we want to emphasize that basal values of adrenaline and noradrenaline outputs (around $100\,pg\,kg^{-1}\,min^{-1}$) were very low suggesting a fairly complete denervation. We observed that pinacidil induced a significant increase in basal secretion of catecholamines from the gland. This pinacidil-induced increase in adrenal plasma flow rate ($R_{2/1}$ was 1.51) is insufficient to explain the excitatory effect of pinacidil on the function of the adrenal medulla, since the ratios $R_{2/1}$ were 2.16 and 3.96 for adrenaline and noradrenaline respectively. The increase in adrenal plasma flow rate is probably due to the vasodilator properties of pinacidil.

How did the drug induce an increase in plasma and adrenal catecholamines? This effect is unexpected as the opening of K^+ channels, by causing hyperpolarization (Cook, 1988), inhibits calcium influx into the cells where calcium is required for exocytosis of catecholamines; especially sympathetic nerve endings and the adrenal medulla. Consequently, pinacidil should elicit the opposite effect, i.e. a decrease in catecholamine release. Cook (1988) demonstrated that K^+ channel opening reduces beta pancreatic cell electrical activity and inhibits insulin secretion. One possibility is that pinacidil directly affects, in a non-specific manner, the storage sites for catecholamines, as does tyramine (Lindmar *et al.*, 1967). Calcium channel blockers, like nifedipine or

diltiazem, do have such properties (Wolchinsky & Zsoster, 1985; Zsoster & Wolchinsky, 1986). Moreover, this hypothesis of a 'non-specific' effect agrees with a recent communication from Cook *et al.* (1989) who found, in *in vitro* experiments, that the vasodilator activity of pinacidil cannot be solely explained by potassium channel opening. Other less likely mechanisms include the haemodynamic effect of hypotension within the central nervous system, but this is known to be activated only when blood pressure falls below 50 mmHg (Meyer, 1983) and that was not the case under our experimental conditions.

However, the pinacidil-induced release was not observed during splanchnic nerve stimulation. One possible explanation is that this phenomenon was counteracted by an opposite inhibitory effect on catecholamine release. In this case, K^+ channels may be involved as Wada *et al.* (1987) demonstrated that adrenal medullary cells have at least three distinct types of K^+ permeability mechanisms.

In conclusion, we hypothesize that the effects of pinacidil on sympathetic tone are the consequence of three different mechanisms: the first two (baroreceptor reflex and another non-specific direct effect) induce an increase in sympathetic tone, while the third (opening of K^+ channels) results in a decrease in catecholamine release. The relative importance of these three effects can vary according to physiological circumstances.

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The effect of atrial natriuretic peptide on human isolated resistance arteries

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1 The action of (1-28) α-human atrial natriuretic peptide (ANP) was studied on human isolated resistance arteries.

2 Renal, skeletal muscle, omental and subcutaneous resistance arteries were taken from tissue removed at surgery and isometric tension responses were measured with a myograph.

3 ANP (10^{-9}–10^{-6} M) relaxed precontracted segments of renal and skeletal muscle arteries in a concentration-dependent manner. ANP failed to relax isolated omental or subcutaneous arteries.

4 The effect of ANP on human isolated resistance arteries varies depending on the site of origin of the artery.

Introduction

The discovery of a vasodilator and natriuretic substance, atrial natriuretic peptide (Kangawa & Matsuo, 1984), has excited much interest in the physiological role of this peptide in the control of blood pressure and extracellular fluid volume (Needleman *et al.*, 1985). Alpha human (1–28) atrial natriuretic peptide (ANP) has been shown to circulate in human plasma (Theiss *et al.*, 1987) and to possess natriuretic effects in man at low doses (Anderson *et al.*, 1986a), whilst higher doses have been shown to be hypotensive in man (Richards *et al.*, 1985). Recent work in man, *in vivo*, has shown a local vasodilator effect of ANP infusion into the forearm arterial vasculature (Bolli *et al.*, 1987; Hughes *et al.*, 1988). Studies in several species including man (Winquist, 1985; Rappoport *et al.*, 1986; Hughes *et al.*, 1988) have demonstrated a vasorelaxant effect of ANP on isolated arterial smooth muscle from a variety of sites.

Most of these studies demonstrating a vasorelaxant effect of ANP on isolated vasculature have been conducted on isolated vessels of internal diameter greater than 0.5 mm. Such arteries do not play a significant role in the control of peripheral vascular resistance (Haddy, 1960; Mulvany, 1983) and it is recognised that considerable differences exist between the pharmacological responsiveness of large 'conduit' arteries and smaller resistance arteries (Altura, 1974). Recent studies in the rat (Aalkjaer *et al.*, 1985; Osol *et al.*, 1986) and rabbit (Edwards & Weidley, 1987) in which the effect of ANP on arteries small enough to influence peripheral resistance was examined, have generally failed to show direct vasorelaxant effects of ANP in a variety of sites with the exception of the renal arcuate artery of the rat (Aalkjaer *et al.*, 1985). Consequently we have examined the effect of ANP on a variety of human isolated resistance arteries. Some of these results have been presented previously to the British Pharmacological Society (Sever *et al.*, 1988).

Methods

Tissue was obtained from 14 patients (8 male) undergoing surgery (age range 40–78 years). Omentum and subcutaneous tissue was obtained from abdominal surgery, macroscopically normal renal tissue from nephrectomies for renal carcinoma and skeletal

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Table 1 Characteristics of arteries used in the study

Site	*Normalised diameter* (μm)	*Active tension induced by KDS* ($N\,m^{-1}$)	n
Renal	291 ± 74	2.2 ± 0.5	7
Skeletal	262 ± 44	2.5 ± 0.7	5
Omental	227 ± 35	2.4 ± 0.2	4
Subcutaneous	339 ± 96	3.6 ± 1.5	4

Values are mean ± s.e.mean.

muscle from hip surgery. Tissue was collected in physiological saline solution (PSS) of composition (mM): NaCl 118, KCl 4.7, $MgCl_2$ 1.2, $NaHCO_3$ 21, glucose 20, NaH_2PO_4 1, $CaCl_2$ 2.5, Na_2EDTA 0.03 and used immediately for studies; arteries from all sites were treated similarly. Arteries were dissected from surrounding tissue with a dissecting microscope and mounted on two 40 μm stainless steel wires in a myograph to allow the measurement of isometric tension (Mulvany & Halpern, 1977). The myograph contained 10 ml PSS at 37°C aerated with 95% O_2: 5% CO_2. Following 30 min equilibration, vessels were normalised on the basis of their passive internal circumference to tension relationship to an internal circumference (0.9 L_{100}), that is, 0.9 times the internal circumference which they would possess if distended by 100 mmHg pressure on the basis of the Laplace relationship (pressure = tension/radius) Under these conditions the contractile responses of

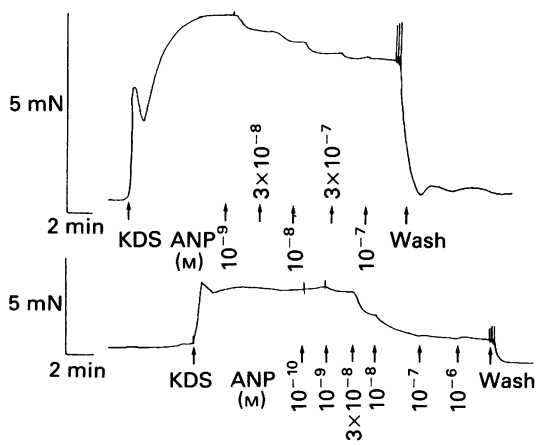

Figure 1 Representative traces showing the effect of cumulative addition of (1–28) α-atrial natriuretic peptide (ANP) (10^{-10}–10^{-6} M) to human isolated resistance arteries taken from kidney (internal diameter = 197 μm) and skeletal muscle (internal diameter = 226 μm). Arteries were contracted with a potassium depolarizing solution (KDS) and ANP added cumulatively as indicated by the arrows.

such small vessels are near to optimal (Mulvany & Halpern, 1977).

Prior to any study the viability of vessels was investigated by exposing them to a sequence of a depolarizing potassium solution (KDS), consisting of PSS containing equimolar KCl substituted for NaCl, followed by washout with PSS and then addition of noradrenaline (NA, 10^{-5} M). Vessels not generating active tension in response to KDS equivalent to or greater than 90 mmHg effective active pressure (calculated by Laplace's relationship) were not used for further studies; 3 out of a total of 23 vessels studied did not satisfy this criterion.

Following washout, arteries were allowed to equilibrate for a further 30 min in PSS before experiments were started. Since arteries rarely possessed much intrinsic tone in PSS, tone was induced with either NA (10^{-5} M) or KDS in order to demonstrate relaxation. ANP (10^{-10}–10^{-6} M) was added cumulatively and responses were calculated as % reduction of induced tone. Concentration-response data from individual arteries was fitted to a logistic function with a computer programme (Barlow, 1984) to derive values for EC_{50}. EC_{50} values are presented as geometric means (95% confidence intervals) and maximum responses (max) as mean ± s.e.mean.

ANP and noradrenaline were obtained from Sigma Chemical Co. Ltd., UK.

Results

The characteristics of the vessels used in these studies are shown in Table 1. Addition of ANP relaxed contracted segments of renal and skeletal muscle arteries in a concentration-dependent manner (Figure 1). The potency and efficacy of ANP was similar in renal and skeletal muscle arteries irrespective of the contractile agonist used, (EC_{50} ($\times10^{-9}$ M) = 11 (3–37) and 8 (3–21); max = 66 ± 5% and 69 ± 16% using KDS and EC_{50} ($\times10^{-9}$ M) = 9 (4–20) and 10 (5–17); max = 73 ± 4% and 69 ± 13% using NA for renal and skeletal muscle arteries respectively). The concentration-response relationships derived from these studies are shown in Figure 2.

In contrast to the results obtained in renal and skeletal muscle vasculature, ANP failed to relax subcutaneous ($n = 4$) or omental arteries ($n = 4$) precontracted by either NA or KDS (Figure 3).

Discussion

This study demonstrates that ANP relaxes human isolated renal and skeletal muscle resistance arteries. The maximum contractile responses of these small human arteries are similar to those reported by others for similar sized arteries obtained from man

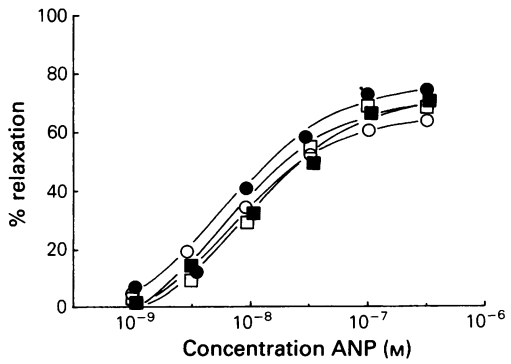

Figure 2 Concentration-response relationships for (1–28) α-atrial natriuretic peptide (ANP)-induced relaxation in renal and skeletal muscle resistance arteries. Arteries were contracted by potassium depolarizing solution (open symbols) or noradrenaline (closed symbols). Points represent means of *n* observations. Some points have been offset and s.e.means have been omitted for clarity: (○) renal arteries ($n = 7$); (●) renal arteries ($n = 3$); (□) skeletal muscle arteries ($n = 5$); (■) skeletal muscle arteries ($n = 5$).

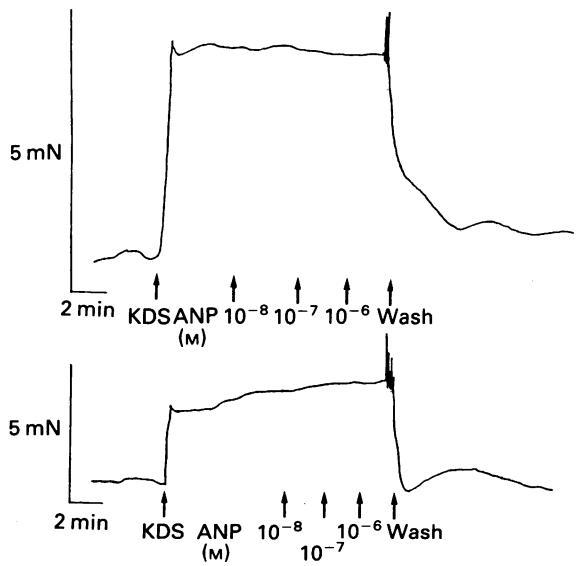

Figure 3 Trace illustrating the failure of (1–28) α-atrial natriuretic peptide (ANP) to induce relaxation in human isolated omental (internal diameter = 264 μm) and a subcutaneous resistance artery (internal diameter = 238 μm). Arteries were contracted with a potassium depolarizing solution (KDS) and ANP added cumulatively as indicated by the arrows. Representative of four separate experiments.

(Aalkjaer & Mulvany, 1983) or other species (Mulvany & Halpern, 1976). The vasorelaxant action of ANP probably involves a direct relaxant effect on vascular smooth muscle cells, as has been suggested by others (Winquist *et al.*, 1984). A notable feature of these results is that the effect of ANP on human isolated resistance arteries varies between arteries taken from different vascular beds. This finding is in contrast to previous studies with large human arteries from a variety of sites *in vitro* (Rappoport *et al.*, 1986; Hughes *et al.*, 1988).

Similar differences with respect to the pharmacological responses of conduit and resistance arteries have been reported previously with respect to neurohypophyseal peptides in the rat (Altura, 1974) and α-adrenoceptor agonists in human vasculature (Nielsen *et al.*, 1987). The findings presented here emphasize the differences between conduit and resistance arteries and indicate caution in extrapolating results obtained in one to the other.

However, these results obtained in human isolated resistance arteries should not be interpreted as definitely excluding the existence of ANP receptors on vascular smooth muscle cells of the omentum and subcutaneous tissue. A recent report by Cauvin and colleagues (1988) suggests that in the rat, at least, although it is not possible to demonstrate vasorelaxant effects of ANP on precontracted mesenteric resistance arteries, ANP does attenuate the phasic component of the response to noradrenaline. This was not studied in our experiments and it is possible that our failure to demonstrate relaxation in response to ANP in subcutaneous and omental arteries similarly reflects the inability of the peptide to relax tonic contraction in these sites. The relaxant effects of ANP in renal and skeletal muscle under the same conditions may therefore reflect a greater efficacy of the peptide in these tissues or some other factor. Further studies will be necessary to establish whether this is the case. The results obtained in these human resistance arteries are nevertheless very similar to those reported by Aalkjaer and colleagues (1985) investigating the vasorelaxant effect of ANP in rat isolated resistance arteries. However, some differences exist, in that skeletal muscle arteries from the rat were found to be unresponsive to ANP, whereas in this study arterioles supplying human skeletal muscle were found to relax in response to ANP. It is possible that this may represent a species difference. In this context, it is interesting that increased central venous pressure, a stimulus to ANP release (Ogihara *et al.*, 1986) is reported to increase skeletal muscle blood flow in man in contrast to some other species (Donald & Shepherd, 1978). However, an involvement of ANP in this response is clearly speculative at present. Indeed the physiological significance of the vasorelaxant effects

of ANP is currently unclear, since the concentrations of ANP necessary, both to produce vasodilatation *in vivo* (Richards *et al.*, 1985; Bussein *et al.*, 1986) or to relax arterial smooth muscle *in vitro* exceed the levels seen circulating under physiological conditions (Anderson *et al.*, 1986b; Ogihara *et al.*, 1986). Further work will be necessary to define the precise role of the receptors for ANP sited on vascular smooth muscle cells and the conditions under which they may be activated.

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Effects of capsaicin pretreatment on neuropeptides and salivary secretion of rat parotid glands

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1 'Atropine-resistant' secretion of saliva in response to parasympathetic stimulation may reflect antidromic activation of sensory nerve fibres. In this investigation, the effect of pretreatment in the rat with capsaicin (total dose of 125 mg kg^{-1}, s.c.), was determined.

2 In the parotid glands substance P (SP)/calcitonin gene-related peptide (CGRP)-containing nerve fibres around ducts and blood vessels disappeared after capsaicin, while periacinar SP-containing fibres (devoid of CGRP) and CGRP-containing fibres (devoid of SP) remained. Vasoactive intestinal peptide (VIP)-containing nerve fibres seemed to be unaffected. The parotid content of SP and CGRP was reduced by 11 and 36% respectively, while that of VIP remained unchanged.

3 The weights of the parotid glands and their sensitivity to the secretagogues methacholine and SP, injected intravenously, were unchanged as was the response to stimulation of the auriculo-temporal nerve in the presence and absence of atropine.

4 In contrast to capsaicin pretreatment, parasympathetic denervation of the parotid gland reduced the weight of the gland and produced an increase in the response to methacholine and SP.

5 For comparison, the effectiveness of the capsaicin treatment on neuropeptide content was determined in the urinary bladder. The bladder of capsaicin-pretreated rats increased in weight (21%) and in VIP content (31%), while the content of SP and CGRP was reduced by 86 and 94%, respectively. SP- and CGRP-containing nerve fibres were virtually eliminated, while VIP-containing nerve fibres seemed unaffected.

6 In conclusion, antidromic activation of primary afferent (capsaicin-sensitive) C-fibres does not contribute significantly to the 'atropine-resistant' secretory response of the parotid gland to stimulation of the parasympathetic nerve.

Introduction

In rats, electrical stimulation of the parasympathetic auriculo-temporal nerve evokes salivary secretion from the parotid gland despite pretreatment with atropine and adrenoceptor blockers. This non-cholinergic, non-adrenergic secretion is about one-third of that evoked in the absence of the blocking agents (Ekström *et al.*, 1983a; Ekström, 1987). Parasympathetic nerve stimulation releases neuropeptides such as substance P (SP), vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP) (Ekström *et al.*, 1985; 1988) so conceivably, these peptides may mediate the 'atropine-resistant' response. SP injected into the blood stream causes a profuse secretion of saliva (Lembeck & Starke, 1968; Ekström & Wahlestedt, 1982), VIP evokes a small secretion, rich in protein and amylase (Ekström *et al.*, 1983b; Ekström & Olgart, 1986), while CGRP does not evoke any flow of saliva (Ekström *et al.*, 1988). However, CGRP and VIP both enhance the secretory response to SP (Ekström & Olgart, 1986; Ekström *et al.*, 1988).

Stimulation of the auriculo-temporal nerve presumably activates both efferent and afferent nerve fibres and the 'atropine-resistant' nerve-evoked secretion of saliva may therefore reflect antidromic stimulation of sensory nerve fibres. In order to test this

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possibility adult rats were pretreated with capsaicin which is toxic to primary afferent C-fibres (see Buck & Burks, 1986). One to two weeks later we examined the secretory response of the parotid gland to auriculo-temporal nerve stimulation and to injections of various secretagogues. We also examined the effect of capsaicin pretreatment on the content of CGRP, SP and VIP in the parotid gland. A preliminary report of some of these findings has been presented (Ekman *et al.*, 1988).

Methods

Altogether 59 female Sprague-Dawley rats were used. They were killed by an overdose of diethyl ether or, at the completion of the secretion experiments, by air embolus under chloralose anaesthesia. The rats to be treated with capsaicin or to be denervated were paired with age- and weight-matched controls (litter mates). The seven capsaicin-treated rats used for immunocytochemistry were matched against five controls in the same weight range (about 150 g).

Capsaicin pretreatment

Capsaicin (25 mg ml^{-1} in 20% (v/v) ethanol, 10% (v/v) Tween 80 in isotonic saline, stock solution) was injected s.c. in a total dose of 125 mg kg^{-1}: 25 and 50 mg kg^{-1} on the 1st day and 50 mg kg^{-1} on the 2nd day (Holzer-Petsche & Lembeck, 1984). Fifteen min before each capsaicin injection the rats received aminophylline 5 mg kg^{-1} i.p., terbutaline 0.1 mg kg^{-1} i.p. and ketamine 50 mg kg^{-1} i.m. These drugs and the vehicle were also injected in the controls. All the rats survived the capsaicin pretreatment and were killed 1–2 weeks after the last capsaicin injection. The effectiveness of the capsaicin pretreatment was tested (1–2 weeks after the last injection) by instilling a drop of capsaicin solution (0.01% (v/v), dilution in saline from the stock solution) into the eye of the chloralose-anaesthetized animals and counting the number of eye blinks. In pretreated rats this procedure caused only a few blinks of the eye (0.3 ± 0.2, $n = 16$), whereas in control rats blinks were numerous (72 ± 9, $n = 16$, $P < 0.001$). The body weights of the capsaicin-pretreated rats (278 ± 7 g, $n = 16$), used in functional studies, did not differ from those of the controls (283 ± 5 g, $n = 16$).

Parasympathetic denervation

The rats were anaesthetized with diethyl ether and the auriculo-temporal nerve was cut on the right side, where it emerges from the base of the skull (Ekström, 1974; Alm & Ekström, 1976). One week after sectioning the nerve the body weights of the operated rats (319 ± 20 g, $n = 5$) were not different from those of unoperated controls (325 ± 6 g, $n = 5$).

Immunochemistry

Specimens from the parotid gland and urinary bladder were fixed by immersion in an ice-cold mixture of 2% (v/v) formaldehyde and 15% (v/v) of a saturated aqueous picric acid solution in 0.1 M phosphate buffer (pH 7.2) overnight and thoroughly rinsed in Tyrode solution containing 10% sucrose. They were then frozen on dry ice and sectioned at 10 μm thickness in a cryostat. The sections were processed for the immunocytochemical demonstration of SP, CGRP and VIP. The SP antiserum (code SP 8, kind gift from P.C. Emson, Cambridge, UK) was used in a dilution 1:320. The CGRP antiserum (code 8427, Milab, Malmö, Sweden) was used at a dilution of 1:1280. The VIP antiserum (code 7852, Milab, Malmö, Sweden) was used at a dilution of 1:640. The sections were exposed to either of the antisera for 24 h in a moist chamber. The site of the antigen-antibody reaction was revealed by application of fluorescein isothiocyanate (FITC)-labelled antibodies against immunoglobulin G (Milab) at a dilution 1:320 for 1 h at room temperature. Control sections were exposed to peptide antiserum that had been preabsorbed with an excess amounts of the antigen (10–100 μg of synthetic peptide, Peninsula Labs, Belmont, U.S.A.) per ml diluted antiserum. The antibodies used do not cross-react with other known neuropeptides (Sundler *et al.*, 1985; Grunditz *et al.*, 1986). Nevertheless, cross-reaction with still unknown peptides or proteins containing amino acid sequences recognized by the different antisera cannot be excluded and it is appropriate, therefore, to refer to the immunoreactive material as SP-like and CGRP-like. For simplicity, however, the shorter terms 'SP' and 'CGRP' are used henceforth. In order to reveal coexistence of SP and CGRP a simultaneous immunostaining method was used. A CGRP antiserum raised in guinea-pig (code 8513, Milab; dilution 1:1280) was used in combination with the rabbit antiserum against SP and the labels of the second antibodies were FITC and tetramethyl rhodamine isothiocyanate (TRITC), respectively. Sections were first incubated with one of the pair of peptide antibodies and then with the FITC-labelled anti-guinea-pig IgG antibodies. The sections were then incubated with the other peptide antibody. The latter antibodies were visualized using TRITC-labelled anti-rabbit IgG antibodies. The sections were examined in a fluorescence microscope fitted with the appropriate filter settings for viewing FITC and TRITC fluorescence alternately.

Radioimmunoassay

Preparation of samples for radioimmunoassay (RIA) The glands were minced while frozen and extracted in 10 volumes of 0.5 M acetic acid at 100°C for 15 min. After homogenization (Polytron) and centrifugation (3000 *g* for 20 min), the supernatants were collected and lyophilized. The samples were reconstituted and diluted in 0.05 M phosphate buffer (pH 7.4), containing 0.25% human serum albumin (Behringwerke, Marburg, FRG) and assayed for CGRP, SP and VIP in at least two different dilutions.

Radioimmunoassay of calcitonin gene-related peptide A rabbit antiserum (K-8429, Milab, Malmö, Sweden), raised against synthetic rat CGRP conjugated to bovine serum albumin, was used. The final dilution of the antiserum was 1:37,500 and ^{125}I-Tyr^{0}-CGRP served as tracer. The RIA has a detection limit of 20 pmol l^{-1} of standard rat CGRP and the antiserum does not recognize calcitonin, VIP, somatostatin, gastrin-releasing peptide, enkephalins or tachykinins (Grunditz *et al.*, 1986).

Radioimmunoassay of substance P Antiserum SP-2 (kind gift from E. Brodin, Karolinska Institute, Stockholm, Sweden), which is directed against the C-terminal part of SP was used in a final dilution of 1:350,000 with ^{125}I-Tyr^{0}-SP as tracer. The assay detects 10 pmol l^{-1} of synthetic SP and the antiserum does not cross-react with other known tachykinins (Brodin *et al.*, 1986).

Radioimmunoassay of vasoactive intestinal peptide Antiserum 7582 (Milab, Malmö, Sweden) recognizes the N-terminal 15-amino acid sequence of VIP and does not cross-react with peptide histidine isoleucine amide or any known regulatory peptide. The assay detects 5 pmol l^{-1} (Ekström *et al.*, 1984).

Secretory responses

Rats were fasted for 24 h and anaesthetized with chloralose (100 mg kg^{-1} i.v.) after induction with diethyl ether. The trachea was cannulated. The body temperature was maintained at 38°C. The duct of the parotid gland was exposed and cannulated with a fine glass cannula. In experiments on the effect of denervation the ducts were cannulated bilaterally. Saliva was collected in (pre-weighed) plastic tubes and weighed; the specific density of the saliva was taken to be 1.0 g ml^{-1}. Saliva was analysed for amylase by an enzymatic colorimetric test (Boehringer Mannheim, Mannheim, F.R.G.) using α-4-nitrophenyl maltoheptaoside (4NP-G_7) as substrate (Hägele *et al.*, 1982). One unit (u) of catalytic activity of α-amylase is defined as the hydrolysis of 1 μmol of 4NP-G_7 min^{-1} ml^{-1}, which is equivalent to one International Unit.

Drugs were injected through the cannulated femoral vein (polyethylene catheter) in volumes of 0.25–0.3 ml. Each injection of drug was immediately followed by an injection of 0.2 ml saline, a volume considerably exceeding that of the cannula dead-space. The interval between each injection of secretagogue was usually 10 min. The tachykinin antagonist [D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11}]SP was injected 10–15 min before the start of the test procedure.

The auriculo-temporal nerve was exposed medial to the mandible and divided at the base of the skull. The peripheral end of the nerve was stimulated at supramaximal voltage with a duration of 0.2 ms using a bipolar electrode and a Grass S48 stimulator: (a) one group of rats was stimulated at various frequencies (0.1–60 Hz) in periods of 2 min in the absence of atropine, (b) another group of rats was stimulated in the presence of atropine at 20 Hz for 1 min before and after administration of the tachykinin antagonist and (c) yet another group was stimulated continuously at 40 Hz, producing a maximal secretory response, in the presence of atropine for 60 min; in the latter series of experiments saliva was collected in 10 min periods. In order to exclude adrenergic effects on the secretory cells the rats were injected with the α-adrenoceptor blocker dihydroergotamine, 1 mg kg^{-1} i.p., and the β-adrenoceptor blocker propranolol 1 mg kg^{-1} i.p. before induction of ether anaesthesia. The dose of atropine used was 2 mg kg^{-1} i.v. The parotid glands and urinary bladder were removed and weighed; the urinary bladder and the parotid gland on the non-stimulated side were taken as controls and analysed for neuropeptides.

Capsaicin in acute experiments

(a) Cotton wool was soaked in a solution of capsaicin (2.5%) and applied locally, onto the peripheral stump of the acutely cut auriculo-temporal nerve as distally as possible for a period of 30 min during which any flow of saliva was monitored. This was followed by electrical nerve stimulation proximal to the site of capsaicin application. (b) The effect of a single subcutaneous injection of capsaicin (25–50 mg kg^{-1}) on the secretion was tested; the observation period was about 1 h. (c) The cervical sympathetic chain and the auriculo-temporal nerve were cut together with the facial nerve (just outside the stylomastoid foramen) in order to minimize reflex effects. Immediately afterwards, the parotid duct was cannulated and atropine (2 mg kg^{-1} i.v.),

Table 1 Parotid gland weights (mg) and secretory responses (μl saliva) to methacholine and substance P (both i.v.) of control rats ($n = 5$) and of operated rats ($n = 5$) subjected to parasympathetic denervation of the right gland one week beforehand

	Weight	*Methacholine* ($\mu g\,kg^{-1}$) 1	2	*Substance P* ($\mu g/kg^{-1}$) 0.1	0.2
Control					
Right	206.3 ± 13.6	2.9 ± 0.6	6.5 ± 0.7	5.1 ± 1.2	15.6 ± 2.2
Left	201.2 ± 8.6	3.1 ± 0.5	6.4 ± 0.5	5.0 ± 1.2	15.8 ± 2.7
Operated					
Right	141.2 ± 14.7$^{\dagger b}$	13.0 ± 2.0$^{\dagger a}$	26.3 ± 1.1$^{\ddagger b}$	22.9 ± 1.6$^{\ddagger b}$	39.3 ± 2.2$^{\ddagger b}$
Left	201.0 ± 14.8	3.6 ± 0.3	7.4 ± 0.6	6.7 ± 1.0	16.6 ± 1.8

Values are means ± s.e.mean.
† $P < 0.01$, ‡ < 0.001 when compared with contralateral gland; ^{a} $P < 0.01$, ^{b} < 0.001 when compared with corresponding gland of control litter mate.

dihydroergotamine ($1\,mg\,kg^{-1}$ i.v.) and propranolol ($1\,mg\,kg^{-1}$ i.v.) were injected. The secretory response to a standard dose of substance P was tested, about 15 min before an intraductal injection of capsaicin. Thereafter, the parotid cannula was replaced by another cannula of a similar type, producing drops of the same size and supplied with a three-way tap to which a syringe could be attached. The dead-space of the cannula was filled with capsaicin. Capsaicin (0.01%) in 0.04 ml saline was injected retrogradely into the duct over a 5 s period; 5–10 s later, the tap was turned so as to allow saliva to flow out (see Alm & Ekström, 1976). Immediately a small volume of fluid (0.3–1.9 μl) appeared at the tip of the cannula; this was discarded as it was attributed to overfilling of the system. The standard dose of substance P was injected repeatedly, over a period of 40 min, after cessation of capsaicin-evoked salivary flow. In this type of experiment the concentration of Tween 80 in the capsaicin solution was as low as 0.01%, and the solution was devoid of ethanol. The effect of saline alone (0.04 ml) injected into the duct was also tested.

Drugs

Atropine sulphate (Sigma Chemical Co, MO, St Louis, U.S.A.), capsaicin (Fluka Chemie AG, Buchs, Switzerland), dihydroergotamine methansulphonate (Sandoz, Basel, Switzerland), ketamine (Parke-Davis, Morris Plains, NJ, U.S.A.), methacholine chloride (Sigma), propranolol hydrochloride (ICI Pharmaceuticals, Macclesfield, U.K.), SP (Peninsula Labs, Belmont, CA, U.S.A.), [D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11}]SP (Peninsula), aminophylline (ACO Läkemedel AB, Solna, Sweden) and terbutaline (AB Draco, Lund, Sweden) were used.

Statistics

Values are means ± s.e.mean. Student's t test for paired data was used.

Results

Parasympathetic denervation

The denervated parotid glands weighed about 30% less ($P < 0.01$) than the contralateral glands and glands of unoperated rats (Table 1). The volume of saliva secreted from the denervated glands in response to i.v. injections of methacholine and SP was 3–4 ($P < 0.01$ or 0.001) and 2–3 ($P < 0.001$) times larger, respectively, than that of the contralateral glands and of glands in unoperated rats (Table 1). There were no differences in either weight or secretory responses between right and left glands of unoperated rats or between these glands and the contralateral glands of the operated rats.

Capsaicin pretreatment

Peptide-containing nerve fibres in the parotid gland In control glands SP-containing nerve fibres were very numerous around acini, and quite prominent around ducts and blood vessels (see also Ekström *et al.*, 1988). CGRP-containing nerve fibres were few by comparison around acini and fairly numerous around ducts and blood vessels. Periacinar SP- and CGRP-containing fibres were fine and varicose, whereas those occurring around ducts and blood vessels were more coarse and less beaded. VIP-containing nerve fibres were numerous around acini, ducts and blood vessels. Double immunostaining showed that the bulk of SP-containing fibres around acini did not contain CGRP, whereas most SP-containing fibres around ducts and blood vessels did. Capsaicin pretreatment resulted in a marked reduction in the number of SP/CGRP-containing nerve fibres around ducts and blood vessels leaving only a few mostly smooth fibres, together with the network of delicate SP-containing fibres around acini. The delicate periacinar CGRP

Table 2 Parotid gland weights (mg, left gland) and secretory responses (μl saliva, right gland) to methacholine and substance P (both i.v.) in control rats ($n = 16$) and in capsaicin-pretreated rats ($n = 16$)

		Methacholine ($\mu g\,kg^{-1}$)		*Substance P* ($\mu g\,kg^{-1}$)	
	Weight	1	2	0.1	0.2
Control	186.3 ± 7.0	2.8 ± 0.4	5.2 ± 0.5	10.6 ± 2.2[a]	17.6 ± 1.7
Capsaicin	191.3 ± 5.4	2.1 ± 0.3	4.8 ± 0.5	9.2 ± 2.3[a]	17.4 ± 1.6

Values are mean ± s.e.mean.
There were no significant differences when the gland of the capsaicin-pretreated rat was compared with the corresponding gland of control litter mate. [a] $n = 7$.

fibres were also persistent. The latter fibres were devoid of SP and the many SP-containing fibres around acini were devoid of CGRP. The VIP-containing nerve fibres were apparently unaffected by treatment with capsaicin.

Neuropeptide content of the parotid gland In the control glands ($n = 9$), the total amounts (pmol) of CGRP, SP and VIP were 0.14 ± 0.02, 1.0 ± 0.2 and 2.0 ± 0.2, respectively. In the glands of the capsaicin-pretreated rats ($n = 9$) the total amounts of CGRP and SP were 63.7 ± 6.6% ($P < 0.02$) and 88.6 ± 6.6% ($P < 0.05$), respectively, of controls, whereas the total amount of VIP was 102.7 ± 11.8% of controls.

Secretory responses to secretagogues and parasympathetic nerve stimulation

The parotid gland weights and the secretory responses of capsaicin-pretreated rats to methacholine and SP did not differ from control rats (Table 2). The volume of saliva secreted in response to parasympathetic nerve stimulation for periods of 2 min (0.1–60 Hz) was the same in five capsaicin-pretreated and five control rats (Figure 1); at 40 Hz the glands of pretreated rats secreted 136 ± 8 μl saliva ($n = 5$) and those of controls 131 ± 4 μl saliva ($n = 5$). The volume of saliva secreted and the output of amylase in response to continuous maximal (40 Hz) parasympathetic nerve stimulation did not differ between capsaicin-pretreated rats and controls (Figure 2). The fluid secretion and the amylase output during the initial period of stimulation (0–10 min) was 174 ± 26 μl and 201 ± 36 u, respectively, in seven pretreated rats and 158 ± 18 μl and 185 ± 25 u in seven controls. The total volume of saliva secreted over a period of 60 min and the total output of amylase over 40 min were the same in the capsaicin-pretreated (381 ± 82 μl and 291 ± 61 u) and control rats (383 ± 65 μl and 279 ± 60 u).

Effects of a tachykinin antagonist

The tachykinin antagonist [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]SP (0.75–1 $mg\,kg^{-1}$, i.v.) abolished the secretory effect of SP, 0.2 $\mu g\,kg^{-1}$ i.v., in four capsaicin-pretreated rats and in four control rats. The atropine-resistant parasympathetic nerve-evoked secretion was also reduced by the tachykinin

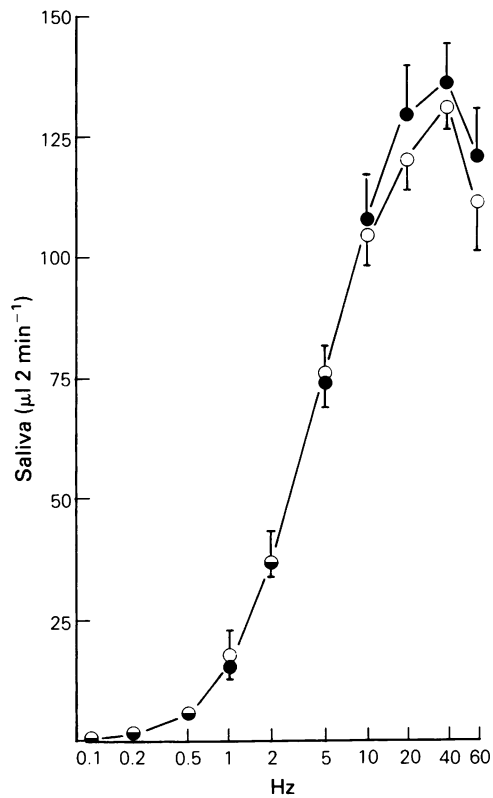

Figure 1 Amount of parotid saliva secreted in capsaicin-pretreated ($n = 5$, ●) and control rats ($n = 5$, ○) in response to stimulation of the parasympathetic (auriculo-temporal) nerve at various frequencies in the presence of adrenoceptor blockers. Ordinate scale: amount of saliva, in μl, per 2 min. Abscissa scale: frequency of stimulation in Hz. Values are means, and vertical bars indicate s.e.mean; in some cases s.e.mean was smaller than the size of the symbols.

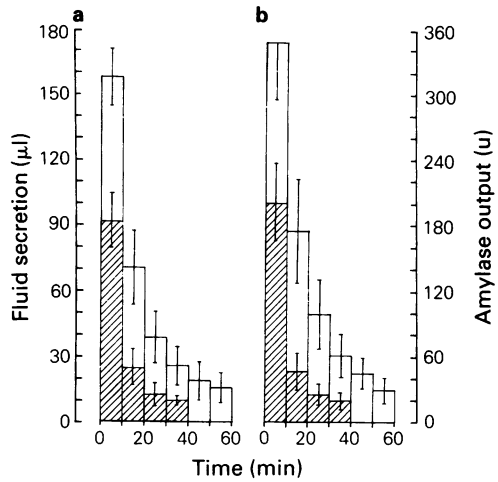

Figure 2 Amount of parotid saliva secreted and output of amylase in capsaicin-pretreated ($n = 7$, b) and control rats ($n = 7$, a) in response to continuous stimulation of the parasympathetic nerve (40 Hz) for 60 min in the presence of atropine and adrenoceptor blockers. Saliva was collected in 10 min periods; the two last samples were not analysed for amylase. Ordinate scales: left, the amount of saliva, in μl, per 10 min; right, the output of amylase, in units, per 10 min. Abscissa scale: time in min. Open columns: mean amount of saliva secreted. Hatched columns: mean output of amylase. Vertical bars indicate s.e.mean.

antagonist: in the capsaicin-pretreated rats the tachykinin antagonist reduced the secretion in response to stimulation for 1 min at 20 Hz to 70.3 ± 5.4% ($n = 4$, $P < 0.05$) of that obtained before administration of the antagonist, while the corresponding figure in control rats was 65.2 ± 6.2% ($n = 4$, $P < 0.05$).

Acute effects of capsaicin

Capsaicin, in a 2.5% solution, applied locally to the auriculo-temporal nerve ($n = 4$) evoked no salivation. Subsequent stimulation of the nerve (40 Hz, 2 min) proximal to the site of the capsaicin application caused a secretory response of the same magnitude as that obtained before the toxin was applied. Capsaicin, in a dose of 25–50 mg kg^{-1}, injected s.c. evoked no secretion from otherwise unstimulated glands ($n = 5$). In three out of four rats where capsaicin was injected retrogradely into the duct a small amount of saliva (0.8–5.8 μl) was secreted; the secretion started 1–2 min after the injection and ceased 2–6 min later. SP, 1 μg kg^{-1} i.v., was injected 15 min before the intraductal capsaicin injection and again after the capsaicin-induced secretion had stopped. In

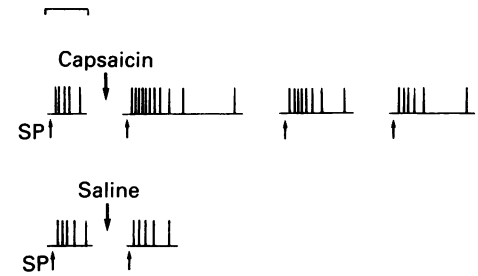

Figure 3 Effects of capsaicin (0.01%), in a volume of 0.04 ml, (upper tracing) or saline, 0.04 ml, (lower tracing) injected retrogradely in the parotid duct on the secretory response to substance P, 1 μg kg^{-1} i.v., in one rat. Substance P was given (arrow) 15 min before the intraductal injection, and then 8, 15, and 25 min after the injection (at the two last time of observations only after capsaicin). Each mark on the tracing represents one drop of saliva (corresponding to 10 μl). Vertical bar indicates one min. The tracings show that after capsaicin, but not after saline, the response to substance P was increased. The auriculo-temporal nerve, the facial nerve and the sympathetic cervical chain had been cut, and atropine and adrenoceptor blockers had been given just before the start of the experiment.

all four of these rats the SP-evoked response was enhanced after capsaicin, by between 100 and 30% over the next 25 min (Figure 3). About 45 min after the capsaicin injection the SP response was the same as before the capsaicin injection. Injection of saline into the duct evoked no secretion, and the SP-evoked response was not enhanced (Figure 3).

Urinary bladder weight, peptide-containing nerve fibres and neuropeptide content

The bladder weights of the capsaicin-pretreated rats (113.7 ± 5.9 mg, $n = 9$) were 21% higher ($P < 0.05$) than those of the untreated controls (89.6 ± 3.2 mg, $n = 9$). In controls SP- and CGRP-containing nerve fibres were numerous in the bladder wall, particularly in the subepithelial layer, where they formed a dense plexus with single fibres penetrating into epithelium. Double immunostaining showed that most CGRP-containing fibres contained SP and *vice versa*. VIP-containing fibres were moderate in number and occurred predominantly in the smooth muscle layer and around blood vessels. Capsaicin treatment virtually abolished the SP- and CGRP-containing fibres, whereas those storing VIP remained unaffected. In the control bladders ($n = 9$) the total amounts (pmol) of CGRP, SP and VIP were 2.6 ± 0.2, 0.31 ± 0.03 and 0.17 ± 0.02, respectively. In the bladders of the capsaicin-pretreated

rats ($n = 9$) the total amounts of CGRP and SP were $5.5 \pm 1.5\%$ ($P < 0.001$) and $13.8 \pm 2.0\%$ ($P < 0.001$), respectively, of controls. The total amount of VIP in the bladders of capsaicin-pretreated rats was $130.7 \pm 10.6\%$ ($P < 0.05$) of that in controls.

Discussion

This study did not provide evidence for any involvement of primary afferent C fibres in the response of the parotid gland to stimulation of the auriculo-temporal nerve as judged by fluid secretion and amylase output. The 'atropine-resistant' response to nerve stimulation persisted undiminished after pretreatment with capsaicin. Further, in the absence of atropine the responses over a whole range of frequencies in capsaicin-pretreated rats were the same as those in the controls. Local application of capsaicin to the auriculo-temporal nerve had no apparent effect on parotid secretion *per se* and did not modify the secretory response to subsequent nerve stimulation. A tachykinin antagonist reduced the 'atropine-resistant' nerve-evoked response just as effectively in capsaicin-pretreated rats as in controls.

There were no signs of 'denervation supersensitivity' following capsaicin treatment. In untreated rats, there is probably a positive interaction not only between certain peptides but also between acetylcholine and VIP as well as CGRP released in response to parasympathetic nerve stimulation (Ekström, 1987). However, the secretory cells of the parotid glands in the capsaicin-pretreated rats were just as sensitive to i.v. injections of the muscarinic agonist methacholine and to SP as those in the control rats. This makes it unlikely that acetylcholine, SP or other substances released during nerve stimulation acted on sensitized cells thereby masking a sensory contribution to the response of the gland.

It is difficult to avoid the conclusion that capsaicin-sensitive sensory nerve fibres in the parotid gland have little or no effect on the secretory activity of the gland. Neither do they seem to play a trophic role, since capsaicin failed to affect the weight of the gland. The time which passed between capsaicin pretreatment and the subsequent acute experiment (1–2 weeks) was thought to be long enough to allow any changes in weight or sensitivity of the secretory cells to secretagogues to reveal themselves because there were marked changes in these parameters within one week following parasympathetic denervation (see also Ekström, 1980; Ekström & Wahlestedt, 1982).

Several peptides have been demonstrated in capsaicin-sensitive primary afferent nerve fibres (Lembeck, 1985; Ju *et al.*, 1987). Assuming that capsaicin eliminates all SP and all CGRP in primary afferent C-fibres, these results suggest that only a minor proportion of SP (11%) and a rather larger proportion of CGRP (36%) are located in primary afferent C-fibres in the parotid gland of the rat. VIP content was unaffected by capsaicin which suggests that VIP-containing fibres either do not have a sensory origin or are insensitive to capsaicin. SP-containing nerve fibres in the parotid gland of control rats were found around acini, ducts and blood vessels (in order of predominance), while CGRP-containing nerve fibres seemed to be mainly localized around ducts and blood vessels; periacinar CGRP-containing fibres were comparatively few (see also Ekström *et al.*, 1988). Double immunostaining showed that most CGRP-containing fibres around ducts and blood vessels contained SP. However, most periacinar SP-storing nerve fibres were devoid of CGRP and most periacinar CGRP-containing fibres were devoid of SP. Following capsaicin treatment, the bulk of SP/CGRP-containing nerve fibres around large ducts and blood vessels had disappeared. Nerve fibres that contain both SP and CGRP are believed to be sensory (Saria *et al.*, 1985; Sundler *et al.*, 1985).

Systemic administration of capsaicin failed to give rise to salivation. However, when capsaicin was injected retrogradely into the duct secretion, albeit very sparse, did occur. When this secretion had ceased, the secretory response to SP was found to be enhanced for about 30 min. The secretory response to SP is known to be enhanced by CGRP (Ekström *et al.*, 1988). It is therefore tempting to speculate that the small secretion that occurred in response to ductal injection of capsaicin involved the combined action of CGRP and SP released from the sensory nerve endings, and that the CGRP that was released enhanced the effect of exogenous SP.

Although the capsaicin-evoked reduction in neuropeptide content was relatively small (11% for SP and 36% for CGRP) and the loss of immunoreactive nerve fibres was modest in the parotid gland we conclude that the capsaicin treatment was effective in eliminating capsaicin-sensitive nerve fibres because it almost abolished the sensitivity of the eye to chemical irritation and more particularly, it greatly reduced the content of SP and CGRP (but not VIP) in the urinary bladder and virtually eliminated nerve fibres storing these two peptides.

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Dual effects of diazoxide on ATP-K^+ currents recorded from an insulin-secreting cell line

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1 The effects of diazoxide on ATP-K^+ channel currents, recorded from the insulin-secreting cell line, CRI-G1, were studied using patch-clamp techniques.

2 Under current-clamp recording conditions diazoxide (0.6 mM), inhibited action potential activity and hyperpolarized CRI-G1 cells with a concomitant increase in membrane conductance. Recordings from voltage-clamped whole-cells and isolated patches indicate that activation of ATP-K^+ channel currents underlie these effects.

3 Diazoxide elicited an activation of ATP-K^+ channels which had been partially inhibited by ATP, on application to either surface of the plasma membrane, although it was more effective when applied directly to the cytoplasmic side. Activation of the ATP-K^+ currents involves an increase in the single channel open-state probability and an apparent increase in the number of functional channels.

4 Activation was observed only when Mg-ATP was present in the cytoplasmic bathing solution. There was no activation of currents by diazoxide when ATP, in the absence of Mg^{2+} ions, or Mg-AMP-PNP was present to inhibit the ATP-K^+ channels.

5 In the absence of ATP and Mg^{2+} ions in the cytoplasmic bathing solution, diazoxide (0.6 mM) produced an inhibition of ATP-K^+ currents.

6 Cromakalim (BRL 34915) at 10 μM and 100 μM had no significant effects on ATP-K^+ currents.

7 It is concluded that diazoxide-induced activation of ATP-K^+ channel currents probably involves phosphorylation of the channel or some closely associated membrane protein.

Introduction

The adenosine 5'-triphosphate (ATP)-sensitive potassium (ATP-K^+) channel has been shown to exist in mammalian cardiac muscle, pancreatic β-cells and amphibian skeletal muscle (Ashcroft, 1988) and, more recently, mammalian central neurones (Ashford *et al.*, 1987; 1988). In insulin-secreting cells, the ATP-K^+ channel current is a major influence on the resting electrical characteristics of the cell, even though the channels are approximately 99% inhibited (Cook *et al.*, 1988a). Although the ATP-K^+ current will act to clamp the potential towards the potassium equilibrium potential (about −80 mV), the resting membrane potential of β-cells in low glucose (3–5 mM) is closer to −60 mV (Croghan *et al.*, 1986) and in glucose-free solution is approximately −70 mV (Rorsman & Trube 1985; Ashcroft *et al.*, 1988). Hence a counter-balancing depolarizing current must exist, although its identity has not yet been elucidated. Any significant increase or decrease induced in the ATP-K^+ current will therefore cause a hyperpolarization or depolarization respectively with concomitant changes in calcium entry and therefore insulin secretion (i.e. decrease and increase respectively).

Alterations in the concentrations of substrates able to be metabolized (e.g. glucose or leucine) extracellularly will produce such effects (Ashcroft *et al.*, 1984; Trube & Hescheler, 1984; Misler *et al.*, 1986; Sturgess *et al.*, 1988). In addition, the sulphonylureas, tolbutamide and glibenclamide by inhibiting ATP-K^+ channels (Sturgess *et al.*, 1985; Ashford *et al.*, 1986; Trube *et al.*, 1986) induce depolarization and insulin secretion (Sturgess *et al.*, 1988), whereas the benzothiadiazine, diazoxide by activating

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ATP-K^+ channels (Trube *et al.*, 1986; Zunkler *et al.*, 1988; Dunne *et al.*, 1987; Sturgess *et al.*, 1988) induces hyperpolarization and inhibition of insulin release (Sturgess *et al.*, 1988).

It is likely that the sulphonylureas act directly on the ATP-K^+ channel or a closely associated protein by virtue of their potent inhibition of channel activity on isolated membrane patches (Sturgess *et al.*, 1985; Ashford *et al.*, 1986; Trube *et al.*, 1986; Sturgess *et al.*, 1988). However, the evidence is less clear with respect to the mechanism by which diazoxide activates ATP-K^+ conductance. Trube *et al.* (1986) demonstrated, using whole-cell recording and isolated patch techniques, that diazoxide activated the ATP-K^+ current and that this effect was related to the concentration of ATP within the cell (high levels of ATP prevented the action of diazoxide), and Sturgess *et al.* (1988) showed that diazoxide only activated ATP-K^+ channels under inhibition by ATP or tolbutamide (but not glibenclamide). Similar results were also obtained by Dunne *et al.* (1987) using the open cell-attached patch configuration. These authors concluded that diazoxide does not act as a 'genuine channel activator', but interferes with the inhibition of the ATP-K^+ channel produced by the ATP^{4-} ion. Recent evidence suggests that it is the ATP^{4-} ion and not Mg-ATP which acts to inhibit the opening of ATP-K^+ channels (Ashcroft & Kakei 1987a; Dunne *et al.*, 1987). We have re-examined whether diazoxide activation of ATP-K^+ channels is dependent upon ATP^{4-} ions or upon the presence of Mg-ATP, and suggest, contrary to the conclusion of Dunne *et al.* (1987), that the Mg-ATP complex is required for diazoxide-induced activation of the ATP-K^+ conductance. Furthermore, we show that diazoxide, in the absence of Mg-ATP, produces an inhibition of the ATP-K^+ current. The effectiveness of another putative K^+-channel activator (Hamilton *et al.*, 1986), cromakalim, was tested. This compound (also known as BRL 34915) is, like diazoxide, a potent antihypertensive agent and there are conflicting results on whether it reduces insulin secretion or not (Wilson *et al.*, 1988; Cook *et al.*, 1988b). In this study we show that cromakalim has no observable effect on the ATP-K^+ current recorded from this insulin-secreting cell line. Some of these results have been communicated to the Physiological Society (Ashford *et al.*, 1989).

Methods

Cell culture

Cells of the rat pancreatic islet cell line (CRI-G1) were cultured and passaged at 2–4 day intervals as previously described (Carrington *et al.*, 1986). Cells used for patch clamp experiments were plated onto 3.5 cm petridishes (Sterilin) at a density of 1.5×10^5 cells per dish. The cells were used 2–4 days (inclusive) after plating.

Electrical recording and analysis

This study employed both the cell-free and whole-cell configurations of the patch clamp recording technique, as described by Hamill *et al.* (1981). Recording electrodes were pulled from borosilicate glass capillaries, and when filled with electrolyte had resistances of 8–12 MΩ for isolated patch experiments, and 3–6 MΩ for whole-cell recording. Single channel events were detected using a Dagan 8900 patch clamp amplifier or an EPC-7 (List Electronics) and were stored on magnetic tape (Racal 4D tape recorder). Records used for illustrative purposes were replayed into a chart recorder (Gould 2200) which filtered the data at 140 Hz. The potential across the membrane is described following the usual sign convention for membrane potential (i.e. inside negative). Outward current (defined as the current flowing from the intra to extracellular side of the membrane) is shown as upward deflections on all traces. The single channel current analysis was determined off line using a programme that incorporated a 50% threshold crossing parameter to detect events (Dempster, 1988) and run on an IBM-AT microcomputer or an Apricot XEN-i286/45. Data segments between 30 and 90 s duration were replayed at the recorded speed and filtered at 1.0 kHz using a 8-pole Bessel filter and digitized at 5.0 kHz using a Data Translation 2801 interface. The open state probability was determined by measuring the total time spent at each unitary current level and expressed as a proportion of the total time recorded; the number of channels observed in the patch was taken into consideration. To obtain whole cell currents, the cell was voltage clamped at a holding potential of −70 mV, and alternate ±10 mV pulses of 200 ms duration were elicited at 2 s intervals as described previously (Trube *et al.*, 1986; Sturgess *et al.*, 1988). Drug effects were quantified by measuring the amplitudes of the current responses (I) during drug exposure and comparing them with those observed under control conditions (I_c) immediately preceding drug administration. Because of the transient activation induced by diazoxide, the value for I was taken at the peak of the current. As it was not possible to compensate for rundown and because the inhibitory effects of diazoxide were progressive and did not reach a maximum, the inhibition was quantified by taking a value 2.5 min after diazoxide application.

Solutions

Before use cells were washed thoroughly with solution A which consisted of (mM): NaCl 135.0, KCl 5.0, $CaCl_2$ 1.0, $MgCl_2$ 1.0, HEPES 10.0, pH 7.4 with NaOH. For whole-cell voltage clamp studies, cells were bathed in solution A and the pipette contained (mM): KCl 140.0, $MgCl_2$ 1.0, $CaCl_2$ 2.0, K-EGTA 10.0, HEPES 10.0; pH 7.2 with KOH which resulted in free Ca^{2+} and Mg^{2+} concentrations of 20 nM and 0.65 mM respectively (solution B). When 0.1 mM ATP was added to the pipette solution the Mg^{2+} concentration was raised to 1.1 mM to allow for Mg^{2+} chelation by ATP, thus the free Mg^{2+} concentration was maintained at 0.65 mM. In whole-cell experiments where the cell was dialysed with a Mg^{2+}-free solution the pipette contained (mM): KCl 140.0, $CaCl_2$ 4.6, K-EDTA 10.0, HEPES 10.0; pH 7.2 with KOH which resulted in free Ca^{2+} and Mg^{2+} concentrations of 20 nM and <3.0 nM respectively (solution C). In some early experiments Ca^{2+} was omitted entirely from this solution and trace amounts chelated with EGTA 1.0 mM or EDTA 1.0 mM. This variation did not affect the results obtained.

In experiments on outside-out patches, the bath solution was A and the pipette solution was B, C or one composed of (mM): KCl 140.0, $MgCl_2$ 1.0, K-EGTA 1.0, ATP 0.1 and HEPES 10.0; pH 7.2 with KOH which resulted in free Ca^{2+} and Mg^{2+} concentrations of <0.2 nM and 0.85 mM respectively (solution D). For inside-out patches the pipette contained solution E, composition (mM): KCl 140.0, $MgCl_2$ 1.0, $CaCl_2$ 1.0, HEPES 10.0, pH 7.2 with KOH. The bath contained solution F, composition (mM): KCl 140.0, $CaCl_2$ 0.9, $MgCl_2$ 1.0, K-EGTA 1.0, HEPES 10.0; pH 7.4 with KOH which resulted in free Ca^{2+} and Mg^{2+} concentrations of 0.4 μM and 1 mM respectively. When 0.1 mM ATP was included in solution F the concentration of $MgCl_2$ was raised to 1.1 mM, thus Mg^{2+} was kept constant. Experiments conducted in the absence of Mg^{2+} utilised solution G, composition (mM): KCl 140.0, $CaCl_2$ 0.9, K-EDTA 1.0, HEPES 10.0; pH 7.4 with KOH which resulted in free Mg^{2+} and Ca^{2+} concentrations of <75 nM and 0.1 μM respectively. The concentrations of free ATP and divalent cations were determined by using a programme for calculating metal ion/ligand binding 'METLIG' (England, P. & Denton, R., University of Bristol). The ATP^{4-} concentration was calculated by using a pK_a value of 6.80 (Perrin, 1979).

Solutions containing 0.6 mM diazoxide were prepared freshly before each experiment (from a 30–40 mM stock solution in 0.1 M KOH) and maintained at pH 7.4 due to its long term instability in solution. Changes in pH and potassium concentration were thus compensated for in the control solutions. Cromakalim (BRL 34915) was added from a 25 mM stock solution prepared in 70% ethanol/H_2O v/v. (In control experiments 0.3% ethanol, equivalent to 100 μM BRL 34915, was shown to have no effect on recordings.) K_2-ATP and Li_4-adenylyl- imidodiphosphate (AMP-PNP) were prepared as 20–30 mM stock solutions in H_2O. When AMP-PNP was included in either the pipette or bath solution the appropriate divalent cation adjustments were made as described above. The metal chelating properties of AMP-PNP were assumed to be similar to those of ATP (Yount, 1975).

Drugs were applied to membrane patches or whole-cells by superfusing the bath, by a gravity feed system, at a rate of approximately 0.5 ml s^{-1} which allowed complete solution exchange within 45 s. ATP (K^+ salt, vanadium free) and Li_4-AMP-PNP were obtained from Sigma (Poole, Dorset). Diazoxide was donated by Glaxo Pharmaceuticals (Greenford, England) and cromakalim (BRL 34915) by Beecham Pharmaceuticals (Harlow, Essex, England). All experiments were performed at room temperature, 22–25°C.

All data in text and figures are presented as mean values ± s.e.mean unless otherwise stated.

Results

On recording from CRI-G1 cells under current-clamp conditions, the initial resting membrane potential was -44.7 ± 1.8 mV ($n = 31$) and subsequently slowly became more negative as an outward K^+ current developed, until eventually it stabilized at a value (only measured occasionally so as not to interrupt the voltage-clamp experiments) of -72.2 ± 0.8 mV ($n = 6$), close to the equilibrium potential for potassium ions. It is generally accepted that this current develops as the cell interior (and hence the ATP) is slowly dialysed with pipette solution, which results in a progressive activation of ATP-K^+ channels. In order to test the action of diazoxide on these cells under current-clamp, 0.1 mM ATP was present in the pipette solution resulting in a much reduced and slower activation of this current. Under these conditions the CRI-G1 cells were occasionally observed to fire action potentials spontaneously. A typical example of such a current-clamp recording is shown in Figure 1a, where hyperpolarizing current pulses (10 pA and one second duration) were given every four seconds in order to assess changes in cellular conductance (an action potential was usually elicited at the termination of these pulses). Application of 0.6 mM diazoxide to the cell resulted in a cessation of action potential activity, and a progressive hyperpolarization of the cell

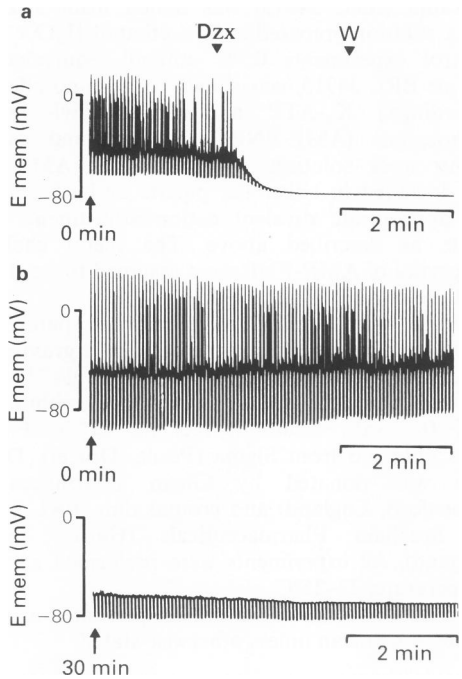

Figure 1 Whole cell current-clamp records from CRI-G1 cells, comparing the time course of hyperpolarization induced by diazoxide (a) with that by cell dialysis (b). Hyperpolarizing current pulses (10 pA and 1 s duration) were applied every 4 s, often resulting in anodal-break action potentials (for the first 5–10 min after formation of the whole cell configuration) although spontaneous action potentials were also evident. (a) The initial resting membrane potential was −41 mV. Bath application of 0.6 mM diazoxide (Dzx) produced an increase in membrane conductance, hyperpolarization (to a value of −74 mV) and inhibition of firing, effects not reversible on wash (W). Compare this with (b) which shows a current-clamp recording from a separate cell, having an initial resting potential of −51 mV. In this experiment no diazoxide was applied and it is apparent from the figure that the time course of increased membrane conductance, hyperpolarization (to a value of −74 mV) and inhibition of firing are much reduced, a steady membrane potential was not attained until 35 min after whole cell formation (lower trace). In both (a) and (b) the extracellular solution was A and the pipette contained solution B and 0.1 mM ATP. The zero minute mark (a) and (b) is equivalent to approximately 30 s elapsed time from the formation of the whole cell to achieving a stable current clamp.

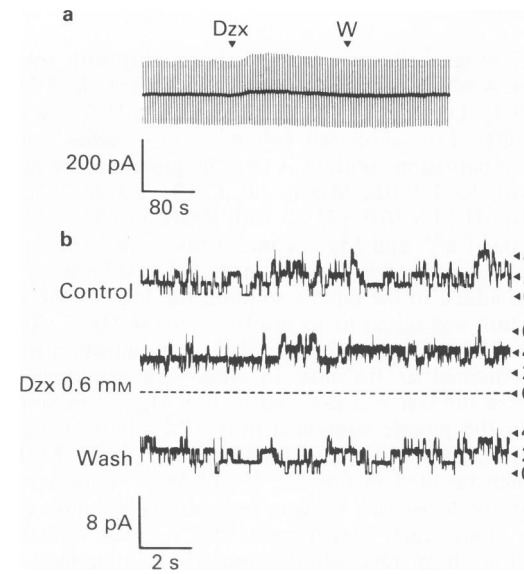

Figure 2 (a) Recording of whole-cell voltage-clamped ATP-K^+ currents. The cell membrane was clamped at −70 mV and the voltage alternately stepped by +10 and −10 mV. The current pulses are denoted by vertical lines. Diazoxide (Dzx, 0.6 mM) induced an increase in current which waned with time of exposure. (b) Activation of ATP-K^+ channels by 0.6 mM diazoxide applied to an outside-out membrane patch, held at a membrane potential of zero mV. Channel openings are denoted by upward deflections (outward currents). The closed state (C) and the number of channels open at a given time are indicated to the right of this and all subsequent isolated patch figures. The values of $N_f \cdot P_o$ were as follows: control 2.208; diazoxide (0.6 mM) 3.359; wash 2.336. In both (a) and (b) the extracellular solution was A and the pipette contained solution B and 0.1 mM ATP.

membrane potential with a concomitant increase in its resting conductance. We found that removal of the diazoxide rarely resulted in any recovery of the resting membrane potential or resting conductance, although, if no diazoxide was applied, at the equivalent time in control experiments (Figure 1b) the cell exhibited only a much slower hyperpolarization and increase in conductance, presumably due to washout of the ATP. In many experiments a transient depolarization preceded the slow hyperpolarization (Figure 1b), an effect not yet understood. The hyperpolarization and increased conductance induced by diazoxide are indicative of activation of a potassium current. This is illustrated more clearly under voltage clamp conditions, where the cell membrane potential is held at −70 mV and alternate ± 10 mV pulses (200 ms duration at 2 s intervals) are given in order to assess the magnitude of the ATP-K^+ current (Trube *et al.*, 1986; Sturgess *et al.*, 1988). As previously shown, the ATP-K^+ current increases slowly as the cell interior is dialyzed, until a peak is reached, thereafter the current declines slowly with time, due to a phenomenon known as channel run-

down (Trube *et al.*, 1986; Sturgess *et al.*, 1988). In the present study the effects of diazoxide were tested on the ATP-K^+ currents after the peak had occurred. Figure 2a shows a voltage-clamp recording from a cell where the pipette contained 100 μM ATP. Addition of 0.6 mM diazoxide produced an increase in the ATP-K^+ current which slowly declined with time and was not reversed on washing. This increase in K^+ current is due to an activation of ATP-K^+ channels. This is shown in Figure 2b where diazoxide (0.6 mM) applied to an outside-out membrane patch (with 0.1 mM ATP present in the electrode) produced a reversible increase in the ATP-K^+ channel activity. Hence, these data clearly indicate that diazoxide induces an increase in the ATP-K^+ current which results in a hyperpolarization of the cell and inhibition of action potential activity. The consequence of these actions would be an inhibition of insulin secretion and this has been observed to occur in these cells (Sturgess *et al.*, 1988).

Diazoxide is also capable of activating ATP-K^+ channels when applied to the cytoplasmic membrane surface, as shown in Figure 3a which is a recording from an inside-out membrane patch. Initially, in control conditions (no ATP present) at least four channels were observed to be active. Application of 0.1 mM ATP to the bath (in the presence of 1.0 mM Mg^{2+}) resulted in an inhibition of channel activity such that only two channels could now be discerned. But following the addition of 0.6 mM diazoxide in the presence of 0.1 mM ATP, there was an increase in the ATP-K^+ channel activity such that it was again possible to observe the opening of four channels simultaneously. This effect of diazoxide was partially reversed on substitution of the drug with the 0.1 mM ATP solution, although it was noticeable that channel activity did not immediately return to pre-exposure levels (Figure 3a). However, when this experiment was repeated using 0.1 mM ATP in the absence of Mg^{2+} ions (by EDTA chelation and omission of $MgCl_2$), it was found that the inhibition of the channels by 0.1 mM ATP was greater than when Mg^{2+} ions were present and that diazoxide did not produce an activation of channel activity (Figure 3b).

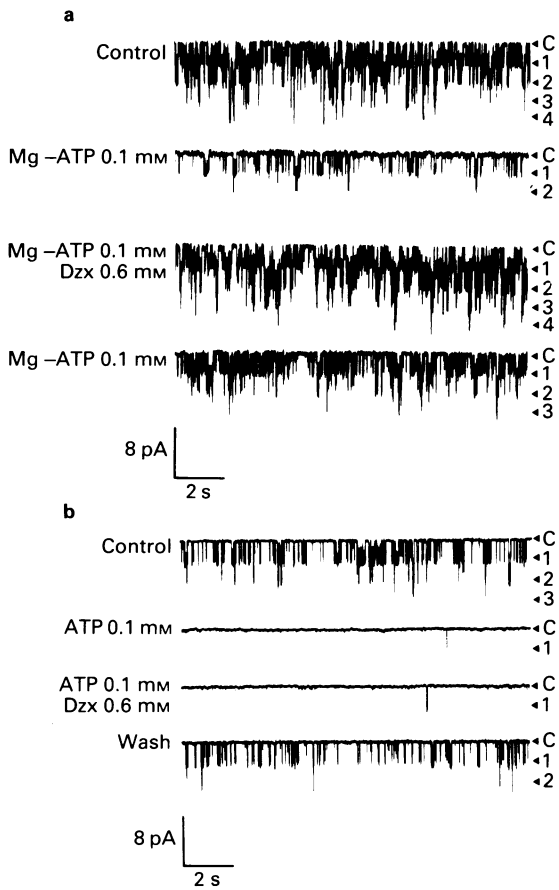

Figure 3 Single channel currents recorded from inside-out membrane patches exposed to symmetrical 140 mM KCl held at a membrane potential of −50 mV. Single channel openings are denoted by downward deflections (inward currents). (a) Activation of ATP-K^+ channel currents by diazoxide (Dzx, 0.6 mM) in the presence of Mg-ATP. The values of $N_f \cdot P_o$ were as follows: control 0.988; Mg-ATP 0.145; Mg-ATP and diazoxide 0.903; Mg-ATP 0.619. (b) Lack of activation of ATP-K^+ channels by diazoxide in the presence of ATP and no Mg^{2+}. Note the increased potency of the ATP producing channel inhibition compared to (a). The values of $N_f \cdot P_o$ were as follows: control 0.207; ATP 0.0005; ATP and diazoxide 0.001; wash 0.072. The electrode solution was E in both (a) and (b) and the bath solution was F in (a) and G in (b).

Involvement of Mg^{2+} ions versus ATP^{4-} for activation

There are two possible explanations for the results shown in Figure 3, that activation requires the presence of Mg^{2+} ions or that the lack of activation in Figure 3b was simply due to an enhanced level of the species ATP^{4-} producing a greater (and therefore non-reversible) degree of block. Dunne *et al.* (1987) have recently put forward evidence supporting the latter possibility and suggest that diazoxide in some way interferes directly or indirectly with the inhibitory effect of ATP^{4-}. The experiments shown in Figure 3 certainly do not distinguish between the possibilities as in Figure 3a, with Mg^{2+} present, the ATP^{4-} concentration (6 μM) was an order of magnitude lower than that for the patch shown in Figure

Table 1 The effects of diazoxide (0.6 mM) on whole-cell voltage-clamped ATP-K^+ currents

Pipette solution	*I/Ic*	
B + 0.1 mM ATP	1.27 ± 0.05	(7)
C + 100 μM ATP	0.87 ± 0.03	(6)
C + 10 μM ATP	0.84 ± 0.03	(4)
B	0.88 ± 0.05	(4)
C	0.75 ± 0.05	(7)

The results are expressed as the mean relative change in current ± s.e.mean, with the number of cells tested in parentheses.
Solution B contains Mg^{2+} ions whereas solution C is effectively Mg^{2+} free (see Methods).

3b (80 μM). Hence, it was decided to test the actions of diazoxide in conditions where the concentrations of ATP^{4-} in the absence or presence of Mg^{2+} ions were more comparable. This was achieved by reducing the total ATP level to 10 μM in the absence of Mg^{2+} ions resulting in an ATP^{4-} concentration of 8 μM.

However, even at this lowered ATP^{4-} concentration diazoxide did not produce an activation of the ATP-K^+ currents, when tested on whole cell currents (Figure 4a and Table 1) or on inside-out patches (Figure 4b and 6b). Replacement of the 10 μM Mg-free ATP with 0.1 mM Mg-ATP (ATP^{4-} = 6 μM) in the presence of diazoxide in the inside-out patch experiment resulted in an activation (Figure 4b). In addition, even with a lowered total ATP concentration of 50 μM ([ATP^{4-}] = 36 μM in Mg^{2+}-free and 4 μM in the presence of Mg^{2+}) activation only occurred if the Mg-ATP species was present (data not shown). Indeed, it appears that, if anything, diazoxide inhibits the currents under Mg-free conditions (Figure 4a,b and Table 1). Altering the levels of total ATP and the Mg^{2+} concentration with EDTA also has the effect of changing the concentration of calcium ions in the solutions. Thus, because the free calcium concentrations varied so widely in these experiments (from approximately 5×10^{-7} M to 2×10^{-8} M), we decided to determine whether diazoxide produced an activation of the ATP-K^+ current in an essentially calcium-free environment. This experiment was performed using outside-out membrane patches rather than whole cells, in an attempt to obviate possible contributions from intracellular storage/release sites. Figure 4c illustrates that diazoxide (0.6 mM) clearly produces an activation of ATP-K^+ channels in the virtual absence of calcium ions (free calcium $< 10^{-9}$ M) but in the presence of Mg-ATP. Thus, from these data, it appears that diazoxide is not capable of activating ATP-K^+ channel currents in the absence of Mg^{2+}

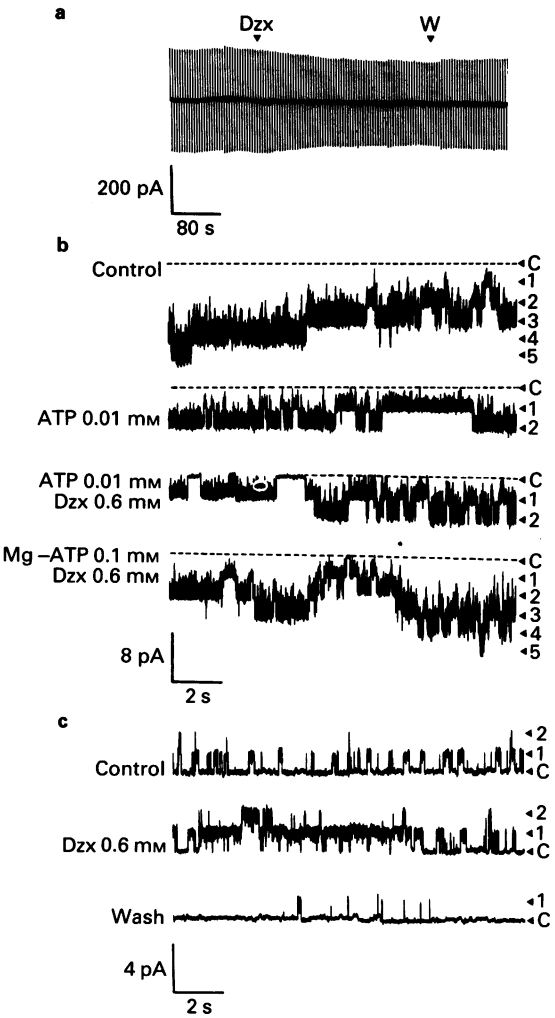

Figure 4 Diazoxide (Dzx) did not activate ATP-K^+ currents with 10 μM ATP and no Mg^{2+} in the 'intracellular' solution, either in (a) voltage-clamped cells where the extracellular solution was A and the solution in the electrode was C plus 10 μM ATP, or (b) an inside-out membrane patch held at a membrane potential of −50 mV (electrode solution was E and bath solution G for 10 μM ATP and F for 0.1 mM Mg^{2+}-ATP). Note that in (b) the ATP-K^+ channels could be activated by diazoxide if the 10 μM ATP was substituted with 0.1 mM Mg-ATP. The values for $N_f \cdot P_o$ in (b) were: control 3.778; ATP 2.202; ATP and diazoxide 1.142; Mg-ATP and diazoxide 1.921. ATP-K^+-channels could be activated by diazoxide in the virtual absence of calcium ions, and this is shown in (c) where an outside-out membrane patch and solution D plus 0.1 mM ATP in the electrode were used. The values for $N_f \cdot P_o$ were: control 0.201; diazoxide 0.394; wash 0.015. The membrane potential was zero mV and the bath solution was A.

ions, regardless of whether whole-cells or isolated patches are used.

The changes in the channel activity induced by ATP and diazoxide could be due to an alteration in the average open-state probability (P_0) of the channels and/or the number of functional channels (N_f). Unfortunately we could not obtain an isolated patch with only one active channel and so performed a binomial analysis of the average current recorded from an inside-out patch in order to try to determine which of these parameters was changing. The result of such an analysis is illustrated in Figure 5 where the probability that a given number of channels are open (P_n) is plotted against the number of channels open (n). Figure 5a shows that the channels open independently of one another (i.e. a good fit is observed to that predicted by the binomial theorem) in an ATP-free solution assuming five channels are active at a measured P_o of 0.196. In the presence of 0.1 mM ATP (Figure 5b), the distribution can now only be well-fitted if it is assumed that two channels are active and at a reduced P_o of 0.072. This is consistent with a reduction in the individual open-state probability and with some of the channels entering a long-lived closed state (an apparent reduction in N_f), as this effect of ATP is reversible on wash (data not shown). Diazoxide (0.6 mM) (in the presence of 0.1 mM ATP) partially reversed this action of ATP (Figure 5c), the distribution being well-fitted by four active channels at a P_o of 0.226. Thus a recovery of the open-state probability and of the number of functional channels is observed.

Therefore in order to present the single channel data quantitatively, and as no change in the single channel amplitude (i) was observed, $N_f \cdot P_o$ was used ($I = N_f \cdot P_o \cdot i$) as the above analysis indicates that both parameters appear to be affected (at least over the time periods used for data analysis). Figure 6 illustrates the combined data from outside-out membrane patches (Figure 6a) for activation of the ATP-K$^+$ current by diazoxide, and for inside-out membrane patches (Figure 6b) and shows the inhibition induced by 0.1 mM total ATP and 10 μM Mg-free ATP and the effect of diazoxide. It is clear from this figure that there is a marked contrast in the action of diazoxide depending upon the presence of Mg^{2+} ions. This is also evident from the whole-cell voltage-clamp data (Table 1).

Diazoxide and Mg^{2+} inhibit ATP-K$^+$ currents

The inhibition of the ATP-K$^+$ current by diazoxide observed in Figure 4 was investigated further in order to determine whether this was also Mg^{2+}- and/or nucleotide-dependent. In the absence of ATP, but with 1 mM Mg^{2+} still present in the solution bathing the cytoplasmic membrane surface, diazo-

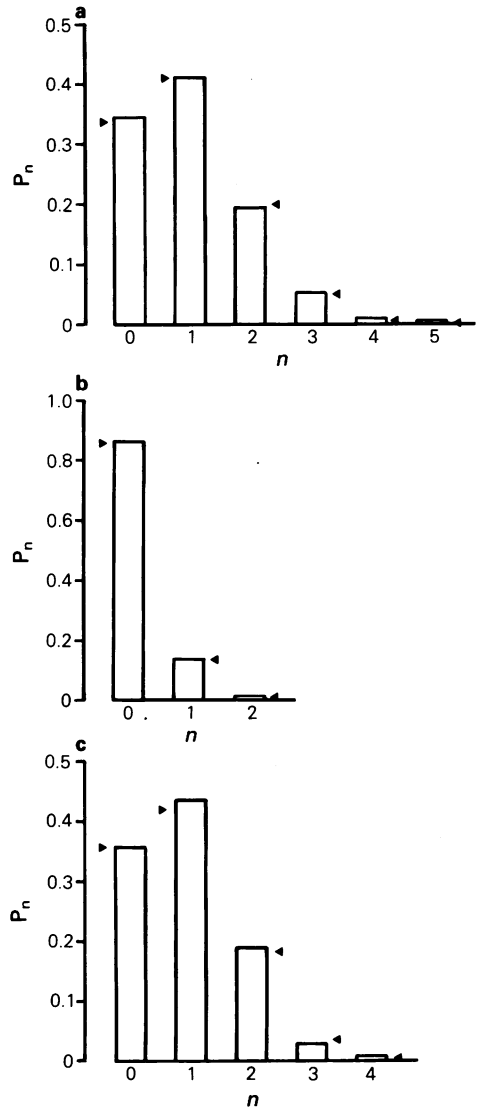

Figure 5 Binomial analysis of single channel data from the inside-out membrane patch shown in Figure 3a. Ordinate scales: probability that a given number of channels, n, are open (P_n). Abscissa scales: the number of channels open, n. The columns show the experimental probabilities obtained using the measured values for each unitary current level. The arrows denote the probabilities predicted by the binomial theorem by use of the measured open-state probability for each channel and the number of channels observed. The experimental probabilities were in (a) ATP-free solution, the arrows indicating the values predicted using a measured P_o = 0.196 and N_f = 5; (b) in a solution containing 0.1 mM Mg-ATP, measured P_o = 0.072 and N_f = 2; (c) in a solution containing 0.1 mM Mg-ATP and 0.6 mM diazoxide, measured P_o = 0.226 and N_f = 4.

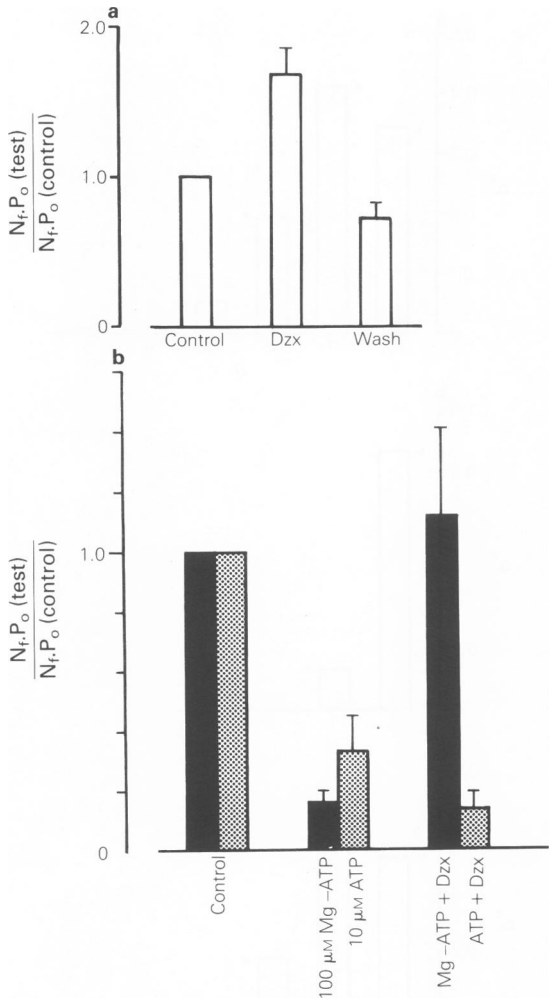

Figure 6 Quantitative effects of diazoxide (Dzx, 0.6 mM) on ATP-K^+ channels recorded from isolated membrane patches. (a) Outside-out membrane patches. The electrode solution was A and the membrane potential was zero mV. The value of $N_f \cdot P_o$ is plotted relative to the control value (in the presence of nucleotide) prior to addition of diazoxide. Values are means of five patches, and vertical lines indicate s.e. (b) Inside-out membrane patches. Electrode solution was E and bath solutions were G for 10 μM ATP and F for 0.1 mM Mg-ATP. The membrane potential was −50 mV. $N_f \cdot P_o$ is plotted relative to control value (i.e. in the absence of nucleotide). Solid columns refer to data from patches exposed to Mg-ATP ($n = 7$) whereas stippled columns refer to those exposed to ATP^{4-} only ($n = 4$). Vertical lines indicate s.e.mean.

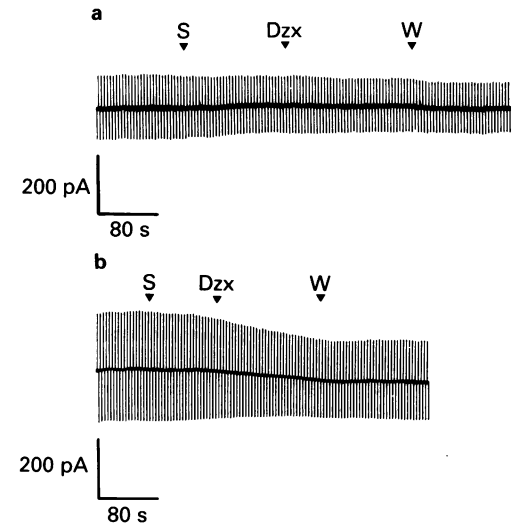

Figure 7 (a) Recording of whole-cell voltage-clamped ATP-K^+ currents (solution B in electrode (i.e. 1 mM Mg^{2+} and no ATP) and solution A in the bath). Diazoxide (Dzx, 0.6 mM) produced little effect on the currents. Inhibition of ATP-K^+ currents by diazoxide was more readily observed in the absence of Mg^{2+} ions and ATP. This is illustrated in (b) using whole-cell voltage-clamp recording (solution C in the electrode and A extracellularly).

xide (0.6 mM) produced little inhibition of the ATP-K^+ current recorded by whole cell clamp (Figure 7a and Table 1). Whereas on outside-out membrane patches the inhibition was seemingly greater (data not shown), the value of $N_f \cdot P_o$ (test)/$N_f \cdot P_o$ (control) being 0.45 ± 0.06 ($n = 5$), although reversibility was not always observed upon washout of the drug, indicating that a substantial run-down of channel activity had occurred during these experiments.

The inhibitory effects of diazoxide were more readily apparent when neither Mg^{2+} nor ATP was present in the 'intracellular' solution. This is shown on whole-cell clamp currents (Figure 7b and Table 1) and was also observed for inside-out patches (data not shown) where $N_f \cdot P_o$ (test)/$N_f \cdot P_o$ (control) was 0.33 ± 0.13 ($n = 4$) in the presence of diazoxide (0.6 mM). Although, again, there was no consistent reversal on removal of the drug indicating that a substantial run-down of channels may have also occurred. The presence of Mg^{2+} ions themselves also produced an inhibition of the ATP-K^+ single channel activity, when present in the solution bathing the cytoplasmic side of the membrane (inside-out patch, and this effect was reversible on wash, $n = 3$). A typical example produced values for

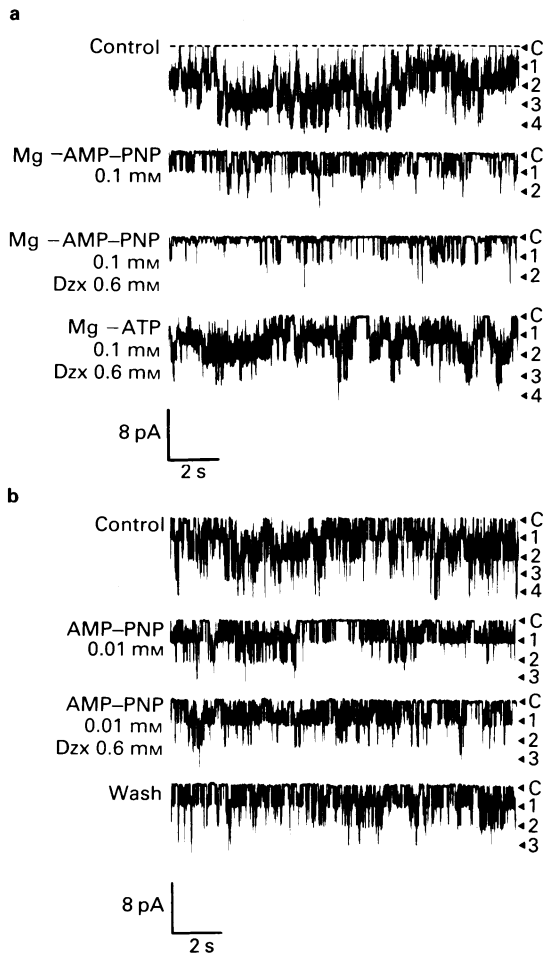

Figure 8 Single channel currents recorded from inside-out membrane patches exposed to symmetrical 140 mM KCl held at a membrane potential of −50 mV. (a) Li_4-adenylyl-imidodiphosphate (AMP-PNP) (0.1 mM) in the presence of Mg^{2+} ions inhibited the activity of ATP-K^+ channels. Addition of diazoxide did not activate the channels but caused further inhibition. Replacement of the Mg-AMP-PNP with an equivalent concentration of Mg-ATP in the presence of diazoxide did induce channel activation (solution E in the electrode and F in the bath). The values for $N_f \cdot P_o$ were as follows: control 2.285; Mg-AMP-PNP 0.204; Mg-AMP-PNP and diazoxide 0.109; Mg-ATP and diazoxide 0.503. (b) A similar experiment in which 10 μM AMP-PNP and no Mg^{2+} was used, clearly indicates that this ATP analogue also inhibits the ATP-K^+ channel activity and, in agreement with the ATP data (Figures 2, 3 and 5), diazoxide induces further inhibition. This effect was also not readily reversed on wash (solution E in the electrode and G in the bath). The values for $N_f \cdot P_o$ were: control 2.397; AMP-PNP 0.805; AMP-PNP and diazoxide 0.325; wash 0.368.

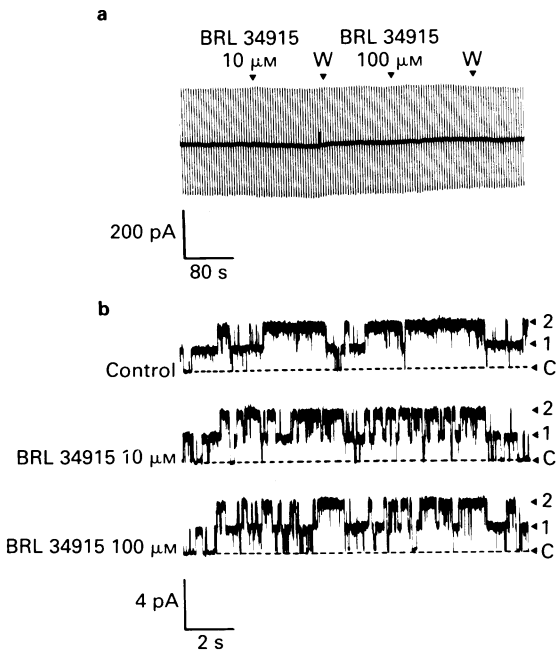

Figure 9 Cromakalim (BRL 34915), tested at concentrations of 10 μM and 100 μM, did not activate ATP-K^+ currents with 0.1 mM ATP present in the intracellular solution either in (a) voltage-clamped cells or (b) outside-out membrane patches (held at a membrane potential of zero mV). In both (a) and (b) the electrode solution was B plus 0.1 mM ATP and the extracellular solution was A. The values for $N_f \cdot P_o$ in (b) were: control 1.161; cromakalim 10 μM 0.875; 100 μM 0.885.

$N_f \cdot P_o$ of, control, 0.368; 1.0 mM Mg^{2+}, 0.049; and wash 0.153. Thus, diazoxide can not only activate ATP-K^+ currents when Mg-ATP is present, but can also produce a depression of these currents in the absence of this species and this is especially marked if neither ATP^{4-} nor Mg^{2+} is present.

Does activation of the ATP-K^+ current require hydrolysis of ATP?

The preceding data therefore show that the diazoxide-induced activation of ATP-K^+ channels is dependent upon the presence of Mg-ATP and cannot simply be ascribed to a displacement of ATP^{4-}. This raises the possibility that nuclectide hydrolysis is required for activation, diazoxide perhaps acting to stimulate an endogenous kinase (which would have to be closely associated with cytoplasmic membrane for the effect to be observed in isolated patches). The observation that diazoxide is more effective in producing activation when applied to the cytoplasmic side of isolated patches (Figure 6) supports this possibility.

Thus the non-hydrolysable analogue, AMP-PNP was substituted for ATP and the effect of diazoxide tested on inside-out membrane patches. This analogue inhibited the ATP-K^+ channels in a manner similar to ATP. Addition of diazoxide (0.6 mM) in the presence of either Mg-AMP-PNP or Mg-free AMP-PNP induced only a further inhibition of channel activity (Figure 8a,b). On replacement of the 100 μM Mg-AMP-PNP with 100 μM Mg-ATP, diazoxide was shown to induce ATP-K^+ channel activation (Figure 8a). Thus, these data suggest that magnesium-dependent hydrolysis of ATP may well be a prerequisite for diazoxide-induced ATP-K^+ channel activation.

Cromakalim does not affect the ATP-K^+ current

The putative K^+ channel activator cromakalim (BRL 34915) was also tested for its actions on the ATP-K^+ currents. This compound was chosen for comparison with diazoxide because certain of its vasorelaxant effects (Wilson *et al.*, 1988) can be inhibited by glibenclamide (a potent and, as far as has been shown, selective ATP-K^+ current inhibitor) and there are conflicting results regarding its effect on insulin and glucose levels *in vivo* (Wilson *et al.*, 1988; Cook *et al.*, 1988b). Application of cromakalim at concentrations of 10 μM and 100 μM to ATP-K^+ whole-cell channel currents, or to single channel currents recorded from outside-out membrane patches, was totally ineffective at inducing either an activation or a significant inhibition with 0.1 mM ATP present in the 'intracellular' environment (Figure 9a,b; Table 2). Similar data were obtained in the absence of Mg^{2+} and/or ATP in the electrode solutions (data not shown).

Discussion

Diazoxide, a sulphonamide derivative which is closely related to the hypoglycaemic sulphonylureas, produces hyperglycaemia in mammals. This increase in blood glucose results mainly from its action to suppress insulin release, although stimulation of catecholamine release and hepatic glycogenolysis contribute to its hyperglycaemic activity (Bowman & Rand, 1980). Recent studies have indicated that diazoxide reduces insulin release from freshly isolated β-cells and insulin-secreting cell lines by activating ATP-K^+ channels (Trube *et al.*, 1986; Dunne *et al.*, 1987; Sturgess *et al.*, 1988; Zunkler *et al.*, 1988). It is argued that activation of this channel hyperpolarizes the cell membrane, thus making it less likely that the cell will fire action potentials in response to stimuli (e.g. nutrients, transmitters or hormones) and so reduce calcium entry and hence insulin secretion. We have shown in this study, using an insulin-secreting cell line, that under current-clamp whole-cell recording conditions, application of diazoxide causes the cessation of action potential activity and this was associated with an increase in resting membrane conductance and hyperpolarization. These effects were due to activation of a potassium current and, in agreement with the previous studies, this was shown to be an ATP-K^+ current.

Diazoxide activates the ATP-K^+ current when applied to either side of the membrane, apparently being more effective when applied directly to the cytoplasmic side. This is evident on comparing data from inside-out membrane patches (Figure 5b) to that of outside-out patches (Figure 5a) or voltage-clamp whole-cell recordings (Table 1). Diazoxide produced approximately a 7 fold increase in current in inside-out patches compared to less than a 2 fold increase when applied at the extracellular membrane (outside-out and whole-cell recording). Diazoxide also activates ATP-K^+ channels in cell-attached patches when applied to the bath solution (Trube *et al.*, 1986; Sturgess *et al.*, 1988). As there is no direct route to the channels due to the diffusion barrier created by the giga-ohm seal (Sakmann & Neher, 1984), diazoxide must partition into the membrane before diffusing to its site of action. Thus, the receptor site for diazoxide appears to be accessed more easily from the intracellular side of the membrane.

In previous studies, diazoxide-induced ATP-K^+ channel activation has been shown to depend upon the presence and concentration of ATP in the intracellular environment (Trube *et al.*, 1986; Dunne *et al.*, 1987; Sturgess *et al.*, 1988). The ATP-K^+.channels were not activated in the absence of ATP or if high concentrations of ATP were present. Dunne *et al.* (1987) have suggested a mechanism for the action of diazoxide, namely that the drug interferes in some way with the inhibitory effect of ATP^{4-}, the purported active species of ATP (Ashcroft & Kakei 1987a; Dunne *et al.*, 1987). However, the data presented here do not concur with this suggestion. On changing the total ATP levels and maintaining the

Table 2 The lack of effect of cromakalim (BRL 34915) on ATP-K^+ currents

[*Cromakalim*] (μM)	*Whole-cell* *I/Ic*	*Outside-out* $N_f \cdot P_o$ (*test*) / $N_f \cdot P_o$ (*control*)
10	0.99 ± 0.02 (6)	0.74 ± 0.13 (5)
100	0.90 ± 0.03 (6)	0.77 ± 0.26 (5)

Results are expressed as the mean relative change in current or $N_f \cdot P_o \pm$ s.e.mean. The number of cells/patches used are shown in parentheses. The electrode solution for both whole-cell and outside-out voltage clamp configurations was B plus 0.1 mM ATP, the extracellular solution was A.

ATP^{4-} concentration relatively constant, we showed that ATP-K^+ channel activation only occurs if the magnesium-ATP species is present, otherwise diazoxide has no effect or produces an inhibition (see below). These effects were observed regardless of whether isolated patches or voltage-clamped whole-cell recordings were used. Thus there is a requirement for the presence of magnesium ions for diazoxide to evoke an activation of ATP-K^+ channel activity. In addition, activation could not be elicited when the ATP was replaced by AMP-PNP either in the presence or absence of magnesium ions. These data indicate that a phosphorylation process may well underlie diazoxide-induced activation. This is different from nucleotide-induced inhibition of channel activity which can be mimicked by non-hydrolysable analogues and probably involves simple nucleotide binding not linked to a metabolic process (Cook & Hales, 1984; Ashcroft, 1988). It has been shown that, for isolated membrane patches, in order to maintain ATP-K^+ channel activity Mg-ATP must be present (Findlay & Dunne, 1986; Misler *et al.*, 1986; Ohno-Shozaku *et al.*, 1987) and that non-hydrolysable analogues are unable to mimic this effect. This suggests that phosphorylation of the ATP-K^+ channel may be required to maintain the channel in an excitable state. If this is the case then the protein kinase responsible must be closely associated with the cytoplasmic membrane for the activation to occur in isolated patches. Diazoxide may elicit activation by stimulating this kinase to increase the activity of the channels in a magnesium-dependent but calcium-independent manner.

The binomial analysis of the single channel data obtained from inside-out membrane patches indicate that diazoxide partially reverses the effects of ATP on the channels. Addition of ATP to inside-out membrane patches produced a reduction in the open state probability and an apparent decrease in the number of functional channels. It is likely that this effect of ATP is due to some channels entering a very long-lived closed state over the time period of our recordings, which manifests itself as an apparent reduction in the number of functional channels. This has also been observed for frog skeletal muscle ATP-K^+ channels (Spruce *et al.*, 1987). Diazoxide seemingly reverses this effect of ATP such that there is an increase both in the number of channels active and in the individual open state probability.

In the absence of Mg-ATP diazoxide was found to produce an inhibition of the ATP-K^+ channel currents and this was especially marked if neither ATP nor magnesium ions was present at the cytoplasmic aspect of the membrane. Thus it is possible that the activation produced by diazoxide may be somewhat underestimated or masked by inhibition. The inhibitory effect of diazoxide is similar to the effect of tolbutamide (a sulphonylurea), although much less potent. However, one difference from tolbutamide block is the lack of reversal of the diazoxide inhibition. This inhibition appears to be progressive (Figure 7b for example) and does not reach steady-state. On wash-out of diazoxide the current level remains relatively constant (but will decline slowly due to run-down). Hence, one explanation for this action of diazoxide is that it enhances the rate of channel run-down, removal of the drug allowing the previous run-down rate to be re-established. The inhibition of the ATP-K^+ channel by diazoxide may also be magnesium-dependent, as in the presence of magnesium ions this action was less noticeable. In agreement with other investigators, we also found that magnesium ions themselves decreased the single-channel activity when applied to the intracellular environment. This has been demonstrated for ATP-K^+ channels, in β-cells (Findlay, 1987a; Ashcroft & Kakei, 1987b) and cardiac muscle (Findlay, 1987b).

Cromakalim (BRL 34915) was found to have no significant effect on ATP-K^+ channels, producing neither an activation nor an inhibition. This agent is thought to produce its anti-hypertensive action by acting as a potassium channel activator. At present it is not clear which potassium channel this drug acts upon. However, from the data presented here it is unlikely that diazoxide and cromakalim act by a similar mechanism upon ATP-sensitive potassium channels in insulin-secreting cells. These data support the conclusions of Wilson *et al.* (1988) that cromakalim had no effect on plasma insulin levels.

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A purified lipocortin shares the anti-inflammatory effect of glucocorticosteroids *in vivo* in mice

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1 The injection of a suspension of a polyacrylamide gel (bio gel) into the dorsal subcutaneous area of mice induced an inflammatory reaction and the migration of neutrophils towards the inflamed site.

2 The intravenous administration of anti-inflammatory drugs (dexamethasone, indomethacin and lysine-acetylsalicylate) to polyacrylamide gel-treated mice inhibited the accumulation of neutrophils in the inflamed site.

3 A similar administration of a 36 K mouse lipocortin, induced a strong dose-dependent inhibition of neutrophil accumulation in the inflamed site.

4 Dexamethasone and lipocortin inhibited the production of eicosanoids, prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) in the inflamed site of polyacrylamide gel-treated mice.

5 Lipocortin impaired both phospholipase A_2 (PLA_2) activity and chemotaxis of isolated inflammatory neutrophils.

6 The present studies show an *in vivo* anti-inflammatory effect of lipocortin similar to that of glucocorticosteroids. In agreement with recent data on the extracellular effects of various lipocortins, these results might implicate lipocortin(s) in the anti-inflammatory effects of glucocorticosteroids.

Introduction

Glucocorticosteroids are widely used as potent anti-inflammatory drugs (Lewis & Piper, 1981; Townley & Suliaman, 1987). The mechanism of action of glucocorticosteroids as anti-inflammatory agents has received a tentative explanation over the last ten years. These steroids induce the synthesis of phospholipase A_2 (PLA_2) inhibitory proteins now referred to as lipocortins (Hirata *et al.*, 1980; Blackwell *et al.*, 1980; Cloix *et al.*, 1983; Errasfa *et al.*, 1985). Lipocortins have been shown to inhibit soluble phospholipase A_2 *in vitro* and eicosanoid synthesis in whole cells. These effects could be responsible for part of the anti-inflammatory effects of glucocorticosteroids. To support this hypothesis, it has been shown that the anti-inflammatory effects of glucocorticosteroids involve gene expression and protein synthesis (Tsurufuji *et al.*, 1979) and are correlated with inhibition of eicosanoid production (Parente *et al.*, 1984). Recently, the full cDNA sequence of lipocortin I was obtained (Wallner *et al.*, 1986). It showed that this protein belonged to a family of at least six homologous intracellular proteins (Pepinsky *et al.*, 1988) and had no leader peptide to permit its secretion. Although lipocortins are intracellular proteins, extracellular lipocortin(s) have also been found in the supernatant of glucocorticosteroid-treated cells (Hirata *et al.*, 1980; Blackwell *et al.*, 1980; Cloix *et al.*, 1983; Errasfa *et al.*, 1985), suggesting that these proteins may be secreted and may interact with other cells. Indeed, the recombinant lipocortin I was shown to inhibit, both thromboxane A_2 release from guinea-pig activated lungs (Cirino *et al.*, 1987) and prostacyclin production in human endothelial cells (Cirino &

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Flower, 1987). Moreover, we could demonstrate that lipocortins were able to inhibit cellular phospholipase A_2 from mouse thymocytes (Rothhut *et al.*, 1987; Errasfa *et al.*, 1989) and guinea-pig alveolar macrophages (Errasfa *et al.*, 1988). In parallel, we have shown that a 36 K mouse lipocortin was able to impair the production of leukotriene B_4 (LTB_4) and Paf (platelet-activating factor) in rat inflammatory neutrophils (Fradin *et al.*, 1988). These cellular effects of lipocortins have not been explained yet in terms of their molecular mechanism, although their inhibitory effect on PLA_2 *in vitro* has been attributed to their ability to bind to acidic phospholipids (Davidson *et al.*, 1987; Aarsman *et al.*, 1987). Moreover, the ability of lipocortins to reproduce the anti-inflammatory effect of glucocorticosteroids *in vivo* needs to be studied with highly purified lipocortins.

In this study, we present results which indicate that lipocortins share the anti-inflammatory effects of glucocorticosteroids *in vivo*. Our data might implicate lipocortin(s) in the anti-inflammatory effects of glucocorticosteroids.

Methods

Lipocortin preparation

A 36 K lipocortin was purified either from mouse thymuses or lungs. Protein purification was performed as described previously (Errasfa *et al.*, 1988; Errasfa & Russo-Marie, 1988a). Briefly, thymuses or lungs were removed from mice, then homogenized and sonicated. A first centrifugation allowed the elimination of a crude material. From the resulting supernatant, proteins were extracted by calcium precipitation-EGTA solubilization, then purified on a Mono Q column HR 5/5 using fast protein liquid chromatography (Pharmacia). Biochemical and immunological analysis showed that the two 36 K proteins purified either from mouse lungs or thymuses were identical. Therefore, they are referred to as 36 K lipocortin, this lipocortin is identical to a 32 K lipocortin from human peripheral blood mononuclear cells (Errasfa & Russo-Marie, 1988a). Human recombinant lipocortin I was a gift from Dr Jeff Browning (Biogen, Cambridge, MA, U.S.A.).

Inflammatory model

The experimental inflammatory model has been described in detail previously (Errasfa & Russo-Marie, 1988b). Briefly, mice were anaesthetized with ether, and 2 ml of a 150 mg ml^{-1} polyacrylamide gel suspension were injected into the dorsal subcutaneous tissue of mice. After a 4–7 h period, mice were killed and the subcutaneous area washed with phosphate buffered saline (PBS) in order to collect the gel and the migrated inflammatory cells (polymorphonuclear leucocytes (PMNs) > 90% of total cells). The gel was decanted, then the cells present in the supernatant (Sn1) were counted using a Coulter counter ZBI. Sn1 was then centrifuged at 300 *g* at 4°C. Prostaglandin E_2 (PGE_2) and LTB_4 were measured in the resulting supernatant (Sn2) by a specific radioimmunoassay according to the method of Salmon *et al.* (1982).

Drug treatment of mice

(i) Mice received intravenously 200–300 μl of either dexamethasone (0.15 and 1.5 mg kg^{-1}), indomethacin (0.5 and 5 mg kg^{-1}) or lysine-acetylsalicylate (50 and 150 mg kg^{-1}). Control mice received ethanol at an appropriate dilution for matching dexamethasone- and indomethacin-treated mice, and saline for matching lysine-acetylsalicylate-treated mice. Two hours after dexamethasone or 1 hour after acetylsalicylate or indomethacin administration, mice received the polyacrylamide gel suspension.

(ii) Two hundred to three hundred μl of the protein (P) or its vehicle (V) (saline containing the same concentration of the buffer mixture used to purify the protein) were injected intravenously into mice. After a 0.5–1 h period, mice received the polyacrylamide gel suspension subcutaneously.

After administration of the anti-inflammatory drugs or the protein, and injecting the gel, mice were killed after a predetermined period (4–7 h). The number of migrated cells was counted and PGE_2 and LTB_4 measured as described above.

Neutrophil phospholipase A_2 activity estimation

Mice were killed 6 h after receiving the polyacrylamide gel. Cells were obtained as described above. They were washed with PBS and labelled with [^{3}H]-arachidonic acid (AA) (5 μCi per 10^8 cells per 20 ml of MEM-HEPES medium) for 30–40 min at 37°C. Cells were then washed with PBS containing 0.5% fatty acid free-bovine serum albumin (FAF-BSA) to eliminate unbound [^{3}H]-AA from the incubation medium. Labelled PMNs (25,000–30,000 c.p.m. per 3–4 $\times$ 10^6 cells in 0.45 ml) were resuspended in MEM-HEPES medium, pH 7.4 and preincubated 20 min at 37°C with 5 μg of mouse 36 K lipocortin. Control cells received the vehicle of the protein. The reaction was started by the addition of 0.45 ml of the

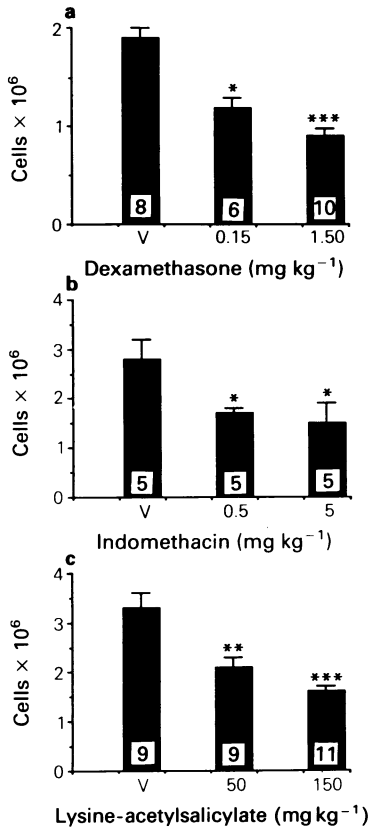

Figure 1 Inhibition of cell migration induced by anti-inflammatory agents in polyacrylamide gel-induced inflammation in mice. Mice received, intravenously, (a) dexamethasone, (b) indomethacin or (c) lysine-acetylsalicylate. Then, they received 2 ml of a 150 mg ml^{-1} gel suspension subcutaneously. Five hours later, the animals were killed and the number of the migrated PMNs counted as described in Methods. The number of animals is indicated inside each column. Each column represents the mean number of cells per animal. Vertical bars show s.e.mean. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

cell suspension to 50 μl of the calcium ionophore A23187 or the chemotactic peptide N-formyl-methionyl-leucyl-phenylalamine (fMLP) at a known concentration. Unstimulated cells received the solvent (DMSO) at the appropriate concentration (0.01%). The reaction was terminated by the addition of 500 μl of 10 mM EGTA containing 0.5% FAF-BSA, pH 7.4. The samples were centrifuged for 5 min at 10,000 g, and aliquots of the supernatants were counted by liquid scintillation spectrometry to determine the released radioactivity. The cellular PLA_2 activity of PMNs was estimated as the release of [^{3}H]-AA from the cells.

Measurement of neutrophil chemotaxis

The chemotaxis assay was performed as described by Harvath *et al.* (1980). PMNs were obtained from mice that had received the suspension of polyacrylamide gel 6 h previously. The cells were washed with PBS pH 7.4 and adjusted to 10^6 cells ml^{-1} in the following buffer (mM): NaCl 145, KCl 5, $MgSO_4$ 1, HEPES 10, glucose 10, $CaCl_2$ 1, pH 7.4. The cells (0.45 ml) were incubated with increased concentrations of the protein or with its vehicle (50 μl) at 37°C for 20–30 min. Then 50 μl of the cell suspension was subjected to the chemotaxis assay using 1 μM FMLP for 40 min at 37°C in 5% CO_2 in a humidified atmosphere. A 48-well micro chemotaxis assembly (Neuro Probe, Inc) was used fitted with 25 × 80 mm polycarbonate membranes of 3 μm pore size (Nucleopore). The migrating cells which traversed and adhered to the membrane were stained with Diff-Quik staining reagents. The cells were counted by use of a light microscope. The result of a single well is expressed as a mean of counts from ten separate fields observed at 40×. The final result of each assay is expressed as the mean ± s.e.mean of several replicate wells.

Measurement of leukocytes and platelets in mouse whole blood

Blood samples (50 μl) were collected from the retroorbital vein of mice. Total leukocyte and platelet counting was performed with a Coulter counter ZBI.

Chemicals

The following products were purchased from Sigma; dexamethasone, calcium ionophore A23187, FMLP, BSA-FAF, EGTA and indomethacin. Lysine-acetylsalicylate (Aspegic) was from Egic Joullié, France. Polyacrylamide gel (Bio Gel, P4, 200–400 mesh) was purchased from Biorad (Paris, France). DMSO was from Merck (Paris, France). HEPES and MEM were purchased from Eurobio (Paris, France). Prostaglandin E_2 (PGE_2) was a gift from J. Pike (The Upjohn company, Kalamazoo, Michigan, U.S.A.). [^{125}I]-PGE_2 and antibodies against PGE_2 were from Pasteur Institute (Paris, France). [^{3}H]-LTB_4 and [^{3}H]-arachidonic acid were purchased from Amersham (Paris, France) and the kit for LTB_4 radioimmunoassay from Wellcome (Paris, France).

Statistical analysis

The results are expressed as mean ± s.e.mean. Means were compared by use of Student's t test. In

our experimental conditions, differences were considered to be significant when P values were less than 0.05.

Results

Effect of anti-inflammatory drugs on cell migration

The injection of a suspension of polyacrylamide gel into the dorsal subcutaneous tissue of mice induced an inflammatory reaction, the latter was characterized by the accumulation of PMNs, and the production of inflammatory mediators such as PGE_2 and LTB_4 in the inflamed site of mice. The intravenous administration of all anti-inflammatory drugs (dexamethasone, indomethacin, lysine-acetylsalicylate) reduced the migration of PMNs toward the polyacrylamide gel (Figure 1).

Effect of two lipocortins (LCI and 36 K) on cell migration

Intravenous injection of the 36 K lipocortin in mice reduced dose-dependently the migration of PMNs to the subcutaneous tissue of mice (Figure 2a). A comparable inhibitory effect on cell accumulation was observed when mice received an intravenous injection of human recombinant lipocortin I (15 μg per animal): the number of migrated cells was $(10.1 \pm 1.9) \times 10^6$ in vehicle-treated mice versus $(4.1 \pm 0.5) \times 10^6$ in lipocortin I-treated mice (mean $\pm$ s.e.mean, $n = 6$, $P < 0.01$).

Intravenous administration of boiled 36 K lipocortin (10 μg per animal) to the animals had no effect on the inflammatory reaction: $(9.1 \pm 2.5) \times 10^6$ cells in vehicle-treated mice versus $(8.1 \pm 2.0) \times 10^6$ cells in boiled lipocortin-treated mice ($n = 4$). Administration of the 36 K lipocortin to the animals did not induce any change in their whole blood platelet and leukocyte counts. Platelets and leukocytes were $(6.7 \pm 1.6) \times 10^8$ and $(1.7 \pm 0.3) \times 10^6$ cells ml^{-1} respectively in 36 K lipocortin-treated mice versus $(6.4 \pm 1.2) \times 10^8$ and $(1.5 \pm 0.4) \times 10^6$ cells ml^{-1} respectively in vehicle-treated mice ($n = 10$). Moreover, the percentage of circulating blood neutrophils and mononuclear cells was not changed (data not shown).

Effect of dexamethasone and 36 K lipocortin on eicosanoid production

The inhibition of cell accumulation induced by the protein was accompanied by an inhibition of PGE_2 and LTB_4 production in the inflamed site (Figure 2b). When results were expressed as ng per 10^6 cells, the amounts of LTB_4 decreased significantly ($P < 0.001$) from 24.6 ± 1.5 (vehicle) to 13.9 ± 2.5

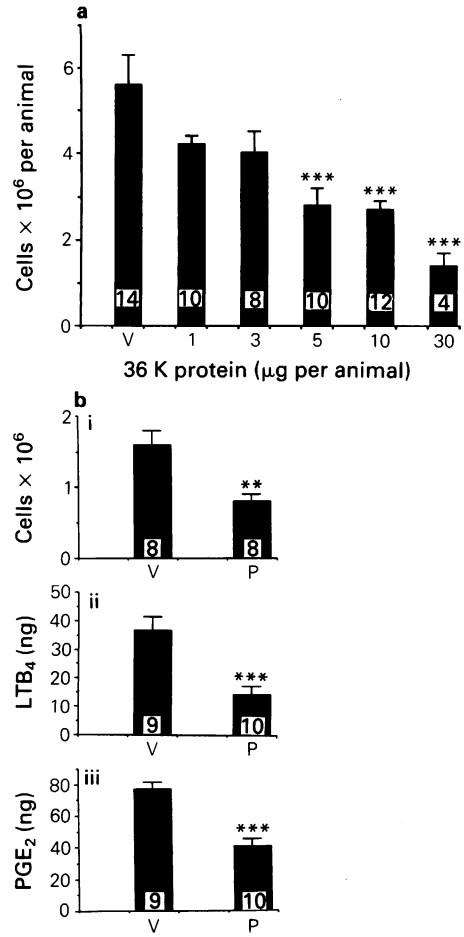

Figure 2 Effect of intravenous administration of the 36 K lipocortin on the migration of PMNs in inflamed mice. Mice received the protein 0.5–1 h before administration of 2 ml of a 150 mg ml^{-1} polyacrylamide gel suspension. (a) Inhibition of cell migration (expressed as cells per animal) by increasing doses of the 36 K lipocortin. Mice were killed 7 h after receiving the polyacrylamide gel suspension. (b) Inhibition of cell migration (i) and of production of leukotriene B_4 (LTB_4) (ii) and prostaglandin E_2 (PGE_2) (iii) by 10 μg of the 36 K lipocortin. Mice were killed 4 h after receiving the polyacrylamide gel suspension. The number of animals per group is indicated inside the columns. Protein-treated (P) mice were compared to vehicle-treated (V) mice. Values are expressed as means with vertical bars showing s.e.mean. ** $P < 0.01$ and *** $P < 0.001$.

Table 1 Amounts of leukotriene B_4 (LTB_4) and prostaglandin E_2 (PGE_2) in the inflamed site of mice receiving either dexamethasone (0.15 and 1.50 mg kg^{-1}) or its vehicle

	Vehicle	*Dexamethasone* (mg kg^{-1}) *0.15*	*1.50*
LTB_4			
ng per exudate	67 ± 5	47 ± 5*	37 ± 7**
ng per 10^6 cells	36 ± 4	34 ± 2	28 ± 3
PGE_2			
ng per exudate	19 ± 2	nd	13 ± 2*
ng per 10^6 cells	12 ± 1	nd	16 ± 2

The experimental protocol is the same as described in Figure 1. The amounts of LTB_4 and PGE_2 are expressed as ng per exudate and as ng per 10^6 cells. Results are shown as mean ± s.e.mean; n = between 5 and 9 animals per group. ND: not done. * $P < 0.05$, ** $P < 0.01$.

(36 K protein), whereas the amounts of PGE_2 were not different (47.0 ± 4.6 and 52.0 ± 5.0) in vehicle and protein-treated mice, respectively.

Similarly, dexamethasone induced an inhibition of eicosanoid production (ng per mice) in the inflamed site of mice. However, when eicosanoids were expressed as ng per 10^6 cells, the amount of LTB_4 was decreased whereas levels of PGE_2 were not (Table 1).

Effect of 36 K lipocortin on [^{3}H]-arachidonic acid release from isolated neutrophils

As shown in Figure 3, when the 36 K lipocortin was incubated with inflammatory mouse PMNs, it inhibited [^{3}H]-arachidonic acid release from both A23187- and FMLP (0.1 μM)-stimulated PMNs. The boiled protein was inactive (data not shown).

Effect of 36 K lipocortin on neutrophil chemotaxis

The effect of the 36 K lipocortin on PMN chemotaxis was investigated *in vitro*. As shown in Figure 4, 36 K lipocortin impaired, in a dose-dependent manner, PMN migration toward the chemotactic stimulant FMLP.

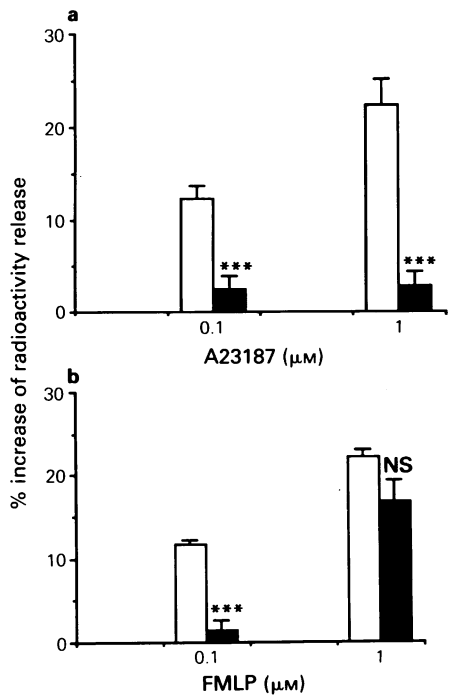

Figure 3 Inhibition of PMN phospholipase A_2 (PLA_2) activity by 5 μg 36 K lipocortin. PMNs were obtained from mice after they had received, 6 h before, 2 ml of a 150 mg ml^{-1} gel suspension. The cells were labelled with [^{3}H]-arachidonic acid, then incubated with the 36 K lipocortin for 20 min. Cell stimulation by (a) the calcium ionophore A 23187 or (b) N-formyl-methionyl-leucyl-phenylalanine (FMLP) and measurement of the released radioactivity are described in Methods. Results are expressed as % increase of radioactivity released from stimulated cells versus DMSO-treated cells. Vehicle-treated cells (open columns, $n = 3$) were compared to protein-treated cells (solid columns, $n = 6$). Results are expressed as mean with vertical bars showing s.e.mean. *** $P < 0.001$.

Discussion

Our findings show that purified lipocortin(s) can reproduce some of the anti-inflammatory effects of glucocorticosteroids in mice with an inflammatory lesion, since: (i) purified mouse 36 K lipocortin and recombinant lipocortin I decreased the accumulation of PMNs at the injured site; (ii) the mouse 36 K lipocortin decreased the amounts of LTB_4 and PGE_2 in the exudate of the injured animals; (iii) the mouse 36 K protein impaired, as glucocorticosteroids do (Hirata *et al.*, 1980), both cellular PLA_2 activation and chemotaxis of mouse PMNs *in vitro*. Taken together, these data suggest that lipocortins might be related to glucocorticosteroids in some way. Indeed, lipocortins were initially found as extracellular proteins after glucocorticosteroid treatment both in cell

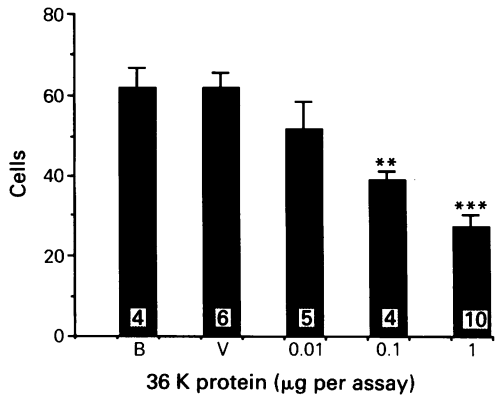

Figure 4 Inhibition of PMN chemotaxis by the 36 K lipocortin. PMNs were obtained from mice after they had received, 6 h before, 2 ml of a 150 mg ml^{-1} gel suspension. They were prepared as indicated in Methods, and incubated with increasing concentrations of the 36 K lipocortin. Cells (50 μl) were then subjected to the migration assay in the chemotaxis assembly. The number of cells which migrated toward the chemoattractant peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP) were counted as described in Methods. The number of replicates is indicated inside the columns. B: buffer medium-treated cells, V: cells incubated with vehicle for the protein. Protein-treated cells were compared to vehicle-treated cells. ** $P < 0.01$, *** $P < 0.001$.

supernatants (Hirata *et al.*, 1980; Cloix *et al.*, 1983; Errasfa *et al.*, 1985) and rat peritoneal lavage fluid (Blackwell *et al.*, 1980). In addition, the first complete purification and partial sequencing of lipocortin I were performed on extracellular lipocortin (Pepinsky *et al.*, 1986), although the cDNA sequence of lipocortin I showed that this protein had no leader peptide to permit its secretion. Moreover, recently it has been found that recombinant lipocortin I inhibits both thromboxane release from guinea-pig isolated, perfused lungs (Cirino *et al.*, 1987) and prostacyclin from human endothelial cells (Cirino & Flower, 1987), and that other lipocortins are able to inhibit PLA_2 activity of mouse isolated thymocytes (Rothhut *et al.*, 1987; Errasfa *et al.*, 1989) and guinea-pig alveolar macrophages (Errasfa *et al.*, 1988). Taking these results into account, we hypothesized that if lipocortins were able to inhibit cellular PLA_2 activity when added exogenously, they may also be able to reproduce part of the anti-inflammatory effects of glucocorticosteroids when injected into the whole animal *in vivo*. Our results showing similar effects for lipocortins and glucocorticosteroids support such a hypothesis but do not give us any insight into the putative mechanism of action. We, therefore, tried to estimate how lipocortins might be anti-inflammatory.

Lipocortins inhibited both cell migration and eicosanoid production *in vivo* suggesting that at least two inflammatory parameters were altered: (i) cell migration i.e. chemotaxis and/or (ii) LTB_4 and PGE_2 production i.e. PLA_2 activity. In order to estimate more precisely whether *in vivo* PLA_2 activity was altered by lipocortin, we compared both the amounts of eicosanoids at the inflamed site and the ratio of these amounts correlated to the number of migrated PMNs. When results were expressed per cell, the amounts of LTB_4 decreased significantly, whereas the amount of PGE_2 were unchanged. Since LTB_4 is known to derive mainly from PMNs, these results suggested that PLA_2 activity of PMNs was impaired in lipocortin- and dexamethasone-treated mice, although the amounts of PGE_2 were not altered. This discrepancy between inhibition of PGE_2 and LTB_4 production may be due to the fact that PGE_2 originates from cells, located in the subcutaneous tissue, not taken into account when results were expressed per cell. To analyse further this effect, the same lipocortin used *in vivo* was assayed *in vitro* to check its ability to alter the cellular PLA_2 activity of neutrophils isolated from the inflammatory tissue. Indeed the 36 K lipocortin was able to inhibit the release of [^{3}H]-AA induced by both the calcium ionophore A23187 and 0.1 μM FMLP, indicating that on those neutrophils, lipocortin was able to inhibit PLA_2. The absence of effect for the higher dose of FMLP is not clear, it might be because at a high dose FMLP induces the release of AA through another activation pathway not involving PLA_2.

The second parameter likely to be altered by lipocortin was PMN chemotaxis. We therefore used the same approach to check whether lipocortin could alter the *in vitro* chemotaxis of isolated neutrophils. The 36 K lipocortin was able to inhibit the FMLP-induced chemotaxis in a dose-dependent manner. Therefore, the *in vitro* impairment of both PMN chemotaxis and PLA_2 activity by the 36 K lipocortin could occur *in vivo* and could participate in the *in vivo* inhibition of cell migration and eicosanoid production at the inflamed site in mice. Recently, we have shown (Fradin *et al.*, 1988) that the same 36 K lipocortin was able to impair the production of two chemoattractant agents; LTB_4 and Paf in rat PMNs. This effect could contribute to the anti-inflammatory effect induced by the 36 K lipocortin.

The ability of lipocortins to bind to acidic phospholipids *in vitro* in a calcium-dependent manner is known to be responsible for *in vitro* inhibition of both PLA_2 activity (Aarsman *et al.*, 1987; Davidson *et al.*, 1987) and blood coagulation (Funakoshi *et al.*,

1987; Chap *et al.*, 1988). Hence, we hypothesize that lipocortins bind to membrane phospholipids of activated cells within the vessel, inducing a modification of the membrane which leads to protection against a further PLA_2 attack of membrane phospholipids. PMNs would then become unable to migrate towards the inflamed site and to release PLA_2-derived mediators. The possibility that the inhibitory effect of lipocortins on PMN chemotaxis is related to PLA_2 inhibition is plausible but is not yet proven. Further investigations are required to explain the physiological presence of extracellular lipocortins and their *in vivo* effect. Nevertheless, the recently described phospholipase A_2 inhibitory effect by lipocortins in whole cells allows us to speculate that these proteins may play a role in the protection of cells against their activation by different stimuli.

Addendum

After completion of this manuscript, a paper was published (L. Miele *et al.*, 1988) describing the anti-inflammatory effect of lipocortin I and uteroglobin in the carrageenan-induced rat paw oedema.

We thank Dr Babette Weksler and Dr Robert Fauve for their valuable discussions.

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Sodium pentobarbitone enhances responses of thalamic relay neurones to GABA in rat brain slices

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1 In rat isolated thalamic slices, intracellular recordings were made of responses to electrophoretically applied γ-aminobutyric acid (GABA) in the absence and presence of sodium pentobarbitone (NaPb).

2 Responses to electrophoretically applied GABA were biphasic in the majority of neurones studied. They consisted of an early, negative-going and later, positive-going phase, when recorded close to reversal potential.

3 An increase in the GABA ejection current caused an enhancement of the late positive-going phase, along with a shift in the reversal potential of the whole response to a more positive value. These changes were accompanied by an increase in GABA-induced conductance (decrease in resistance) and an increase in the duration of the response.

4 Application of NaPb to the GABA response produced similar effects, namely an enhancement of the late positive-going phase, a shift in the reversal potential to a more positive value, an increase in conductance and an increase in duration.

5 GABA-mediated inhibitory postsynaptic potentials (i.p.s.ps) were evoked by electrical stimulation of the nucleus reticularis thalami. Application of NaPb caused an increase in the duration of the i.p.s.p. and a shift in its reversal potential to a more positive value.

Introduction

A model, derived from experiments by Steriade and coworkers (Steriade *et al.*, 1971; 1977; 1986; Deschenes *et al.*, 1982; 1984; Roy *et al.*, 1984) implicates γ-amino-butyric acid (GABA)-mediated inhibition, in the mechanism underlying the transition from tonic to burst firing in thalamic relay neurones at the onset of slow wave sleep. Since the *in vivo* studies that give the most comprehensive model for slow wave sleep generation to date, were performed under barbiturate anaesthesia, it was of interest to determine whether barbiturates enhance $GABA_A$-receptor mediated responses in thalamus, and thus contributes to the ability of these drugs to promote slow wave sleep. *In vitro* studies in cultured mouse spinal neurones (Ransom & Barker, 1975; 1976), in hippocampal pyramidal neurones (Nicoll 1975; Scholfield, 1977; Gage & Robertson, 1985) and in sympathetic ganglion cells (Brown & Constanti, 1978) have shown that barbiturates primarily increase $GABA_A$ activated Cl^- channel open time.

Thalamic relay neurones disconnected from the nucleus reticularis thalami did not display the rhythmic, long-lasting inhibitory postsynaptic potentials (i.p.s.ps) typical of thalamic neurones during slow wave sleep (Steriade *et al.*, 1985). The reticularis thalami is therefore proposed to be the origin of these i.p.s.ps. Stimulation of the reticularis thalami *in vitro* (Thomson, 1988a,b), elicited i.p.s.ps in thalamic relay cells. These i.p.s.ps and responses to GABA were biphasic, the early component displaying a more negative reversal potential than the later component, although both components appeared to be mediated by $GABA_A$-receptors and Cl^- channels. The later component was, however, more sensitive to antagonists. Biphasic responses to GABA have also been recorded in the hippocampus (Alger & Nicoll, 1979; Anderson *et al.*, 1980). Here, the later phase was seen more clearly in the presence of barbiturates or benzodiazipines (Biscoe & Duchen, 1985a,b; Blaxter *et al.*, 1986). It was therefore of interest to

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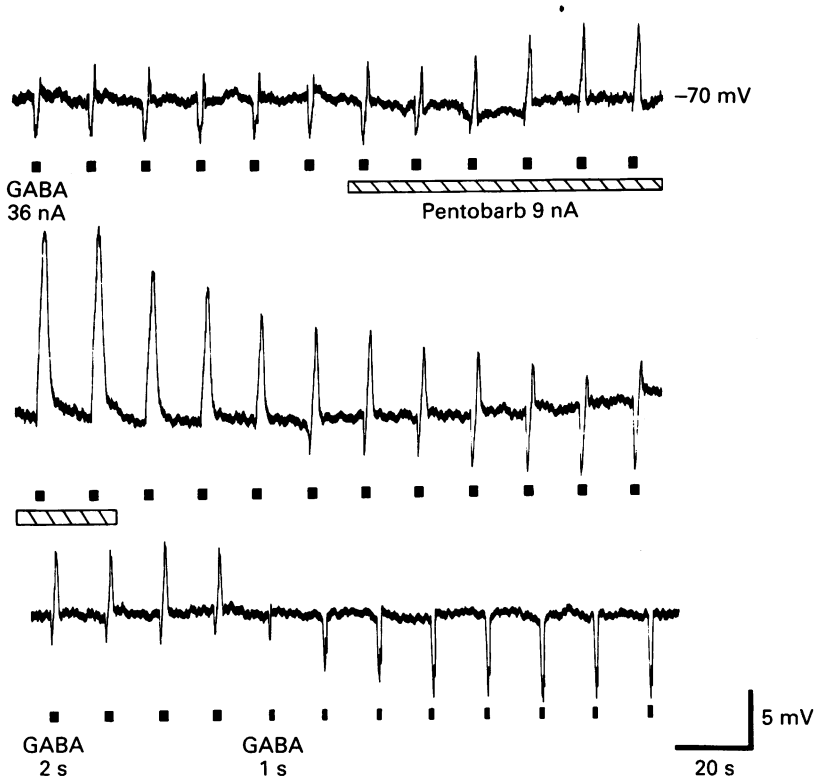

Figure 1 Responses of a thalamic relay neurone to pulses of γ-aminobutyric acid (GABA) applied electrophoretically. All pulses were 36 nA. The top record illustrates the transition from a biphasic response in which the early, negative component predominated, to one in which the later positive-going component predominated during the application of Na pentobarbitone (9 nA, hatched bar). Twenty minutes later (middle record) the response was large and entirely positive-going, but the negative component reappeared on termination of the pentobarbitone. Approximately one hour later (bottom record) the control biphasic response to a 2 s GABA pulse became entirely negative-going when the pulse duration was reduced to 1 s. Membrane held at −70 mV by negative current injection throughout.

determine whether the later component of the i.p.s.p. and the GABA response was similarly more sensitive to barbiturate in thalamus. Accordingly, experiments were designed in which the i.p.s.ps evoked by stimulation of reticularis thalami and responses to GABA in thalamic relay neurones were challenged with sodium pentobarbitone (NaPb). A preliminary account of this work has been published (Sykes *et al.*, 1988).

Methods

Methods similar to those used here have been described previously (Thomson, 1988a). Female rats, body weight 120 to 180 g, were anaesthetised with halothane and decapitated. Coronal slices of brain, 400 μm thick, containing the anterior portion of the thalamus were cut and maintained at 35°C at the interface between warm humidified gas (O_2/CO_2 95%/5%) and medium containing (mM): NaCl 124, $NaHCO_3$ 25.5, KCl 3.3, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.0 and D-glucose 15. Intracellular recordings were made using 3 M K-acetate-filled micropipettes with tip resistances of 60–100 MΩ. Conventional current clamp recording methods were used. Drugs were applied electrophoretically from a multibarrel pipette whose 3 barrels contained GABA (200 mM, pH 3.5), NaPb (20 mM, pH 9.5; Curtis & Lodge, 1977, or 100 mM, pH 9.5) and NaCl (150 mM, balance barrel). GABA was ejected with pulses of 1–4 s duration. NaPb when applied, was applied continuously. Ejection currents between 10 and 75 nA for GABA and between 10 and 45 nA for NaPb were found to be effective. I.p.s.ps were evoked by stimulating the reticularis thalami via a twisted bipolar wire stimulating electrode (50 μm, Ni/Cr).

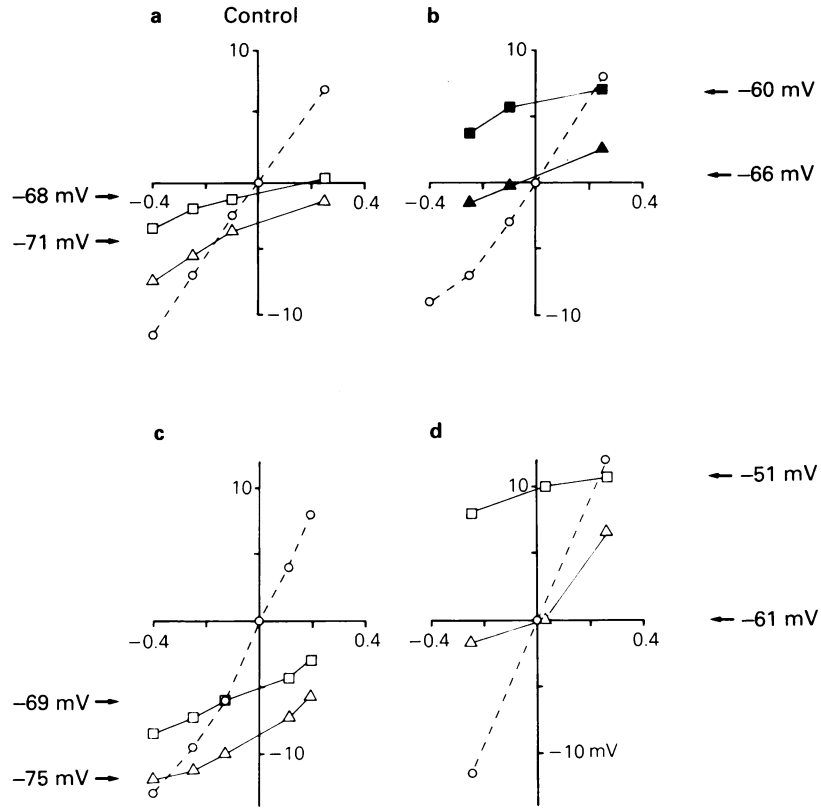

Figure 2 Current-voltage relations for a thalamic relay neurone obtained in the absence of γ-aminobutyric acid (GABA; ○- - -○) and during the application of GABA (36 nA, 2 s). (△) Current-voltage relation obtained during the early, more negative going phase of the GABA response and (□) points obtained during the later, more positive going phase. These plots are repeated during the application of Na pentobarbitone (b, solid symbols), during recovery from pentobarbitone (c) and following an increase in the GABA ejection current to 74 nA (d). Arrows indicate the reversal potentials for the two phases.

Membrane potential was altered by continuous injection of current and accurate electrode balance monitored by observing voltage responses to small, relatively brief current pulses. Slope resistance was obtained from I-V plots, by measuring voltage responses to steady state current injection under control conditions and during GABA and/or NaPb applications.

Results

Electrical properties of thalamic neurones

Twenty neurones were recorded in anterior and intralaminar thalamic nuclei that displayed slow spike activity typical of thalamic relay neurones (Jahnsen & Llinas, 1984a,b; Crunelli *et al.*, 1987). Their mean resting potential (RP) was $-60.8\,\text{mV} \pm 8.8\,\text{mV}$ (s.d.) and input resistance (R_i) was between 40 and 80 ($53 \pm 30\,\text{M}\Omega$), when measured close to RP. Fast spike amplitude measured from threshold was approximately 55 mV and spikes overshot zero potential by $>5\,\text{mV}$. Spike width at half amplitude was $\leqslant 0.75\,\text{ms}$.

Responses evoked by GABA

Responses to brief electrophoretic pulses of GABA under control conditions and in the presence of NaPb, were recorded from 9 cells. Accurate electrode placement was found to be important. Small changes in the position of the electrophoretic pipette produced large changes in the amplitude of responses evoked by a given GABA pulse. Changes in position as small as 10 μm from the optimal could reduce the response by >80%. Under control conditions,

typical responses were close to monophasic if the GABA pulse duration was short (1 s) (see Figure 1, lower records). These responses reversed close to RP (between 5 mV negative and 3 mV positive of RP) and produced a 20 to 50% drop in slope resistance measured close to reversal potential. Increasing the ejection current, and/or time, resulted typically in a biphasic response in which the early component reversed at a more negative membrane potential (mean -62.4 ± 7.1 mV) than the later component (-56.4 ± 5.39 mV) (see Figure 2). The mean RP for these cells was -61.7 ± 7.6 mV. Figure 1 shows the transition from a biphasic response to a monophasic response, when the GABA pulse was reduced from 2 s to 1 s. The decrease in slope resistance associated with the response to GABA was also enhanced when the GABA current was increased (see Figure 3). For example, in a cell whose control R_i was 50 MΩ, 10 nA GABA reduced R_i to 10 MΩ, and 20 nA GABA reduced R_i to 3.3 MΩ. Equivalent figures for another cell, were 54 MΩ (control), 37.4 MΩ (12 nA GABA) and 7.0 MΩ (20 nA GABA). Increasing the GABA ejection current beyond that required to evoke a biphasic response augmented the late phase, apparently at the expense of the early phase and shifted the reversal potentials of both phases in the positive direction. For example, in one cell whose RP was -60 mV, the early component reversed at -64 mV and the later at -61 mV with a GABA current of 12 nA (4 s). When the GABA current was increased to 40 nA, the two components reversed at -57 mV and -55 mV respectively. Another example is illustrated in Figure 2. Further increases in GABA application resulted in even more complex, e.g. triphasic responses, in some cells. Such complex responses were not studied in detail and are not described here.

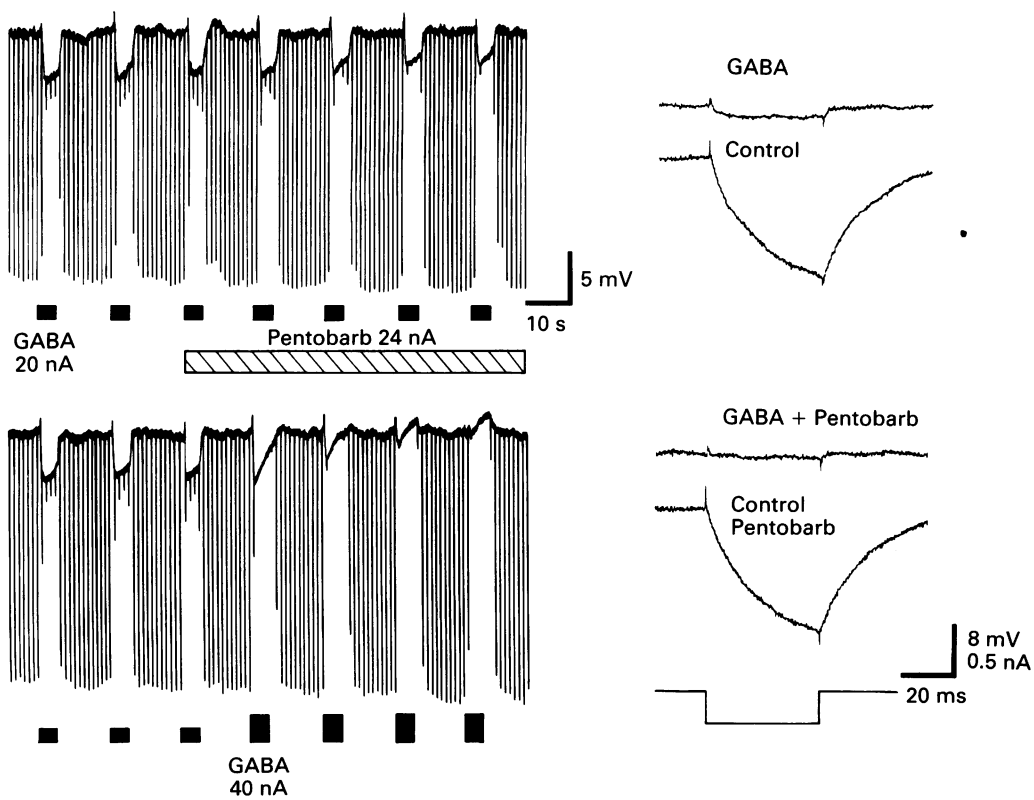

Figure 3 Responses of a thalamic relay neurone to pulses of γ-aminobutyric acid (GABA, 20 nA, 4 s). The downward deflections are voltage responses to brief current pulses injected into the cell throughout the recording. Application of Na pentobarbitone (24 nA, hatched bar) decreased the amplitude of the voltage responses during the GABA response, i.e. it increased the conductance produced by GABA. Averages (each of 4) of these voltage responses are shown at a faster time base on the right. The lower record shows a similar increase in the conductance produced by GABA, when the GABA ejection current was increased from 20 to 40 nA. The transition from a negative going response to the 20 nA pulse, to a positive going response to the 40 nA pulse, was gradual.

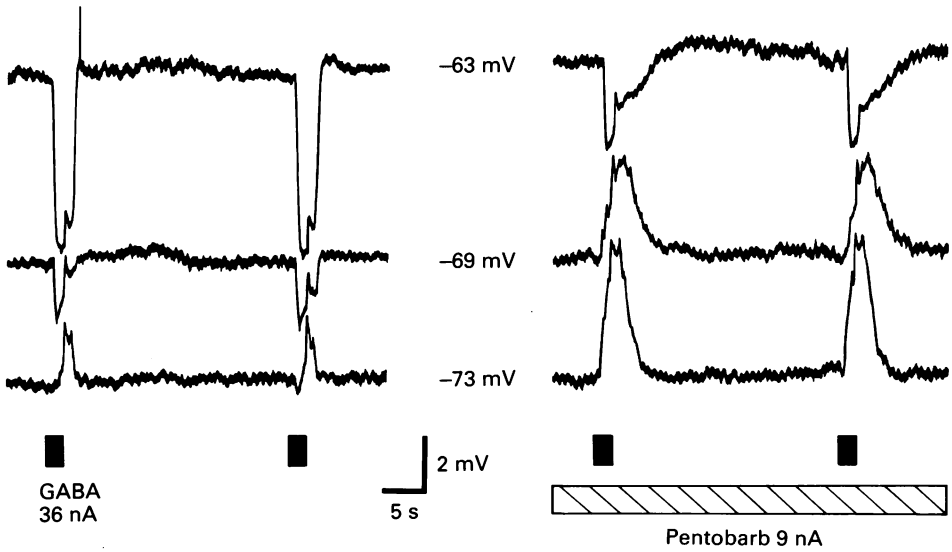

Figure 4 Responses of a thalamic relay neurone to pulses of γ-aminobutyric acid (GABA, 36 nA, 2 s), recorded at four different membrane potentials in the absence (left) and presence of Na pentobarbitone (9 nA, hatched bar). Pentobarbitone increased the duration of the response and shifted its reversal potential in the positive direction.

Gradual transition from monophasic to biphasic responses when the GABA current is increased

The transition from monophasic to biphasic, following an increase (Figure 3), or from biphasic to monophasic response shape, following a decrease in GABA ejection (Figure 1), was gradual. In addition, in some cells there was a gradual change in response shape during the earliest 10 to 30 GABA applications. This typically involved the development of the later, more positive-going component. It is possible that this gradual change paralleled a gradual increase in the amount of GABA ejected with each pulse. However, GABA was always pulsed during the placement of the multibarrel pipette, i.e. several tens of GABA pulses had already been ejected from that barrel before the optimal position, from which responses to GABA could be obtained, was found. The first pulse applied in the final position was therefore not the first pulse from that pipette. This gradual change in response shape was not matched by a similarly gradual rise in the evoked conductance. The evoked conductance was enhanced with the first increased GABA pulse (see Figure 3), and plateaued before the change in response shape. A gradual redistribution of Cl^- could also account for the change in reversal potential. However, even responses that were apparently totally positive-going became more positive-going at rest and reversed at a more depolarized membrane potential when the GABA current was increased.

Enhancement of GABA responses by Na pentobarbitone

In brief, the effect of applying NaPb was similar to that produced by increasing the GABA pulse. Responses to small, brief pulses of GABA that were predominantly monophasic under control conditions, became biphasic, with the gradual development of a later, more positive going phase (see Figure 1). Responses that were biphasic under control conditions also changed, with a gradual increase in the later, positive-going phase and the apparent loss, or obscuring of the early phase (see Figures 1 and 4). Reversal potentials for both components shifted in the positive direction (mean ($\pm$s.d.) for early phase to -58.1 ± 9.0 mV and for the later phase to -51.7 ± 6.6 mV) (see Figure 2). In addition, response duration and the conductance change evoked by GABA both increased. In 8 cells studied, the slope resistance decreased from a control value of 43 ± 19.0 MΩ to 16.97 ± 13.0 MΩ with GABA alone and to 7.85 ± 1.7 MΩ with GABA and NaPb. These effects gradually reversed on termination of the NaPb application (Figure 1).

Direct effects of Na pentobarbitone on recorded cells

Sodium pentobarbitone affected RP or R_i only when large currents were applied. Doses of NaPb that augmented responses to GABA produced no measurable effect on these parameters in the majority of

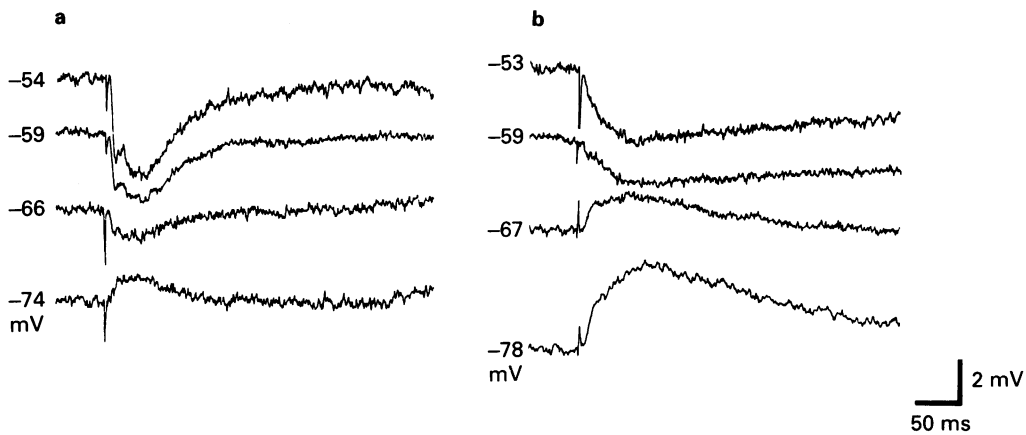

Figure 5 Inhibitory postsynaptic potentials (i.p.s.p.) recorded from a thalamic relay neurone in response to electrical stimulation of the nucleus reticularis thalami (see stimulus artifacts for stimulus timing). I.p.s.ps were recorded at several membrane potentials in the absence (a) and presence (b) of Na pentobarbitone. Each record is an average of 4 single sweeps. Pentobarbitone increased the duration of the i.p.s.p. and shifted its reversal potential in the positive direction.

neurones (e.g. Figures 1 and 3). There was no significant change in slow spike threshold, amplitude or duration in the presence of effective NaPb doses.

Stimulus-evoked i.p.s.ps: enhancement by NaPb

In two cells, electrical stimulation of the reticularis thalami evoked i.p.s.ps. These were similar to those described previously (Thomson, 1988a). One of these is illustrated in Figure 5. The effect of NaPb on these i.p.s.ps was similar to its effects on responses to GABA. The duration, amplitude and conductance increase associated with the i.p.s.p. increased and there was a positive shift in its reversal potential. In addition, trains of i.p.s.ps evoked by low frequency, repetitive stimulation (10 Hz) summed more readily in the presence of NaPb, with little or no repolarization between stimuli.

Discussion

The similarity of the responses to GABA described here, to those in the previous study (Thomson, 1988b) suggest that the majority of these were also predominantly mediated by $GABA_A$-receptor operated Cl^- channels. It is these channels that have been shown to be sensitive to barbiturates (Ransom & Barker, 1975; Nicoll, 1975; Scholfield, 1977; Brown & Constanti, 1978; Gage & Robertson, 1985; Olsen *et al.*, 1986). In the present study, the augmentation of GABA responses that was seen when NaPb was applied, was similar in several respects to the augmentation produced by simply increasing the GABA application, a result that is in agreement with previous studies (Ransom & Barker, 1976). In both cases, the response increased in duration and in associated conductance. This is consistent with the proposal that barbiturates enhance the open time of the activated channel. Again, in both conditions, there was a shift in the reversal potential of the response in the positive direction, which appeared to be associated with a profound augmentation of the late component. Preferential augmentation of the later component of the GABA response is also apparent in hippocampus, where the early, more negative going phase predominates in control conditions, but the later, more positive phase is revealed in the presence of barbiturates, or benzodiazepines (Biscoe & Duchen, 1985b; Blaxter *et al.*, 1986).

The present results confirm our previous suggestion (Thomson, 1988b), that under the present conditions, much of the response of thalamic relay neurones to electrophoretically applied GABA is mediated by $GABA_A$-receptors. These results also demonstrate that a major action of NaPb on thalamic relay cells is augmentation of GABA mediated i.p.s.ps. The results are consistent with the view that enhancement of GABA-mediated inhibition may play a role in barbiturate promotion of slow wave sleep. They do not, however, dispute a role for $GABA_B$-receptors in thalamus, or in the initiation of burst firing in slow wave sleep. We have recorded i.p.s.ps and in one cell a response to GABA, that differed greatly from the responses described here, in that they had reversal potentials around -90 mV (unpublished results, see also Crunelli *et al.*, 1987).

The precise mechanisms underlying the biphasic nature of i.p.s.ps and responses to GABA remains a matter for debate. From the results obtained here it seems probable that at, or close to the soma, the Cl^- equilibrium potential is close to the RP and more negative than it is in the dendritic tree. The early, negative component was always the lowest threshold when the multibarrel pipette was positioned close to the soma, while monophasic depolarizing responses were more commonly observed when the pipette was some distance from the soma. It does, however, seem unlikely that the site of activation of the later component is very far from the soma, since this component was the most sensitive to GABA antagonists (Thomson, 1988b) and to barbiturates applied proximally. Whatever the geometrical arrangement, it appears that the later, more positive-going component is the one that is most readily enhanced by NaPb.

The results presented here indicate that the later component develops gradually with time. Although it is possible that the NaPb takes some time to reach full effect and similarly, some time to wash off, it seems less likely that an increase, or decrease in GABA ejection current would take several minutes and tens of applications to stabilize. Indeed, augmentations of the conductance produced by GABA were generally seen to precede changes in response shape, when the GABA ejection current was increased. Two relatively simple explanations could account for the gradual shift in reversal potential. Firstly, enhancement of the later component is gradual and as it develops, it slowly obscures the early component and leads to an apparent shift in the reversal potential for the whole response, without any significant redistribution of Cl^-. This explanation would require some time-dependent modification in the responsiveness of the receptor-channel complex produced by a change in its level of activation.

The second explanation involves a gradual redistribution of Cl^- ions due to augmentation of the hyperpolarizing component. A negative-going response would involve an influx of Cl^-. Increasing the conductance would increase influx and successive GABA responses would have an additive effect if Cl^- pumping were saturated. However, responses that were apparently purely positive-going under control conditions also became more strongly positive-going and their reversal potential shifted. It remains possible, however, that a large positive-going component obscures the smaller negative-going component which causes Cl^- loading of the cell when augmented. This explanation would require more powerful Cl^- pumping in some parts of the cell. For example, relatively weak outward Cl^- pumping would occur at the soma, which would be saturated by augmented Cl^- influx, while powerful inward pumping in the dendrites would not be saturated by augmented Cl^- efflux. This does not seem entirely unlikely since the apparently different equilibrium potentials for Cl^- in the different cell compartments would require differences in Cl^- pumping anyway. Further experiments are, however, required to determine the precise mechanism underlying the time-dependence of this effect.

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The cardiovascular actions of clonidine and neuropeptide-Y in the ventrolateral medulla of the rat

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1 The cardiovascular responses to neuropeptide-Y (NPY) (25 and 50 pmol) and clonidine (10 and 20 nmol) were examined following microinjection into the rostral ventrolateral medulla (RVLM) and the caudal ventrolateral medulla (CVLM). Mean arterial pressure (MAP) and heart rate (HR) were measured in anaesthetized rats, pre- and post-injection.

2 The α_2-adrenoceptor agonist clonidine (10 and 20 nmol) reduced MAP and HR significantly when microinjected into the CVLM and RVLM.

3 NPY (25 and 50 pmol) microinjected into the CVLM decreased MAP and HR. However, in the RVLM neither dose had a significant cardiovascular effect.

4 The possibility of a functional interaction between the adrenergic system and NPY was examined by co-administration of clonidine and NPY in doses that gave submaximal blood pressure responses. In the CVLM this produced hypotension and bradycardia which was similar in magnitude to the sum of their individual responses, indicating that in this area their actions appear to be independent.

5 In the RVLM, where NPY has no significant cardiovascular effects, co-administration with clonidine, did not alter the response to clonidine.

6 It appears that in the areas investigated, there is no functional interaction between NPY and clonidine.

Introduction

The ventrolateral medulla (VLM) is now considered to be a major centre in the tonic and reflex control of blood pressure (Ciriello *et al.*, 1986). On the basis of neuronal content and different actions on blood pressure this area can be separated into two regions, the rostral ventrolateral medulla (RVLM) and the caudal ventrolateral medulla (CVLM).

The RVLM includes the C1 group of adrenaline-containing neurones which project to the intermediolateral cell column of the spinal cord, where the preganglionic sympathetic neurones are situated (Hokfelt *et al.*, 1974; Ross *et al.*, 1981). Electrical or chemical stimulation of these C1 neurones elevates mean arterial pressure (MAP) while neuronal blockade lowers MAP to similar levels reached by spinal cord transection, thus suggesting a tonic influence (Ross *et al.*, 1984; Guertzenstein *et al.*, 1978; Routledge & Marsden, 1987). In addition a series of lesioning experiments by Granata *et al.* (1983; 1985a) has shown that this region is also involved in the vasodepressor baroreflex response via an inhibitory projection from the nucleus tractus solitarius.

Caudal to this region are the noradrenergic A1 neurones (Dahlstrom *et al.*, 1964). In contrast to the C1 group, these neurones when activated elicit a reduction in MAP and when inhibited an increase (West *et al.*, 1981; Blessing *et al.*, 1982). It has been suggested that a component of this response is due to a reduction in sympathetic nerve activity possibly via an indirect inhibitory input to the C1 region (Willette *et al.*, 1984; Granata *et al.*, 1985b; Dampney *et al.*, 1987).

Further characterization of these areas through immunohistochemical studies has revealed that neuropeptide-Y like immunoreactivity (NPY-IR) co-exists with noradrenaline and adrenaline in most of the cell bodies and some terminals of the A1/C1 region respectively (Everitt *et al.*, 1984; Chronwall *et al.*, 1985). NPY, a 36 amino acid peptide and member of the pancreatic polypeptide family, was

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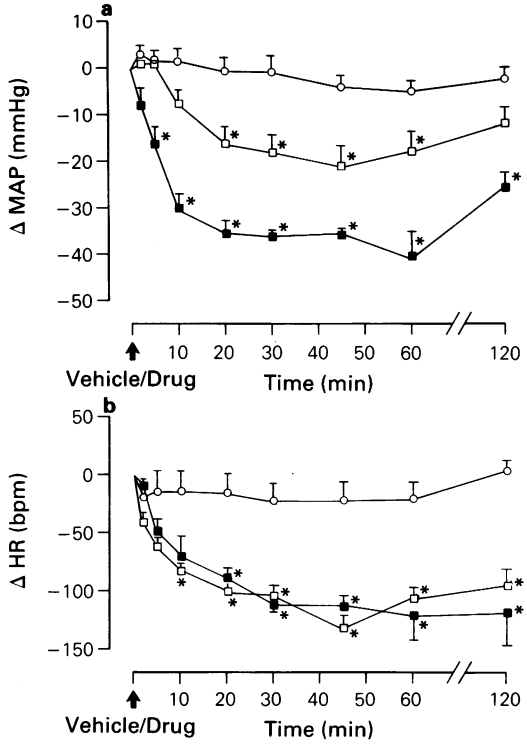

Figure 1 Effect of 10 nmol (□) and 20 nmol (■) clonidine microinjections into the caudal ventrolateral medulla (CVLM) on (a) mean arterial pressure (MAP) and (b) heart rate (HR). Points represent mean changes compared to saline control (○); vertical bars show s.e.mean. Pre-injection values ± s.e.mean are as follows; saline ($n = 6$), MAP = 96.13 ± 2.57, HR = 369 ± 14.1; 10 nmol clonidine ($n = 10$), MAP = 96.8 ± 2.50, HR = 373 ± 8.57; 20 nmol clonidine ($n = 5$), MAP = 94.4 ± 1.69, HR = 339 ± 13.27. *Significant differences from saline control value ($P < 0.05$).

originally isolated and characterized in porcine brain by Tatemoto *et al.* (1982). A functional role for NPY in the medulla was supported by the observation that intracisternally administered NPY in rats, results in hypotension and bradycardia (Fuxe *et al.*, 1983; Macrae & Reid, 1988). In addition, modulation of the hypotensive responses to NPY and adrenaline given separately, has been reported after intracisternal coadministration (Harfstrand *et al.*, 1984). This may be a result of a change in receptor number or an interaction at the level of the receptor coupling or post receptor events. (Agnati *et al.*, 1983a; Fuxe *et al.*, 1984).

The aim of the present study was to determine the haemodynamic effects of NPY and the α_2-adrenoceptor agonist clonidine following microinjection into the CVLM and RVLM, two important areas of cardiovascular regulation. Furthermore we examined the possibility of a functional interaction between NPY and α_2-adrenoceptors in these regions.

Methods

Experiments were carried out on male, 250–320 g Wistar-Kyoto rats (Harlan Olac, Bicester, U.K.) anaesthetized with a mixture of urethane (1 g kg^{-1}, i.p.) and α-chloralose suspension (60 mg kg^{-1}, i.p.). The rats were placed in a David Kopf stereotaxic frame. For the CVLM the incisor bar was set at 5 mm above the intra-aural line (IAL) and the co-ordinates were as follows: 4.5 mm caudal to the IAL; 1.7 mm lateral to the midline and 9.9 mm ventral to the cerebellar surface. To locate the RVLM the incisor bar was set at 5 mm below the IAL and the co-ordinates were: 3.5 mm rostral to the IAL; 2.4 mm lateral to the midline and 8.5 mm ventral to the cerebellar surface (Paxinos & Watson 1986). A small craniotomy was performed and unilateral microinjections of drug or vehicle (0.9% saline), were delivered via a 30-gauge needle with a 1 μl microsyringe (Hamilton). All injections were made in a total volume of 0.1 μl over a 20 s interval. MAP and heart rate (HR) were measured via a heparinised catheter positioned in the abdominal aorta, connected to a Gould pressure transducer and linked to a Grass polygraph, with a tachograph for direct HR recording. The catheter was surgically implanted the previous day under a combination of sodium pentobarbitone (60 mg kg^{-1}) and chloral-hydrate (100 mg kg^{-1}) anaesthesia and used to monitor MAP and HR for 30 min pre- and 120 min post-injection. Temperature was maintained at 37°C with a homoeothermic blanket. At the end of each experiment the animal was killed by an overdose of sodium pentobarbitone. The brain was then fixed by transcardiac perfusion with FAGLU (formaldehyde 4% and gluteraldehyde 0.5% in 0.1 M sodium phosphate buffer pH 7.0), excised and 50 μm sections were cut and mounted for verification of injection site. Experiments where the injection site was not within the A1 or C1 region were excluded from the study. Statistical analysis was carried out on the changes in MAP and HR by use of ANOVA with Bonferoni test for multiple comparisons. All values were expressed as mean ± s.e.mean.

Drugs

Drugs used were porcine neuropeptide-Y (Cambridge Research Biochemicals) and clonidine

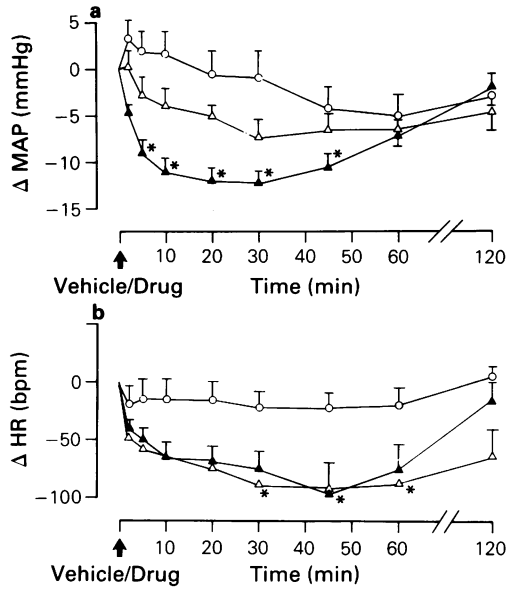

Figure 2 Effect of 25 pmol (△) and 50 pmol (▲) neuropeptide-Y (NPY) microinjections into the caudal ventrolateral medulla (CVLM) on (a) mean arterial pressure (MAP) and (b) heart rate (HR). Points represent mean changes compared to saline control (○); vertical bars show s.e.mean. Pre-injection values ± s.e.mean are as follows; saline ($n = 6$), MAP = 96.13 ± 2.57, HR = 369 ± 14.1; 25 pmol NPY ($n = 9$), MAP = 101 ± 1.91, HR = 388 ± 11.3; 50 pmol NPY ($n = 9$), MAP = 102 ± 2.67, HR = 368 ± 9.17. *Significant differences from saline control value ($P < 0.05$).

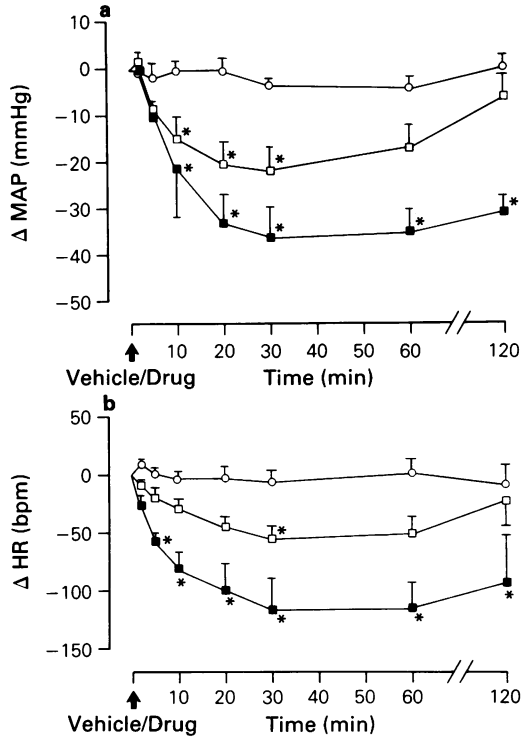

Figure 3 Effect of 10 nmol (□) and 20 nmol (■) clonidine microinjections into the rostral ventrolateral medulla (RVLM) on (a) mean arterial pressure (MAP) and (b) heart rate (HR). Points represent mean changes compared to saline control (○); vertical bars show s.e.mean. Pre-injection values ± s.e.mean are as follows; saline ($n = 6$), MAP = 89.17 ± 3.27, HR = 368.3 ± 18.3; 10 nmol clonidine ($n = 8$), MAP = 97.25 ± 1.86, HR = 375 ± 15.9; 20 nmol clonidine ($n = 5$), MAP = 88 ± 6.04, HR = 347 ± 29.14. *Significant differences from saline control value ($P < 0.05$.

hydrochloride (Sigma Chemicals Co.). Drugs were dissolved in sterile saline, aliquoted and stored at −70°C.

Results

No significant haemodynamic response was observed after injection of 0.1 μl of vehicle (0.9% saline) into the CVLM or the RVLM. Microinjection of the peptide or clonidine into nearby control areas lateral or dorsal to the Al or Cl area produced no significant haemodynamic response

Effects of clonidine and neuropeptide-Y microinjections into the CVLM

Clonidine Microinjections of clonidine (10 and 20 nmol) in 0.1 μl elicited a significant dose-related fall in MAP when microinjected into the CVLM. This effect reached a nadir at 45 min (−21.0 ± 4.59 mmHg; −40.8 ± 6.53 mmHg respectively) and the responses took 2–3 h to return to pre-injection levels. The maximum reduction in HR, was similar for both doses of clonidine (−131 ± 17.42 bpm; −121 ± 22.11 bpm respectively). As shown in Figure 1, these falls were also sustained for 2–3 h.

Neuropeptide-Y The results after microinjection of NPY (25 and 50 pmol) into the CVLM are summarized in Figure 2. Both doses of NPY microinjected into this area produced a fall in MAP which reached a maximum at 45 min (−7.44 ± 2.16 mmHg; −12.33 ± 3.01 mmHg respectively) and returned to control saline values after 1 h. In addition the bradycardia produced by NPY (25 and 50 pmol) resembled that after clonidine in this area. Both con-

centrations of NPY produced similar falls in HR with the nadir reached at 45 min (-91.1 ± 21.45 bpm; -94.4 ± 28.14 bpm respectively) and sustained for 1 h.

Effects of clonidine and neuropeptide-Y microinjections into the RVLM

Clonidine A profound fall in MAP and HR was observed after clonidine (10 and 20 nmol) was injected into the RVLM. This was similar to the response of clonidine in the CVLM. The results are shown in Figure 3. The maximum reduction in MAP (-21.63 ± 5.85 mmHg (10 nmol) and -36.0 ± 3.55 mmHg (20 nmol)) occurred at 30 min with the hypotension lasting 2–3 h at the highest concentration. However in this area, unlike the caudal region, the bradycardia induced by clonidine was dose-related, reaching a nadir of -54.6 ± 12.17 bpm (10 nmol) and -110 ± 16.2 bpm (20 nmol) at 30 min post-injection.

Neuropeptide-Y In the RVLM neither concentration of NPY studied (25 and 50 pmol) influenced blood pressure or heart rate and results were not significantly different from the saline control (Figure 4). Although clonidine in the RVLM produced a dose-related hypotension and bradycardia similar to its effects in the caudal region, NPY appears to have no significant action on these parameters in this area.

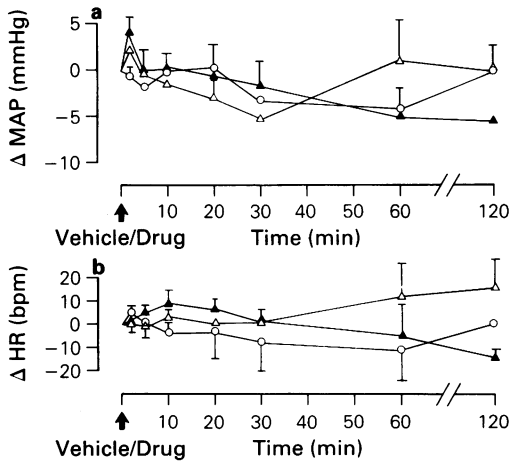

Figure 4 Effect of 25 pmol (△) and 50 pmol (▲) neuropeptide-Y (NPY) microinjections into the rostral ventrolateral medulla (RVLM) on (a) mean arterial pressure (MAP) and (b) heart rate (HR). Points represent mean changes compared to saline control (○); vertical bars show s.e.mean. Pre-injection values ± s.e.mean are as follows; saline ($n = 6$), MAP = 89.17 ± 3.27, HR = 368.83 ± 18.3; 25 pmol NPY ($n = 7$), MAP = 97.86 ± 4.86, HR = 382.8 ± 18.35; 50 pmol NPY ($n = 6$), MAP = 90 ± 2.58, HR = 360 ± 13.9. *Significant differences from saline control value ($P < 0.05$).

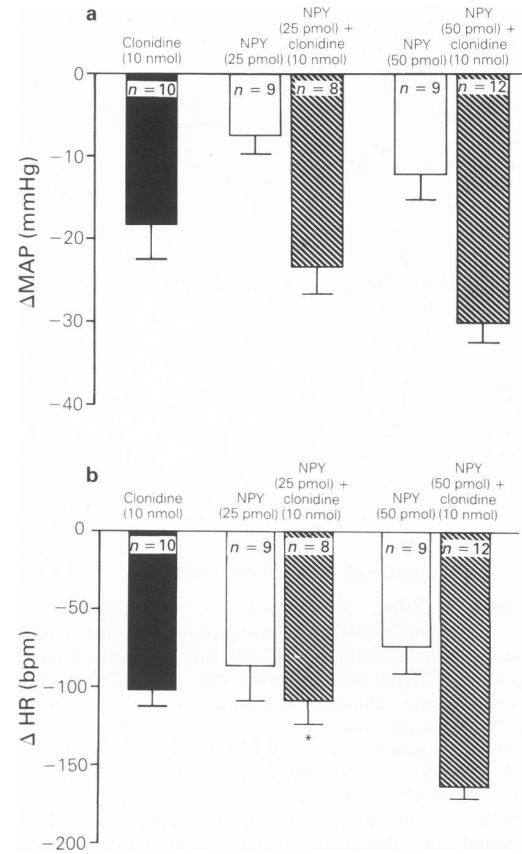

Figure 5 Co-administration of 25 pmol or 50 pmol neuropeptide-Y (NPY) together with 10 nmol clonidine into the caudal ventrolateral medulla. Columns show change in (a) mean arterial pressure (MAP) and (b) heart rate (HR) (with s.e.mean shown by vertical bars) 30 min post injection. *Significant difference compared to sum of individual responses ($P < 0.05$). n values in columns.

Effects of the co-administration of neuropeptide-Y and clonidine in the rostral and caudal ventrolateral medulla

The results presented for both brain areas represent the responses obtained 30 min after injection of clonidine or NPY injected separately and when co-administered.

CVLM Co-administration of clonidine (10 nmol) and NPY (25 pmol) in the CVLM reduced MAP

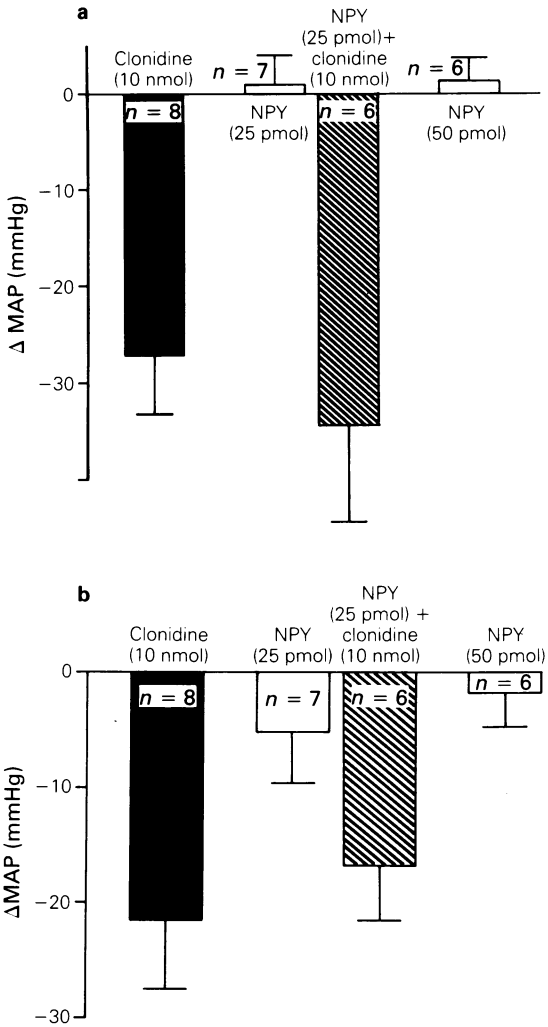

Figure 6 Co-administration of 25 pmol neuropeptide-Y (NPY) and 10 nmol clonidine into the rostral ventrolateral medulla. Columns show change in (a) mean arterial pressure (MAP) and (b) heart rate (HR) (with s.e.mean shown by vertical bars) 30 min post injection. *Significant difference compared to sum of individual responses ($P < 0.05$).

by -23.5 ± 3.3 mmHg which was similar to the sum of their individual responses (Figure 5). However the bradycardia was different. The combination was significantly less than the sum of their separate response (-109.0 ± 13.6 bpm). However, when clonidine (10 nmol) was injected with the higher concentration of NPY (50 pmol) into the CVLM both the fall in MAP and HR which resulted could be considered additive (-30 ± 2.36 mmHg; -163.8 ± 8.0 bpm).

RVLM Although in the RVLM, NPY (25 and 50 pmol) alone produced no significant cardiovascular effect, it is possible that it could modulate the response to other agents which have an effect here. Therefore, the cardiovascular effects of NPY (25 pmol) and clonidine (10 nmol) injected together (-16.7 ± 4.6 mmHg; -68.8 ± 20.1 bpm) were investigated (Figure 6). For both blood pressure and heart rate, the response to co-administered clonidine and NPY was not significantly different from clonidine alone (-21.6 ± 5.9 mmHg; -54.6 ± 12.2 bpm). There is therefore no evidence to suggest an interaction between NPY and α_2-adrenoceptors at this site.

Discussion

In this study further evidence has been presented which supports a vasodepressor role for NPY in the medulla of the rat (Fuxe *et al.*, 1983; Macrae & Reid, 1988). NPY (25 and 50 pmol), when injected by a discrete microinjection technique, into the CVLM of the anaesthetized rat, evoked a dose-related hypotension. This effect may be mediated through the specific NPY receptors which have been demonstrated in the medulla by high affinity binding studies (Harfstrand *et al.*, 1986). Although NPY elicited a pronounced fall in heart rate in the CVLM, it was not dose-related. One explanation for this may be the close proximity and possible involvement of the nucleus ambiguus, an area with cardio-inhibitory vagal projections (Ciriello & Calaresu, 1979; Paxinos & Watson, 1986). Macrae & Reid (1988) have shown that the bradycardia elicited by CVLM microinjections of NPY in vagotomized rats, is significantly reduced compared to control rats. It is possible that the mechanisms involved in the regulation of heart rate are more sensitive to NPY than the response to blood pressure. Thus although the blood pressure response to the lower dose of NPY was submaximal, the heart rate response may have been maximal.

The hypotension and bradycardia observed following the injection of clonidine (10 and 20 nmol) into the RVLM and CVLM is consistent with reports by Punnen *et al.* (1987) and previous studies where other regions of the medulla have been investigated (Chan & Koo, 1978; Carter *et al.*, 1985). However, in the CVLM, the bradycardia produced by clonidine was not dose-related. In this respect the response resembled the action of NPY in this area and may again reflect a greater sensitivity in the mechanisms controlling heart rate compared to those controlling blood pressure.

The haemodynamic effects produced by clonidine are thought to involve α_2-adrenoceptors which are

widely distributed in the medulla. Unnerstal *et al.* (1984) demonstrated that the distribution of these α_2-adrenoceptors in the CNS, as identified by [^{3}H]-*p*-aminoclonidine binding, correlates well with the distribution of central noradrenergic and adrenergic neurones.

Some investigators believe that clonidine lowers MAP via an α_2-presynaptic mechanism which inhibits the release of noradrenaline or adrenaline from the medullary neurones (Scatton *et al.*, 1979; Atkinson *et al.*, 1984). In addition, Bousquet *et al.* (1984) proposed that clonidine may act on 'imidazole preferring sites', which possess a pharmacologically distinct profile from that of α_2-adrenoceptors. However, local brainstem networks containing γ-aminobutyric acid, acetylcholine or catecholamines can also modify the actions of ventrolateral medullary neurones and may account for the similar actions of clonidine that we observed in both a vasopressor (Cl) and vasodepressor (Al) area (Reis, 1987).

Central immunohistochemical studies show that many of the classical catecholamine neurones also store and probably release biologically active peptides as cotransmitters (Everitt *et al.*, 1984; Hokfelt *et al.*, 1987). Evidence for a functional role of NPY/adrenaline or NPY/noradrenaline coexistence in neurones in cardiovascular centres of the medulla, comes from *in vitro* studies by Agnati *et al.* (1983a,b) and Fuxe *et al.* (1986; 1987) who describe a reciprocal modulation of receptor number and affinity. Moreover, in *in vivo* experiments the fall in MAP and HR after intraventricular NPY was unaltered by α_2-blockade, suggesting an independent mechanism of action (Harfstrand *et al.*, 1984). However, the response to intraventricular NPY when coadministered with adrenaline was not additive implying a functional interaction (Harfstrand & Fuxe, 1987). We have employed a method of discrete microinjections which allowed us to study the possibility of *in vivo* interactions in the CVLM and RVLM. In the CVLM the combination of NPY together with clonidine produced a hypotensive effect which was additive. The bradycardia elicited after coadministration of these agents was also additive after combination of clonidine with the highest dose of NPY. In the RVLM however, NPY alone had no effect on either parameter measured and when coadministered with clonidine NPY did not alter the response to the latter. Thus, the two agents did not appear to antagonize the action of each other, as previously claimed (Fuxe *et al.*, 1986). In conclusion, we have demonstrated a fall in blood pressure and heart rate after NPY and clonidine given alone into the caudal ventrolateral medulla and for clonidine alone into the rostral ventrolateral medulla, areas of importance in the tonic and reflex control of blood pressure. However, there was no apparent interaction between clonidine and NPY when coadministered suggesting that, at least in these regions, their effects are independent.

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Sympathetic vascular control of the pig nasal mucosa: adrenoceptor mechanisms in blood flow and volume control

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1 The adrenoceptor mechanisms influencing the total blood flow, volume and superficial blood flow in the nasal mucosa of pigs anaesthetized with pentobarbitone have been characterized by use of various agonists and antagonists.

2 Local intra-arterial bolus injection of the selective α_1-agonist phenylephrine, the selective α_2-agonist UK 14.304, the mixed α_1/α_2-agonist oxymetazoline and the mixed α/β-agonists noradrenaline (NA) and adrenaline induced dosed-related reduction of nasal arterial blood flow (BF), nasal mucosal volume (V, reflecting capacitance vessel function) and the laser Doppler flowmetry signal (LDF, reflecting superficial movement of blood cells). The rank order of α-agonist potency regarding BF reduction was UK 14.304 > oxymetazoline > NA > phenylephrine = adrenaline. For the volume response the potency order was UK 14.304 > oxymetazoline = NA = adrenaline > phenylephrine while for the reduction of the LDF signal the potency was UK 14.304 = NA = adrenaline > oxymetazoline > phenylephrine. The selective β_2-agonist terbutaline caused dose-dependent increase of BF whereas only a small augmentation of the V was obtained upon the highest dose (40 nmol) while no modification of the LDF signal was observed.

3 After pretreatment with the selective α_1-antagonist prazosin, the response to phenylephrine was abolished while the selective α_2-antagonist idazoxan attenuated the effect of UK 14.304. After pretreatment with α-antagonists, both NA and adrenaline caused biphasic effects with constriction followed by vasodilatation for BF, but not for V or LDF. This vasodilatation was blocked by the β-antagonist propranolol.

4 The reduction in nasal BF and V upon sympathetic nerve stimulation was attenuated both by prazosin and idazoxan. Propranolol enhanced the remaining reduction of BF but not of V in the presence of α-antagonists.

5 It is concluded that α_2-adrenoceptor mechanisms in the pig nasal mucosa are dominating for the BF, V and LDF responses to exogenous agonists. α_1-Adrenoceptors also seem to be involved in the sympathetic control of BF, V and LDF. Activation of β_2-receptors increases mainly BF and does not influence the LDF signal.

Introduction

The nasal mucosal vascular bed is composed of arteries, arterioles (resistance vessels) and a sub-epithelial capillary network which is drained into collecting veins and large venous sinusoids (capacitance vessels) (Cauna, 1982). Nasal blood vessels receive a very dense sympathetic innervation (Dahlström & Fuxe, 1965) and noradrenaline (NA) is considered as the classical postganglionic transmitter (Salem, 1972; Malm, 1974a; Änggård & Edwall, 1974; Hall & Jackson, 1978; Eccles 1982; Berridge & Roach, 1986). In the pig (Lacroix *et al.*, 1988a, b; 1989) and cat (Lundblad *et al.*, 1987) there is also evidence that non-adrenergic mechanisms are involved in the sympathetic vascular control of the nasal mucosa. The classification of adrenoceptors into α_1- and α_2-subtypes has been a result of the development of various selective agonists and antagonists (see Starke, 1981; 1987). Non-selective α-adrenoceptor agonists acting on both α_1- and/or α_2-receptors are of common clinical use as nasal decongestants (Empey & Medder, 1981). Andersson & Bende (1984) reported that in the human nasal mucosa, α_2-receptors are most important for control of resistance vessels while β-receptor mechanisms

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have minor influence on nasal blood flow (BF) as measured by the ^{133}Xe washout method. However, a detailed characterization of α- as well as β-adrenoceptor mechanisms for exogenous agents and endogenously released NA involved in the control of BF and volume (V, i.e. capacitance vessel function) as well as superficial blood flow as revealed by the laser Doppler flowmeter signal (LDF) remain to be established in the nasal mucosa. By means of a recently developed experimental model which allowed simultaneous recordings of nasal arterial BF, V (Lacroix *et al.*, 1988a) and the LDF signal (Lacroix *et al.*, 1989), we have therefore aimed to characterize adrenoceptor mechanisms in the different compartments of the pig nasal vascular bed.

Methods

Surgical procedure

The experiments were performed on 12 pigs of either sex (body weight 25–30 kg). After premedication with ketamine hydrochloride (20 mg kg^{-1}, i.m.) and atropine (0.05 mg kg^{-1}, i.m.), the anaesthesia was induced with sodium pentobarbitone (20 mg kg^{-1}, i.v.) and maintained with continuous i.v. infusion of the same agent (3 mg kg^{-1} h^{-1}). For skeletal muscle relaxation, pancuronium bromide (0.25 mg kg^{-1}, i.v.) was given at regular intervals. The animals were artificially ventilated with a volume regulated ventilator (Engström, model 150, Sweden) and the body temperature was kept between 37–38°C. Catheters were placed in the femoral artery for systemic blood pressure monitoring and sampling for blood gas analysis, and in the femoral vein for drug and fluid administration. Briefly, the external carotid artery and the superficial jugular vein were dissected up to the sphenopalatine fossa. All the arterial branches located down stream to the superficial temporal artery were ligated and cut (for details of the surgical approach see Lacroix *et al.*, 1988a). Nasal arterial blood flow was recorded with a Transonic flow probe (2-4RB143) of 2–4 mm diameter placed around the internal maxillary artery in the portion proximal to the superficial temporal branch. The flow probe was connected to a T202S ultrasonic blood flowmeter (Transonic system Inc, NY, USA). The superficial temporal artery was cannulated with a PE 90 catheter which allowed selective injections of agents into the main nasal arterial blood supply. Volume changes of the nasal cavity (mainly reflecting blood content of the venous sinusoids) were recorded with the latex balloon method (Malm, 1973; Lacroix *et al.*, 1988a). The superficial mucosal blood flow was recorded with a laser Doppler flowmeter probe (Periflux PF 2B, Perimed KB, Sweden) placed on the surface of the anterior tip of the inferior turbinate in a portion which was not in contact with the balloon. Moving blood cells in the capillaries change the frequency of the back-scattered light according to the Doppler principle. The output signal is a function of the product of red cell volume and their mean velocity. The movement of blood cells is measured to a tissue depth determined by the absorption coefficient of the tissue studied as well as by the wavelength used. By means of this LDF method, former studies on the superficial mucosal blood flow, the red light (632.8 nm, He-Ne, 2 mW) from the PF2, has been shown to penetrate the intestinal mucosa to a depth of 2–4 mm (Ahn *et al.*, 1985) and to about 1 mm depth in dark tissue such as the renal cortex (Stern, 1975). The LDF signal was not secondarily influenced by the balloon volume change *per se* as shown by filling or removing 10 ml of saline from the balloon in the presence of the LDF probe within the nasal cavity. Arterial blood flow, volume of the mucosa and the LDF signal were recorded in parallel on a Grass polygraph 7B (Grass Instrument Co., USA). The ipsilateral cervical sympathetic trunk was dissected and cut 2–3 cm below the superior cervical ganglion and the cranial portion of the nerve was placed on a bipolar platinum electrode connected to a Grass S 88 stimulator.

Cervical sympathetic nerve stimulations (15 V, 5 ms) were performed with single impulses or at a continuous frequency of 2 Hz for 2 min (giving a total of 240 impulses).

Experimental procedure

The nasal vascular effects (BF, V and LDF) upon sympathetic nerve stimulation (single pulse and 2 Hz) and local i.a. injection of adrenaline (0.055–55 nmol), NA (0.06–60 nmol), the selective α_2-agonists UK 14.304 (0.05–50 nmol) and oxymetazoline (0.04–40 nmol), the selective α_1-agonist phenylephrine (0.05–50 nmol) and the selective β_2-agonist terbutaline (0.04–40 nmol) were studied by the following procedure:

Group 1 ($n = 4$): Nerve stimulations and local i.a. injections were first performed under control conditions and then repeated 30 min after inhibition of neuronal uptake of monoamines by infusion of desipramine (DMI) (0.5 mg kg^{-1}, i.v.), 10 min after subsequent infusion of the selective α_1-antagonist prazosin (0.1 mg kg^{-1}, i.v.) and 15 min after subsequent administration of the β_1- and β_2-antagonist propranolol (0.5 mg kg^{-1}, i.v.).

Group 2 ($n = 4$): Nerve stimulations and local i.a. injections were performed under control conditions then 30 min after infusion of DMI, 10 min after sub-

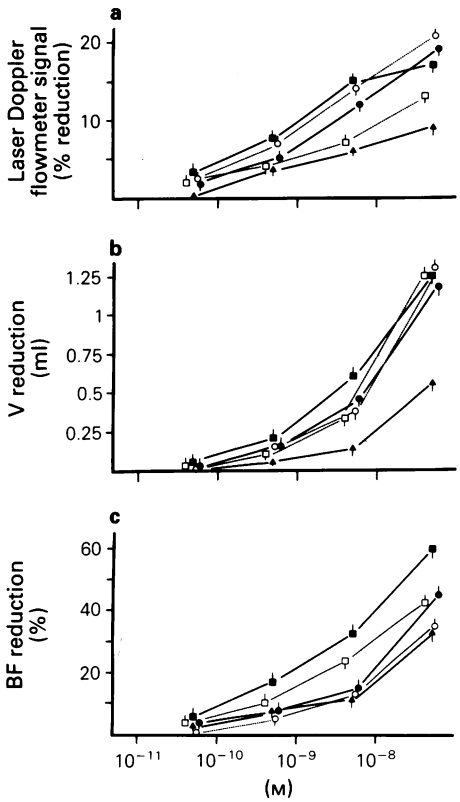

Figure 1 Effects of local i.a. bolus injections of UK 14.304 (■—■), oxymetazoline (□--□), noradrenaline (●—●), adrenaline (○--○) and phenylephrine (▲—▲) on the laser Doppler flowmeter signal (% reduction) the volume (V, in ml) of the nasal mucosa and the nasal arterial blood flow (BF, % reduction). Data are given as mean with s.e.mean shown by vertical lines; $n = 8$.

sequent infusion of the selective α_2- antagonist idazoxan (0.5 mg kg^{-1}, i.v.) and 15 min after propranolol (0.5 mg kg^{-1}, i.v.).

Group 3 ($n = 4$): Thirty min after infusion of DMI, nerve stimulations and local i.a. injections were performed 10 min after combined i.v. pretreatment with idazoxan (0.5 mg kg^{-1}) and prazosin (0.1 mg kg^{-1}), and 15 min after subsequent addition of propranolol (0.5 mg kg^{-1}, i.v.).

Calculations

Blood flow reduction is expressed as the percentage decrease compared to zero flow (100% reduction) obtained by clamping the external maxillary artery upstream to the flow probe. Calibration of the nasal cavity volume changes was achieved by removal of 1 ml from the reservoir directly connected to the latex balloon placed in the nasal cavity (see Lacroix *et al.*, 1988a). Under control conditions, the balloon contained 5 ± 1.5 ml. The LDF signal is expressed as a percentage of the maximal internal calibration signal (100%). The duration of the vascular responses is given in seconds and calculated as the time for recovery of basal values from the end of the sympathetic stimulation or, for local i.a. injections of exogenous α- and β-agonists, from the beginning of the vascular effects until return to former baseline. Data are given as means ± s.e.mean and statistical differences were estimated with Student's paired t test or Kruskal-Wallis analysis of variance with multiple comparisons (Theodorsson-Norheim, 1986).

Drugs

The following drugs (source) were used: ketamine hydrochloride (Ketalar, Parke-Davis, U.S.A.), sodium pentobarbitone (Mebumal), atropine (ACO, Sweden), pancuronium bromide (Pavulon, Organon, Netherlands), noradrenaline hydrochloride ((±)-arterenol), adrenaline hydrochloride ((±)-adrenaline) and phenylephrine hydrochloride (Sigma Chemical Company, USA), desipramine hydrochloride (DMI) and propranolol (Inderal, Ciba, Basel, Switzerland), oxymetazoline hydrochloride, terbutaline (Draco, Sweden). UK 14.304 (5-bromo-6-(imidazoline-2-yl amino-quinoxaline tartrate), prazosin hydrochloride (Pfizer, Sandwich), idazoxan (RX 781094, Reckitt & Colman, Hull). All solutions except prazosin (which was initially dissolved in ethanol) were freshly prepared before each experiment by dissolving the compounds in sterile 0.9% w/v NaCl solution.

Results

The local i.a. administration of the adrenoceptor agonists or the sympathetic nerve stimulations did not significantly affect the heart rate (HR) or the mean systemic arterial blood pressure (MAP). The HR was, however, increased by 22 ± 4% after i.v. infusion of idazoxan and by 16 ± 2% after prazosin. Prazosin induced a MAP reduction of 10 ± 2%. However, the basal value of BF (75 ± 10 ml min^{-1}), the V and LDF signal (50 ± 10%) were not significantly affected by the administration of prazosin, idazoxan or propranolol.

Control effects of adrenoceptor agonists

Local i.a. injections of the five α-adrenoceptor agonists studied gave dose-related reductions of BF, V and LDF (Figure 1). The relative potency of the

Table 1 Local i.a. bolus doses required of various adrenoceptor agonists to reduce, under control conditions, the nasal arterial blood flow by 25% (BF 25), the nasal mucosal volume by 0.5 ml (V 0.5) and the laser Doppler flowmeter signal by 10% (LDF 10) in pigs anaesthetized with pentobarbitone

	Blood flow (25%) (nmol)	*Mucosal vol.* (0.5 ml) (nmol)	*LDF* (10%) (nmol)
Agonist			
UK 14.304	1.6 ± 0.3*	3.2 ± 0.4*	1.1 ± 0.2**
Oxymetazoline	5.1 ± 0.5*	5.7 ± 0.5	12.0 ± 1.5
Noradrenaline	8.7 ± 0.7*	7.0 ± 0.6***	3.1 ± 0.5***
Phenylephrine	18.0 ± 3.0	39.0 ± 5.0	50.0 ± 6.0
Adrenaline	20.0 ± 4.0	7.5 ± 0.6	1.5 ± 0.4

* $P < 0.05$; ** $P < 0.001$, *** $P < 0.001$ (Student's paired t test) when compared to the following agent. Data are given as mean ± s.e.mean, $n = 8$.

agonists under control conditions were somewhat different for each vascular parameter studied, as shown in Table 1. The rank order potency regarding BF reduction was UK 14.304 > oxymetazoline > NA > phenylephrine = adrenaline. For the V response the order was UK 14.304 > oxymetazoline = NA = adrenaline > phenylephrine while for the reduction of the LDF signal the potency order was UK 14.304 = NA = adrenaline > oxymetazoline > phenylephrine. Local i.a. administration of terbutaline caused a dose-dependent increase of BF whereas a small augmentation of V was obtained only upon the highest dose (40 nmol) (Figure 2). The LDF signal was not affected by terbutaline (not shown).

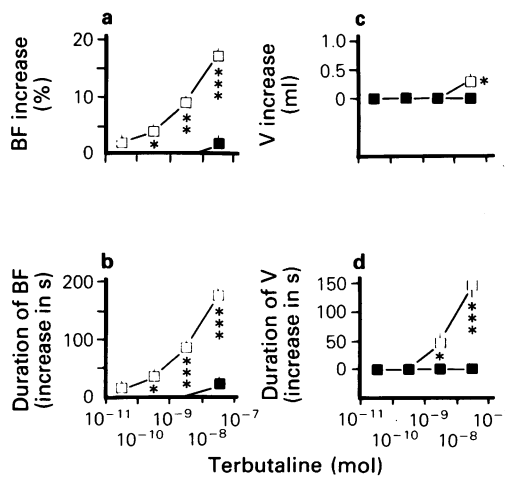

Figure 2 Effects of local i.a. bolus injections of the β_2-agonist terbutaline before (□—□) and after (■—■) pretreatment with propranolol (0.5 mg kg^{-1}, i.v.). (a) Nasal arterial blood flow (BF) % increase, (b) duration (in s) of the BF increase, (c) nasal mucosal volume (V, in ml) increase and (d) duration (in s) of the V increase. Mean (with s.e.mean shown by vertical lines) are given, $n = 8$. * $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$ (Student's paired t test).

Effects of desipramine and adrenoceptor antagonists

Under control conditions, a parallel reduction of the LDF signal, BF and V of 19 ± 4%, 44 ± 3.5% and 1.17 ± 0.13 ml, respectively, was observed upon local i.a. injection of NA (60 nmol) (Figures 1, 3, Table 2). The peak LDF, BF and V responses to NA were enhanced by 18 ± 3.5% (NS), 38 ± 6% ($P < 0.05$) and 27 ± 5% ($P < 0.05$) after DMI pretreatment (Table 2, BF and V not shown). The BF and V responses to NA and adrenaline were significantly prolonged after DMI (not shown) while DMI did not influence the reduction of LDF signal, BF and V induced by UK 14.304, oxymetazoline or phenylephrine (not shown).

Compared to control, the LDF signal, BF and V responses to NA (60 nmol) were reduced by 70 ± 10%, 60 ± 7% and 60 ± 9%, respectively, after pretreatment with prazosin (Figure 3a–b). The remaining BF response to NA after prazosin was biphasic (Figure 3b) and the reduction of BF (by 16 ± 2%) was followed by a 8 ± 1.5% increase. After idazoxan pretreatment the LDF, BF and V responses evoked by NA were reduced by 40 ± 5%, 11 ± 2% and 60 ± 7.5%, respectively, compared to control (Table 2, Figure 3c). A short lasting BF reduction was then followed by a 22 ± 3% increase which lasted for 120 ± 15 s (Figure 3c). When prazosin and idazoxan were combined, the LDF and V responses to NA were reduced by 80 ± 15% and 87 ± 17% whereas a 13 ± 2% increase of BF occurred (Table 2, Figure 3d). Following concomitant pretreatment with propranolol, the increase of the BF response observed to NA (60 nmol) after α-antagonists was absent (Figure 3e, Table 2).

The three nasal vascular parameters studied upon local i.a. injection of adrenaline were, in principle,

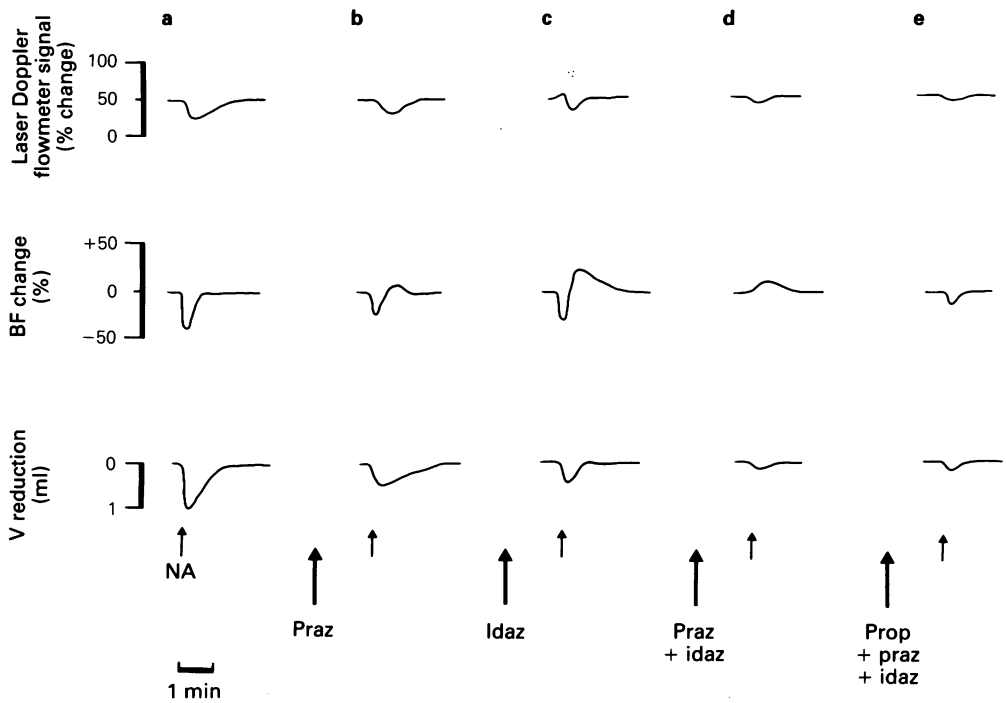

Figure 3 Original pen-trace recordings of the laser Doppler flowmeter (LDF) signal (% change), the blood flow (BF) change (%) and the nasal mucosal volume (V) reduction (in ml) to local i.a. bolus injection of NA (60 nmol). (a) under control conditions, (b) after pretreatment with prazosin (Praz, 0.1 mg kg^{-1}, i.v.), (c) after pretreatment with idazoxan (Idaz, 0.5 mg kg^{-1}, i.v.), (d) after simultaneous pretreatment with idazoxan and prazosin, and (e) after simultaneous pretreatment with idazoxan, prazosin and propranolol (Prop, 0.5 mg kg^{-1}, i.v.).

Table 2 Variations of the laser Doppler flowmeter (LDF) signal upon preganglionic stimulation (5 ms, 15 V) of the superior cervical sympathetic trunk with single impulses or at 2 Hz for 2 min as well as changes to local intra-arterial injections of noradrenaline, adrenaline, UK 14.304, phenylephrine or oxymetazoline

	Laser Doppler flowmeter (Peak change %)					
	Control	*+DMI*	*+Idazoxan*	*+Prazosin*	*Prazosin +idazoxan*	*Prazosin +idazoxan +propranolol*
Single pulse	+4.6 ± 1***	−13.5 ± 1.5***	+4 ± 1	+7 ± 2*	+2.7 ± 0.5	+4 ± 1
2 Hz	−25 ± 5	−33 ± 6**	−15.4 ± 4	−12.6 ± 2.5	−8.7 ± 2	−8.7 ± 2
Noradrenaline	−19 ± 4	−23 ± 3*	−11.4 ± 3*	−5.6 ± 1.5	−4 ± 1.5	−5 ± 1.5
Adrenaline	−21 ± 4	−15 ± 2	−13 ± 3	−7.6 ± 2	−6 ± 0.5	−5.3 ± 1
UK 14.304	−17 ± 3	−18.5 ± 4*	−8.3 ± 2	−5.5 ± 1	−3 ± 0.5	−5 ± 1.5
Phenylephrine	−10 ± 2	−8.5 ± 2	−5 ± 1.5*	0	0	0
Oxymetazoline	−13 ± 2.5	−10 ± 2.5	−8 ± 2	−6 ± 1.5	−4.5 ± 0.5	−6 ± 1.5

The LDF signal responses under control conditions have been compared to those 30 min after pretreatment with the monoamine uptake inhibitor desipramine (0.5 mg kg^{-1}, i.v.), the competitive α_2-antagonist idazoxan (0.5 mg kg^{-1}, i.v.), the competitive α_1-antagonist prazosin (0.1 mg kg^{-1}, i.v.), simultaneous pretreatment with idazoxan and prazosin, and simultaneous pretreatment with idazoxan, prazosin and the β-antagonist propranolol (0.5 mg kg^{-1}, i.v.). Values are mean ± s.e.mean; n = 4–8.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Kruskal-Wallis analysis of variance with multiple comparisons).

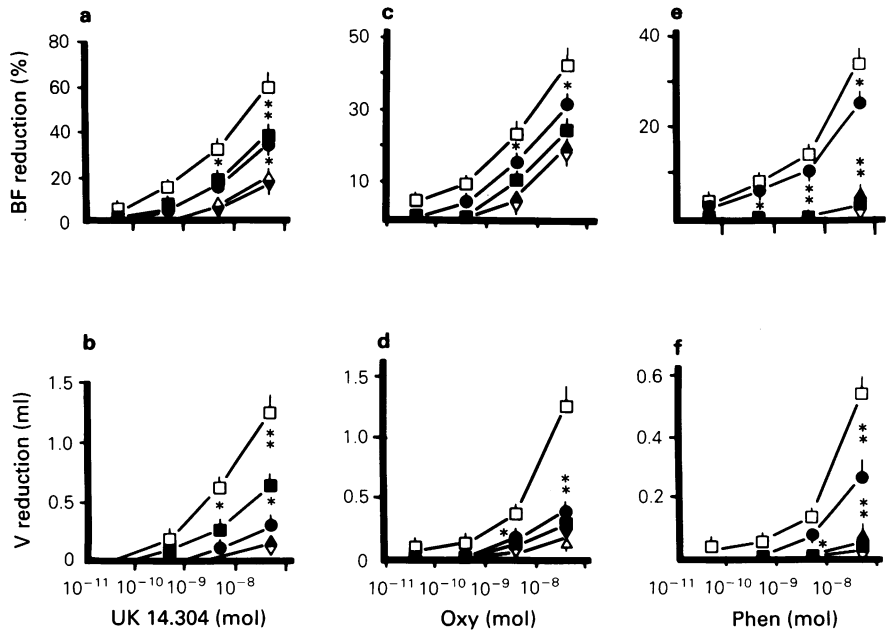

Figure 4 Dose-response curves to local i.a. bolus injections of UK 14.304 (a, b), oxymetazoline (Oxy, c, d), phenylephrine (Phen, e, f) on the nasal arterial blood flow (BF, in %) and on the nasal mucosal volume (V, in ml). (□—□) under control conditions, (■—■) after pretreatment with prazosin (0.1 mg kg^{-1}, i.a.), (●—●) after pretreatment with idazoxan (0.5 mg kg^{-1}, i.v.), (△—△) after simultaneous pretreatment with idazoxan and prazosin, and (▲—▲) after simultaneous pretreatment with idazoxan, prazosin and propranolol (0.5 mg kg^{-1}, i.v.). Means (with s.e.mean shown by vertical lines) are given, $n = 4$–8. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ using Kruskal-Wallis analysis of variance with multiple comparisons.

not significantly different compared to NA and an 18 ± 3% increase of the nasal BF was observed after concomitant prazosin and idazoxan pretreatment. This BF increase was abolished when propranolol was added to the α-adrenoceptor antagonists (not shown).

The reduction of BF and LDF by UK 14.304 (55 nmol) was attenuated to about the same extent after separate pretreatment with either prazosin or idazoxan (Figure 4a, Table 2). When these antagonists were given in combination, both the BF and LDF responses were further attenuated. The V reduction induced by UK 14.304 was more sensitive to idazoxan pretreatment than to prazosin ($P < 0.01$, Figure 4b).

Prazosin and idazoxan were equally effective in attenuating the LDF, BF and V responses to oxymetazoline (Figure 4c and 4d). Simultaneous administration of these antagonists did not further reduce the responses to oxymetazoline (Figure 4c–d, Table 2).

After idazoxan pretreatment the effects on BF and V observed upon phenylephrine administration were only marginally influenced and significantly reduced only at the highest dose (50 nmol) whereas after prazosin less than 10 ± 3% of these responses to phenylephrine remained (Figure 4e and f). Phenylephrine induced no modification of the LDF signal after prazosin (Table 2).

The LDF, BF and V responses to phenylephrine, UK 14.304 and oxymetazoline were not further modified after addition of propranolol (Figure 4a–f, Table 2).

Effects of adrenoceptor antagonists on the responses to sympathetic nerve stimulation

Single impulse stimulation of the superior cervical sympathetic trunk caused a BF and V reduction by 47 ± 5% and 0.41 ± 0.08 ml, respectively (Figure 5a, b), whereas a 4.6 ± 1% increase of the LDF signal occurred (Table 2). Following DMI pretreatment the BF response was prolonged more than 5 fold whereas the increase of the peak BF reduction was not significant. On the other hand, after DMI, the V

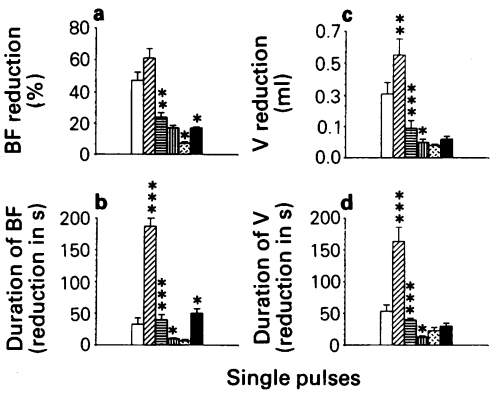

Figure 5 Effects of superior cervical sympathetic trunk stimulation (5 ms, 15 V) with single shocks on (a) the blood flow (BF, in %) reduction, (b) the duration of the BF response (in s), (c) the mucosal volume (V, in ml) reduction and (d) the duration of the V reduction (in s). Under control conditions (open columns), 30 min after pretreatment with desipramine (0.5 mg kg^{-1}, i.v., hatched columns), after pretreatment with prazosin (0.5 mg kg^{-1}, i.v., horizontally lined columns), after pretreatment with idazoxan (0.1 mg kg^{-1}, i.a., vertically lined columns), following simultaneous pretreatment with idazoxan and prazosin (stippled columns), and simultaneous pretreatment with idazoxan, prazosin and propranolol (0.5 mg kg^{-1}, i.v., solid columns). Values represent mean of 4–8 experiments and vertical bars denote s.e.mean * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ when compared to the preceding stimulation by Kruskal-Wallis analysis of variance with multiple comparisons.

response upon single impulse was significantly enhanced by 38 ± 8% ($P < 0.01$) (Figure 4c) and the LDF signal then showed a reduction by 13.5 ± 1.5% ($P < 0.001$) (Table 2). After prazosin pretreatment 48 ± 5% and 46 ± 6% respectively of the BF and V responses observed to a single impulse in control animals remained (Figure 5a, b). Idazoxan also reduced the BF response and was a more effective blocking agent than prazosin regarding the V response to a single impulse. A cumulative effect of these adrenoceptor antagonists was seen on the BF response to single pulse but not on the V. An enhancement of the reduction in the remaining vascular responses was observed only for BF when propranolol was added (Figure 5). Prazosin and idazoxan attenuated the reduction of the LDF response to single impulse stimulation after DMI (Table 2). Thus, the negative LDF response observed after DMI pretreatment was then changed to an increase of the LDF signal (Table 2).

Sympathetic stimulation at 2 Hz caused a BF and LDF reduction of 87 ± 12 and 25 ± 4%, respectively, whereas a 2.37 ± 0.26 ml decrease of the V was observed (Figure 6, Table 2). No significant enhancement of the LDF signal, BF and V peak values upon 2 Hz stimulation occurred after DMI pretreatment but all these parameters were markedly prolonged ($P < 0.001$) (Figure 6). In the presence of prazosin the vascular responses upon 2 Hz stimulation were attenuated by 30–40% ($P < 0.01$). The BF and LDF reduction at 2 Hz were not affected by pretreatment with idazoxan when compared to the effects observed after DMI, whereas the V response was significantly reduced (Figure 6, Table 2). Simultaneous pretreatment with idazoxan and prazosin had no cumulative effect on the attenuation of the vascular responses at 2 Hz when compared to prazosin given alone (Figure 6, Table 2). However, when propranolol was added after these agents, a significant increase ($P < 0.05$) of the BF and V responses occurred while the LDF recordings remained unchanged (Table 2).

Discussion

The present study gives a detailed description of the adrenoceptor mechanisms for endogenously released transmitter and exogenous agonists in three different compartments of the nasal mucosal vascular bed of the pig. The duration of the vascular responses to nerve stimulation, both regarding BF and V, were enhanced after neuronal reuptake inhibition by DMI (see also Eccles & MacLean, 1977). This suggests that noradrenergic mechanisms play an important role in the sympathetic vascular control especially at low frequency stimulation (Lacroix *et al.*, 1988b; 1989). Although both the agonists and antagonists studied have relative α_1-, α_2- and β-receptor selectivity, some comparative conclusions can be drawn regarding the occurrence of adrenoceptor subtypes in the different vascular compartments studied. For the resistance vessels it is likely that both α_1- and α_2-adrenoceptor mechanisms are present. Thus, the BF response to single impulse stimulation, when the 'non-adrenergic component' is relatively small (see Lacroix *et al.*, 1988b), was reduced to a similar extent by both prazosin and idazoxan. It is generally assumed that α_1-receptors are located close to the neuronal release sites for NA while α_2-receptors are mainly activated by circulating catecholamines (Docherty & McGrath, 1980; Langer *et al.*, 1980; Yamaguchi & Kopin, 1980; Langer & Shepperson, 1982). In the present study where DMI pretreatment was used, the released NA may have diffused away from the release sites, escaping neuronal reuptake to activate also α_2-receptors. A second argument for the presence of α_1-receptors in the resistance vessels was

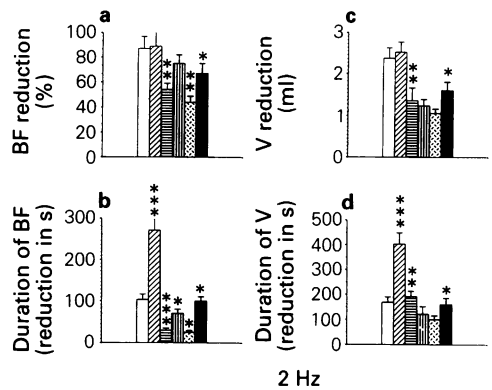

Figure 6 Effects of superior cervical sympathetic trunk stimulation (5 ms, 15 V) at 2 Hz on (a) the blood flow (BF, in %) reduction, (b) the duration of the BF response (in s), (c) the volume (V, in ml) reduction and (d) the duration of the V reduction (in s). Under control conditions (open columns), 30 min after pretreatment with desipramine (0.5 mg kg^{-1}, i.v., obliquely hatched columns), after pretreatment with prazosin (0.1 mg kg^{-1}, i.v., horizontally lined columns), after pretreatment with idazoxan (0.5 mg kg^{-1}, i.v., vertically lined columns), following simultaneous pretreatment with idazoxan and prazosin (stippled columns), and simultaneous pretreatment with idazoxan, prazosin and propranolol (0.5 mg kg^{-1}, i.v., solid columns). Values represent mean of 4–8 experiments and vertical bars denote s.e.mean. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ when compared to the preceding stimulation using Kruskal-Wallis analysis of variance with multiple comparisons.

the finding that the BF response to low doses of the preferential α_1-agonist phenylephrine was markedly reduced by prazosin but only to a small extent by idazoxan. The similar reduction of the BF response to the selective α_2-agonist UK 14.304 by both prazosin and idazoxan may be related to the prazosin dose used, which was at the upper limit of α_1-selectivity.

The BF response to nerve stimulation (both single impulse and 2 Hz) after α-adrenoceptor blockade was enhanced by addition of propranolol, suggesting that neuronally released NA activated β-receptors on resistance vessels. Furthermore, both exogenous NA and adrenaline caused biphasic BF effects after prazosin or idazoxan with an initial decrease followed by an increase. When combined pretreatment of these α-blocking agents with propranolol were done, both the NA and adrenaline induced increase of BF was attenuated. Finally, the selective β_2-agonist terbutaline caused a dose-dependent increase of BF. The presence of β_2-adrenoceptors in resistance vessels of the pig nasal mucosa therefore seems to be well documented. β_2-Adrenoceptors may also be present in resistance vessels of the nasal mucosa of cat (Malm, 1974b).

Regarding capacitance vessels function, α_2-receptors seem to dominate over α_1-mechanisms (see Berridge & Roach, 1986) since the V responses to (1) single impulse stimulation, (2) exogenous NA and (3) UK 14.304 were more attenuated by idazoxan than prazosin. Only a minor contribution by β-adrenoceptors seemed to be present for the capacitance function since the V response to single impulse nerve stimulation was not influenced by propranolol. Furthermore, adrenaline, which has potent β-stimulating actions, was a relatively more potent agent in reducing V than the BF. Moreover, terbutaline caused only a minor increase of the nasal mucosa V (see also Malm, 1974b). This last observation also suggests that 20% increase in BF via dilatation of the resistance vessels only marginally influenced the capacitance vessels of the nasal mucosa.

Andersson & Bende (1984), postulated that α_1-adrenoceptors are functionally more important for capacitance function than for control of BF, based on experiments with local applications of phenylephrine and α_2-agonists in the human nasal mucosa. However, the present data suggest that α_2-mechanisms clearly dominate in the pig nasal mucosa. Thus, the ratio between α_1- and α_2-receptor mechanisms (as revealed by phenylephrine and UK 14.304 effects, respectively) was about 1:10 both regarding BF (BF 25) and V (V 0.5) control (see Table 1). In our study, the α-agonists were given i.a. which may give a different access to receptor sites compared to when the agents are applied locally to the nasal epithelium. Furthermore, Andersson & Bende (1984), observed only systemic effects of the β_2-agonist terbutaline but no influence on local nasal blood flow. In addition, the ^{133}Xe washout method used may mainly reflect superficial nasal BF and the LDF signal was also not influenced by terbutaline in the pig nasal mucosa. β-Adrenoceptor mechanisms seemed to be of minor importance for the LDF signal since in addition: (1) propranolol did not change the nerve stimulation evoked response, and (2) adrenaline caused only a strong reduction of the LDF signal. The absence of a β-adrenoceptor mediated response therefore separates the LDF signal from the BF recording. Both α_1- and α_2-receptor mechanisms influenced the LDF signal, however, since the response to single impulse nerve stimulation was reduced to a similar extent by either prazosin or idazoxan. It is notable that the LDF signal was increased upon single impulse stimulation under control conditions in spite of a parallel BF reduction. Whether this apparent contradiction between the total BF recording and the LDF signal

is related to shunting of blood flow from deeper to more superficial layers of the nasal mucosa or some other mechanism such as increased velocity of blood cells in contracting vessels remains to be established.

Regarding the vascular response that remains to nerve stimulation in the pig nasal mucosa after the competitive adrenoceptor blocking agents used in this study, this is also observed after local i.a. infusion of a very high dose of the irreversible α-adrenoceptor antagonist phenoxybenzamine (Lacroix *et al.*, 1988a, b). Relatively similar responses to single impulse stimulations (almost 20% of BF and V) remain after the combination of prazosin and idazoxan compared to phenoxybenzamine (Lacroix *et al.*, 1988b). Furthermore, the response to exogenous NA was blocked to a similar extent by the present combination of competitive adrenoceptor antagonists and phenoxybenzamine (see Lacroix *et al.*, 1988a). Due to interference by idazoxan with the α_2-mediated feedback inhibition of mediator release upon nerve stimulation at 2 Hz (but probably not to single impulse) the increase in NA release (see Berridge & Roach, 1986; Lacroix *et al.*, 1989) may reduce the postjunctional antagonist effects of this drug. The BF and V reduction to sympathetic nerve stimulations, however, persist especially at 2 Hz and higher frequency and also after depletion of NA induced by reserpine pretreatment (Lacroix *et al.*, 1988b). These remaining vascular responses could be due to the release of another vascoconstrictor agent from sympathetic nerves namely neuropeptide Y (NPY) (see Lacroix *et al.*, 1988b; 1989). NPY release, however, is also likely to increase in the presence of α_2-adrenoceptor blockade (see Lacroix *et al.*, 1989; Lundberg *et al.*, 1989).

In conclusion, the occurrence of adrenoceptor mechanisms in the nasal mucosa of the pig varies between different vascular compartments. Thus, whereas both α_1- and α_2-receptor-mediated actions seem to be involved in the sympathetic control of BF, V and the LDF signal, the α_2-effects dominate for exogenous agonists. On the other hand β-receptor-mediated vasodilatation is mainly present in resistance vessels.

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Gastric damage following local intra-arterial administration of reactive oxygen metabolites in the rat

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1 The effects of reactive oxygen metabolites on the rat gastric mucosa following close-arterial infusion into the left gastric artery have been determined by macroscopic and histological assessment.

2 Local intra-arterial infusion of hydrogen peroxide (0.6–1.3 μmol kg^{-1} min^{-1}) induced mucosal injury, characterised by areas of pronounced disruption and haemorrhage, which was prevented by concurrent intravenous administration of catalase.

3 Local infusion of the superoxide generating system xanthine-oxidase and hypoxanthine likewise induced extensive haemorrhagic damage and necrosis of the mucosa. Prolonged incubation of this mixture (10 min) before administration, significantly reduced the degree of injury, indicating the lability of the products so formed.

4 The gastric mucosal injury induced by the superoxide generating system was inhibited by concurrent local infusion of superoxide dismutase (96 u kg^{-1} min^{-1}), as was the associated increase in mucosal permeability to radiolabelled albumin.

5 Administration of catalase did not inhibit the gastric mucosal damage induced by infusion of xanthine oxidase-hypoxanthine, yet augmented the protective effects of a low dose of superoxide dismutase (46 u kg^{-1} min^{-1} i.a.).

6 These findings directly confirm that reactive oxygen metabolites can induce extensive gastric mucosal injury, supporting their role in the pathogenesis of gastric damage following ischaemia and hypotensive shock.

Introduction

The local generation of oxygen-derived free radicals, predominantly superoxide (O_2-) and hydroxyl radicals (OH.) are considered to be involved in the gastrointestinal damage associated with haemorrhagic shock or ischaemia-reperfusion conditions. Thus, inhibition of the enzyme involved in free-radical generation and radical scavengers greatly reduce the changes in vascular permeability that occur after ischaemia in the intestine (Parks *et al.*, 1982; Parks & Granger, 1983; Perry *et al.*, 1986). Furthermore, pretreatment with oxygen radical scavengers decreases macroscopic gastric damage and reduces blood loss after haemorrhagic shock or ischaemia (Itoh & Guth, 1985; Perry *et al.*, 1986). More direct evidence for the tissue damaging actions of such oxygen metabolites in intestinal tissue has come from studies where local generation of superoxide radical induced substantial permeability changes (Parks *et al.*, 1984). Recently, local infusion of a superoxide generating system into the rat coeliac artery, has been shown to cause gastric mucosal bleeding, as assessed by the leakage of radiolabelled erythrocytes (Wadhwa & Perry, 1987).

Although superoxide and hydroxyl radicals are considered to be the major cytotoxic oxygen-derived moieties, recent *in vitro* experiments have also suggested that hydrogen peroxide (H_2O_2), itself not a free radical but a reactive oxygen metabolite, is more damaging to rat isolated gastric mucosal cells (Hiraishi *et al.*, 1987). In the present study, we have directly assessed the haemorrhagic damage to the rat gastric mucosa, using macroscopic and histological techniques, as well as the leakage of radiolabelled plasma albumin in the gastric tissue, following local

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intra-arterial infusion of H_2O_2 and of the superoxide generating system, hypoxanthine-xanthine oxidase. In addition, the effects of catalase, which reduces H_2O_2 to water and molecular oxygen, and of the enzyme superoxide dismutase (SOD), which scavenges superoxide radicals, on such a damage have been evaluated.

A preliminary account of this work has been presented to the British Pharmacological Society (Esplugues & Whittle, 1988a).

Methods

Male Wistar rats (230–260g), deprived of food but not water for 18–20 h before the experiments, were anaesthetised with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and the stomach exposed by a midline incision. The left gastric artery was carefully freed from connective tissue under a stereomicroscope and cannulated with a modified short 23 g teflon cannula as described in detail by Esplugues & Whittle (1988b,c). After ligation of the oesophagus and the duodenum, 3 ml of 0.1 M HCl in saline was instilled into the gastric lumen, via a 25 g needle inserted through the forestomach wall.

Hydrogen peroxide (H_2O_2; 0.65 and 1.3 μmol kg^{-1} min^{-1}) or isotonic saline was infused into the left gastric artery for 10 min at a rate of 10 μl min^{-1} using a Braun Perfuser pump. The effects of 5 min pretreatment with catalase (5×10^4 u kg^{-1} i.v. bolus, followed by a continuous infusion of 0.2×10^4 u kg^{-1} min^{-1} i.v.), were evaluated on H_2O_2-induced damage; the dose used was derived from preliminary dose-ranging studies.

Oxygen radicals were generated by separately infusing (20 μl min^{-1} of each) a solution of xanthine oxidase (0.125 u ml^{-1} in saline) and hypoxanthine (1.4 mg ml^{-1} in buffered saline, pH 7.4) into a mixing microchamber immediately before the intra-arterial teflon cannula, the doses being 0.01 u kg^{-1} min^{-1} and 112 μg kg^{-1} min^{-1} respectively. The period of infusion was chosen from preliminary dose- and time-ranging studies, as 30 min. In control rats, the vehicle alone was infused into the mixing microchamber, at the same rate as during radical generation.

Experiments were carried out to confirm that the gastric damage induced by the hypoxanthine-xanthine oxidase infusion was caused by the local generation of labile oxygen radicals, and was not the consequence of a non-specific effect of the infusion technique or the agents employed. Thus, in one study, a 10 min delay chamber between the mixing of the enzyme-substrate and its reaching the arterial cannula was introduced. Additional studies were performed where superoxide dismutase (SOD) was added to the hypoxanthine solution immediately before infusion (final dose, 48 or 96 u kg^{-1} min^{-1}). In order to clarify a possible role of H_2O_2 in the gastric damage induced by this local generation of oxygen radicals, the effects of catalase (5×10^4 u kg^{-1}, i.v. bolus; followed by a continuous infusion of 0.2×10^4 u kg^{-1} min^{-1}, i.v.) were also evaluated in this system.

Assessment of damage

Twenty minutes after terminating the local intra-arterial infusion, the stomachs were removed and opened along the greater curvature. The stomachs were pinned out, mucosal side up, to a wax block and immersed in neutral buffered formalin and then photographed on colour transparency film. The extent of macroscopically-visible damage was determined from these projected transparencies in a randomized manner via computerised planimetry. The mucosal damage was calculated as the % of the glandular mucosa showing macroscopically-visible damage.

Two samples of the fundus (corpus) were excised from comparable regions in each stomach and were processed by routine techniques before embedding in paraffin. Sections (4 μm) were stained with haematoxylin and eosin and examined under a light microscope. The 1 cm length of each histological section was divided into 3 fields. Each field was histologically scored on a 0 to 4 scale according to the following criteria: 0 = normal; 1 = epithelial cell damage; 2 = glandular disruption, vasocongestion or oedema in the upper mucosa; 3 = mucosal disruption, vasocongestion or oedema in the mid-lower mucosa; 4 = extensive mucosal disruption involving the full thickness of the mucosa. The overall mean value of the scores for each of the 3 fields was taken as the histological index for that section. All determinations were performed in a randomised manner with both transparencies and histological sections coded to eliminate observer bias.

Plasma leakage

To determine the effects of local intra-arterial infusion of oxygen metabolites on vascular permeability, ^{125}I-labelled human serum albumin (10 μCi, 3.7×10^{-6} GBq) was injected intravenously 10 min before the start of the local infusion. At the end of the study (20 min after local infusion), the abdomen was opened and 1 ml of blood was drawn from the inferior vena cava. The blood sample was centrifuged (2 min; 9000 *g*) and a 100 μl aliquot of the plasma was transferred to a plastic test tube for counting on a

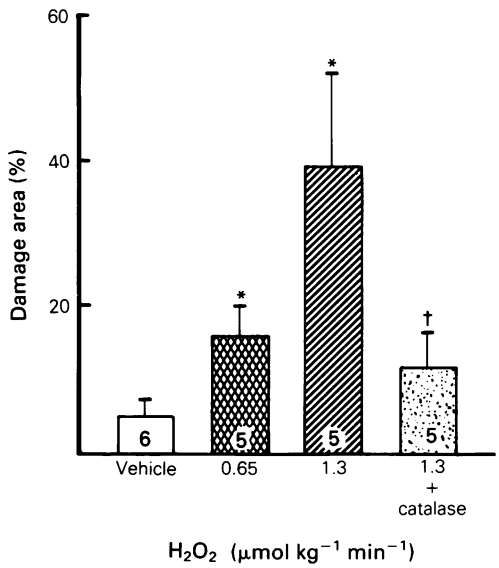

Figure 1 Gastric mucosal damage induced by a 10 min local intra-arterial infusion of hydrogen peroxide (H_2O_2; 0.65–1.3 μmol kg^{-1} min^{-1}) in the rat, and its inhibition by concurrent intravenous administration of catalase (5×10^4 u kg^{-1} bolus, followed by 0.2×10^4 u kg^{-1} min^{-1}). Results, shown as the macroscopically assessed damage area expressed as % of the total mucosal area, are mean of n (number in column) experiments. Bars show s.e.mean. Significant difference from the vehicle is shown as * $P < 0.05$ and from the higher dose of H_2O_2 as † $P < 0.05$.

gamma-spectrometer. After the stomach had been excised, sections of the corpus were taken. These tissues were blotted dry and weighed (wet weight, 250–350 mg), then placed in glass tubes for quantification of tissue levels of radioactivity. The volume of plasma present in the tissue samples was calculated by reference to the radioactivity of the plasma sample. This determination of interstitial albumin, used as an index of plasma leakage, would also include any intravascular albumin (Wallace *et al.*, 1987).

Table 1 Histologically assessed gastric damage following the local intra-arterial infusion of hydrogen peroxide (H_2O_2, 1.3 μmol kg^{-1} min^{-1} for 10 min), with the stomach removed 20 min later

Treatment	(n)	*Histological index*
Saline	7	0.8 ± 0.3
H_2O_2	7	2.1 ± 0.3*
H_2O_2 + calalase	6	1.1 ± 0.2†

In one group, rats were treated with catalase (5×10^4 u kg^{-1} bolus, followed by 0.2×10^4 u kg^{-1} min^{-1}; i.v.). Results, expressed as a histological index (scale, 0–4) are shown as mean ± s.e.mean. Significant difference from control (local intra-arterial saline infusion for 10 min) is given as * $P < 0.05$ and from the H_2O_2 group as † $P < 0.05$.

Drugs

Hydrogen peroxide (Analar, B.D.H. 100 volumes) was diluted in saline immediately before the beginning of each perfusion. Catalase (from bovine liver), hypoxanthine, xanthine oxidase and superoxide dismutase (Sigma Chemical Co.) were dissolved in isotonic saline. ^{125}I-labelled human serum albumin (2.5 μCi mg^{-1}, Amersham) was dissolved in isotonic saline to give a final activity of 40 μCi ml^{-1}.

Statistical analysis

All data are expressed as mean ± s.e.mean. Comparisons between groups of parametric data were made by Student's t test for unpaired data. Comparisons between groups of non parameteric data (histological index) were made by the Mann-Whitney U-test. P values of less than 0.05 were taken as significant.

Results

Gastric mucosal damage by H_2O_2

Following intragastric instillation of 3 ml 0.1 M HCl in saline, close intra-arterial infusion of isotonic saline (10 μl min^{-1}) for 10 min induced minor macroscopic damage to the rat gastric mucosa, when assessed 20 min later, involving 4.9 ± 2.3% ($n = 6$) of the total mucosal area. Local intra-arterial infusion of H_2O_2 (0.65 and 1.3 μmol kg^{-1} min^{-1}) for 10 min resulted in a significant ($P < 0.05$) and dose-dependent increase in the macroscopically assessed damage (Figure 1). This damage was located in the upper regions of the stomach corpus and in the antrum. The nature of the damage, particularly in the corpus, was characterised as areas of mucosal disruption, sometimes giving the appearance of deep penetrating ulcers, and intense haemorrhage.

The effect of catalase on the gastric damage after local infusion with H_2O_2 was investigated. Intravenous bolus administration of catalase (5×10^4 u kg^{-1} i.v. bolus, followed by 0.2×10^4 u kg^{-1} min^{-1} i.v.) caused a significant reduction ($P < 0.05$) in the degree of mucosal damage induced by local intra-arterial infusion of

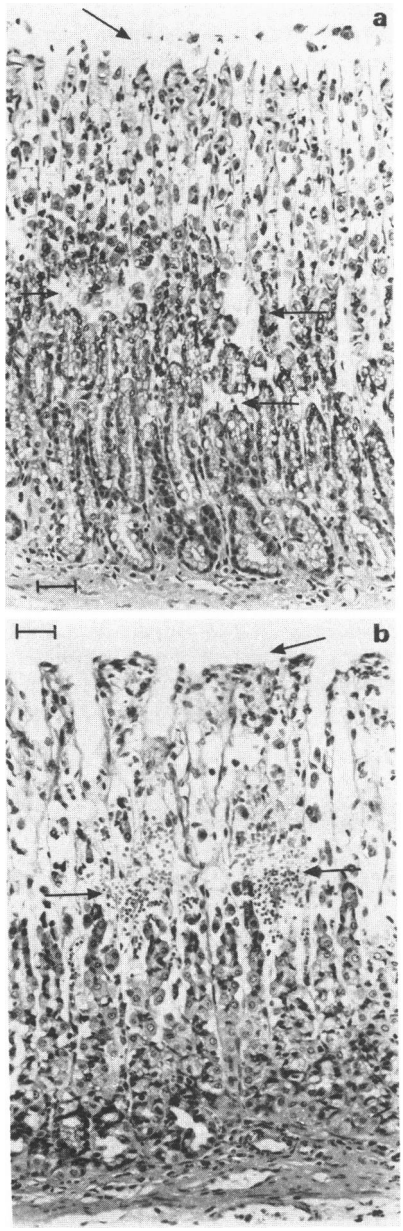

Figure 2 Histological appearance of the corpus region of the rat gastric mucosa following the local intra-arterial infusion of (a) hydrogen peroxide (H_2O_2, 1.3 μmol kg^{-1} min^{-1}) for 10 min and (b) the superoxide generating system, xanthine oxidase (0.01 u kg^{-1} min^{-1}) and hypoxanthine (112 μg kg^{-1} min^{-1}) for 30 min. Sections are stained with haematoxylin and eosin. The upper arrow denotes epithelial disruption while the lower arrows denote areas of deeper damage and extravasation of erythrocytes. Marker bar represents 40 μm.

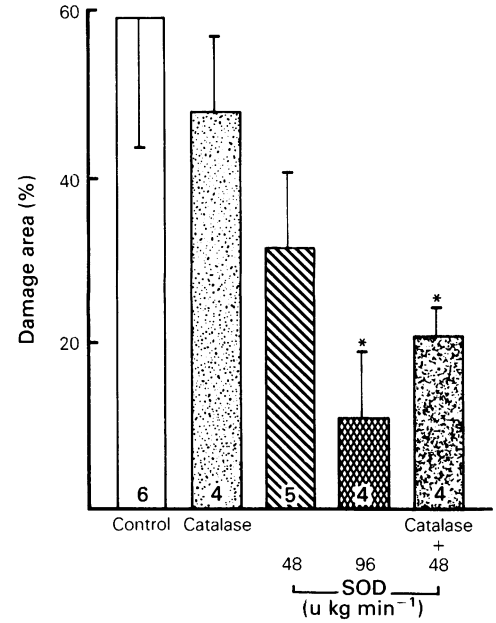

Figure 3 Effect of intravenous administration of catalase (5×10^4 u kg^{-1} bolus followed by 0.2 $\times 10^4$ u kg^{-1} min^{-1}) and local intra-arterial infusion of superoxide dismutase (SOD, 48 or 96 u kg^{-1} min^{-1}) on gastric mucosal damage induced by a 30 min local infusion of xanthine oxidase (0.01 u kg^{-1} min^{-1}) and hypoxanthine (112 μg kg^{-1} min^{-1}). Results, shown as the macroscopically assessed damage area expressed as % of total mucosal area, are mean of n (numbers in columns) experiments. Bars show s.e.mean. Significant difference from the control damage group (open column) is shown as * $P < 0.05$.

H_2O_2 (1.3 μmol kg^{-1} min^{-1} for 10 min) as shown in Figure 1. In control experiments, this dose of catalase alone had no significant effect on the macroscopic appearance of the gastric mucosa ($n = 3$).

Local infusion of H_2O_2 also increased the histological index (Table 1). This H_2O_2-induced damage was unevenly located with small patches of normal mucosa co-existing with areas of extensive damage to the surface epithelium, dilatation of the gastric glands and, in some cases, haemorrhagic damage and necrosis to the mid and lower mucosa (Figure 2). Intravenous administration of catalase significantly reduced ($P < 0.05$) the histologically assessed mucosal damage induced by H_2O_2 (Table 1).

Gastric mucosal damage by superoxide generation

Local intra-arterial infusion of isotonic saline (40 μl min^{-1}) for 30 min, with 3 ml of 0.1 N HCl

instilled intraluminally, produced minimal damage that involved 10.1 ± 5.3% ($n = 6$) of the total area of the mucosa. Generation of oxygen radicals by intra-arterial infusion of the mixture of hypoxanthine-xanthine oxidase (112 $\mu g\,kg^{-1}\,min^{-1}$ and 0.01 $u\,kg^{-1}\,min^{-1}$ respectively), induced a significant ($P < 0.05$) increase in the level of damage, which affected 59 ± 16% ($n = 6$) of the total area of the mucosa (Figure 3). Although there were focal areas of severe injury, the damage was generally diffuse and of haemorrhagic nature, particularly in the corpus, and a thick layer of mucous material covered the damaged area.

There was a 70 ± 24% reduction ($P < 0.05$) in the mucosal damage area when a 10 min delay was introduced between the mixing of xanthine oxidase and the hypoxanthine in the microchamber and its reaching of the left gastric artery (Table 2). Furthermore, concurrent local infusion of SOD (96 $u\,kg^{-1}\,min^{-1}$), in a dose alone not affecting the macroscopic appearance of mucosa ($n = 3$), significantly reduced the damage induced by the superoxide generating system by 81 ± 15% ($n = 4$; $P < 0.05$), as shown in Figure 3. Treatment with catalase ($5 \times 10^4\,u\,kg^{-1}$, i.v. bolus, followed by $0.2 \times 10^4\,u\,kg^{-1}\,min^{-1}$, i.v.) did not significantly reduce the gastric damage determined after superoxide generation (Figure 3). However, when administered simultaneously with a lower dose of SOD (48 $u\,kg^{-1}\,min^{-1}$), which itself did not significantly reduce the damage, the degree of mucosal damage was significantly reduced by 65 ± 6% ($n = 4$, $P < 0.05$).

Histologically, the damage induced by this oxygen radical generating system was characterised by severe damage to the surface epithelium, disruption of the glandular structure, extravasation of erythrocytes and, in some cases, damage and necrosis in the deeper parts of the mucosa (Figure 2). Concurrent treatment with SOD reduced the levels of histologically assessed damage to values similar to that of the control (Table 2). Under these conditions, damage was limited to the epithelium of the mucosa, and only in the most severe cases, was there some dilatation of the gastric glands and/or subepithelial oedema.

Table 2 Macroscopically assessed area of mucosal damage, histologically assessed mucosal damage and the gastric interstitial leakage of ^{125}I-labelled human serum albumin following the local intra-arterial administration of the superoxide-generating system, xanthine oxidase (XO; 0.01 $u\,kg^{-1}\,min^{-1}$) and hypoxanthine (HX; 112 $\mu g\,kg^{-1}\,min^{-1}$) infused at a rate of 20 $\mu l\,min^{-1}$ each for 30 min

Treatment	*Damage area* (%)	*Histological index*	*Tissue albumin* ($\mu l\,g^{-1}$)
Saline	10 ± 5	1.0 ± 0.3	32.8 ± 2.3
XO + HX (10 min)	18 ± 14	1.3 ± 0.6	NT
XO + HX (1 min)	59 ± 16*	2.5 ± 0.4*	52.7 ± 6.1*
XO + HX + SOD (1 min)	11 ± 9†	1.3 ± 0.4†	33.5 ± 2.5†

The period (1 or 10 min) represents the flow delay between the mixing microchamber and the agents reaching the left gastric artery. A further group of rats received a concurrent local infusion of superoxide dismutase (96 $u\,kg^{-1}\,min^{-1}$, SOD). Results are expressed as % area of total mucosa exhibiting damage, as a histological index (scale, 0–4) and tissue albumin content ($\mu l\,g^{-1}$) and shown as mean ± s.e.mean of 4–6 experiments (NT represents not tested). Significant difference from control is given as * $P < 0.05$ and from the XO + HX group as † $P < 0.05$.

Gastric plasma leakage

Control levels of plasma in the mucosal tissue, determined from the radiolabelled albumin content, following local intra-arterial infusion with saline (40 μl for 30 min), and with 3 ml of 0.1 M HCl instilled intraluminally, were 32.8 ± 2.3 $\mu l\,g^{-1}$ of wet tissue. These values were not significantly different from those obtained in other control experiments after ligation of the left gastric artery without intra-arterial infusion, following intraluminal instillation of either 3 ml of isotonic saline or of 0.1 M HCl (28 ± 1.8 and 29.5 ± 2.9 $\mu l\,g^{-1}$ of plasma, $n = 3$ and 4 respectively). Local intra-arterial infusion of the superoxide-generating system resulted in a 61 ± 19% ($P < 0.05$) increase in the level of albumin in the tissue, reflecting plasma leakage (Table 2). Concurrent local administration of SOD (96 $u\,kg^{-1}\,min^{-1}$) significantly ($P < 0.05$) reduced this plasma leakage to control levels (Table 2).

Discussion

The release of reactive oxygen-derived moieties has been implicated in the intestinal injury that follows periods of ischaemia (Parks *et al.*, 1982; Parks & Granger, 1983). The gastric mucosal damage that follows hypotensive shock in the rat has likewise been associated with the local generation of these cytotoxic oxygen metabolites (Itoh & Guth, 1985). Thus, such macroscopically assessed gastric damage

could be reduced by pretreatment with superoxide dismutase (SOD) which scavenges the superoxide radical, and by allopurinol which inhibits xanthine oxidase, the enzyme considered to generate the superoxide radical. Subsequent studies with this model have indicated that although the injury seen after a period of ischaemia followed by reperfusion was susceptible to such attenuation by allopurinol, the damage induced by the period of ischaemia alone was not, suggesting the involvement of oxygen radicals in reperfusion injury but not in the initial ischaemic damage (Yasue & Guth, 1988). In other studies in this hypotensive shock model, administration of a tungstate diet to inactivate xanthine oxidase, as well as SOD, reduced gastric damage as assessed by the leakage of chromium-labelled erythrocytes (Smith *et al.*, 1987a). Studies in the cat using mechanical occlusion to reduce gastric blood flow, likewise demonstrated that allopurinol, superoxide dismutase or dimethyl sulphoxide (which scavenges the hydroxy radical) reduced the resulting gastric mucosal damage, as determined by leakage of radiolabelled albumin and erythrocytes (Perry *et al.*, 1986).

The damaging actions of oxygen radicals on the intestine have been directly demonstrated using a superoxide generating system, following its intraluminal perfusion in the dog or intravascular infusion in the cat (Grogaard *et al.*, 1982; Parks *et al.*, 1984). Likewise in the rat, infusion of this system, comprising the enzyme xanthine oxidase and the substrate hypoxanthine, into the coeliac artery induced gastric mucosal bleeding, as determined by the leakage of radiolabelled erythrocytes (Wadhwa & Perry, 1987). This bleeding was of comparable magnitude to that following local ischaemia induced by mechanical occlusion of the coeliac artery. Mucosal damage was also noted by direct observation, although not quantitated in that study (Wadhwa & Perry, 1987). In the present investigation, macroscopic and histological techniques were utilized to evaluate the gastric damage induced by this superoxide generating system, when infused directly into the left gastric artery. Extensive diffuse haemorrhagic damage was observed throughout the mucosal tissue, with disruption of the epithelium and deep necrosis. Similar characteristics of tissue damage have been observed previously following local intra-arterial infusion of the pro-inflammatory mediator, platelet-activating factor (Paf) and the vasoconstrictor thromboxane mimetic, U-46619 (Esplugues & Whittle, 1988b,c). Although the area of damage was greater using the present superoxide generating system than with these other vasoactive agents, the degree of observed vasocongestion was less. These damaging effects were substantially reduced when the generating system was allowed to incubate for 10 min rather than the 1 min before its delivery to the gastric vasculature, indicating the lability of the pro-ulcerogenic products so formed.

Hydrogen peroxide (H_2O_2), which can react with the superoxide ion to generate the highly reactive hydroxyl radical, has itself been considered to be less cytotoxic than the oxygen radicals. However, intragastric application of a concentrated solution of H_2O_2 has been demonstrated to induce mucosal necrosis in a preliminary study in the rat (Schurer-Maly & Halter, 1986). Furthermore, studies *in vitro* have implicated H_2O_2 as the more toxic species that induced damage to rat cultured gastric epithelial cells following incubation with a xanthine-oxidase and hypoxanthine mixture, since this damage was reduced by catalase but not by SOD (Hiraishi *et al.*, 1987). In the present study *in vivo*, we have demonstrated that local arterial infusion of H_2O_2 induced areas of extensive haemorrhagic necrosis in the gastric mucosa, which were substantially reduced by concurrent intravenous administration of catalase. This dose of catalase did not reduce the mucosal damage induced by the superoxide generating system, suggesting that local generation of H_2O_2 by this system is not the major cytotoxic metabolite *in vivo*. It is not known whether higher doses of catalase would affect such damage. Local infusion of SOD did substantially reduce the extent of such macroscopic and histological damage, as well as the changes in vascular permeability to albumin, indicating the significant involvement of the superoxide ion, while catalase and low doses of SOD also interacted to inhibit the mucosal damage. A complex interaction between the generation of superoxide, H_2O_2 or the subsequent hydroxyl radical may thus underlie the mucosal damage induced by local infusion of this enzyme-substrate system.

The initial site of injury following local infusion of these oxygen metabolites is likely to be the microvascular endothelium, which could also lead to disturbances in mucosal blood flow, prime factors in the regulation of gastric integrity. These moieties are considered to induce cellular disruption by lipid peroxidation of the cell membrane constituents and by destroying the interstitial matrix following degradation of collagen and hyaluronic acid (Fridovich, 1978, Del Maestro *et al.*, 1980). In addition, damage to the endothelial cells would alter the release and actions of endogenous vasoactive mediators involved in the regulation of the mucosal microcirculation. Indeed, the formation of prostacyclin, which is a potent vasodilator in the gastric microcirculation and can protect against mucosal injury may be directly impaired, since the enzyme involved in its endogenous synthesis is readily inhibited by lipid peroxidases and free radicals (see Whittle & Vane, 1987). Furthermore, if endothelium-derived relaxing

factor (EDRF), now characterized as nitric oxide (Palmer *et al.*, 1987) should also play a role in the regulation of the gastric microcirculation, then these effects would be attenuated since this factor is highly susceptible to destruction by the superoxide ion (Gryglewski *et al.*, 1986).

The present study in the rat directly confirms the disruptive actions of the superoxide ion as well as H_2O_2 or subsequent metabolites on the gastric mucosa, supporting their involvement in the pathogenesis of the gastric damage seen after haemorrhagic shock or ischaemia followed by reperfusion. The source of these reactive oxygen moieties remains to be identified, but recent studies have implicated the circulating neutrophil, since anti-neutrophil serum reduced the erythrocyte leakage into the rat gastric lumen following haemorrhagic shock (Smith *et al.*, 1987b). It is of relevance that the pro-ulcerogenic lipid mediator, Paf can induce neutrophil aggregation and microaggregates of white cells can be observed in the mucosal and submucosal microcirculation (Rosam *et al.*, 1986; Whittle *et al.*, 1986). Thus, the release of oxygen metabolites from aggregating neutrophils by Paf may contribute to the resulting gastric mucosal damage induced by administration of this endogenous phospholipid. Furthermore, Paf has been implicated as a mediator of the gastro-intestinal damage induced by endotoxin or septic shock (Rosam *et al.*, 1986; Wallace *et al.*, 1987), while administration of SOD can attenuate the gastric damage in sepsis (Arvidsson *et al.*, 1985). Thus, the release of reactive oxygen moieties may form one link in the chain of events leading to gastric mucosal damage in a number of pathological situations including those involving ischaemia, shock or the endogenous release of vasoactive and tissue destructive mediators.

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Electrophysiological abnormalities and enhanced reperfusion arrhythmias in the isolated hearts of hyperthyroid rats

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1 The influence of hyperthyroidism on electrophysiological characteristics and on reperfusion arrhythmias was examined in rat hearts.

2 Electrophysiological studies were performed with glass microelectrodes, and the experiments on reperfusion arrhythmias were done in isolated perfused hearts.

3 Ventricular muscle from hyperthyroid rats was more prone than that from euthyroid rats to develop triggered activity under conditions believed to cause myoplasmic Ca^{2+} overload.

4 The severity of reperfusion arrhythmias was significantly enhanced in hyperthyroid preparations as compared with euthyroid ones.

5 The enhanced reperfusion arrhythmias in hyperthyroid rats were significantly reduced by propranolol (3×10^{-7} M), lignocaine (1×10^{-5} M) and verapamil (3×10^{-8} M), but not by nadolol (3×10^{-7} M) or prazosin (3×10^{-7} M).

6 These results suggest that increased heart rate due to hyperthyroidism and responses mediated via either α- or β-adrenoceptors were not dominant causes of enhanced reperfusion arrhythmias in hyperthyroid hearts.

7 The increased tendency to develop triggered activity which was observed in the electrophysiological study, may be one possible explanation of enhanced reperfusion arrhythmias in hyperthyroid hearts.

Introduction

Myocardium from pathophysiological models in rats such as streptozotocin-induced diabetes (Nordin *et al.*, 1985) and cardiac hypertrophy induced by renal hypertension (Gulch *et al.*, 1979; Aronson, 1981) has a propensity to develop Ca^{2+} overload as reflected by the appearance of delayed afterdepolarizations and triggered activity. It has been reported that reperfusion of the myocardium after ischaemia elicits accumulation of intracellular Ca^{2+} (Whalen *et al.*, 1974) and malignant ventricular dysrhythmias (Penkoske *et al.*, 1978). Therefore, it might be expected that the myocardium which has such abnormal electrophysiological properties would develop altered reperfusion arrhythmias. From this point of view, we examined first the electrophysiological abnormality in the ventricular muscles from hyperthyroid rats and then investigated the effects of hyperthyroidism on reperfusion arrhythmias in rat isolated perfused hearts. Furthermore, thyroxine increases myocardial sensitivity to catecholamines and other biogenic amines (Coville & Telford, 1970). Catecholamines are released from the myocardium during reperfusion after an ischaemic period *in vivo* (Riemersma, 1982) and *in vitro* (Rochette *et al.*, 1980), and it has been suggested that catecholamine release and α-receptor stimulation are involved in the development of reperfusion arrhythmias (Sheridan *et al.*, 1980). Hence, it is conceivable that reperfusion arrhythmias may also be affected by thyroid state-induced receptor alteration. In additional experiments, the effects of some drugs on reperfusion arrhythmias were also investigated to clarify the relationship of some mediators to the arrhythmias.

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Methods

Animals

Male Wistar rats weighing 220–240 g were randomly assigned to a euthyroid or hyperthyroid group. In the latter, a hyperthyroid state was induced by daily intraperitoneal injection of 1 mg kg^{-1} sodium L-thyroxine suspended in 0.5% carboxymethyl cellulose sodium salt for 8–12 days (Hashimoto & Nakashima, 1978). The euthyroid group received an equivalent volume of the vehicle. Animal care and feeding were identical in both groups.

Electrophysiological study

After rats were anaesthetized with ether, hearts were rapidly removed and the left ventricular papillary muscles were excised. The papillary muscle was mounted in a tissue chamber and superfused with modified Tyrode solution which was equilibrated with 95% O_2 : 5% CO_2 and maintained at 35°C. Control modified Tyrode solution had the following composition (mM): NaCl 137, KCl 2.7, $CaCl_2$ 2.4, $MgCl_2$ 1.0, NaH_2PO_4 0.5, $NaHCO_3$ 12.0 and glucose 5.5. Transmembrane action potentials were recorded by glass microelectrodes filled with 3 M KCl and having a tip resistance of 10 to 20 MΩ. The microelectrode was coupled with an Ag-AgCl electrode and connected to an amplifier. External electrical stimulation was delivered to the tissue through bipolar silver electrodes. Stimuli were rectangular pulses, 1 ms in duration and 2 times the threshold voltage. Action potential parameters were measured at a cycle length of 1000 ms after 60 min equilibration of muscle in control Tyrode solution. Continuous recordings of electrical activity were obtained on a recorder and analysis of action potentials was performed by a microcomputer interfaced with an analogue-digital converter for pulse analysis (Nihon Kohden, ATAC-450).

In the study of triggered activity, each muscle was equilibrated in control Tyrode solution (Ca^{2+} = 2.4 mM) for about 60 min, and then exposed sequentially to Tyrode solution containing increased concentrations of Ca^{2+}; 4.8 and 9.6 mM. The stimulation protocol during superfusion with each solution was as follows: 20 stimuli at a cycle length of 2000 ms, followed by stimulation at a cycle length of 150 ms with trains of 10, 20 and 40 stimuli; a quiescent period of 20 s was allowed after each stimulation sequence (Nordin et al., 1985). Continuous recordings were obtained on a chart recorder.

Reperfusion arrhythmias

Rats were pretreated with heparin sodium (200 iu, i.v.), anaesthetized with ether and the hearts were quickly excised and placed in chilled, heparinized and preoxygenated Krebs-Henseleit (K-H) solution. After a glass cannula filled with the solution had been inserted into the aorta, the heart was mounted in a water-jacketed chamber and perfused retrogradely by the Langendorff technique with K-H solution at a constant pressure (80 mmHg) at 37°C (Sakai & Shiraki, 1978). The K-H solution had the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 1.2, $MgSO_4$ 1.18, KH_2PO_4 1.18, $NaHCO_3$ 24.88 and glucose 11.1. The solution was saturated with 95% O_2 : 5% CO_2, and the pH was 7.4 at 37°C. A polyethylene cannula was inserted via the left atrium into the left ventricle to record the left ventricular cavity pressure (LVP). Silver wire electrodes were attached at the origin of the aorta and the apex of the ventricle to record the electrocardiogram (ECG). In some euthyroid heart preparations, the heart rate was fixed by electrical pacing. The isolated heart was perfused for 20 min to stabilize cardiac function. Drug treatment was started after stabilization by changing the perfusate to one which contained drug and the drug level was then maintained throughout the remainder of the experiment. Fifteen minutes later, the left coronary artery was ligated at a position about 2–3 mm from its origin. Reperfusion arrhythmias were induced by untying the coronary ligature after 10 min of ischaemia. At the end of each experiment, the heart was weighed.

Statistical analysis

The Mann-Whitney U-test was used to compare the data for reperfusion arrhythmias between euthyroid

Table 1 Characteristics of experimental animals

	BW (g)	*HW* (mg)	*HR* (beats min^{-1})	*LVP* (mmHg)
Euthyroid	261 ± 5 (23)	712 ± 19 (21)	299 ± 8 (21)	93.3 ± 2.4 (20)
Hyperthyroid	226 ± 2 (56)	885 ± 10 (48)	366 ± 14 (48)	105.9 ± 4.1 (48)
	$P < 0.001$	$P < 0.001$	$P < 0.01$	NS

Data are given as mean ± s.e.mean. Numbers in parentheses: number of preparations measured. BW: body weight; HW: heart weight; HR: heart rate; LVP: left ventricular pressure; NS: not significant at $P = 0.05$. Statistical significance was determined by Student's t test.

Table 2 Electrophysiological characteristics of papillary muscle cells

	RMP (mV)	*AMP* (mV)	$\dot{V}_{max}$ ($V\ s^{-1}$)	APD_{50} (ms)	APD_{90} (ms)	*ERP* (ms)
Euthyroid ($n = 8$)	-77.0 ± 1.0	99.9 ± 1.3	156 ± 6	11.1 ± 0.8	44.6 ± 3.1	31.7 ± 1.3
Hyperthyroid ($n = 8$)	-74.1 ± 1.4 NS	98.5 ± 1.3 NS	153 ± 9 NS	15.3 ± 0.6 $P < 0.01$	71.9 ± 2.6 $P < 0.01$	58.1 ± 5.2 $P < 0.01$

Results are expressed as mean ± s.e.mean.
RMP: resting membrane potential; AMP: amplitude of action potential; $\dot{V}_{max}$: maximum rate of depolarization; APD_x: duration of action potentials to x% of complete repolarization; ERP: effective refractory period; NS: not significant at $P = 0.05$. Statistical significance was determined by Student's t test.

and hyperthyroid groups. The Chi-squared test was used to analyze the data on the tendency of the myocardium to develop triggered activity. Student's t test was used to compare various other characteristics between each group. A probability value of less than 0.05 was considered to be statistically significant.

Results

Characteristics of experimental animals

The characteristics of experimental animals are summarized in Table 1. Hyperthyroid rats failed to gain their body weight. In the hyperthyroid group, the hearts were significantly hypertrophied and the heart rate was significantly greater than that observed in the euthyroid group.

Electrophysiological characteristics

The electrophysiological characteristics of papillary muscle cells obtained from euthyroid and hyperthyroid rat hearts are detailed in Table 2. There were no significant differences in resting membrane potential (RMP), amplitude of action potential (AMP) and maximum rate of depolarization ($\dot{V}_{max}$) between euthyroid and hyperthyroid groups. The most obvious effects of hyperthyroidism on action potential parameters were marked prolongation of the action potential duration (APD) and of the effective refractory period (ERP).

Triggered activity

Figure 1 shows typical recordings of action potentials from papillary muscle cells from euthyroid and hyperthyroid rats superfused with modified Tyrode solution containing 2.4 and 9.6 mM Ca^{2+}. In the

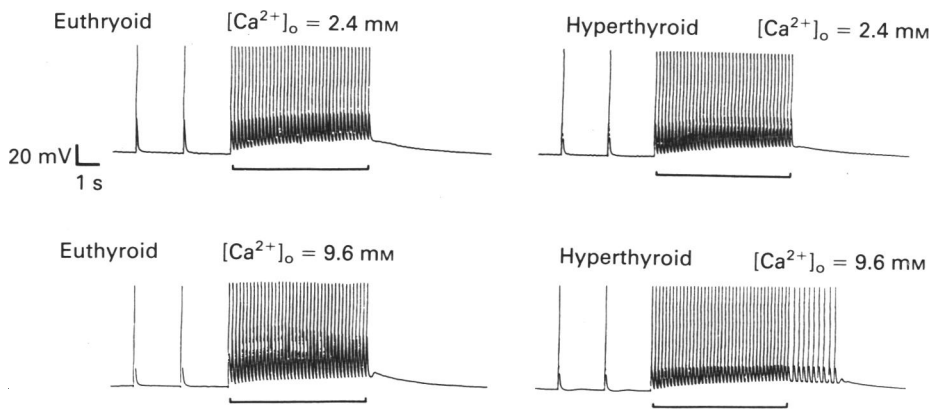

Figure 1 Typical recordings of action potentials after stimulation with trains of pulses in rat papillary muscle cells. In each record, the first two action potentials were the last of 20 action potentials elicited at the control cycle length of 2000 ms. The rapid train stimulation developed 40 action potentials at a cycle length of 150 ms (horizontal line below record). Under the condition of $[Ca^{2+}]_o = 9.6$ mM, the hyperthyroid preparation developed several triggered action potentials followed by a small delayed afterdepolarization, while the euthyroid preparation developed only a small delayed afterdepolarization. $[Ca^{2+}]_o$ = external Ca^{2+} concentration.

Table 3 Effect of hyperthyroidism on the incidence of triggered activity in papillary muscle cells

$[Ca^{2+}]_o$	*Train pulses**	*No. developing triggered activity*** *Euthyroid*	*Hyperthyroid*	
2.4	10	0	1	NS
	20	0	1	
	40	0	1	
4.8	10	0	1	$P < 0.05$
	20	0	4	
	40	4	9	
9.6	10	0	1	$P < 0.01$
	20	0	6	
	40	3	10	

* Number of pulses per train at a cycle length of 150 ms.
** Each number represents the fraction of ten euthyroid and ten hyperthyroid preparations that developed triggered activity under conditions of altered Ca^{2+} concentration.
$[Ca^{2+}]_o$: external Ca^{2+} concentration (mM); NS: not significant at $P = 0.05$. Statistical significance in the incidence of triggered activity was determined between euthyroid and hyperthyroid groups by the Chi-squared test.

figure, following 40 preceding driven action potentials at a cycle length of 150 ms, triggered responses were evoked and followed by a delayed afterdepolarization in the hyperthyroid preparation exposed to the solution containing 9.6 mM Ca^{2+}, whereas the euthyroid preparation failed to develop triggered activity under the same experimental conditions. Table 3 summarizes the frequency of occurrence of triggered activity when the muscles were exposed to the three solutions in order of increasing concentration of Ca^{2+} (2.4, 4.8 and 9.6 mM). The data show that the hyperthyroid myocardium developed triggered activity more frequently than the euthyroid myocardium under conditions of increased external Ca^{2+} concentration. In the preliminary experiments, triggered activity did not develop in hyperthyroid muscles at cycle lengths of stimulation longer than 300 ms even with the highest concentration (9.6 mM) of external Ca^{2+}. Triggered activity in the muscles from euthyroid rats was also examined during incubation with 1×10^{-6} M L-thyroxine at a calcium concentration of 9.6 mM. These muscles did not show enhanced triggered activity (data not shown).

Reperfusion arrhythmias

During the occlusion period, both euthyroid and hyperthyroid groups exhibited general phenomena associated with ischaemia, i.e., LVP was lessened rapidly just after coronary occlusion with an increase in the height of the QRS wave followed by elevation of the ST-T segment. Under our experimental conditions, no severe arrhythmias apart from a few ventricular premature contractions (VPCs) were observed during the 10 min period of coronary ligation. Comparison of arrhythmias elicited during occlusion and reperfusion periods between euthyroid

Table 4 Effect of hyperthyroidism on arrhythmias during occlusion and reperfusion

	Occlusion VPCs*	VPCs**	*Reperfusion* VT (s)#	VF (s)#	VT + VF (s)#
Euthyroid ($n = 14$)	14.8 ± 3.9	16.8 ± 4.5	5.5 ± 0.9	8.5 ± 1.5	14.0 ± 2.1
Hyperthyroid ($n = 14$)	9.8 ± 5.1 NS	40.7 ± 8.8 $P < 0.01$	4.0 ± 0.8 NS	111.0 ± 38.1 $P < 0.001$	115.1 ± 38.6 $P < 0.001$

* Number of VPCs during 10 min occlusion. ** Number of VPCs during first 10 min after reperfusion. # Duration of arrhythmias during first 10 min after reperfusion.
Results are expressed as mean ± s.e.mean.
VPCs: ventricular premature contractions; VT: ventricular tachycardia; VF: ventricular fibrillation; NS: not significant at $P = 0.05$.
Statistical significance was determined by the Mann-Whitney U-test.

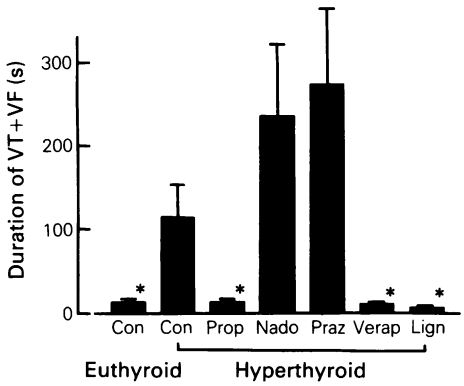

Figure 2 Effect of drugs on enhanced reperfusion arrhythmias in hyperthyroid hearts. The severity of arrhythmias was estimated as the total duration of ventricular tachycardia (VT) and fibrillation (VF). Each column represents the mean, and the vertical bars represent s.e.mean. Con = control (no treatment); Prop = propranolol (3×10^{-7} M); Nado = nadolol (3×10^{-7} M); Praz = prazosin (3×10^{-7} M); Verap = verapamil (3×10^{-8} M); Lign = lignocaine (1×10^{-5} M). Asterisks indicate data significantly reduced from the hyperthyroid control value, $*P < 0.001$ (Mann-Whitney U-test).

and hyperthyroid preparations are represented in Table 4. Ventricular tachycardia (VT) and/or ventricular fibrillation (VF) were induced within 20 s after reperfusion in both of the groups.

Effect of drugs on enhanced reperfusion arrhythmias in hyperthyroid hearts

The effects of drugs on the prolonged duration of arrhythmias (total duration of VT + VF) developed in hyperthyroid preparations are shown in Figure 2. Propranolol (3×10^{-7} M) reduced the duration of arrhythmias significantly, but nadolol (3×10^{-7} M), another β-adrenoceptor blocker which has no membrane stabilizing activity, failed to shorten the duration of arrhythmias. Prazosin (3×10^{-7} M), an α_1-selective adrenoceptor blocker, did not have any anti-arrhythmic effect. Both verapamil (3×10^{-8} M) and lignocaine (1×10^{-5} M) showed significant reduction of reperfusion arrhythmias in hyperthyroid preparations.

Effect of heart rate on reperfusion arrhythmias

Of the drugs tested, lignocaine decreased heart rate significantly from 358 ± 26 beats min^{-1} (before drug treatment) to 290 ± 13 beats min^{-1} (15 min after drug treatment) ($P < 0.05$, $n = 7$), and verapamil reduced heart rate from 368 ± 8 beats min^{-1} to 333 ± 12 beats min^{-1} ($P < 0.05$, $n = 8$) and LVP from 111.6 ± 5.8 mmHg to 83.1 ± 5.5 mmHg ($P < 0.01$, $n = 8$). The other drugs did not show any statistically significant effect either on heart rate or on LVP at least at the concentration tested. To evaluate the effect of increased heart rate on the severity of reperfusion arrhythmias, euthyroid heart preparations were driven by electrical pacing at the fixed rate of 375 beats min^{-1} which was nearly equal to the heart rate observed in hyperthyroid preparations. The electrically paced group exhibited significant ($P < 0.01$) prolongation of the duration of VT + VF (34.8 ± 6.1 s, $n = 8$) as compared with the non-paced euthyroid control (14.0 ± 2.1 s, $n = 14$), but it was still significantly ($P < 0.01$) milder than that observed in the hyperthyroid control group (115.1 ± 38.6 s, $n = 14$).

Discussion

Comparison of electrophysiological characteristics of papillary muscle cells revealed that APD and ERP were significantly prolonged in the hyperthyroid preparations compared with the euthyroid ones, whereas there were no significant differences in the other parameters measured. Such prolonged APD was also observed in the myocardium from streptozotocin-induced diabetic rats (Nordin *et al.*, 1985) and in hypertrophied myocardium from renal hypertensive rats (Gulch *et al.*, 1979). As prolongation of APD and ERP is one of the explanations of the effect of antiarrhythmic drugs such as quinidine and amiodarone, our results obtained from the experiments with reperfused hearts seem paradoxical. This suggests that other factors may be involved in the increased tendency to develop arrhythmias in hyperthyroid preparations.

Delayed afterdepolarization and triggered activity occur under conditions assumed to increase the concentration of intracellular Ca^{2+}, such as the presence of cardiac glycosides or high Ca^{2+} in the extracellular solution (Hashimoto & Moe, 1973; Ferrier & Moe, 1973; Allen *et al.*, 1984). In our study of triggered activity, the results suggest that the myocardium from hyperthyroid rats is much more prone to develop triggered activity than that from euthyroid ones under our conditions believed to cause intracellular Ca^{2+} overload. The enhanced triggered activity is not caused by thyroid hormone itself but by thyroid hormone-induced hypertrophy or by some modification of myocardial function, because the papillary muscles from the euthyroid rat did not show enhanced triggered activity during the incubation with thyroxine.

The present study with rat isolated perfused hearts demonstrates that hyperthyroidism enhances the severity of reperfusion arrhythmias expressed as the total duration of VT and VF. The enhanced arrhythmias observed in hyperthyroid preparations were significantly reduced by propranolol (3×10^{-7} M). In contrast, nadolol failed to reduce the enhanced reperfusion arrhythmias in hyperthyroid preparations at a concentration of 3×10^{-7} M which was the same concentration as propranolol and was sufficient to block β-adrenoceptors (Vukovich *et al.*, 1979). This discrepancy agrees with results which showed that atenolol (Kane *et al.*, 1984; Rochette *et al.*, 1984) and (−)-propranolol (Lamontagne *et al.*, 1986) were ineffective on reperfusion arrhythmias, although propranolol and acebutolol (Rochette *et al.*, 1984) were effective. As nadolol is a β-adrenoceptor blocker that lacks membrane stabilizing activity (MSA) (Lee *et al.*, 1975), our results may support the explanation that MSA of some β-blocking agents is of importance in reducing reperfusion arrhythmias (Rochette *et al.*, 1984), and that β-adrenoceptors are probably not involved in the genesis of arrhythmias of the reperfused ischaemic myocardium, although the responses mediated by β-adrenoceptors are enhanced by hyperthyroidism (Kunos, 1977).

α_1-Adrenoceptor blockade with prazosin failed to reduce the prolonged duration of reperfusion arrhythmias in hyperthyroid preparations. Inhibitory effects of prazosin on reperfusion arrhythmias were reported in dogs (Lamontagne *et al.*, 1986) and in cats (Sheridan *et al.*, 1980) *in vivo*. However, Bralet *et al.* (1985) showed that prazosin did not prevent reperfusion arrhythmias after a 10 min period of coronary ligation in rat hearts on a Langendorff perfusion system and explained that this contradiction might be derived from the difference in the duration of the occlusion period during which the increase of the number of α_1-adrenoceptors took place. In the experiments in dogs and cats, the occlusion period was 20 and 30 min respectively, while in the experiments in rats *in vitro*, the period was only 10 min which was equal to our experimental protocol. It has also been reported that the increase in the number of α_1-adrenoceptors in ischaemic myocardium began to occur after 15 min of ischaemia without an alteration of affinity (Corr *et al.*, 1981). Thus, our result could be explained in a similar manner to the result of Bralet *et al.*, but because responses mediated by α-adrenoceptor are attenuated and those mediated by β-adrenoceptor are potentiated in hyperthyroid animals (Kunos, 1977; Hashimoto & Nakashima, 1978), there is the possibility that attenuation of responses mediated by α-adrenoceptors may conceal the effect of prazosin on α_1-adrenoceptors. Although the detailed reason why prazosin is ineffective in our model is not clear, it seems apparent that α- and β-adrenoceptors may not participate in the enhancement of reperfusion arrhythmias in hyperthyroid preparations under our experimental conditions.

Lignocaine caused a marked reduction of the prolonged duration of reperfusion arrhythmias in hyperthyroid preparations. Similar results have been reported in experiments in normal rats; drugs that can block the fast inward sodium current in cardiac tissue (Class I of the Vaughan Williams classification), such as Org 6001, bepridil (Kane *et al.*, 1984), lignocaine, quinidine and procainamide (Lubbe *et al.*, 1978; Bergey *et al.*, 1982), reduced the severity of reperfusion arrhythmias.

On the other hand, the effect of the calcium entry blocker verapamil in hyperthyroid rats produced a conflicting result compared with the results obtained from the euthyroid rat by other authors. Verapamil reduced the enhanced reperfusion arrhythmias in our model, whereas calcium entry blockers such as verapamil, nifedipine and prenylamine failed to show antiarrhythmic effects on the reperfusion arrhythmias in the euthyroid rat (Kane *et al.*, 1984). Nevertheless, it is generally accepted that calcium antagonists inhibit triggered activity (El-Sherif *et al.*, 1983), which may be involved in the antiarrhythmic effect of verapamil observed in the present study. Considering the enhanced triggered activity and the antiarrhythmic effect of the calcium antagonist in the hearts of hyperthyroid rats, electrophysiological alteration, especially an increased propensity to develop triggered activity, may be involved in the enhanced reperfusion arrhythmias in our hyperthyroid model.

On the other hand, ventricular arrhythmias which developed during the 10 min period of coronary ligation were not enhanced by hyperthyroidism. As the dominant mechanism of genesis, at least of the first phase of early arrhythmias in ischaemic hearts is that of re-entry (Russell, 1982), hyperthyroidism may not affect re-entrant activation within the myocardium during the ischaemic period under our experimental conditions.

As hyperthyroid hearts have faster heart rates than euthyroid hearts, and lignocaine and verapamil significantly reduced this, we investigated whether increased heart rate was responsible for the enhancement of reperfusion arrhythmias in electrically paced euthyroid hearts. Euthyroid hearts which were paced at a rate approximately equal to that of hyperthyroid hearts showed enhanced reperfusion arrhythmias compared with non-paced euthyroid controls, but these were still less severe than those observed in hyperthyroid hearts. Moreover, propranolol shortened the prolonged duration of arrhythmias without a significant fall in heart rate in the hyperthyroid hearts under our experimental conditions. It has also

been reported that atenolol, which had a marked negative chronotropic effect, failed to reduce reperfusion arrhythmias in normal rats *in vivo* (Kane *et al.*, 1984). Thus, increased heart rate may be a part of the cause, but other factors may be more important in the enhancement of reperfusion arrhythmias in hyperthyroid hearts.

In summary, the severity of reperfusion arrhythmias in perfused rat hearts was significantly enhanced in hyperthyroid preparations compared with that observed in euthyroid ones. Neither α- nor β-adrenoceptors were involved in the genesis of enhanced reperfusion arrhythmias, and the increased heart rate could only be a part of the cause. As cardiac hypertrophy occurs in our hyperthyroid model, it is uncertain whether the enhanced reperfusion arrhythmias are the result of hyperthyroidism itself and/or cardiac hypertrophy. Although the precise mechanism of enhancement of reperfusion arrhythmias in hyperthyroid hearts is not apparent, increased suseptibility to development of triggered activity, which was observed in our electrophysiological study under conditions assumed to increase intracellular Ca^{2+}, may be at least one possible explanation for the cause of enhanced reperfusion arrhythmias in the hyperthyroid preparations.

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Non classical, multiple-site interaction of [^{3}H]-prazosin with the α_1-adrenoceptor of intact BC_3H_1 cells

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1 In intact BC_3H_1 cells the EC_{50} of noradrenaline (NA) for the inositol phosphate response measured at 37°C (EC_{50} = 193 nM) was much lower than its apparent dissociation constant ($K_i^{37°C}$ = 83.211 μM) determined at this temperature by [^{3}H]-prazosin binding.

2 After pretreatment of the cells with NA at 37°C for 45 min, the time used in binding assays at this temperature, this difference between EC_{50} and $K_i^{37°C}$ did not decrease significantly. An agonist-induced reduction in α_1-adrenoceptor affinity can therefore not explain the very high $K_i^{37°C}$ value.

3 NA pretreatment at 37°C decreased the number of [^{3}H]-prazosin binding sites (assessed by whole cell binding at 2°C) by only 49%; not by 100%, the value expected if agonist-induced receptor internalization were the origin of the very low $K_i^{37°C}$.

4 The EC_{50} of NA for the inositol phosphate response in the presence of 156 pM [^{3}H]-prazosin was 1.841 μM but the IC_{50} of NA for the inhibition of [^{3}H]-prazosin binding (126 pM) was 316 μM. As there is no α_1-adrenoceptor reserve in these cells we propose that at 37°C [^{3}H]-prazosin interacts, not only with the catecholamine recognition site (site 1) of the receptor, but also reacts weakly with another site from which it cannot be directly displaced by catecholamine-like substances (site 2).

Introduction

The apparent affinities of noradrenaline (NA) for the [^{3}H]-prazosin binding sites on intact BC_3H_1 cells are much higher in the cold than at 37°C (Sladeczek *et al.*, 1983). In a recent study we found that the EC_{50} values at 37°C for several adrenoceptor agonists which stimulate inositol phosphate accumulation were situated between their dissociation constants at 2°C ($K_i^{2°C}$) and at 37°C ($K_i^{37°C}$) (Sladeczek *et al.*, 1988b). The $K_i^{37°C}$ values were all in the same range (0.1 to 1 mM) and showed no simple correlation with the EC_{50} values. The $K_i^{2°C}$ values for almost all of these agonists, correlated linearly with the EC_{50} values. They were about 20–100 times lower than the respective EC_{50} values. This led us to suggest that the $K_i^{2°C}$ values might correspond to a high affinity state of the drug recognition site for catecholamines. As this high affinity site was not found in a study in which another radioligand ([^{125}I]-BE 2254) was used (Hoyer, 1984), we proposed a model in which this state was induced by [^{3}H]-prazosin.

However, the difference between EC_{50} and $K_i^{37°C}$ values still needs to be explained. Agonist degradation is not at the origin of these high $K_i^{37°C}$ values. Several arguments exclude the possibility of lysosomal uptake of [^{3}H]-prazosin being the origin of the low apparent agonist affinities observed in whole cell competition experiments. Inhibitors of this uptake did not affect [^{3}H]-prazosin binding in intact BC_3H_1-cells (Sladeczek *et al.*, 1983). Dissociation of specific [^{3}H]-prazosin binding from intact BC_3H_1-cells is monophasic (Amitai *et al.*, 1984) and

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not multiphasic as would be expected for a combined binding/uptake phenomenon. The number of [^{3}H]-prazosin binding sites in intact BC_3H_1-cells is very close to that found in broken cell preparations (Sladeczek *et al.*, 1983).

Another explanation for a much lower EC_{50} than $K_i^{37°C}$ value is an important receptor reserve. This would mean in our case that the occupation of less than 1% of the α_1-adrenoceptors of intact BC_3H_1-cells by NA is sufficient to elicit the full response to inositol phosphate. However, in a previous paper we demonstrated that there is an almost linear coupling between receptor occupation and IP-response measured in our conditions (Sladeczek *et al.*, 1988b). The 'true' affinity of agonists at 37°C corresponds therefore to their EC_{50} and not to their $K_i^{37°C}$ values.

What could be the origin of the very high $K_i^{37°C}$ values observed? Figure 1 depicts three possible mechanisms which might account for these unusual $K_i^{37°C}$ values.

(a) Agonist-induced reduction in affinity. The agonist initiates the IP-response with an affinity corresponding to its EC_{50} value. During the 45 min incubation period the affinity of the receptor for the agonist would shift to its $K_i^{37°C}$ value. As the $K_i^{37°C}$ values for all the agonists tested were very similar (Sladeczek *et al.*, 1988b) this means that the drug recognition site of the α_1-adrenoceptor would lose its drug selectivity after prolonged incubation with an agonist.

(b) The second possible explanation for the differences in the EC_{50} and $K_i^{37°C}$ values is that agonists might induce internalization of the receptor. Lipophilic substances like prazosin are thought to be able to cross the structures separating the internalized receptor from the extracellular medium whereas hydrophilic substances like NA, cannot. In competition experiments at 37°C, internalized receptors would be much less accessible to NA than to [^{3}H]-prazosin. This could explain the very high concentrations of NA required to inhibit [^{3}H]-prazosin binding at this temperature. In the cold, internalization cannot occur and real competition between [^{3}H]-prazosin and NA for plasma membrane receptors is observed. In this model $K_i^{37°C}$ values would depend on the agonist-activity (necessary for internalization) and the lipophilicity (necessary for penetration of the lipid bilayer) of the competing drug and on the lipophilic character of the radioligand used.

Agonist-induced receptor internalization is a well-known phenomenon for a great number of receptors including: insulin, EGF (epidermal growth factor) (Goldstein *et al.*, 1979; Steinman *et al.*, 1983) or β-adrenoceptors (Hertel & Perkins, 1984; Sibley & Lefkowitz, 1985). Agonist-induced receptor loss has also been described for α_1-adrenoceptors in BC_3H_1-cells (Hughes & Insel, 1986). It was a very slow process, only detectable after 1 h. However, in these experiments the receptor number was assessed by [^{3}H]-prazosin saturation experiments on membrane fractions. In addition to cell surface receptors, internalized but not yet degraded receptors would be detected by this method. More recently a much more rapid ($t_{1/2}$ = 2 min) NA-induced disappearance of [^{3}H]-prazosin binding sites was detected in DDT_1 cells in the cold (Leeb-Lundberg *et al.*, 1987; Fratelli & De Blasi, 1987). It was suggested that this rapid disappearance of [^{3}H]-prazosin binding sites was due to receptor sequestration, which is often

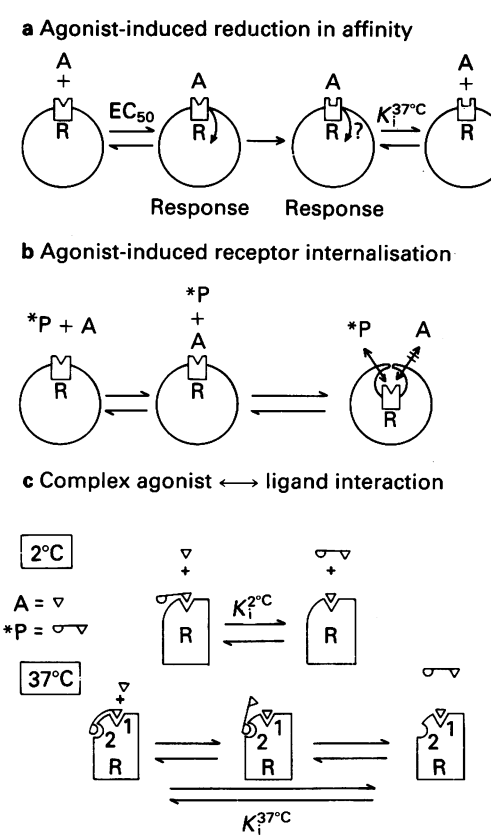

Figure 1 Three simple models which could explain the temperature dependence of apparent receptor affinities for noradrenaline (NA) in BC_3H_1 cells. The question mark between low affinity receptor and 'response' in (a), indicates that the desensitized receptor may still be coupled, or already uncoupled from the effector system. For explanation of the diagrams see Introduction and Results sections. Abbreviations: R = receptor, A = agonist, *P = [^{3}H]-prazosin.

Table 1 Effect of temperature and pretreatment with noradrenaline (NA) and phorbol 12-myristate 13-acetate (PMA) on [^{3}H]-prazosin binding characteristics

	K_D (pM)	B_{max} (sites per cell)	*Number of experiments*
Control (37°C)	26.4 ± 3.6	18740 ± 2782	5
Control (2°C)	44.7 ± 8.5	18379 ± 2674	8
Control (2°C, washed)	46.4 ± 14.1	16802 ± 3372	3
NA-pretreatment	61.7 ± 11.9	8587 ± 2373	3
Control (2°C, DMSO)	31.0 ± 11.7	15657 ± 2408	3
PMA-pretreatment	75.3 ± 13.7	16380 ± 2650	3

K_D and B_{max} values were determined in saturation experiments as described in Methods. For PMA pretreatment, cells were incubated at 37°C for 10 min with 100 nM PMA. Dishes were then put on ice and saturation experiments initiated 30 min later. For NA desensitization experiments, cells were incubated for 45 min at 37°C with 10 μM NA and then put on ice. Thirty min later, dishes were washed three times with 1 ml ice-cold washing buffer and saturation experiments were initiated thereafter. Incubation time at 2°C was 7 h. Control (2°C, washed) dishes were treated as NA dishes. 0.1% DMSO was added instead of PMA to control (2°C, DMSO) dishes and they were then treated as PMA dishes.

observed after stimulation of adenylate cyclase-coupled receptors (Hertel & Perkins, 1984; Sibley & Lefkowitz, 1985). If almost 100% of the α_1-adrenoceptors in BC_3H_1 cells disappeared from the cell surface during the 45 min incubation period used for the binding assays at 37°C, the very high $K_i^{37°C}$ values could be explained by this phenomenon.

(c) A third explanation for the differences in the EC_{50} and $K_i^{37°C}$ values could be that prazosin interacts at 37°C (but not at 2°C), with both the catecholamine recognition site and with a second site, not accessible to catecholamines. These agonists would therefore compete at 37°C with [^{3}H]-prazosin in a non-classical manner and with artificially low affinity. Antagonists such as phentolamine had access to both sites. This would explain why there was no great difference between the $K_i^{37°C}$ and the $K_i^{2°C}$ values for these substances (Sladeczek *et al.*, 1983). In this model the $K_i^{37°C}$ values of the competing drugs would depend on their structure and their ability to interact with both or with only one of the [^{3}H]-prazosin binding sites. The purpose of the present study was to investigate which of these hypotheses could explain the differences between the EC_{50} and $K_i^{37°C}$ values of agonists for α_1-adrenoceptors of intact BC_3H_1 cells.

Methods

The BC_3H_1 muscle cell line was a gift from Dr J. Patrick (The Salk Institute, San Diego, CA, U.S.A.). The cells were grown in 35 mm tissue culture dishes as previously described (Mauger *et al.*, 1982) with the exception that no penicillin or streptomycin sulphate was used. Cells were used 7 to 10 days after seeding without any change of culture medium.

[^{3}H]-prazosin binding experiments on intact BC_3H_1 cells were performed as described previously (Sladeczek *et al.*, 1983). Briefly: culture medium was replaced by 2 ml of HEPES-buffered saline and cells were allowed to equilibrate for at least 15 min at the respective incubation temperature. The assay was initiated by addition of the radioligand and the indicated drugs. The incubation periods were 7 h at 2°C, 5 h at 6°C, 4 h at 10°C, 2 h 30 min at 14°C, 60 min at 18°C and 45 min at 22, 26, 30 and 37°C; it had been checked previously that these periods were sufficient for equilibrium to be achieved. The incubation was terminated by aspiration of the incubation medium followed by washing the culture dishes three times with 3 ml of phosphate buffered saline. Cells were collected from the dishes by the addition of 1 ml of 0.5% Triton X-100/0.2 M NaOH, transferred into a counting vial, neutralised and counted in scintillation fluid. Specific binding was defined as the binding displaced by 10 μM phentolamine. The maximal number of binding sites was almost the same at 2 and 37°C (Table 1).

For IP experiments, cells were seeded in 35 mm plastic dishes in 1.5 ml culture medium containing 1.33 μCi ml^{-1} of [^{3}H]-inositol. Before each experiment the culture medium was replaced by HEPES-buffered saline and the cells were pre-incubated for 10 min with 5 mM Li_2SO_4 in order to block IP-degradation (Sherman *et al.*, 1981; Berridge *et al.*, 1982). The HEPES buffer did not contain any inositol. Reactions were started by the addition of ligands, yielding the indicated concentrations in a

final volume of 1 ml. In standard experiments the reaction was stopped after 45 min by replacing the incubation medium with 5% perchloric acid (PCA). The IPs were extracted as described by Bone *et al.* (1984) and separated by Dowex anion exchange chromatography (Sladeczek *et al.*, 1988a,b).

Results

In order to test the three hypothesis which might explain the low apparent agonist affinities at 37°C (Figure 1), we performed the following experiments.

Agonist induced reduction in affinity

If an agonist-induced reduction in affinity (Figure 1a) was at the origin of the difference between the EC_{50} and $K_i^{37°C}$ values, either a cellular response mediated by NA should not be detectable any more (receptor uncoupled from its effector system) after pretreatment of the cell with NA at 37°C or, if the response could still be stimulated by NA the EC_{50} value should approach the $K_i^{37°C}$ value.

In Figure 2 we compare the effects of NA on [^{3}H]-prazosin binding at 2°C and 37°C with its ability to induce the formation of IPs at 37°C. Pretreatment of the cells with the indicated concentrations of NA resulted in a very weak rightward shift of the dose-response curve for IP accumulation, but the concentrations of NA required to stimulate IP formation remained about 1000 fold less than those required to compete with [^{3}H]-prazosin binding. Furthermore, the maximal IP-response to NA was not significantly changed by the pretreatment, indicating that NA did not induce desensitization of this response.

A more direct way to test the desensitization hypothesis is by direct binding experiments. It has been shown that, in intact hepatocytes, adrenaline and NA exhibit biphasic competition curves when [^{3}H]-prazosin is used as a ligand at 37°C (Schwarz *et al.*, 1985; Corvera *et al.*, 1986). A small fraction of the [^{3}H]-prazosin sites showed high affinity (9–25 nM) for the agonists whereas the majority of the sites exhibited low agonist affinities (about 2000 nM). Pretreatment of the cells with 100 nM of the phorbol ester phorbol 12-myristate 13-acetate (PMA) shifted all the sites to the low affinity state (Corvera *et al.*, 1986). In the cold only high affinity sites were detected (Schwarz *et al.*, 1985). It was concluded that the agonist affinity measured in the cold corresponds to the initial affinity of the receptor for agonists (Schwarz *et al.*, 1985) and activation of protein kinase C by PMA at 37°C provoked receptor phosphorylation, thus transforming all high affinity sites into low affinity sites (Corvera *et al.*, 1986).

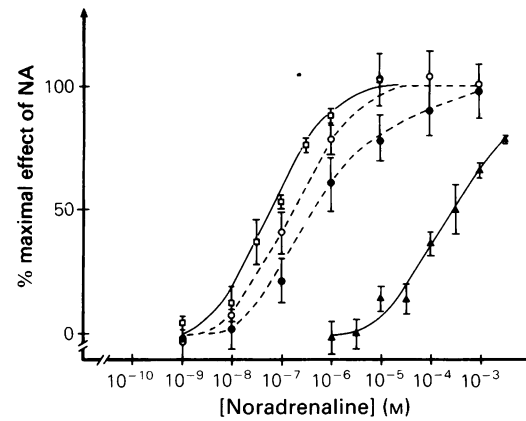

Figure 2 Comparison of the effects of noradrenaline (NA) on [^{3}H]-prazosin competition experiments at 2°C (□) and 37°C (▲) and inositol phosphate (IP) accumulation under normal (○) and desensitized (●) conditions. IP accumulation was measured after preincubating BC_3H_1 cells for 45 min in the presence ('desensitized') (●) or absence (control) (○) of the indicated concentrations of NA at 37°C. IP accumulation was initiated by addition of 10 mM $LiSO_4$ ('control') or 10 mM $LiSO_4$ and NA at the indicated concentrations ('desensitized'). Reactions were stopped after a further 45 min incubation by replacing the medium with 5% perchloric acid. IP extraction and separation and [^{3}H]-prazosin binding experiments were performed as described in Methods. Results are expressed as a percentage of the maximal effect of NA and are the means of at least 6 independent determinations performed on at least 3 different culture preparations. Vertical lines show s.e.mean. The mean concentration of [^{3}H]-prazosin used in these experiments was 126 ± 10 pM.

If the high affinity sites detected at 2°C in BC_3H_1 cells corresponded to the PMA-sensitive sites in hepatocytes, pretreatment of the cells at 37°C with 100 nM PMA should transform all the high affinity sites into low affinity agonist sites. For this reason, we pretreated BC_3H_1 cells at 37°C for 10 min with 100 nM PMA, a concentration needed to obtain maximal phosphorylation of α_1-adrenoceptors in DDT_1 cells (Leeb-Lundberg *et al.*, 1985; 1987). As in DDT_1 cells, this protocol inhibited 100% of the α_1-adrenoceptor-mediated response in our system (data not shown). In NA competition experiments at 2°C, we obtained a K_i of 9.5 nM, corresponding to the usual high affinity state at this temperature. This was not due to a reversal of the PMA-induced desensitization, since the PMA blockade of the IP

response was still effective after the 7 h incubation period at 2°C (data not shown). Furthermore, cells preincubated at 37°C for 45 min with varying concentrations of NA (from 100 pM to 1 mM) and then put on ice, showed the classical high affinity NA site (11.3 nM) in competition experiments with [^{3}H]-prazosin.

Model 1a does not therefore explain the differences observed between EC_{50} and $K_i^{37°C}$ values.

Agonist-induced receptor internalisation

In order to test the internalisation hypothesis (Figure 1b) we performed [^{3}H]-prazosin saturation experiments on whole cells in the cold after pretreating them for 45 min at 37°C with a maximally effective concentration of NA (10 μM). The results shown in Table 1 indicate a loss of about 49% of the receptors with no significant change in their K_D for [^{3}H]-prazosin. The remaining surface receptors kept their high affinity for NA in the cold ($K_i^{2°C} = 4$ nM). If such a receptor sequestration was the reason for the appearance of the low affinity NA state at 37°C during the incubation period of 45 min, in equilibrium binding experiments at this temperature we should obtain a clearly biphasic competition curve. The results presented in Figure 2 show that this is not the case. Model 1b is therefore not able to explain the high temperature dependence of the apparent affinities of NA for α_1-adrenoceptors in intact BC_3H_1 cells.

The concentration of PMA (100 nM) which was maximally active in phosphorylating the α_1-adrenoceptor in DDT_1 cells (Leeb-Lundberg *et al.*, 1985) decreased the affinity of the receptor for [^{3}H]-prazosin by a factor of 2, but had no effect on the number of maximal binding sites (Table 1) in our system.

Complex agonist-ligand interactions

Another possibility which could explain the temperature-dependence of the affinities for NA is shown in Figure 1c. Prazosin and NA have quite different structures. Their structural similarities are limited to the catechol head region in which the hydroxyl groups of NA are exchanged by methoxy groups in the prazosin molecule. The hydroxylated amino ethyl tail of NA is replaced in prazosin by a rigid pyrimidine cycle containing, in position 2, a large tail consisting of a cyclic aliphatic diamine, a ketone and a furane group. It is therefore possible that the prazosin molecule may interact with both, the catecholamine acceptor site (site 1 in Figure 1c) and with another site accepting its large tail region (site 2 in Figure 1c). NA might only be able to displace the part of the prazosin molecule fixed in site 1 and the radioligand would remain attached to the receptor by its interaction with site 2. There would be, as in every equilibrium reaction, continuous association and dissociation of the drugs interacting with the receptor. Thus as soon as NA dissociated from site 1, prazosin would very quickly reassociate with this site due to the fact that it remained attached to site 2. In this case, NA would displace prazosin from its receptor (in a way detectable in binding experiments) only when its concentration was high enough to produce a mean NA occupation time of site 1 greater than the prazosin dissociation rate from site 2. If site 2 was not accessible at 2°C much lower concentrations of NA would be able to displace prazosin from its receptor.

A way to test this hypothesis is to perform NA dose-response curves in the presence and absence of prazosin at 37°C. Figure 3 shows that 156 pM [^{3}H]-prazosin shifted the IP dose-response curves in an almost competitive manner to the right. The observed EC_{50} of 1585 nM was very close to the EC_{50} of 1841 nM, calculated by the Cheng Prusoff equation (Cheng & Prusoff, 1973). Therefore at 37°C NA is able to compete in an almost classical manner with [^{3}H]-prazosin for the biologically active site

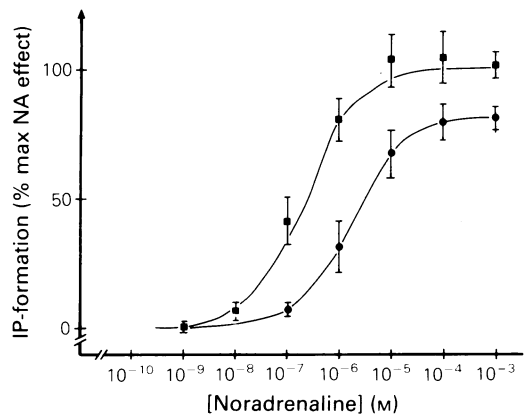

Figure 3 Effect of [^{3}H]-prazosin on the dose-response curves for noradrenaline (NA)-induced formation of inositol phosphate (IP) in BC_3H_1 cells. Cells were preincubated for 45 min in the absence (control, ■) or presence (●) of 156 pM [^{3}H]-prazosin and experiments were performed as described in Methods. Results are expressed as % of maximal effect of NA and are the means of six independent determinations performed on three different culture preparations. Vertical lines show s.e.mean. EC_{50} values = 263 nM, control, and 1585 nM in the presence of prazosin.

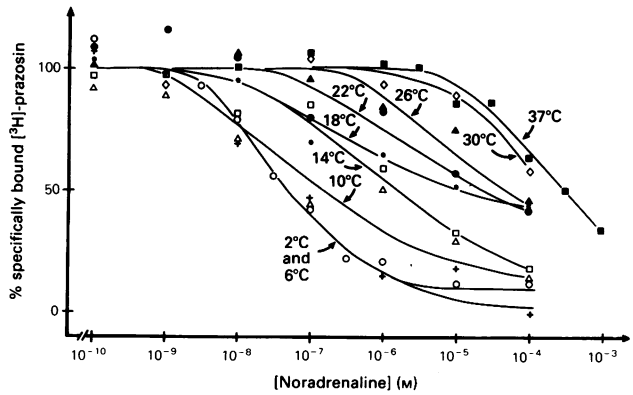

Figure 4 [^{3}H]-prazosin/noradrenaline competition experiments at different temperatures in BC_3H_1 cells: (○) 2°C, (+) 6°C, (△) 10°C, (□) 14°C, (*) 18°C, (●) 22°C, (▲) 26°C, (◇) 30°C and (■) 37°C. Results are expressed as % of specifically bound [^{3}H]-prazosin and are the means of eight independant determinations performed on four different culture preparations.

(site 1) (Figure 3), but is much less efficient in displacing 126 pM [^{3}H]-prazosin from the receptor at this temperature (Figure 2, IC_{50} = 316 μM).

In order to determine if the appearance of site 2 is an all or nothing phenomenon which occurs at a particular temperature or appears progressively as the temperature increases, we performed NA competition experiments at different temperatures. The competition curves obtained at 2°C and 6°C were almost identical (Figure 4). Between 10°C and 30°C, there was a progressive shift to lower apparent affinities of NA. At 30°C, the apparent affinity of NA was the same as that usually observed at 37°C.

Discussion

BC_3H_1 cells exhibit a loss of 49% of [^{3}H]-prazosin binding sites, compared to those measured in the cold, after pretreatment with NA for 45 min at 37°C. This loss of binding sites was not reproduced by application of PMA (Table 1) at a concentration (100 nM) which completely inhibited the IP-response in BC_3H_1 cells. A similar observation was recently made in DDT_1-cells (Leeb-Lundberg *et al.*, 1987), where the kinetics of PMA-induced α_1-adrenoceptor phosphorylation paralleled the kinetics of loss of the inositide phospholipid response. It can therefore be concluded that the failure of protein kinase C to induce α_1-adrenoceptor sequestration is not limited to DDT_1 cells but seems to be a general phenomenon.

There is one difference between our work on BC_3H_1 cells and the work on DDT_1-cells. Leeb-Lundberg *et al.* (1987) observed a significant reduction of the agonist-induced IP response in DDT_1-cells after pretreatment with NA. In BC_3H_1 cells we saw no such effect after a similar pretreatment (Figure 2). However, it should be noted that Leeb-Lundberg *et al.* (1987) utilized shorter periods of pretreatment and stimulation and measured ^{32}P labelling of the phospholipids rather than the accumulation of IPs, which is a more direct measure of lipid hydrolysis.

Non-equilibrium competition experiments have previously been done to demonstrate an initial high affinity agonist state of α_1-adrenoceptors (Toews, 1987; Fratelli *et al.*, 1987). However the postulates at the origin of such a procedure have never been seriously investigated. One of these postulates is that the radioligand and agonist both interact in a simple monostep procedure (Drug + Receptor = Drug-Receptor) with the receptor. However, our results indicate that the [^{3}H]-prazosin α_1-adrenoceptor interaction is much more complicated. The K_D of an equilibrium reaction is defined as $K_D = k_-/k_+$. The temperature dependency of the rate constant $k_\pm$ is given by the Arrhenius law: $k_\pm = A_\pm e^{-E_{a\pm}/RT}$ with: $A_\pm$ = activation energies of association or dissociation, T = absolute temperature (°K) and R = universal gas constant = 1.99 cal mol^{-1} K^{-1}. The energy liberated or consumed by an equilibrium reaction (ΔG) is given by the equation $\Delta G = RT \ln K_D$. The K_D values listed in Table 1 allow the determination of ΔG liberated by the [^{3}H]-prazosin binding reactions at 2°C (−13 kcal mol^{-1}) and at

37°C (−15 kcal mol^{-1}). For a classical one step equilibrium reaction $E_{a-} = E_{a+} + \Delta G$. ΔG was very important in the equilibrium [^{3}H]-prazosin/α_1-adrenoceptors. The consequence should be that $E_{a-} \gg E_{a+}$. With increasing temperatures k_- should therefore increase much quicker than k_+ and $K_D^{37°C} > K_D^{2°C}$. But in our experiments the $K_D^{37°C}$ of 26.4 pM was even slightly lower than the $K_D^{2°C}$ of 44.7 pM. A detailed kinetic study of the [^{3}H]-prazosin α_1-adrenoceptor interaction indicates that [^{3}H]-prazosin association could be a multistep procedure involving a preassociation state requiring a very high activation energy (Sladeczek *et al.*, unpublished observations). This could explain why K_D of [^{3}H]-prazosin exhibits no important temperature-dependent effect. Non-equilibrium competition experiments are impossible to interpret in such a complicated kinetic system without detailed investigation of association and dissociation kinetics of *all* the drugs used. We therefore used exclusively equilibrium binding studies in the present work.

The results presented here show that neither agonist-induced reduction in affinity nor agonist-induced receptor internalization could account for the very low apparent agonist affinities observed in whole cell competition experiments performed with [^{3}H]-prazosin. The temperature-dependent effect of the apparent affinities of NA shown in Figure 4 further supports this. It is indeed possible that a conformational change of the receptor structure at the origin of agonist-induced reduction in affinity and receptor internalization are impossible at low temperatures, due to the fact that the membrane is in its solid, frozen state. But as soon as the threshold temperature between the solid and liquid state of the membrane is reached, we should observe an immediate appearance of the low affinity state. The appearance of the low affinity state with increasing temperatures should therefore be an all or nothing phenomenon. The progressive appearance of this low affinity state shown in Figure 4 is best explained by the multisite interaction model depicted in Figure 1c. In this case [^{3}H]-prazosin would interact at 2°C and 6°C exclusively with site 1. At higher temperatures the interaction with site 2 progressively appeared. The site 2 region could be part of the receptor molecule. Another possibility is that the site 2 interaction reflects a dissolution of the very lipophilic prazosin molecule in some lipophilic phase of the receptor complex. The progressive nature of the appearance of site 2 with increasing temperature supports such a dissolution phenomenon. If the active site of α_1-adrenoceptors is, similar to that of β-adrenoceptors, on the lipophilic part of the receptor molecule (Bar-Sinai *et al.*, 1986; Yarden *et al.*, 1986; Dixon *et al.*, 1987a,b; Dohlman *et al.*, 1988; Kobilka *et al.*, 1988), such a dissolution phenomenon would not be very surprising. In order to test this hypothesis hydrophilic antagonists for the α_1-adrenoceptor have to be developed.

However, in intact DDT_1 cells (Toews, 1987) only 52% of the [^{3}H]-prazosin binding sites had a low affinity for NA, whereas 48% had a $K_i^{37°C}$ of 1.32 μM close to the EC_{50} value of 0.38 μM for the IP response described by us (Sladeczek *et al.*, 1988b). In [^{3}H]-prazosin competition experiments performed in intact brain slices even low affinity sites for agonists had not been detected (Kendall *et al.*, 1985). When [^{125}I]-BE 2254 or [^{3}H]-dihydroergocryptine was used, lower $K_i^{37°C}$ values for NA were observed than the one described by us in [^{3}H]-prazosin whole cell binding assays (Cornett & Norris, 1982; Cornett *et al.*, 1982; Hoyer, 1984; Leeb-Lundberg *et al.*, 1985). The presence of low affinity interactions is therefore highly dependent on the cell system and the radioligand used.

The interaction of [^{3}H]-prazosin with the active site of α_1-adrenoceptors in intact BC_3H_1 cells at 37°C is as complex as that depicted in Figure 5, if the [^{3}H]-prazosin-induced high affinity agonist state, we recently postulated in order to explain the unusually high apparent agonist affinities observed at 2°C (Sladeczek *et al.*, 1988b), is still present at 37°C. In this model the bulky radioligand is supposed to be a bifunctional molecule and we suggest that it interacts at higher temperatures with 2 sites of the receptor (site 1 and 2 in Figure 5) with the dissociation constant $K_{D1+2} = K_D^{37°C} = 26.2$ pM. The interaction of [^{3}H]-prazosin with the drug-specific site 1 (dotted line in state II) could lead to the proposed slight distortion of the active site region (Sladeczek *et al.*, 1988b). This distortion would increase the apparent affinity of the active site for catecholamine like drugs (state II to state III). The high affinity interaction of NA with this site 1 in the presence of [^{3}H]-prazosin would lead to the dissociation of [^{3}H]-prazosin from site 1 (state III to state IV). As a result of the site 2 interaction, the radioligand would remain attached to the receptor (state IV). The high affinity agonist state would therefore not be detected by binding experiments at 37°C. As soon as [^{3}H]-prazosin dissociates from site 1, the receptor would return to its normal agonist state (state V). The fact that NA competes in a classical manner with the receptor in IP experiments (Figure 3) shows that NA is able to stimulate the IP-response via the normal agonist site 1 in state V even in the presence of [^{3}H]-prazosin on site 2. But as the mean occupation time of site 1 by NA is not high enough to allow dissociation of [^{3}H]-prazosin from site 2 before NA dissociates (state V to state VI), low concentrations of the catecholamine are not able to displace [^{3}H]-prazosin in a way detectable in binding experiments (Figure 2). [^{3}H]-prazosin

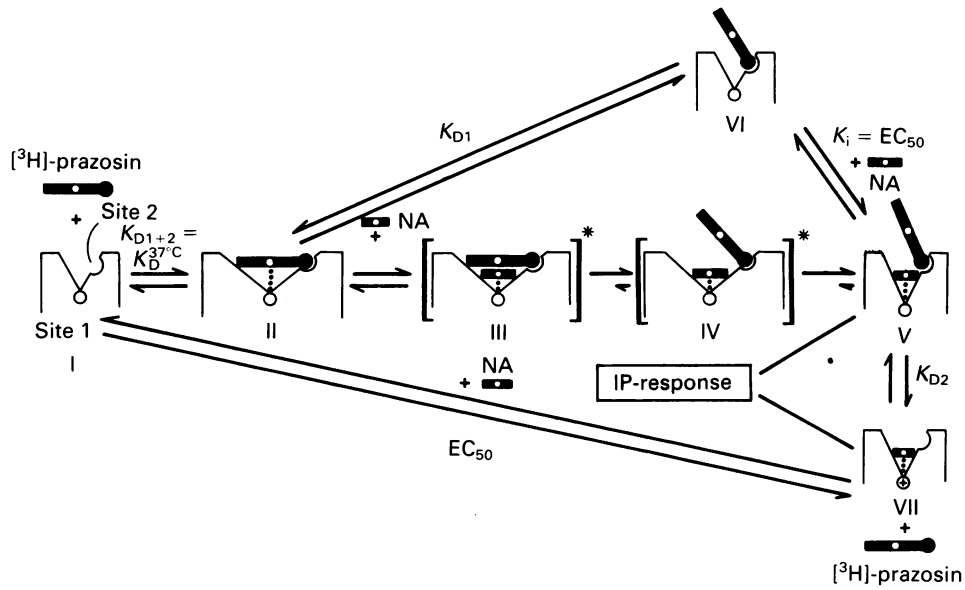

Figure 5 Schematic representation of the hypothetical interaction of [^{3}H]-prazosin with the α_1-adrenoceptor of intact BC_3H_1 cells at 37°C. A detailed description of the model is given in the Discussion. K_i values are the dissociation constants of noradrenaline (NA). EC_{50} is the concentration of NA that evokes 50% of the inositol phosphate (IP) response at 37°C. As there is a linear coupling between receptor occupancy and IP-response (2) the EC_{50} represents the 'true' affinity of NA for its receptor. $K_D^{37°C}$ is the dissociation constant of [^{3}H]-prazosin measured at 37°C. K_{D1}, K_{D2} and K_{D1+2} are the dissociation constants characteristic for the interaction of [^{3}H]-prazosin with site 1, site 2 or site 1 and 2 simultaneously.

remains close to site 1 and reassociates immediately with site 1 as soon as NA dissociates (state VI to state II). Only at very high concentrations can NA occupy site 1 longer than [^{3}H]-prazosin needs to dissociate from site 2 of the receptor (state V to state VII). Agonists are only able to activate the receptor in its normal configuration (states V and VII). [^{3}H]-prazosin displaces the NA dose-response curve for IP accumulation to the right (Figure 3) and not to the left, as would be expected if NA could stimulate the IP response via the postulated [^{3}H]-prazosin-induced high affinity agonist states (states III and IV, which are both transition states).

The [^{3}H]-prazosin-induced distortion of the active site region of α_1-adrenoceptors observed at 2°C, and as site 2 interaction at 37°C, seem to depend on the integrity of the α_1-adrenoceptor transmembrane signal transduction system. Both effects were much lower or even non-existent in systems in which no obvious α_1-adrenoceptor-mediated IP-response could be detected (Sladeczek *et al.*, 1988b).

For β-adrenoceptors, unusually low agonist affinities on intact cells were also obtained when lipophilic radioligands were used (Porzig *et al.*, 1982; Staehelin *et al.*, 1983). If binding was done with the hydrophilic ligand CGP-12177 more physiological agonist affinities were found. Non-competitive interactions between agonist and lipophilic ligands have been suggested in order to explain this finding. Interactions of the type shown in Figure 1c for lipophilic ligands with the β-adrenoceptor might also be at the origin of this non-competitive behaviour.

In conclusion, it is predicted that similar situations to the one described in this paper, might exist for other receptors for which very large, lipophilic ligands are used for the assessment of active sites specific for much smaller, hydrophilic natural hormones and neurotransmitters. This could be the case for 5-hydroxytryptamine receptors when using ketanserin as a radioligand, or cholinoceptors when using quinuclidinyl benzilate as a radioligand. If site 2 type interactions of large artificial ligands is a general feature of high affinity antagonists, this discovery could have important implications in the design of new drugs. Bulky, lipophilic ligands, exhibiting the complex binding behaviour described here and in our previous paper (Sladeczek *et al.*, 1988b), might become precious tools for the defini-

tion of the tertiary structure of the active site regions of functional transmembrane receptors.

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Postnatal changes in response to adenosine and adenine nucleotides in rat duodenum

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1 The effects of adenosine and adenine nucleotides were studied in rat duodenum from postnatal day 1 to day 70. The mechanical activity of duodenal segments was recorded through an isotonic transducer connected to a polygraphic recorder.

2 In rat duodenal segments, adenosine-5′-triphosphate (ATP, 10^{-4} M) and adenosine-5′-diphosphate (ADP, 10^{-4} M) produced a contractile response on postnatal day 1. This response increased with age, peaking on day 7, followed by a gradual decrease and was non-existent by day 21. In contrast, a relaxant response to ATP and ADP was apparent on day 21, and continued to increase up to day 70.

3 The contraction caused by ATP was inhibited by indomethacin or the P_{2y}-purinoceptor antagonist, reactive blue-2 but not by tetrodotoxin or hyoscine. Thus, it may be mediated by production of prostaglandin through the P_{2y}-purinoceptor. The relaxation produced by ATP was inhibited by reactive blue-2 but not by tetrodotoxin, guanethidine or the P_1-purinoceptor antagonist, 8-phenyltheophylline indicating that ATP acts on smooth muscle directly through the P_{2y}-purinoceptor. The pD_2 for the contractile response to ATP was 5.15 on day 7 and that for the relaxant response, 6.64 on day 70.

4 Adenosine (10^{-4} M) and adenosine-5′-monophosphate (AMP, 10^{-4} M) elicited no response before day 14. On day 14, both adenosine and AMP produced a small relaxant response which increased with age. The relaxation produced by adenosine was inhibited by 8-phenyltheophylline but not by tetrodotoxin or guanethidine, indicating that it is mediated by an action on the P_1-purinoceptor of smooth muscle.

5 It is evident from these results that in neonatal rat, a contractile response to ATP and ADP occurs initially in the duodenum, followed by a relaxant response to adenosine and AMP on day 14 and to ADP and ATP on day 21.

6 The smooth muscle of rat duodenum may tentatively be concluded to contain separate purinoceptors for adenosine and AMP (P_1) and ADP and ATP (P_2) and the responses to P_1- and P_2-agonists change during the course of development.

Introduction

Non-adrenergic, non-cholinergic (NANC) nerves are known to be widespread throughout the autonomic nervous system of vertebrates. According to Burnstock (1972), adenosine-5′-triphosphate (ATP) or a related nucleotide may be a neurotransmitter released from some of these nerves which he designated as 'purinergic'. He also proposes that there are two types of receptors for purine compounds, the P_1-receptor for adenosine and adenosine-5′-monophosphate (AMP) and the P_2-receptor for adenosine-5′-diphosphosphate (ADP) and ATP (Burnstock, 1978). This hypothesis has been supported and extended by many experiments including those on the gastrointestinal tract (Burnstock & Buckley, 1985). Manzini *et al.* (1985, 1986) have presented evidence for a NANC inhibitory neuronal mechanism in rat duodenum and suggest that ATP may possibly be a neurotransmitter released from inhibitory NANC nerves in rat duodenum.

The response of rat duodenum to certain neuropeptides including thyrotropin-releasing hormone (Tonoue *et al.*, 1981), methionine[5] enkephalin (Furukawa *et al.*, 1982), and calcitonin-gene related peptide (Furukawa & Nomoto, 1988) was found, in a

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previous study, to change within several weeks following birth and the physiological role of neurotransmitters or neuromodulators in the intestine was thought to change with the progress of development after birth. No data are presently available on the postnatal ontogenesis of purinoceptors. It thus seemed worthwhile to examine the response of rat duodenum to purine nucleosides and purine nucleotides in the developing neonatal rat duodenum.

In the present study, P_2-receptors, which induce contraction through prostaglandin production, were noted in the duodenum of neonatal rats, followed by development of P_1- and P_2-receptors which caused relaxation through a direct action on smooth muscle.

Methods

Animals and preparations

Wistar-Imamichi strain rats aged 1, 3, 7, 14, 21, 28 and 70 (adult) days were used. The day of birth was designated as day 0, with day 1 as 12–24 h after birth. At an age less than 21 days, the duodena of both male and female rats were used and after day 28, only those of male rats were used. The animals were stunned by a blow on the head and bled by cutting the vessels in the neck. Each duodenum was then dissected out. About one third the duodenum from the proximal area was used. The length of a duodenal segment was about 1 cm on days 1 and 3; 1.5 cm on days 7 and 14; 2 cm on days 21 and 28 and 2.5 cm in adult rats.

Mechanical studies

All segments were placed in an organ bath containing 25 ml of modified Locke solution (composition in mM: NaCl 154, KCl 4.02, $CaCl_2$ 1.36, $MgCl_2$ 0.9, $NaHCO_3$ 2.97, glucose 5.56, pH 7.0) saturated with 95% O_2 and 5% CO_2. Experiments were performed at 32°C with continuous bubbling of air. The mechanical activity of the duodenum was recorded through an isotonic transducer (Nihon Koden, TD 112S) attached to a polygraphic recorder (Nihon Koden, WI-640GS); a load of 0.5 g was used for segments of days 1 to 7, 0.75 g for days 14 to 28 and 1 g for segments from adult rats.

Drugs

The following drugs were used: adenosine (Sigma), adenosine-5′-monophosphate sodium salt (AMP, Sigma), adenosine-5′-diphosphate sodium salt (ADP, Sigma), adenosine-5′-triphosphate disodium salt (ATP, Sigma), tetrodotoxin (TTX, free base, kindly supplied by Sankyo), hyoscine (Scopolamine hydrochloride, Sigma), indomethacin (Sigma), reactive blue-2 (RB-2, Sigma), guanethidine sulphate (Ciba-Geigy) and 8-phenyltheophylline (8-PT, Sigma). The drugs were usually dissolved in saline to make a stock solution, 0.5 ml of which was added to the 25 ml organ bath. Indomethacin was dissolved in 70% ethanol and 37.5 μl of the solution thus obtained were added to the organ bath. 8-PT was dissolved in dimethylsulphoxide (DMSO) and 5 μl was added to the organ bath. RB-2 was dissolved in distilled water which was then added in a volume of 25 μl to the organ bath.

Results

Experiments were repeated 5 or more times, and essentially the same results were obtained in each case. Table 1 shows the body weights of the rats and those of the duodenal segments.

Mechanical response to adenosine and adenine nucleotides

Figure 1 and Table 1 show postnatal changes in the duodenal response to adenosine, AMP, ADP and ATP (all at 10^{-4} M). Duodenal preparations from 1 to 7 day-old rats showed no response to the P_1-agonists, adenosine and AMP. However, a weak but distinct contraction, was induced by the P_2-agonists, ADP and ATP in duodena of 1 day-old rats. The contractile response to ADP and ATP increased in intensity with age, peaking on days 7 to 14. The magnitude of this response per mg wet weight of tissue was maximal at day 7 (Table 1). The contraction induced by ATP appeared without a time lag after adding the nucleotide to the bath on day 7. On day 14, a short time lag (14.2 ± 1.1 s, $n = 6$) before the contractile response to ATP was observed and some preparations (5 out of 20) showed a slight relaxant response to ATP before a contraction appeared (Figure 3f). After day 14, contractile response to ATP decreased and in its place, a slight but distinct relaxant response was noted. This relaxation, consistent on day 21 for the first time, continued to increase until day 70 (Figure 1). By day 14, both adenosine and AMP produced a slight relaxant response which increased in segments taken from older rats (Figure 1). On day 70, the magnitude of the relaxant response to adenosine and AMP became about half that of the ADP- and ATP-induced relaxation (Table 1).

Features of the contractile and relaxant responses to ATP

The pD_2 s for the contractile and relaxant responses to ATP were determined on days 7 and 70, respectively. A linear concentration-contractile response

Table 1 Postnatal changes in response to adenosine, AMP, ADP and ATP (each at 10^{-4} M) in rat duodenal segments

Age (days)	*1*	*3*	*7*	*14*	*21*	*28*	*70*
n	8	4	12	8	8	6	6
Body weight (g)	5.7 ± 0.2	8.5 ± 0.2	14.0 ± 0.4	25.1 ± 0.6	36.9 ± 0.8	65.2 ± 0.7	368 ± 7
Preparation weight (mg)	19.7 ± 1.3	24.9 ± 0.8	29.5 ± 1.5	61.1 ± 1.9	115.9 ± 0.5	169.6 ± 4.7	403 ± 21
				Responses (mm)			
Adenosine	0	0	0	−0.45 ± 0.09 (−0.74)	−0.41 ± 0.04 (−0.35)	−0.78 ± 0.18 (−0.46)	−1.30 ± 0.42 (−0.32)
AMP	0	0	0	−0.60 ± 0.10 (−0.98)	−0.41 ± 0.05 (−0.35)	−1.07 ± 0.22 (−0.63)	−1.50 ± 0.31 (−0.37)
ADP	+1.00 ± 0.22 (+5.07)	+2.37 ± 0.37 (+9.5)	+3.29 ± 0.29 (+11.2)	+3.25 ± 0.25 (+5.32)	−0.43 ± 0.05 (−0.37)	−0.77 ± 0.08 (−0.45)	−3.20 ± 0.62 (−0.79)
ATP	+0.82 ± 0.20 (+4.16)	+2.33 ± 0.45 (+9.4)	+3.15 ± 0.30 (+10.7)	+3.18 ± 0.44 (+5.20)	−0.54 ± 0.05 (−0.47)	−0.76 ± 0.07 (−0.45)	−3.20 ± 0.64 (−0.79)

Symbols (+) and (−) indicate contractile and relaxant responses, respectively.
Values are the mean ± s.e.mean. Figures in parentheses were estimated from the equation: (Response (mm)/preparation wet weight (mg)) × 100.

curve was obtained in the range 2.5×10^{-6} to 10^{-4} M ATP and pD_2 was 5.15 ($n = 7$) (Figure 2). In the range 10^{-7} to 10^{-5} M ATP, a linear concentration-relaxant response curve was constructed again and pD_2 for the relaxation was 6.64 ($n = 6$), which was larger than that of the contraction (Figure 2).

Contractile response to ATP The contractile response to 10^{-5} M ATP of duodenal segments taken from rats of days 7 to 14 did not change on preincubation with 10^{-6} M TTX or 2.5×10^{-7} M hyoscine for 15 min, respectively (Figure 3a and b). The presence of subtypes of P_2-purinoceptors, x and y has been proposed by Burnstock & Kennedy (1985) on the basis of agonist potency orders. RB-2 was used to distinguish P_y- from P_x-purinoceptors, since it has been shown to act as a competitive antagonist against P_{2y}-purinoceptor-mediated responses within the concentration range 0.1–10 μM (Burnstock *et al.*, 1986; Burnstock & Warland, 1987). In the presence of 10^{-5} M RB-2, added 5 min before ATP, the duodenal contractile response to ATP was inhibited by 71.0 ± 3.0% (mean ± s.e.mean, $n = 5$) (Figure 3c). The contractile response to ATP of the duodenum on days 7 to 14 was inhibited in most cases in the presence of 10^{-5} M indomethacin added 30 min previously (Figure 3d, e and f) and the residual response to ATP was 22.2 ± 5.2% ($n = 8$). The addition of the vehicle alone (70% ethanol) to the bath failed to have any effect on the response to ATP in rat duodenum. Therefore, the contractile response to ATP is not mediated by cholinergic neurones, but rather by stimulation of prostaglandins synthesis through an effect on P_{2y}-purinoceptors in the duodenum.

Relaxant response to ATP As shown in Figure 4, the relaxant response to 10^{-5} M ATP on day 70 was not affected by preincubation with either 10^{-6} M TTX for 15 min, 6.4×10^{-6} M guanethidine for 30 min, 2.5×10^{-7} M hyoscine for 15 min, 10^{-5} M indomethacin for 30 min or the P_1-antagonist, 8-PT (10^{-6} M) (Griffith *et al.*, 1981) for 5 min (Figure 4a, b, d and e). However, this response was markedly inhibited by preincubation with 10^{-5} M RB-2 for 5 min by 75.5 ± 4.2% ($n = 5$) (Figure 4c). The relaxant response to ATP thus appears to involve an action on the P_{2y}-purinoceptor of smooth muscle but not on adrenergic nerves in the myenteric plexus of the duodenum.

The nature of the response to adenosine

The mechanism of the relaxant response to adenosine 10^{-4} M in the duodenum was examined in adult (about 70 days old) rats. This response to adenosine (10^{-4} M) was inhibited by 68 ± 4% ($n = 5$)

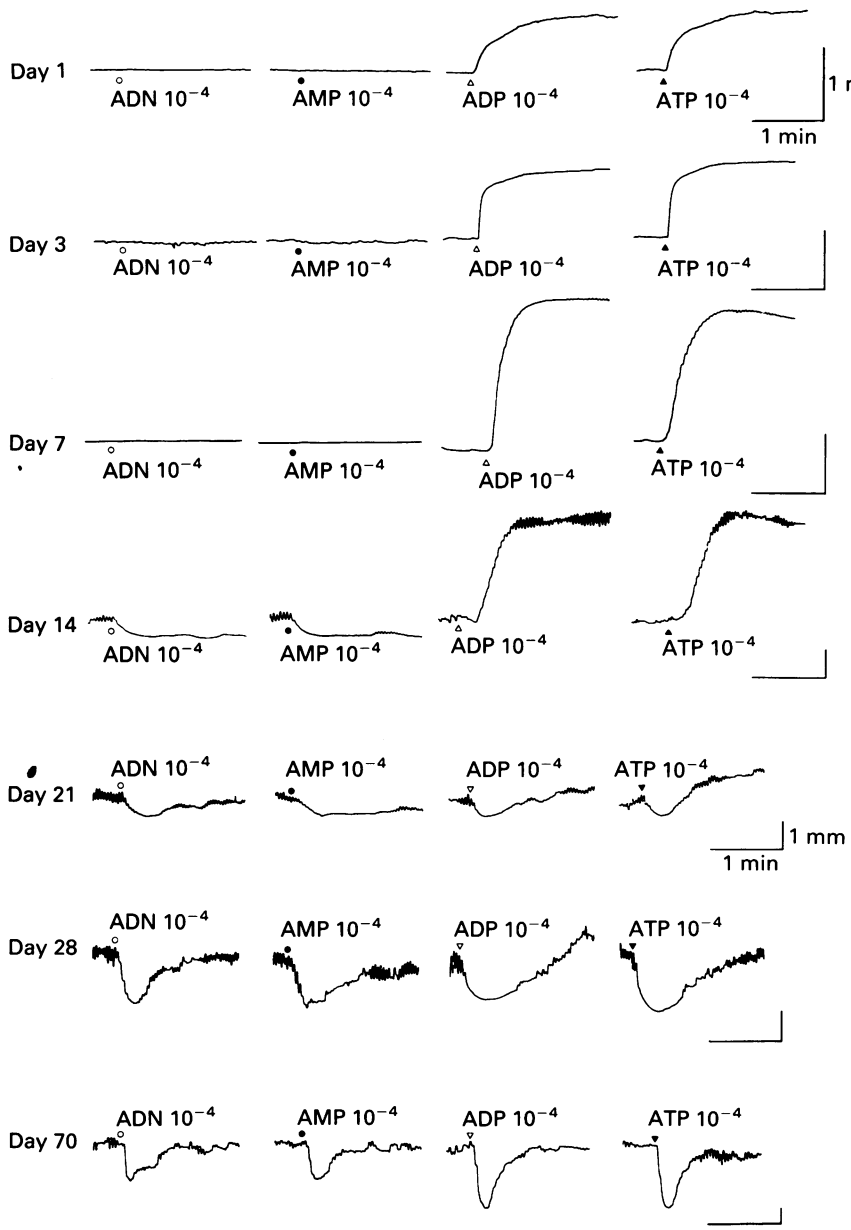

Figure 1 Mechanical responses to adenosine (ADN), adenosine-5'-monophosphate (AMP), adenosine-5'-diphosphate (ADP) and adenosine-5'-triphosphate (ATP), each at 10^{-4} M, in duodenal segments of rats from 1 to 70 days old. Both mechanical activity (mm) and time (min) scales are shown.

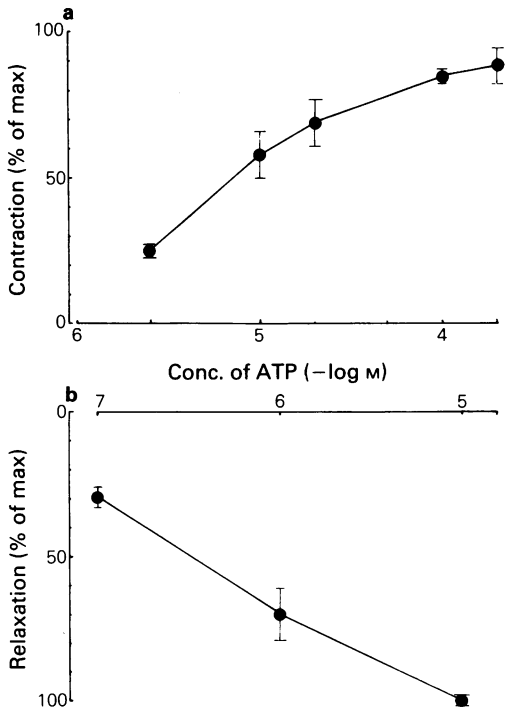

Figure 2 Log concentration-response curves for (a) contraction (day 7, $n = 7$) and (b) relaxation (day 70, $n = 6$) caused by ATP in an isolated segment of rat duodenum. Values are the mean with s.e.mean shown by vertical lines. The pD_2 of ATP for the contractile response was 5.51 and for the relaxant response, 6.46.

by 10^{-6} M 8-PT added 5 min previously (Figure 5c). Preincubation with 10^{-6} M TTX for 15 min or both 6.4×10^{-6} M guanethidine for 30 min and 2.5×10^{-7} M hyoscine for 15 min failed to affect this response (Figure 5a and b). Neither did the vehicle of 8-PT (5 μl DMSO) by itself have any effect. It would thus appear that adenosine acts directly on smooth muscle through the P_1-receptor to induce relaxation.

Discussion

It is evident from the data presented above that, in rat duodenum, the contractile response to P_2-agonists such as ATP and ADP occurs before the myogenic relaxant response. Contractile responses, detectable on day 1, became maximum at days 7 to 14, decreased markedly thereafter and then became non-existent by day 21. In contrast, on day 21, transient relaxation, due to ATP or ADP, was detectable. The relaxant response to ATP and ADP continued to increase until adulthood. This postnatal change in the response of rat duodenum to ATP or ADP is in contrast to the consistent direct myogenic effects of the P_1-agonists, adenosine and AMP, seen on day 14 for the first time as well as in adult rats.

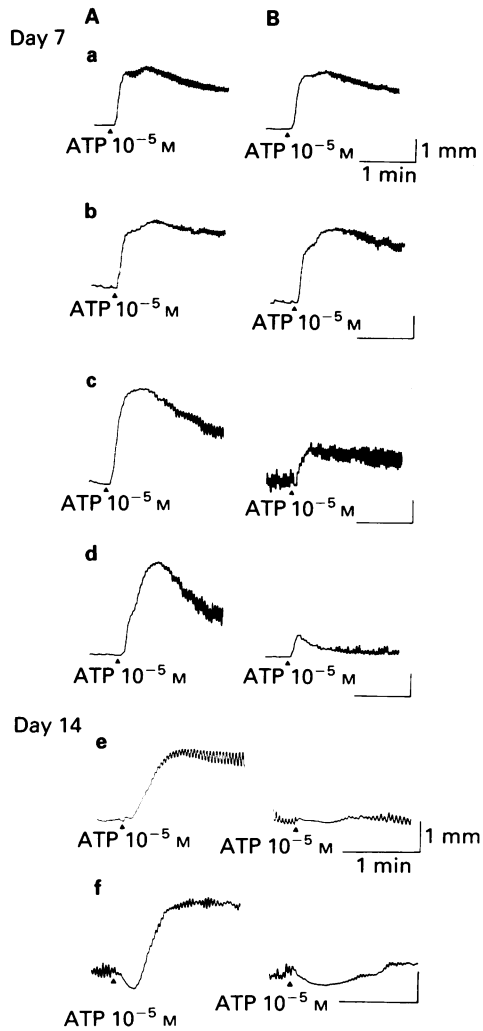

Figure 3 Effects of preincubation with tetrodotoxin (10^{-6} M for 15 min) (a), hyoscine (2.5×10^{-7} M for 15 min) (b), reactive blue-2 (10^{-5} M for 5 min) (c) and indomethacin (10^{-5} M for 30 min) (d, e and f) on the contractile response of rat duodenal segments to ATP on postnatal days 7 (a–d) and 14 (e and f). Contraction caused by ATP was not affected in the presence of tetrodotoxin or hyoscine. The response to ATP was inhibited by reactive blue-2 or indomethacin. Responses before (A) and after (B) incubation with the inhibitors are shown.

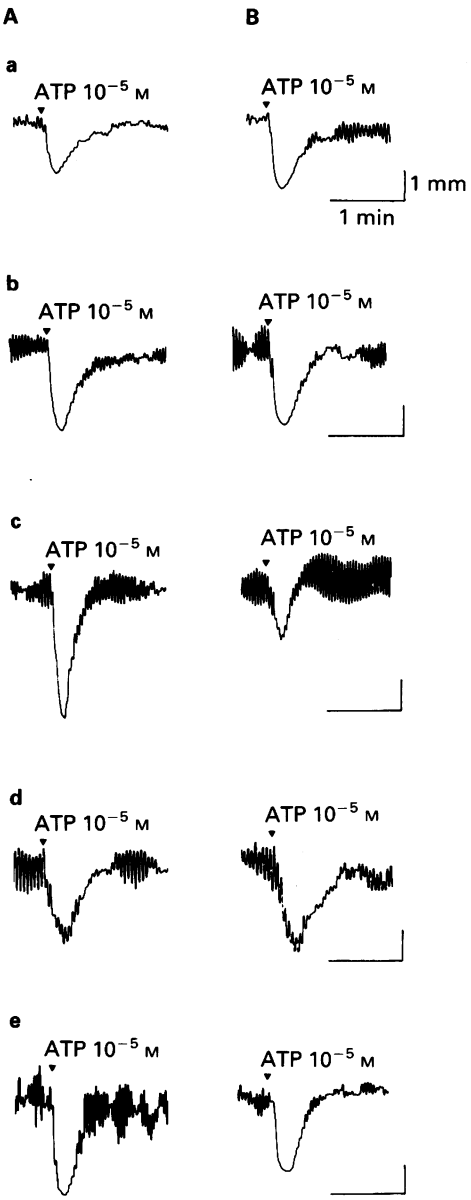

Figure 4 Effects of preincubation with tetrodotoxin (10^{-6} M, 15 min) (a), guanethidine (6.4×10^{-6} M, 30 min) and hyoscine (2.5×10^{-7} M, 15 min) (b), reactive blue-2 (10^{-5} M, 5 min) (c), indomethacin (10^{-5} M, 30 min) (d), and 8-phenyltheophylline (10^{-6} M, 5 min) (e) on the relaxant response to ATP of duodena on day 70. Relaxation produced by ATP showed no change in the presence of tetrodotoxin, guanethidine plus hyoscine or 8-phenyltheophylline, but was inhibited on adding reactive blue-2. Responses to ATP before (A) and after (B) incubation with the inhibitors are shown.

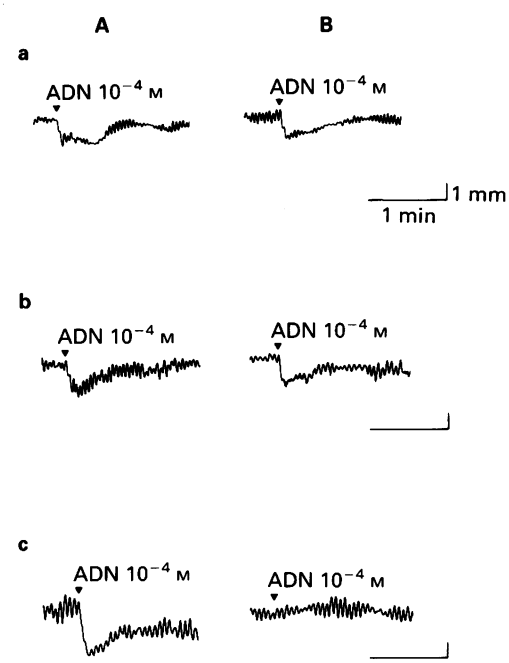

Figure 5 Effects of preincubation with tetrodotoxin (TTX, 10^{-6} M, 15 min) (a), guanethidine (Guan, 6.4×10^{-6} M, 30 min) and hyoscine (Hyos, 2.5×10^{-7} M, 15 min) (b) and 8-phenyltheophilline (8-PT, 10^{-6} M, 5 min) (c) on the relaxant response to adenosine (ADN) of duodena on day 70. The relaxant response to adenosine failed to change in the presence of TTX or Guan plus Hyos, but was inhibited by the addition of 8-PT. Responses to adenosine before (A) and after (B) incubation with the inhibitors are shown.

The contractile response to ATP in the duodena of rats may possibly be mediated by prostaglandins since the prostaglandin synthesis inhibitor, indomethacin (Vane, 1971) inhibits this response and ATP and ADP are potent stimulators of prostaglandin biosynthesis in various organs in mammals (Needleman *et al.*, 1974; Burnstock *et al.*, 1975; Burnstock, 1978; Gordon, 1986). RB-2, a P_{2y}-antagonist, has also been found to inhibit the contractile response to ATP, indicating that ATP stimulates prostaglandin synthesis through an effect on the P_{2y}-purinoceptor in neonatal rat duodenum. The absence of any effect of hyoscine or TTX on the response to ATP indicates that the contractile response is not mediated by cholinergic neurones.

The relaxant as well as the contractile response to 10^{-5} M ATP was markedly inhibited by RB-2 10^{-5} M but not by guanethidine or TTX. The relaxant response to ATP is thus myogenic through postsynaptic P_{2y}-receptors and is not induced by an

effect on adrenergic nerves. The conversion of ATP and ADP to AMP or adenosine, resulting in a relaxant response, would not be possible since the P_1-antagonist, 8-PT, failed to inhibit the response to ATP. The relaxant response to ATP has been found to arise mainly from an increase in the potassium permeability of the smooth muscle membrane (Brown & Burnstock, 1981). Both relaxant and contractile responses to ATP may be mediated through P_{2y}-receptors since each is inhibited by RB-2. From the above findings, the P_{2y}-receptor appears to be linked to two different transduction mechanisms, the early one to prostaglandin synthesis and the later, to potassium ion channels. The affinity of the relaxant response to ATP (pD_2: 6.64) was higher than that for the contractile response (pD_2: 5.51). The proposed different transduction mechanisms may perhaps account for the observed differences in pD_2 values. However, the presence of two subtypes of P_{2y}-receptor in the duodenum of the developing rat remains a possibility. Change in the features of P_{2y}-purinoceptors in the duodenum seems to occur between postnatal days 14 and 21. It is unlikely that the responsiveness of the duodenum to prostaglandins is lost by about day 21, since prostaglandins E_1 and E_2 have been shown to contract the longitudinal muscle of adult rat small intestine through a direct action on it (Bennett *et al.*, 1968).

An increase in muscle tone of the duodenum may be responsible for the change in response to ATP, causing contraction instead of relaxation. The response to ATP has been found to vary according to the tone of the preparation (Burnstock *et al.*, 1970). Cocks & Burnstock (1979) classified this tone in the case of the taenea coli of guinea-pig, based on the response to non-adrenergic, non-cholinergic inhibitory neurone stimulation and exogenous ATP as follows. Preparations have high tone if the response to either stimulus is relaxation without 'rebound contraction', those having medium tone give relaxation followed by 'rebound contraction' to each stimulus, while with low tone preparations, 'rebound contraction' occurs with cessation of the stimulus, with little or no relaxation during stimulation. Based on this classification, it may be concluded that with the progress of postnatal growth, duodenal muscle tone becomes higher and reaches a critical level, where a relaxant response to ATP would be evident, on about day 21. In this study, a contractile response to ATP, preceded by a relaxant response on day 14, was blocked by indomethacin. This seems to be related to the observation of a previous study that indomethacin inhibits rebound contraction on cessation of stimulation of non-adrenergic, non-cholinergic nerves or by the exogenous application of ATP to guinea-pig taenia coli (Burnstock *et al.*, 1975). However, contractile responses to ATP of rat duodenum on days 1 to 7 are not a rebound phenomenon, since exogenous ATP caused contraction without a time lag after adding ATP to the bath, and the contractile response to ATP always occurred in the presence of the nucleotide in the bath but not following its removal. Thus, ATP-induced contraction on day 14 is not a rebound phenomenon but a remnant of that observed at the neonatal stage.

Relaxant responses to adenosine were blocked by the P_1-antagonist, 8-PT, which indicates that adenosine stimulates the P_1-purinoceptor in rat duodenum, leading to relaxation. The involvement of adenosine or P_1-purinoceptors in the regulation of transmitter release from adrenergic, cholinergic and non-adrenergic, non-cholinergic nerves has been reported (Jager & Den Hertog, 1985). We showed that TTX and guanethidine did not affect the response to adenosine, which confirms that adrenergic nerves are not involved in the response to adenosine in rat duodenum. However, a further study should be performed to determine whether adenosine actually regulates transmitter release from myenteric neurones in rat duodenum.

Duodenal responses to AMP and ADP were essentially the same as those to adenosine and ATP, respectively, with respect to ontogenetic change and magnitude of response. This is consistent with the notion that adenosine and AMP are P_1-agonists and ADP and ATP, P_2-agonists (Burnstock, 1978). We thus propose that the smooth muscle of rat duodenum may possess separate purinoceptors for adenosine and AMP (P_1) and ADP and ATP (P_2), as has been indicated in a previous study with the smooth muscle of guinea-pig caecum (Brown & Burnstock, 1981; Satchell & Maguire, 1982; Ferrero & Frischknecht, 1983), and that the response to P_1- and P_2-agonists changes with the progress of development.

A remarkable degree of coordination has been found between the development of various gastrointestinal functions in rats and major developmental changes that occur during the third week following birth (Henning, 1981; Thomson & Keelan, 1986). The present study indicates that changes in the P_2-receptor-mediated response to purine nucleotides in rat duodenum also occur in accordance with this timing. Although the ontogenesis of nerves containing purine nucleotides has not been studied, the present results indicate that P_2-agonists such as ATP and ADP may affect the contractility of smooth muscle of the rat duodenum through synthesis of prostaglandins in neonatal rats before day 14, while at about postnatal day 21 they begin to act as transmitters of non-adrenergic, non-cholinergic inhibitory neurones, causing the appearance of myogenic relaxant responses.

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Nitric oxide synthesized from L-arginine regulates vascular tone in the coronary circulation of the rabbit

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1 The role of nitric oxide (NO) in the regulation of the vascular tone of the coronary circulation of the Langendorff-perfused rabbit heart was investigated.

2 N^G-monomethyl-L-arginine (L-NMMA; 10–100 μM), a specific inhibitor of NO formation from L-arginine (L-Arg), but not its D-enantiomer (D-NMMA; 100 μM) produced a dose-related, sustained increase in the coronary perfusion pressure (CPP). In addition, L-NMMA inhibited the vasodilator responses of acetylcholine (ACh), unmasking in some instances its direct vasoconstrictor effect. These effects of L-NMMA were attenuated by L-Arg.

3 L-NMMA (10 and 30 μM), but not D-NMMA (30 μM), caused a long-lasting inhibition of NO formation which was reversed by L-Arg (30 and 100 μM), but not by D-Arg (100 μM).

4 This study indicates that the formation of NO from L-Arg in the coronary circulation of the rabbit plays a role both as a regulator of vascular tone and as a mediator of the vasodilatation induced by ACh.

Introduction

The role of endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980) in the microcirculation has not yet been established. This is mainly due to its intrinsic instability (Griffith *et al.*, 1984), which makes its detection difficult, and to the problems inherent in obtaining 'endothelium-free' vascular beds. However, the identification of EDRF as nitric oxide (NO; Palmer *et al.*, 1987) and the elucidation of the metabolic pathway to its synthesis from L-arginine (L-Arg; Palmer *et al.*, 1988a; Schmidt *et al.*, 1988) have allowed a fresh approach to the study of the role of NO in the microcirculation.

Nitric oxide is released in the coronary circulation of the isolated perfused rabbit heart and this release accounts for the vasodilatation induced by acetylcholine (ACh; Amezcua *et al.*, 1988) and bradykinin (Kelm & Schrader, 1988). The synthesis of NO by vascular endothelial cells in culture or by fresh vascular tissue is inhibited in a dose-dependent and enantiomerically-specific manner by N^G-monomethyl-L-arginine (L-NMMA; Palmer *et al.*, 1988b; Rees *et al.*, 1989a). This compound also inhibits endothelium-dependent relaxation in rings of rabbit aorta (Rees *et al.*, 1989a) and guinea-pig pulmonary artery (Sakuma *et al.*, 1988).

In view of this, we have now assessed the role of NO synthesized from L-Arg in the coronary circulation of the rabbit isolated heart by studying the effects of L-Arg and L-NMMA on the coronary perfusion pressure (CPP) and on the responses of the coronary circulation to ACh.

Methods

Modified Langendorff-perfused hearts

Male New Zealand White rabbits (1.8 to 2.4 kg) were given heparin (100 u kg^{-1}) and anaesthetized with sodium pentobarbitone (40–50 mg kg^{-1}) via an ear

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vein. The thorax was rapidly opened and the aorta cannulated retrogradely. The heart was removed, flushed with Krebs buffer gassed with 5% CO_2 and 95% O_2 at room temperature and perfused at constant flow with Krebs buffer at 37°C containing indomethacin (5 μM). The Krebs buffer consisted of (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.17, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 8.4. The rate of perfusion was either 15 ml min^{-1} for bioassay and chemiluminescence experiments or 25 ml min^{-1} for measurement of CPP.

The hearts were paced at 180 beats min^{-1} via two platinum pin electrodes inserted into the left ventricular wall and connected to a Grass Stimulator S9 (square wave pulses, 6V, 3 ms). In some experiments the CPP was measured with a pressure transducer connected to a side arm of the perfusion line and was displayed as the average signal on a Gould recorder. The average CPP was raised from a basal level of 15–20 mmHg to 35–70 mmHg by a continuous infusion of 9,11-dideoxy-9α,11α methano epoxyprostaglandin $F_{2\alpha}$ (U46619, 30–150 nM). All experiments were completed within 2 h of starting the perfusion of the heart.

Drugs were administered through an injection port immediately before the aortic cannula. Prostacyclin and ACh were delivered as 1 min infusions, NO as bolus injections of 3 to 10 μl of the appropriate solution and all other drugs, unless otherwise specified, as 20 min infusions starting 10 min before administration to the heart of ACh or prostacyclin. The falls in CPP induced by ACh, prostacyclin and NO were measured as the areas under the curves. The vasoconstrictor responses were expressed as the percentage increase of the CPP above the resting value.

Detection of nitric oxide

NO formation by the heart was measured by chemiluminescence as described previously (Palmer *et al.*, 1987; Amezcua *et al.*, 1988). Briefly, the cardiac effluent (15 ml min^{-1}) was collected into the stem of a funnel closed at the bottom. An aliquot of the effluent (3.5 ml min^{-1}) was continuously transferred into the reaction vessel of the chemiluminescence apparatus while the volume of the sampling pool was kept constant by continuous removal of the excess. The delay between the collecting funnel and the reflux vessel, which contained 75 ml of 1% sodium iodide in glacial acetic acid under reflux, was 15 s. NO was removed under reduced pressure in a stream of N_2, mixed with ozone and the chemiluminescent product was quantified by reference to NO standards.

In some experiments the cardiac effluent (15 ml min^{-1}) was used to superfuse a cascade of three spirally cut strips of rabbit thoracic aorta denuded of endothelium with a pipe cleaner as described previously (Gryglewski *et al.*, 1986) and contracted either with U46619 (150 to 750 nM) or with phenylephrine (0.75 to 2.5 μM). There was a delay of 7 s between the port of injection of drugs into the heart and the uppermost bioassay tissue and a delay of 1 s between this tissue and the heart. The subsequent tissues were separated from each other by 3 s delays.

We have observed previously that superfusion of the aortic strips with the cardiac effluent caused a pronounced relaxation of the tissues which caused a decreased sensitivity to NO. As a result of this, the release of EDRF/NO activity from the heart could not be detected (Amezcua *et al.*, 1988). We have now modified our method so that the rate of infusion of the contracting agent (U46619 or phenylephrine) is increased to restore the tone of the bioassay tissues to the level prior to superfusion with the heart effluent.

Chemicals

Nitric oxide (>99.98% pure, British Oxygen Corporation) solutions were prepared as described previously (Palmer *et al.*, 1987). Glyceryl trinitrate and prostacyclin (Wellcome), phenylephrine, ACh, L- and D-Arg (Sigma), U46619 (Cayman Chemicals), sodium pentobarbitone (May and Baker) and heparin (Abbott) were obtained as indicated. L-NMMA and D-NMMA were synthesized as described previously (Patthy *et al.*, 1977).

Statistics

Differences between means were assessed with Student's t test for paired or unpaired samples as appropriate and considered significant when $P < 0.05$. The results are presented as the mean ± s.e.mean of (n) experiments.

Some of these data were presented at a meeting of The British Pharmacological Society in December, 1988.

Results

Effects on the coronary perfusion pressure

L-NMMA (10, 30, 100 μM) induced a dose-dependent increase in the resting CPP (Figure 1). This increase was long-lasting, usually persisting for the duration of the experiment (1.5 h, $n = 11$).

The vasodilator responses to ACh (0.03 to 3.0 μM) were also inhibited in a dose-related manner by

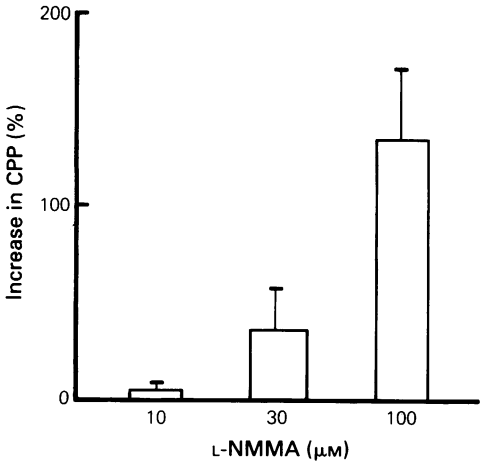

Figure 1 Effect of N^G-monomethyl-L-arginine (L-NMMA) (20 min infusions) on the mean coronary perfusion pressure (CPP) of the Langendorff-perfused rabbit heart. The results are expressed as percentage change from the basal CPP ($n = 4$).

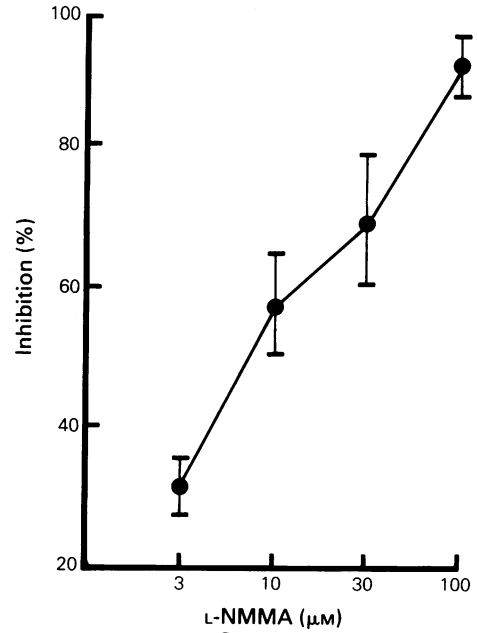

Figure 2 Effect of N^G-monomethyl-L-arginine (L-NMMA) (20 min infusions) on the fall in coronary perfusion pressure (CPP) induced by acetylcholine (ACh) (0.3 μM). The results are expressed as percentage inhibition of the control response to ACh ($n = 4$–6).

L-NMMA (IC_{50} against 0.3 μM ACh was 8.4 ± 1.4 μM, $n = 11$; Figure 2). In addition, L-NMMA (30 and 100 μM) frequently revealed the vasoconstrictor property of ACh (0.3 μM; Figure 3) so that in seven out of eleven experiments the fall in CPP induced by ACh (0.3 μM) was converted into a vasoconstrictor response by L-NMMA. The rise in CPP induced by ACh under these conditions lasted for 3 to 5 min, ranged from 25 to 120% of the resting value and was occasionally preceded by a short-lasting (< 1 min) reduction in CPP. In contrast, the increase in CPP induced by L-NMMA (100 μM) enhanced the vasodilator responses to prostacyclin (1 μM; $n = 3$) and exogenous NO (450 pmol; $n = 4$) to $184 \pm 27\%$ and $208 \pm 36\%$ respectively ($P < 0.05$). The actions of L-NMMA were enantiomerically specific, since D-NMMA (100 μM; $n = 4$) did not exhibit any of the effects described above.

Neither L- nor D-Arg (100 μM) had any effect on resting CPP, nor did they affect the responses to ACh in the absence of L-NMMA. Infusion of L-Arg (100 μM) after administration of L-NMMA (30 μM) partially reversed the increase in CPP induced by L-NMMA and reduced significantly the inhibition of the ACh-induced vasodilatation from 69 ± 9 to $41 \pm 4\%$ ($n = 4$; $P < 0.05$). When L-Arg was infused from the beginning of the experiment it prevented the rise in CPP induced by L-NMMA and reduced significantly (from 69 ± 9 to $34 \pm 12\%$; $n = 4$, $P < 0.05$) the inhibition of the vasodilator response to ACh. The ACh-induced vasodilatation was never

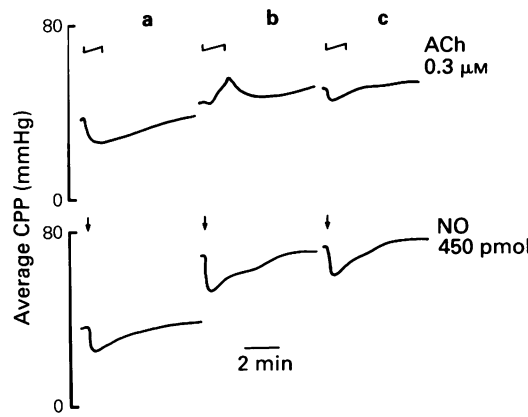

Figure 3 Effects of acetylcholine (ACh, 0.3 μM; 1 min infusions, upper panel) and nitric oxide (NO, 450 pmol; bolus injections, lower panel) on the coronary perfusion pressure (CPP) of the Langendorff-perfused rabbit heart. (a) Control responses; (b) Responses obtained during a 20 min infusion of N^G-monomethyl-L-arginine (L-NMMA, 30 μM); and (c) responses obtained after a 20 min washout with L-Arg (100 μM). Note the elevation in CPP after infusion of L-NMMA. Trace representative of 3 experiments.

converted into the vasoconstriction in the presence of L-Arg ($n = 4$).

Chemiluminescence

Acetylcholine (1 and 3 μM) produced a dose-dependent release of NO, detected by chemiluminescence, which was matched by exogenous NO (450 to 1340 pmol) administered through the heart. The release of NO by ACh (1 μM) was enhanced significantly from 499 $\pm$ 180 pmol to 1031 $\pm$ 270 pmol by L-Arg (30 μM, $n = 4$, $P < 0.05$; Figure 4). L-NMMA (10 μM) inhibited the release of NO induced by a higher concentration of ACh (3 μM) from 1025 $\pm$ 256 to 367 $\pm$ 51 pmol ($n = 3$; $P < 0.05$). This inhibition persisted for more than 1 h but could be reversed by an infusion of L-Arg (30 μM; Figure 5). The ACh-induced release of NO was not affected by either D-Arg (100 μM, $n = 3$) or D-NMMA (30 μM, $n = 3$).

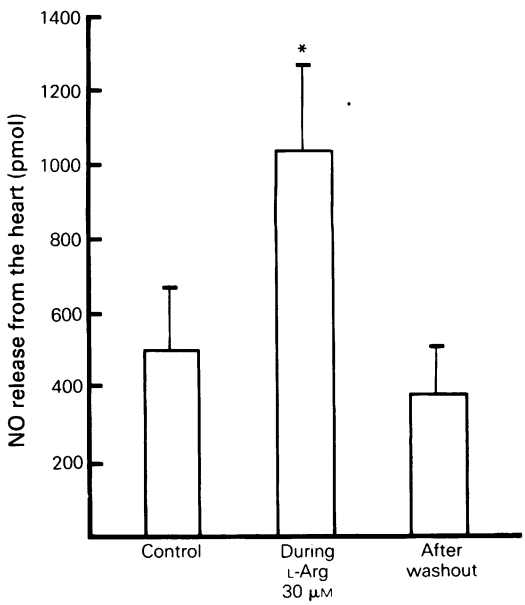

Figure 4 Nitric oxide (NO) formation by the coronary circulation in response to three consecutive challenges with acetylcholine (ACh, 1 μM; 1 min infusions) in the same preparation. The second challenge was given at the midpoint of a 20 min infusion of L-arginine (L-Arg, 30 μM) and the third 20 min after the end of the L-Arg infusion. The amount of NO detected by chemiluminescence was calculated by reference to authentic NO standards injected through the heart ($n = 4$). * Indicates that the values differ significantly ($P < 0.05$) from the control in a paired t test.

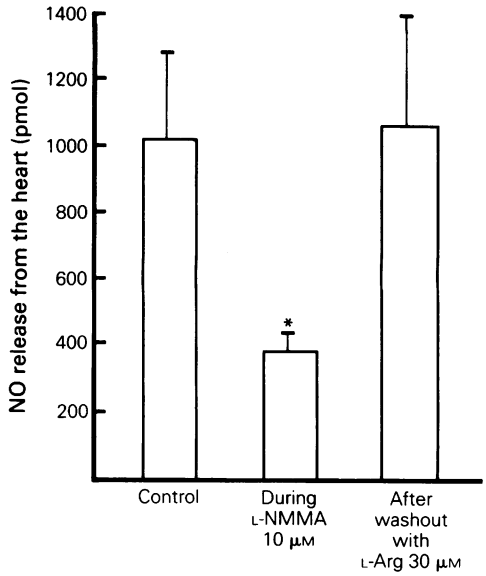

Figure 5 Nitric oxide (NO) formation by the coronary circulation of the Langendorff-perfused rabbit heart in response to three consecutive challenges with acetylcholine (ACh, 3 μM; 1 min infusion) in the same preparation. The second challenge was given at the midpoint of a 20 min infusion of N^G-monomethyl-L-arginine (L-NMMA, 10 μM). The third challenge was given immediately after a 20 min infusion of L-arginine (L-Arg, 30 μM). The amounts of NO detected by chemiluminescence were calculated by reference to authentic NO standards injected through the heart. * Indicates that the values differ significantly ($P < 0.05$) from the control in a paired t test ($n = 3$).

Bioassay

Acetylcholine (3 μM) induced the release of a relaxing factor from the heart which was identified as NO for it decayed during passage down the bioassay cascade at the same rate as NO (45 to 134 pmol) applied over the tissues (OT). Furthermore, its activity was inhibited by a concomitant infusion of haemoglobin (100 nM, OT, $n = 6$, $P < 0.05$) and enhanced by an infusion of superoxide dismutase (15 u ml^{-1}, $n = 6$, $P < 0.05$) administered through the heart.

The release of NO induced by ACh (3 μM) was increased to 175 $\pm$ 15% of control ($n = 6$, $P < 0.05$) by L-Arg (100 μM; Figure 6) and was inhibited significantly by 61 $\pm$ 5% by L-NMMA (30 μM; $n = 3$, $P < 0.05$). This inhibition could be reversed by a 20 min infusion of L-Arg (100 μM, $n = 3$, $P < 0.05$). None of these effects were observed with the D-enantiomers of these compounds ($n = 3$). Neither L-Arg (100 μM) nor L-NMMA (30 μM) had any effect

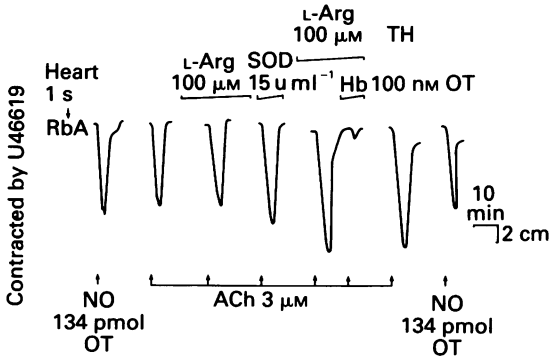

Figure 6 Effect of L-arginine (L-Arg, 100 μM; 30 min infusions), administered over the tissues (OT) and through the heart (TH), on the relaxation of a rabbit aortic strip (RbA) caused by acetylcholine (ACh, 3 μM; 1 min infusion; TH)-induced release of nitric oxide (NO). The effects of superoxide dismutase (SOD; 15 u ml; 10 min infusion; OT) and haemoglobin (Hb, 100 nM; 10 min infusion; OT) are also shown. NO was administered as a bolus injection (134 pmol; OT). The tissue was not affected by ACh (3 μM; OT; not shown). The trace is representative of 6 experiments.

on the bioassay tissues or on their response to exogenous NO.

Discussion

We have shown that L-NMMA, a specific inhibitor of NO formation from L-Arg (Palmer *et al.*, 1988b; Rees *et al.*, 1989a) induces a dose-dependent, long-lasting increase in the CPP. In addition, L-NMMA inhibited the vasodilatation induced by ACh and these effects were enantiomerically specific. These results indicate that NO is formed from L-Arg in the coronary microcirculation to regulate CPP and to mediate the effects of endothelium-dependent vasodilators.

In rabbit aortic rings we have previously shown that L-NMMA induces a small increase (≈20%) in vascular tone ($EC_{50} \approx 4\,\mu M$) and inhibits ACh-induced relaxation. Furthermore, L-NMMA was more potent at increasing basal tone than as an inhibitor of ACh-induced relaxation (Rees *et al.*, 1989a). This is in contrast to our present findings in which lower doses of L-NMMA were required to inhibit the ACh-induced fall in CPP than were required to increase basal CPP. This most probably indicates that in resistance vessels there is a high basal level of NO formation which plays an important role in the regulation of tone in these vessels. This suggestion is supported by the observation of Kelm & Schrader (1988) who have recently reported that basal release of NO is high in the coronary circulation of the guinea-pig heart when compared to that induced by bradykinin.

Exogenous L-Arg had no direct effect on CPP or on the fall in CPP induced by ACh. This is in agreement with previous observations in vascular tissue (Sakuma *et al.*, 1988; Rees *et al.*, 1989a) and is consistent with our suggestion that under basal conditions the amounts of endogenous substrate are sufficient to provide for the basal formation of NO.

Exogenous L-Arg partially reversed the effect of L-NMMA on CPP and on ACh-induced fall in CPP. This is in contrast to the effects in large conduit arteries where a complete reversal of the effects of L-NMMA by L-Arg (Palmer *et al.*, 1988b; Sakuma *et al.*, 1988; Rees *et al.*, 1989a) has been observed. This difference could be due to mechanical or pharmacokinetic changes in the microcirculation caused by the oedema which develops during the experiment and which is enhanced by elevation of CPP. The competitive interaction between L-Arg and L-NMMA could be more clearly seen in experiments in which L-Arg was infused from the beginning of the experiment. Under these circumstances the effects of L-NMMA were greatly reduced.

Acetylcholine releases NO from the coronary circulation, although this decays rapidly to NO_2^- which is biologically inactive (Amezcua *et al.*, 1988). In the present studies we could also detect the release of biologically active NO from the heart and this was inhibited by L-NMMA. L-Arg not only reversed the effects of L-NMMA but, interestingly, enhanced the release of NO induced by a submaximal dose of ACh. The fact that this was not associated with potentiation of the ACh-induced fall in CPP in the absence of L-NMMA may be due to differences between luminal and abluminal release of NO (Bassenge *et al.*, 1987) or to the high doses of ACh required to elicit detectable NO release compared to those that lower CPP.

In summary, our results indicate a major role for EDRF/NO in the regulation of vascular tone and reactivity in the coronary circulation. The speculation that altered responses to endothelium-dependent vasodilators may play a role in the pathogenesis of coronary vasospasm has so far been supported by observations made in conditions associated with gross morphological damage of the endothelium. It is possible that a specific biochemical defect of the endothelium, with or without morphological damage, also plays a role in the development of coronary vasospasm. L-NMMA, which produces what could be considered a selective reversible 'biochemical denudation' of the endothe-

lium, offers the possibility of studying the consequences of removing the synthesis of NO by the vascular endothelium. Indeed, we have recently observed that L-NMMA induces a rise in blood pressure in the anaesthetized rabbit (Rees *et al.*, 1989b).

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Inhibition of neurally-evoked transmitter release by calcium channel antagonists in rat parasympathetic ganglia

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1 Excitatory postsynaptic potentials (e.p.s.ps) were recorded from the submandibular parasympathetic ganglia of newborn rats (10–20 days old), by intracellular microelectrode recording and a suction electrode to deliver stimulus trains to the lingual nerve (15 stimuli at 0.1, 0.3, 1, 3, and 10 Hz, 22°C). Only evoked responses without voltage-dependent action potentials were analyzed (observed at membrane potentials negative to −70 mV), and e.p.s.p. amplitudes were determined for the plateau responses during each train (5–15th response).

2 Cadmium, an inorganic calcium channel antagonist, reduced e.p.s.p. amplitudes in a dose-dependent manner (K_d 74 μM, $P < 0.01$). Nickel (1–300 μM) did not attenuate the amplitude of evoked responses.

3 Verapamil (0.1–30 μM), a phenylamine, had no significant effects upon e.p.s.p. amplitudes at any frequency examined. Higher concentrations of verapamil (100 μM) blocked neurally evoked responses in a manner consistent with the antagonism of voltage-sensitive sodium currents.

4 Diltiazem, a benzothiazepine, reduced e.p.s.p. amplitudes in a dose-dependent manner, the depression being accentuated at high stimulation frequencies (80% block at 30 μM and 10 Hz). The pure (−)-*cis* enantiomer of diltiazem (10–30 μM) was without effect.

5 Amlodipine, a 1,4-dihydropyridine, did not antagonize synaptic transmission at any stimulus frequency examined (10–30 μM, 0.1–10 Hz, $n = 3$).

6 Amiloride, a potassium-sparing diuretic, depressed the amplitudes of evoked responses in a dose-dependent manner (one-site K_d 31 μM, $P < 0.005$), although the extent of the block was alleviated with high stimulus frequencies. The effects of 30 μM amiloride were unlikely to be of postsynaptic origin as both the amplitudes of miniature e.p.s.ps, and the iontophoretic potentials induced by exogenous acetylcholine, were not attenuated by treatment with this compound. The amiloride derivative, 3',4'-dichlorobenzamil was ineffective in reducing the amplitude of e.p.s.ps (30–100 μM).

7 ω-Conotoxin GVIA, a marine neurotoxin, which depressed whole cell calcium currents recorded from cultured rat parasympathetic cardiac neurones (up to 90% block at 10 nM), was ineffective at blocking synaptic transmission in submandibular ganglia (0.1–1 μM).

8 The differential effects of these calcium channel antagonists upon synaptic transmission in rat parasympathetic ganglia, suggest that either more than one type of calcium channel may be involved in transmitter release, or that the presynaptic calcium channels possess pharmacological sensitivities different from those of channel types described in neuronal cell bodies.

Introduction

Calcium channel antagonists continue to be an essential tool for investigating calcium channel function (Janis *et al.*, 1987; Vanhoutte *et al.*, 1988). It transpires that at least three types of calcium antagonist binding sites are associated with certain voltage-sensitive calcium channels, these include sites for benzothiazepines, phenylamines, and dihydropyridines (Wagner *et al.*, 1988). Other distinct sites may exist for the inorganic calcium channel blockers, as well as for neurotoxins such as ω-conotoxin (CGTX)

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GVIA, VIIA, and maitotoxin. Calcium channel antagonists also exhibit modulatory actions upon other ligand binding sites under certain conditions, for example, the phenylamine and benzothiazepine sites interact both in their binding and efficacy (Wagner *et al.*, 1988). The influence of physiological concentrations of cations, such as H^+ and Ca^{2+}, on calcium antagonist binding, and the use-dependence of many of these drugs, also complicate the interpretation of data from different cell types and species. Although several types of calcium channels may be distinguished on the basis of biophysical and pharmacological criteria in some cell types, including chick dorsal root ganglion (DRG) neurones where three calcium channels types (designated L, N, and T) have been described (McCleskey *et al.*, 1986; Fox *et al.*, 1987), the relevance of such a classification for other cell types and species is unclear. Differences may also exist between the types of calcium channels which reside in the cell soma, and in neuronal processes which participate in neurotransmitter release.

Investigation of the role of calcium channel types in neurotransmitter release processes has been prompted by the availability of specific antagonists of presynaptic calcium channels such as the shellfish peptide toxin, ω-CGTX GVIA (Kerr & Yoshikama, 1984; Olivera *et al.*, 1985; McCleskey *et al.*, 1987). The high potency of ω-CGTX in blocking neurotransmitter release at the frog neuromuscular junction (Kerr & Yoshikama, 1984), and noradrenaline release from sympathetic neurones (Hirning *et al.*, 1988), compared with the ineffectiveness of some dihydropyridines, have led to the suggestion that the N-type calcium channels are responsible for mediating transmitter release from nerve terminals. However, one cannot exclude a role for L-type calcium channels in neurotransmission on the basis of dihydropyridines not inhibiting the electrically evoked release of substance P, given the voltage-dependence of dihydropyridine block of calcium channels (Holz *et al.*, 1988). At the lizard neuromuscular junction, presynaptic calcium currents, and neurotransmitter release induced by prolonged presynaptic depolarizations, are attenuated by dihydropyridines, indicative of the modulation of L-type currents (Lindgren *et al.*, 1988). However, like the latter study, many recent investigations of the calcium channel types involved in neurotransmitter release have been conducted under conditions whereby the presynaptic depolarization is prolonged by the application of 4-aminopyridine, tetraethylammonium ions, or by high potassium containing saline (Penner & Dreyer, 1986; Holz *et al.*, 1988; Pun *et al.*, 1988). At the mouse neuromuscular junction more than one type of calcium channel is present (Penner & Dreyer, 1986). The temporal characteristics of activation and inactivation of different types of calcium channels differ, and consequently with these varied methodologies a confusing pharmacological profile of the calcium current(s) involved in transmitter release has emerged.

In this study, the actions of calcium channel antagonists on synaptic transmission were investigated to determine if a pharmacologically distinct calcium channel type may be involved in neurally evoked transmitter release in parasympathetic ganglia. The parasympathetic neurones used in this study belong to that part of the mammalian autonomic nervous system in which preganglionic axons emerge from the cranial region of the CNS. Synaptic transmission was examined between submandibular ganglia, and whole-cell calcium currents were recorded from cultured cardiac parasympathetic neurones. The differential sensitivity of synaptic transmission to a variety of calcium channel antagonists suggest that either more than one type of calcium channel is involved in transmitter release in submandibular ganglia, or that these presynaptic calcium channels possess pharmacological sensitivities different from those channel types described in neuronal cell bodies. A preliminary presentation of some of these results was made at the September meeting of the British Pharmacological Society (Seabrook & Adams, 1988).

Methods

Preparation and solutions

Submandibular ganglia were excised from 10–20 day old Sprague Dawley rats according to the methods of Ascher *et al.* (1979). The preparation was bathed in physiological salt solution which contained (mM): NaCl 119, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, $CaCl_2$ 7.5, KCl 4.7, $MgSO_4$ 5, 346 mOsm. A high extracellular divalent cation concentration was essential to obtain successful (> 1 h) recordings from these neurones. The saline was equilibrated with 95% O_2 + 5% CO_2, and the pH was adjusted to 7.2. Drug solutions were freshly made before each experiment, and were perfused onto the preparation at a rate of ca. 5 ml min^{-1} (bath volume 2 ml) for ca. 10 min, at 22°C. In the case of ω-CGTX GVIA, 1.65 ml of 1 μM stock solution was applied directly to the preparation without any background perfusion. Dichlorobenzamil was applied in 0.1% dimethylsulphoxide, at which concentration no significant effects of the solvent were observed on e.p.s.p. amplitudes.

Parasympathetic cardiac neurones obtained from newborn rat hearts were grown in tissue culture (Adams *et al.*, 1987). A dissociated neuronal cell culture was prepared from explants of the cardiac

ganglion plexus by dissecting out individual ganglia following enzyme treatment (collagenase). Neuronal cell cultures were used 24–72 h after plating. Cells were perfused in external saline of the following ionic composition (mM): NaCl 82, tetraethylammonium. Cl 50, HEPES 10, glucose 20, $CaCl_2$ 10, KCl 2.5, $MgCl_2$ 0.8 and 300 nM tetrodotoxin, adjusted to pH 7.4 with NaOH. The internal (pipette) solution contained (mM): $CsCl_2$ 150, Cs.BAPTA 5, HEPES 10, Mg.ATP 2, adjusted to pH 7.2 with KOH. High resistance seals (>5 GΩ) were formed against the soma membrane and the membrane was ruptured with increased suction to obtain the whole-cell recording configuration.

Electrophysiological recordings and analysis

Intracellular recordings were made from neurones visualized with Hoffman modulation contrast optics (× 400 magnification), using an Axoclamp-2A amplifier and 90–120 MΩ 4 M K-acetate electrodes. Few successful recordings were obtained with alternative intracellular electrode solutions (3 M KCl, 2 M K-citrate pH 7.5). Stimuli were applied to the lingual nerve via a suction electrode at a basal frequency of 0.1 Hz. Stimulus protocols of 15 stimuli at 0.3, 1, 3, and 10 Hz were conducted at 5–7 min intervals (without any significant decline in response amplitudes over 5 protocols) to examine the use-dependence of drug action. Only evoked responses without voltage-dependent action potentials were analyzed (observed at membrane potentials negative to −70 mV), and e.p.s.p. amplitudes were determined for the plateau responses during each train (5th to 15th response). Means are presented ±s.d. of either the sample size, or the averaged responses as indicated.

Responses were recorded on video tape after A-D conversion via a PCM-2 recorder adaptor (Medical Systems, N.Y., U.S.A.). For analysis, waveforms were digitized at 10 kHz on a Tetronix 5223 oscilloscope. After acquisition through a GPIB interface the waveforms were averaged (10 consecutive e.p.s.ps at each frequency), and parameters (including amplitude, rise and decay times) were determined with customized software on an IBM 80286 computer. Waveforms were acquired, their offset zeroed at the rise of the e.p.s.p. and then averaged. The rise times and rate of decay were determined from this averaged waveform. Graphical interpretation of the data was executed using GOS (OLIS Inc.).

The dose-response relationships obtained for the effects of calcium channel antagonists on e.p.s.p. amplitudes were fitted by one-or two-site adsorption isotherms:

$$F(x) = (Y_{max} \times K_d^{-1} \times [X])/(1 + K_d^{-1} \times [X]) + C$$

Where K_d is the equilibrium dissociation constant, [X] is the concentration of the test compound, and F(x) represents the amplitude of the response normalized with respect to the control. The significance of the dose-response curves were tested on ASYSTANT-plus (MacMillan Software Co.) by a Gauss–Newton algorithm.

Membrane potential transients evoked by ionophoretically-applied acetylcholine (ACh) were recorded after pretreatment of ganglia with 1 μM tetrodotoxin (TTX), and 3 μM neostigmine, to block voltage-dependent action potentials and acetylcholinesterase activity, respectively. A microelectrode filled with 2 M ACh (−3 nA retaining current) was positioned proximal to the neurone from which intracellular potentials were being recorded, and +400 nA pulses of 5 ms duration were applied every 30 s. Resultant membrane potential transients were averaged (5 consecutive responses) and digitized at 10 kHz prior to analysis by computer.

Calcium currents were recorded from rat parasympathetic cardiac neurones by use of the whole cell configuration of the patch clamp technique. Membrane current and voltage were recorded with a List EPC-7 patch clamp amplifier (List Electronics), digitized at 20 kHz (Tecmar Labmaster A/D interface) and stored on an IBM/PC AT 80286 computer. Cells were voltage clamped at −70 mV, and whole cell currents were evoked by depolarizing voltage steps applied in 10 mV increments from −80 mV to +80 mV following preconditioning pulses to −100 mV (0.5–1 s). Pulse protocols were provided by pCLAMP 4.1 software (Axon Instruments) at 5 s intervals. Membrane capacitative transients and leak currents were uncompensated.

Drugs

Drugs were obtained from the following companies: amiloride hydrochloride dihydrate (Merck); cadmium chloride 99.999% (Aldrich); diltiazem hydrochloride (Calbiochem); (−)-*cis* diltiazem (gift from Dr J. Stern); 3′,4′-dichlorobenzamil (gift from Dr P. Siegl, Merck Sharp & Dohme, West Point, PA, U.S.A.); neostigmine (Sigma); nickel chloride (Sigma); tetrodotoxin (Sigma); verapamil hydrochloride (Sigma); ω-CGTX GVIA (Calbiochem).

Results

Inorganic calcium channel antagonists

The inorganic calcium channel antagonist cadmium (Cd^{2+}), which blocks neurotransmitter release at the

squid giant synapse (Llinas *et al.*, 1981) and is a potent antagonist of L- and N-type calcium channels (Fox *et al.*, 1987), blocked synaptic transmission in rat parasympathetic ganglia in a dose-dependent manner (Figure 1a, K_d 74 μM, $P < 0.01$). The peak amplitude of control e.p.s.ps was significantly reduced (>50%) by stimulation at 10 Hz compared to 0.1 Hz, this attenuation of e.p.s.p. amplitudes with increasing stimulus frequency was more accentuated when more than five stimulation protocols were performed on any one preparation (see methods). Even so, the block by 300 μM Cd^{2+} was alleviated with increasing stimulus frequency, in that stimulation at 10 Hz did not significantly reduce the amplitude of the evoked response compared to the control (Figure 1b, means ± s.d.; average of 10 e.p.s.ps from the same cell). Bath application of nickel (1–300 μM), a T-type calcium channel antagonist (Fox *et al.*, 1987), did not depress the peak amplitude of neurally evoked e.p.s.ps in parasympathetic neurones (Figure 1c), although the rate of e.p.s.p. decay in 300 μM Ni^{2+} was prolonged.

Organic calcium channel antagonists

Verapamil, a phenylamine calcium channel antagonist which attenuates the high threshold calcium currents (L-type) in cardiac muscle (Lee & Tsien, 1983), had little effect on synaptic transmission in the present study, even at 30 μM and 10 Hz (Figure 2a,b). At 0.1 Hz the effect of 30 μM verapamil on the peak e.p.s.p. amplitude was insignificant ($n = 3$). Higher concentrations of verapamil (100 μM) were observed to block axonal conduction in this preparation, as well as at the rat hemidiaphragm neuromuscular junction. This was apparent from the intermittent failure of normal sized e.p.s.ps, whose high probability of failure in verapamil deviated from the low probability expected according to the binomial theorem for large (≥10) multiquantal discharges (Boyd & Martin, 1956). Unlike the rat diaphragm NMJ (personal observations), and the mouse and frog NMJ (Publicover & Duncan, 1979; Chang *et al.*, 1988), no significant increase in miniature e.p.s.p. (m.e.p.s.p.) frequency was detected after treatment with verapamil.

In contrast to verapamil, the benzothiazepine diltiazem was a relatively effective antagonist of synaptic transmission. Diltiazem depressed e.p.s.p. amplitudes in both a dose-dependent and frequency-dependent manner (Figure 2c,d), without any change in the resting membrane potential. Recovery from treatment with 30 μM diltiazem was observed after prolonged washing, >20 min (Figure 2d). Increasing the stimulus frequency increased the degree of block of evoked release by diltiazem. Almost complete block (>80%) was observed at 30 μM diltiazem with

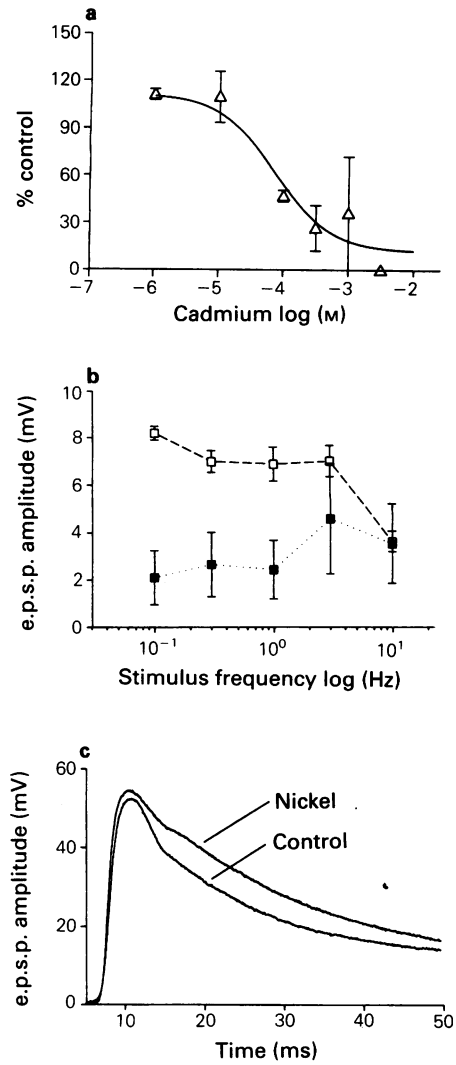

Figure 1 Effects of Cd^{2+} and Ni^{2+} on neurally evoked e.p.s.ps. (a) The attenuation of e.p.s.p. amplitudes by Cd^{2+} was dose-dependent (mean control amplitude 17.4 mV), the relationship was fitted according to a one-site adsorption isotherm (smooth curve) with a K_d of 74 μM ($P < 0.01$, $n = 3$ cells). No change in the resting membrane potential was evident after treatment with cadmium. (b) The depression of e.p.s.p. amplitudes by 300 μM Cd^{2+} was stimulus-dependent, in that by increasing the stimulus frequency to 10 Hz no significant block by cadmium was observed (means ± s.d., 10 consecutive e.p.s.ps from the same cell); (□) = control, (■) = 300 μM Cd^{2+}. (c) Ni^{2+} (300 μM) did not depress the amplitude of neurally evoked e.p.s.ps ($n = 3$), although the rate of decay of the potential was slower than that of the control. Waveforms represent the average of ten consecutive responses at 0.1 Hz, membrane potential −74 mV.

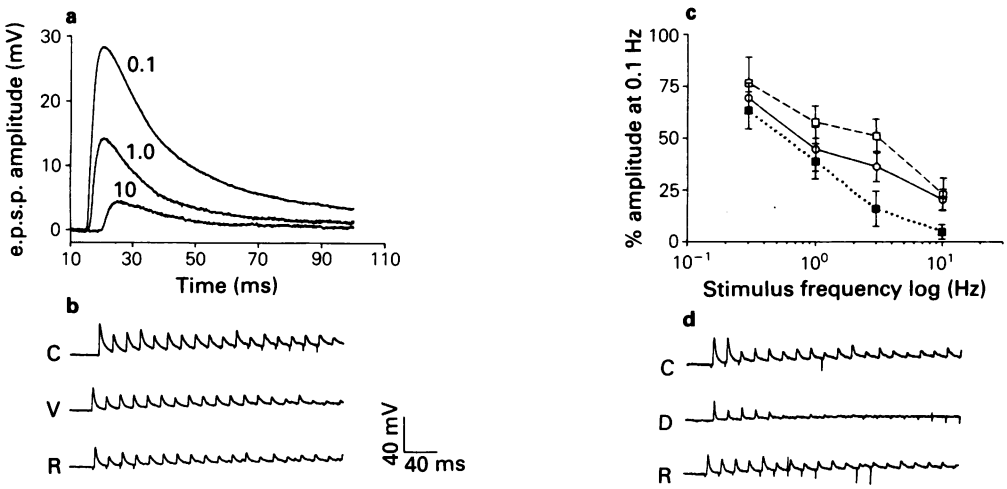

Figure 2 Effects of verapamil and diltiazem on the amplitude of neurally evoked e.p.s.ps. (a) Application of 30 μM verapamil did not block neurally evoked e.p.s.ps at either 0.1, 1.0 or 10 Hz (average 10 consecutive waveforms), resting membrane potential −75 mV. An increase in e.p.s.p. delay was noted in 30 μM verapamil, a phenomenon which may relate to verapamil's non-specific effects on voltage-sensitive sodium channels (see Table 2). (b) At the highest stimulus frequency examined, 10 Hz, no significant reduction in e.p.s.p. amplitude was observed in verapamil (V) compared to the control (C). The slight depression with prolonged stimulation (>15th response) reflects the decrease in quantal efficacy with time (R = recovery). (c) Diltiazem (30 μM) depressed e.p.s.p. amplitudes in a stimulus-dependent manner (■); no significant block by diltiazem was noted at a stimulus frequency of 0.1 Hz. Recovery of e.p.s.p. amplitudes following treatment with diltiazem (○) approached that of control levels (□). Responses were normalised with respect to the amplitude of e.p.s.ps elicited at 0.1 Hz in each test solution. (d) The block by diltiazem at 10 Hz was more pronounced after the first few responses (C = control, D = 30 μM diltiazem, R = recovery), this is consistent with the use-dependence of block seen when different stimulus intensities are compared in (c).

stimulus frequency of 10 Hz. The (−)-*cis* isomer of diltiazem, which blocks a cyclic GMP-activated conductance in salamander retina (Stern *et al.*, 1986), was ineffective in depressing the amplitude of neurally evoked e.p.s.ps in parasympathetic neurones.

Dihydropyridine calcium channel antagonists are considered specific for L-type calcium currents (Fox *et al.*, 1987), but the voltage- and time-dependent antagonist actions of such drugs have been implicated in their inability to inhibit electrically evoked release of substance P from dorsal root ganglia (Holz *et al.*, 1988). Nevertheless, at high stimulus frequencies and with a long lasting dihydropyridine, one may expect to observe antagonist effects of these drugs on synaptic transmission if an L-type calcium current were involved in neurotransmitter release processes. The 1,4-dihydropyridine amlodipine maleate, which is a relatively light-stable and water-soluble derivative of nifedipine, did not depress e.p.s.p. amplitudes at concentrations of up to 30 μM even with prolonged application (Figure 3).

Amiloride, a potassium sparing diuretic which has recently been shown to be an antagonist of T-type calcium channels in DRG neurones and neuroblastoma cells (Tang *et al.*, 1988), blocked synaptic transmission between rat parasympathetic ganglia (one-site K_d 31 μM, $P < 0.005$, $R^2 = 0.974$). However, the dose-response relation between 10 and 100 μM amiloride was not simply due to an inflexion in the dose-response relation (Figure 4a), suggesting that more than one effect of amiloride may contribute to the depression of e.p.s.ps at high concentrations (2-site K_ds of 10 and 560 μM respectively with $R^2 = 0.979$). The block of e.p.s.ps by amiloride (30 μM) was rapid (<1 min) and readily reversible, without any significant depression or prolongation of e.p.s.p. rise or decay times (Figure 4b,c). The depression of the e.p.s.p. amplitudes by 30 μM amiloride was partly relieved by raising the stimulus frequency to 10 Hz (Figure 4d), in that only a 40% depression was observed at 10 Hz compared to the 47% depression at 0.1 Hz. To ensure that the site of action of amiloride was not a consequence of the postsynaptic block of synaptic transmission, the effect of this diuretic upon spontaneous transmitter release and upon ionophoretic-ACh responses were examined. The amplitude and frequency of m.e.p.s.ps (Figure 5a) were unaffected by treatment with 30 μM amiloride

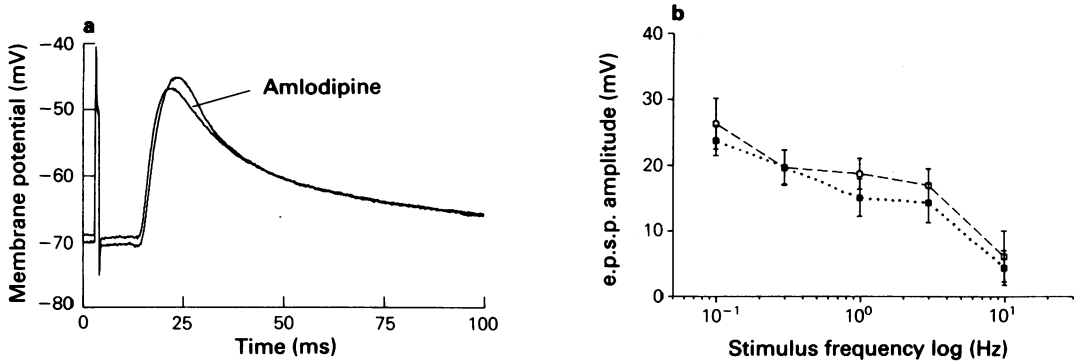

Figure 3 Inability of amlodipine, a 1,4-dihydropyridine, in blocking neurally evoked e.p.s.ps. (a) Averaged e.p.s.ps at 0.1 Hz recorded before and 10 min after application of 30 μM amlodipine. (b) The reduction in e.p.s.p. amplitudes with increasing stimulus frequency was not significantly affected by 30 μM amlodipine (■) compared to the control (□).

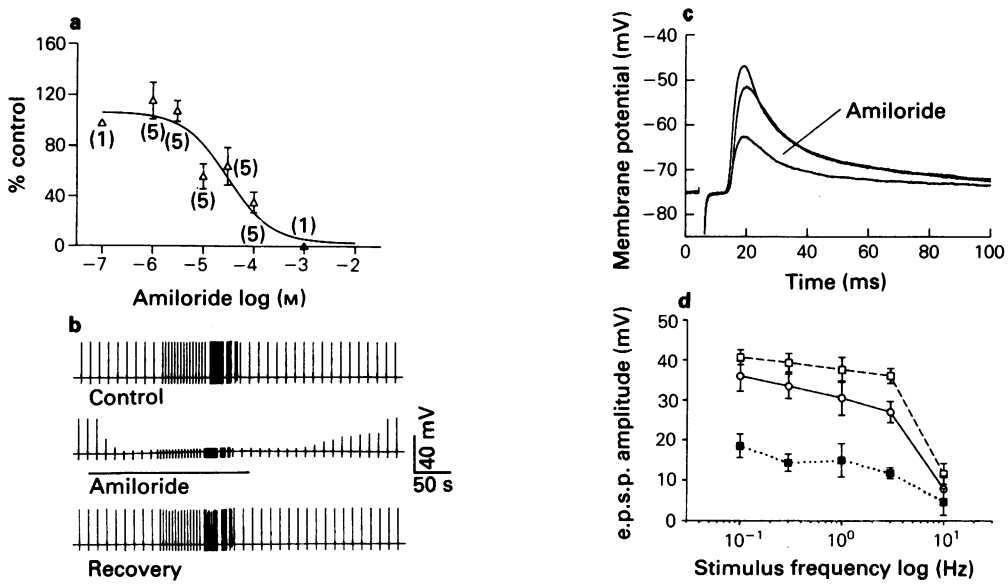

Figure 4 (a) Amiloride reduced e.p.s.p. amplitudes in a dose-dependent manner with complete block at 1 mM, smooth curve represents fit of the data to a single-site adsorption isotherm with a K_d of 31 μM ($P < 0.001$, number of cells in parentheses). (b) Stimulus protocols of 15 stimuli conducted at 0.1–10 Hz in control saline, 1 mM amiloride and after washing. The block by amiloride was rapid and readily reversible. Vertical deflections represent e.p.s.ps superimposed upon stimulus artifacts (which also have a negative component to the transient). In amiloride the responses were completely blocked and the residual transient reflects just the stimulus artifact. In this cell, e.p.s.ps elicited postsynaptic action potentials, hence the large amplitude of the evoked responses. After washing, e.p.s.ps occasionally failed to elicit an action potential at 1.0 Hz due to incomplete washout of amiloride and the normal stimulus-dependent depression of e.p.s.p. amplitudes. (c) The depression of e.p.s.p. amplitudes by amiloride (30 μM) did not involve a distortion of the e.p.s.p. time course, or a change in the resting membrane potential (−76 mV). (d) Increasing the stimulus frequency alleviated the reduction in e.p.s.p. amplitudes by amiloride compared to the amplitude of control e.p.s.ps evoked at an identical stimulus frequency: control = (□); treated = (■); recovery = (○).

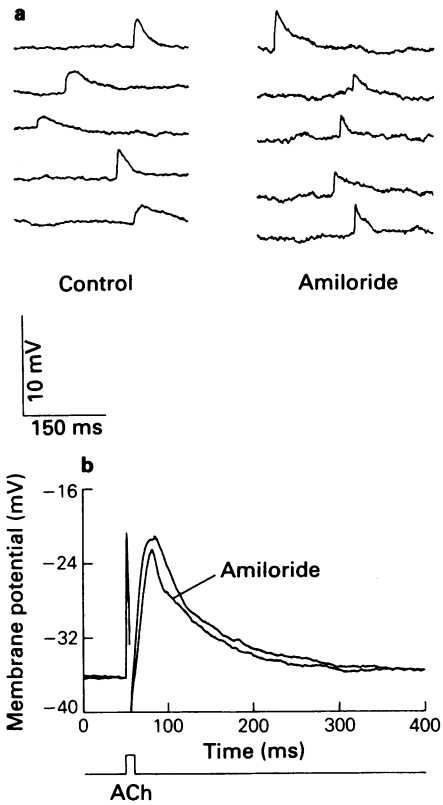

Figure 5 Depression of e.p.s.p. amplitudes by amiloride did not involve a postsynaptic antagonism of synaptic transmission. (a) Treatment of neurones with amiloride (30 μM) did not affect the frequency or amplitude of m.e.s.ps (0.053 Hz, 2.6 $\pm$ 0.7 mV) compared to the control (0.045 Hz, 2.9 $\pm$ 0.8 mV). (b) The amplitude of depolarizations induced by ionophoretic pulses of acetylcholine (ACh, indicated by lower trace) were not significantly reduced by 30 μM amiloride. Waveforms represent the average of 5 consecutive depolarizations in response to ACh pulses (0.033 Hz) in the presence and absence of amiloride, recorded from the same cell in the presence of 3 μM neostigmine and 1 μM tetrodotoxin.

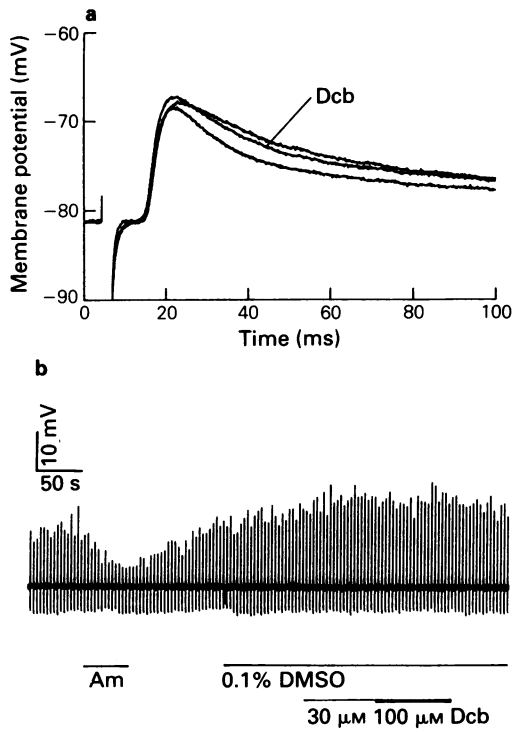

Figure 6 The amiloride derivative dichlorobenzamil (Dcb) did not significantly depress the peak amplitude or alter the waveform of e.p.s.ps. (a) Averaged e.p.s.p. recorded from the same cell before, during and after treatment with 100 μM Dcb. (b) In a cell sensitive to the application of 30 μM amiloride (Am), 30–100 μM Dcb applied in 0.1% DMSO failed to depress the amplitude of neurally evoked responses.

(2.6 $\pm$ 0.7 mV, 0.053 Hz compared to the control 2.9 $\pm$ 0.8 mV, 0.045 Hz). Similarly, membrane potential transients induced by the ionophoretic application of ACh to the soma (Figure 5b) were not significantly affected by 30 μM amiloride (for example control 21.1 $\pm$ 4.8 mV, treated 18.9 $\pm$ 10.2 mV; means $\pm$ s.d. of 5 pulses from the same cell). The amiloride derivative 3',4'-dichlorobenzamil (30–100 μM), which like amiloride blocks Na^+-H^+ exchange, did not suppress e.p.s.p. amplitudes (Figure 6).

The marine neurotoxin, ω-conotoxin GVIA (0.1–1 μM), which has been shown to block neurotransmitter release at the frog neuromuscular junction (Kerr & Yoshikama, 1984) but not at the mouse neuromuscular junction (Anderson & Harvey, 1987), did not block synaptic transmission in rat parasympathetic ganglia ($n = 4$, Figure 7a). Furthermore, increasing the stimulus frequency (0.1–10 Hz) did not affect this result.

To examine the effect of this toxin on the calcium currents in the neuronal soma, whole cell calcium currents were recorded from cultured parasympathetic neurones of the newborn rat heart. Voltage-sensitive calcium currents were recorded in the presence of TTX, as well as internal Cs and external TEA to block voltage-sensitive sodium, and potassium conductances respectively. Inward current amplitude was dependent on the external $[Ca^{2+}]$ and in the following experiments calcium currents were elicited in the presence of elevated, 10 mM,

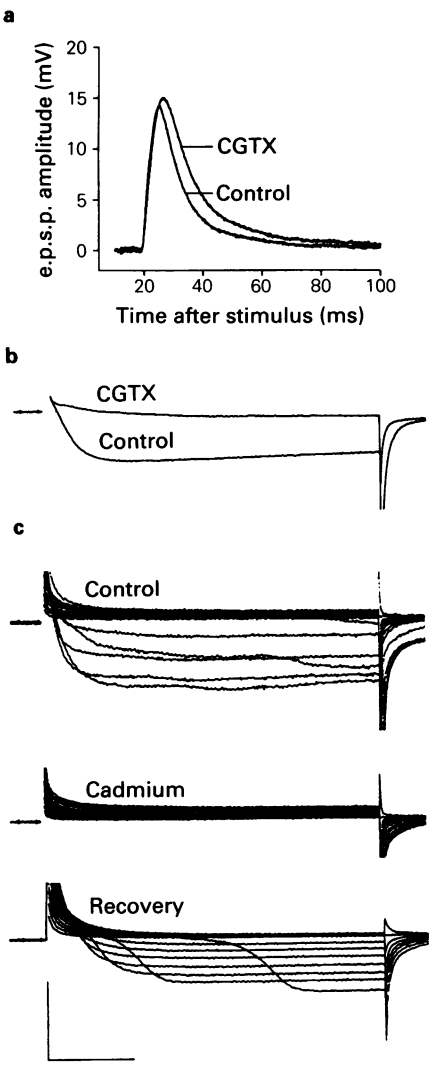

Figure 7 (a) Application of 1 μM ω-conotoxin GVIA (CGTX) did not block neurally evoked e.p.s.ps in parasympathetic ganglia ($n = 4$). Average of ten consecutive e.p.s.ps evoked at 0.1 Hz, membrane potential -72 mV. (b) and (c) Whole cell calcium currents elicited with depolarizing steps to $+10$ mV were strongly attenuated after application of 10 nM ω-CGTX, indicative of the high potency of this conotoxin fraction toward somal calcium currents (scale bar 2 nA, 10 ms). The block by this peptide was irreversible but was distinguishable from calcium current rundown, because over a similar time course the block of somal calcium currents by 100 μM Cd^{2+}•(recorded from a different neurone) was readily alleviated upon washing (scale bar 2 nA, 10 ms), compared to the control (scale bar 1.32 nA, 10 ms). A voltage-dependent increase in the latency of onset of the somal calcium current was observed in washout following treatment with cadmium.

external calcium. The whole-cell calcium currents recorded from cardiac neurones were predominantly high-threshold and exhibited only 20% inactivation during a 500 ms depolarising pulse to $+20$ mV. Application of prior conditioning hyperpolarizations (1–60 s) to -100 mV failed to reveal any transient calcium current. A marked reduction of the whole cell calcium currents (up to 90%) by 10 nM ω-CGTX GVIA was observed (Figure 7b). The irreversibility of calcium current blockade by ω-CGTX was distinct from calcium current 'run-down', evident in many whole-cell patch clamp experiments (Yaari *et al.*, 1987), because the inhibition of soma calcium currents with 100 μM Cd^{2+}, obtained in a different neurone, was readily reversible over a time course similar to the duration of the ω-CGTX experiments (Figure 7c). Calcium currents recorded from the soma of parasympathetic cardiac neurones were also inhibited by the dihydropyridine, amlodipine (10 μM) but not by 30 μM amiloride (data not shown).

Discussion

This study presents pharmacological evidence that the calcium channels involved in synaptic transmission between parasympathetic ganglia are distinct from the calcium channels characterized in dorsal root ganglia and N1E-115 neurones (Table 1). Apart from the insensitivity of neurally evoked transmitter release in parasympathetic ganglia to Ni^{2+}, it appears that the calcium current involved has pharmacological characteristics in common with the T-type currents, although it cannot be unambiguously classified with present criteria (Fox *et al.*, 1987).

Following vagal stimulation, the cardiac interval of spontaneously beating bullfrog heart is prolonged, an effect which is accompanied by an increase in the release of ACh. Application of 5 μM Cd^{2+} attenuates these effects of vagal stimulation leading to the suggestion that Cd^{2+} suppresses the release of neurotransmitter from parasympathetic ganglia (Hayashi & Takayama, 1978). However, Cd^{2+} was much less potent in reducing the amplitude of neurally evoked e.p.s.ps in rat parasympathetic ganglia (K_d 74 μM; $P < 0.01$), than in reducing the somatic L-type currents in cardiac parasympathetic neurones (Figure 7c), neuroblastoma (Narahashi *et al.*, 1987), and in sympathetic neurones (Fox *et al.*, 1987). Increasing the stimulus frequency (from 0.1 to 10 Hz) suppressed the degree of antagonism of e.p.s.p. amplitudes by Cd^{2+}. This finding is consistent with the occluded calcium channels being cleared of this blocker upon repetitive depolarizations. However, one cannot exclude the possibility that additional calcium channels (or types) are being recruited at

Table 1 Potency ranking of calcium channel block

Calcium channel antagonist	*Calcium channel type** L	N	T	*Synaptic transmission in rat parasympathetic ganglia (this study)*
Cadmium (inorganic)	+++	++	+	++(1/S)
Nickel (inorganic)	—	—	++	—
Verapamil (phenylamine)	+++(S)	—	+(S)	—
Diltiazem (benzothiazepine)	+++(S)	?	?	++(S)
(−)-*cis*-Diltiazem (benzothiazepine)	—	—	—	—
Amlodipine (dihydropyridine)	+++	—	—	—
ω-CGTX GVIA (peptide)	+++	+++	—	—
Amiloride	+	?	+++	+++(1/S)

*Criteria used in the characterization of calcium channel types in this table were based upon observations made from the dorsal root ganglia of the frog, rat and chick, as well as from cultured neuroblastoma cell lines (McCleskey *et al.*, 1986; Fox *et al.*, 1987; McCleskey *et al.*, 1987; Narahashi *et al.*, 1987; Hirning *et al.*, 1988; Tang *et al.*, 1988).

Key

—	Negligible	>1 mM, or ineffective at concentrations which block other calcium currents.
+	Low	(IC_{50} 100 μM to 1 mM)
++	Average	(IC_{50} 10 μM to 100 μM)
+++	High	(IC_{50} < 10 μM)

(S) denotes increase in block with increasing stimulus frequency.
(1/S) alleviation of block with increasing stimulus frequency.

high stimulus frequencies, or that the reduced calcium influx in Cd^{2+} may unmask an underlying calcium-dependent mechanism involved in facilitation.

Additional evidence against the involvement of L-type channels in synaptic transmission in rat submandibular ganglia, was the lack of effect of verapamil, even at 30 μM and a stimulus frequency of 10 Hz. Although the binding of calcium channel antagonists like verapamil are sensitive to millimolar concentrations of Ca^{2+} (Kostyuk *et al.*, 1975; Triggle & Swamy, 1983; Triggle & Janis, 1987), the calcium concentration used in this study (7.5 mM) is considered insufficient to account for the failure of verapamil to suppress the e.p.s.p. amplitude. The depression of synaptic transmission by 30 μM diltiazem at high frequencies (10 Hz), would appear indicative of a role for L-type calcium channels in synaptic transmission, due to diltiazem's potent antagonism of slowly-inactivating high-threshold (presumably L-type) calcium currents (Uehara & Hume, 1985; Penner & Dreyer, 1986). However, diltiazem's potency was low compared to these other studies, and at 30 μM the specificity of diltiazem for L-type calcium currents remains questionable (Tables 1 and 2). The absence of a role for L-type calcium currents is further supported by the inability of 1,4-dihydropyridine amlodipine maleate, to block synaptic transmission.

The kinetics of L-type calcium channels would favour the predominance of this conductance at high stimulus intensities, and thus this may support the differential release of neurotransmitters discharged from the same nerve terminal (Hirning *et al.*, 1988). However, as benzothiazepines and phenylamine calcium channel antagonists are markedly voltage-dependent in their efficacy (Uehara & Hume, 1985), facilitation of block by these antagonists at high stimulus frequencies cannot be used alone as an argument for the role of more than one calcium channel type in neurotransmitter release.

The block of neurally-evoked transmitter release in rat parasympathetic ganglia by amiloride may relate to the recent finding that this K^+-sparing diuretic acts as a potent and reversible blocker of low threshold (T-type) calcium channels in neuroblastoma and chick DRG neurones (Tang *et al.*, 1988). However, it is uncertain as to whether or not the depression of synaptic transmission is a consequence of either the block of a population of calcium channels, or an increase in intraterminal pH due to a suppression of Na^+-H^+ exchange. Several lines of evidence indicate that the effect of amiloride may be specific for calcium currents. Varying intraterminal pH (7.8 to 6.7) does not block transmitter release at the squid giant synapse (Adams & Thomas, 1989). In rat brain synaptosomes acidification from pH 7.2 to 5.8 increases the efflux of dopamine in a $[Ca^{2+}]_o$-independent manner (Drapeau & Nashchen, 1988). In addition, the potent Na^+-H^+ exchange inhibitor dichlorobenzamil did not suppress e.p.s.p. amplitudes. The findings that

Table 2 Non-specific effects of various calcium channel antagonists

Calcium antagonist	*'Non-specific' effects (IC_{50}s/K_ds)*	*Tissue*	*Reference*
Cadmium	(1) fast Na current antagonist (180 μM)	Calf purkinje fibres	DiFrancesco *et al.* (1985)
	(2) H^+ activated Na-current (0.5 μM)	Chick DRG neurones	Konnerth *et al.* (1987)
Nickel	(1) nAChR antagonist (10 mM)	Frog NMJ	Magleby & Weinstock (1980)
Verapamil	(1) α-adrenergic ligand (< 1 μM)	Platelets/heart	Motulsky *et al.* (1983)
	(2) mAChR antagonist (> 10 μM)	Rabbit Purkinje fibres	Mubagwa & Carmeliet (1987)
	(3) Na + H-ATPase inhibitor (> 15 μM)	Hog gastric membranes	Blakeman *et al.* (1984)
	(4) nAChR (> 20 μM)	Frog NMJ	Edeson *et al.* (1985, 1988)
	antagonist (> 20 μM)	Mouse NMJ/BC3H1	Wachtel (1987)
	(5) delayed K-current antagonist (≥ 50 μM)	*Helix* neurones	Nishi *et al.* (1983)
	(6) Na channel antagonist (≥ 100 μM)	Frog/crayfish NMJ	Van der Kloot & Kita (1975)
		Mouse NMJ	Chang *et al.* (1988)
		Rat brain/frog NMJ	Nashchen & Blaustein (1979)
	(7) Na-dependent transmitter uptake	PC12 cells	McGee & Schneider (1979)
Diltiazem	(1) nAChR antagonist (≥ 20 μM)	Mouse NMJ/BC3H1 cells	Wachtel (1987)
	(2) K channel antagonist (≥ 30 μM)	Frog atria	Nishi *et al.* (1983)
	(3) Na channel antagonist (≥ 100 μM)	Mouse NMJ	Chang *et al.* (1988)
	(4) H^+ activated Na-current (1 mM)	Chick DRG neurones	Konnerth *et al.* (1987)
(−)-*cis*-Diltiazem	Cyclic GMP conductance antagonist (10 μM)	Salamander retina	Stern *et al.* (1986)
ω-Conotoxin GVIA	(≤ 10 μM)	None-identified	
Amiloride	(1) inhibits Na-fluxes (1 μM)	Epithelia	Dale (1982)
	(2) Na-H exchange inhibitor (1 μM)	Epithelia/frog skin	Dale (1982)
	(3) mAChR antagonist (≥ 40 μM)	Rabbit pancreas	Kuijpers *et al.* (1984)
	(4) Na-Ca exchange inhibitor (≥ 1 mM)	Rat brain	Dale (1982) Drapeau & Nashchen (1988)

NMJ = neuromuscular junction; mAChR = muscarinic acetylcholine receptor; nAChR = nicotinic acetylcholine receptor.

m.e.s.p. amplitudes were unchanged by 30 μM amiloride, and that the amplitude of potentials induced by the iontophoretic application of ACh were unaffected, suggest that the depression of e.p.s.p. amplitudes by amiloride is unlikely to be a postsynaptic effect.

To test the hypothesis that T-type calcium channels may be involved in synaptic transmission the activity of ω-CGTX GVIA, a specific blocker of N- and L-type currents in dorsal root ganglion neurones (McCleskey *et al.*, 1987), was examined. In parasympathetic ganglia, 1 μM ω-CGTX was ineffective in blocking synaptic transmission. It has been demonstrated that the block of calcium currents by a synthetic omega-conotoxin is alleviated by raising the extracellular calcium concentration (Oyama *et al.*, 1987), but even with 10 mM $[Ca^{2+}]_o$, significant block of the calcium conductance still may be obtained with 1 μM ω-CGTX, as seen with 10 nM ω-CGTX in cardiac parasympathetic neurones

(Figure 7). However, in certain tissues such as frog and guinea-pig ventricular muscle, and in rabbit ear artery smooth muscle, high threshold calcium currents (presumably L-type) are reported to be insensitive to this toxin (see McCleskey *et al.*, 1987), and a verapamil-sensitive calcium current in the mouse neuromuscular junction is also unaffected by ω-CGTX (Anderson & Harvey, 1987). Neurotransmitter release at the squid giant synapse, which is sensitive to the inorganic blocker Cd^{2+} (K_d 70 μM), is also insensitive to ω-CGTX (M.P. Charlton personal communication; Llinas *et al.*, 1989). These data are indicative of an additional, but as yet unidentified, calcium conductance involved in neurotransmitter release processes.

Nickel blocks T-type calcium currents approximately ten times more potently than other calcium currents (Fox *et al.*, 1987; Narahashi *et al.*, 1987). Thus, one would predict that if T-type channels were responsible for eliciting e.p.s.ps, a potent antagonism of the e.p.s.p. by low doses of nickel ($< 100\,\mu$M) would be evident. However, no such effect was observed, which may suggest the absence of T-type calcium currents in neurotransmitter release processes. But, this observation contrasts with the block observed with amiloride, and the actions of other calcium channel antagonists used in this study (Table 1). It has also been noted at the lizard neuromuscular junction that 100 μM Ni^{2+} is ineffective in blocking the normal presynaptic calcium currents (Lindgren *et al.*, 1988). This discrepancy may relate to the pharmacological sensitivities of the underlying calcium current involved in transmitter release, or alternatively to differing accessibilities/binding characteristics of this cation relative to Cd^{2+}.

In conclusion, the pharmacological characteristics of synaptic transmission in rat submandibular ganglia are inconsistent with the involvement of only one of the three identified neuronal calcium channel types. The calcium current(s) underlying neurally evoked transmitter release in parasympathetic ganglia share some characteristics with the T-type channels described in chick DRG neurones and neuroblastoma cell lines. However, the possibility of a different calcium current type in parasympathetic nerve terminals exists, as the current cannot be unambiguously defined within the existing pharmacological definitions of calcium channel types.

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Alterations in atrial reactivity in a strain of spontaneously diabetic rats

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1 The present study examined the reactivity of atria from control and spontaneously diabetic rats to various adrenoceptor agonists and to adenosine.

2 Isoprenaline (1.5 nM–1500 nM) produced concentration-dependent increases in inotropy which were unchanged in diabetic atria. However, the sensitivity to isoprenaline-induced changes in chronotropy was reduced in diabetic preparations.

3 In the presence of propranolol (2 μM), phenylephrine (0.2 μM–100 μM) produced concentration-dependent increases in both inotropy and chronotropy; however, atria from diabetic rats exhibited a much greater maximal response. The diabetic state did not alter the sensitivity to phenylephrine.

4 Adenosine (0.15 μM–300 μM) produced concentration-dependent decreases in both inotropy and chronotropy which were unchanged in diabetic atria.

5 Radioligand binding studies revealed that both α_1- and β-adrenoceptor populations were substantially reduced in atria from diabetic rats. However, there was no change in receptor affinity for either adrenoceptor.

6 These results show that diabetes leads to an alteration in atrial reactivity to adrenoceptor stimulation. Future studies examining steps following hormone-receptor coupling are required in order to characterize this defect.

Introduction

Diabetes mellitus is frequently associated with cardiac dysfunction. Clinical and pathological studies along with the epidemiological data from the Framingham study suggest that diabetes mellitus is associated with a specific cardiomyopathy (Kannel *et al.*, 1974; Regan *et al.*, 1977). Animal studies support this concept. Reductions in mechanical performance as well as alterations in myocardial reactivity have been observed in diabetic rats (Fein *et al.*, 1980; Vadlamudi *et al.*, 1982). In particular, animal studies indicate that the responsiveness of the diabetic myocardium to adrenoceptor agonists is changed. However, the degree and nature of this change is still controversial. Foy & Lucas (1976; 1978) observed a decrease in myocardial sensitivity to β-adrenoceptor stimulation under both *in vivo* and *in vitro* conditions. Other studies, however, have failed to detect any difference in either sensitivity or maximum response to β-adrenoceptor stimulation (Ingebretsen *et al.*, 1980; Vadlamudi & McNeill, 1984). Studies examining α-adrenoceptor stimulation are also conflicting. Heyliger *et al.* (1982) demonstrated that methoxamine-stimulated increases in contractile force were significantly attenuated in diabetic preparations. In direct contrast, more recent investigations have revealed an enhanced inotropic and chronotropic response to α-adrenoceptor stimulation in hearts from diabetic animals (Jackson *et al.*, 1986; Canga & Sterin-Borda, 1986). All of the above animal studies have relied on chemical substances as diabetogenic agents. These substances purportedly selectively destroy beta cells of the pancreas and eventually lead to an insulin-deficient state. Undoubtedly differences in experimental protocol, such as the diabetogenic agent used (alloxan or streptozotocin) or the doses of the agent used, contributed to some of the observed discrepancies.

In an attempt to clarify work in this area we examined the reactivity of atria from a strain of spontaneously diabetic rats to both β- and α-adrenoceptor stimulation. In addition, atrial reactivity to adenosine, an important cardiac regulator, was studied.

Methods

Animal model

Experiments were conducted on a strain of spontaneously diabetic Bio-Breeding (BB) rats. The BB rats

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were obtained from a colony maintained at the C.H. Best Institute, University of Toronto. They were initially derived by crossing Long Evans rats ($RT1^u$ haplotype) with seventh generation BB rats from the University of Massachusetts, Worcester. Subsequently, the colony was maintained by random matings between a diabetic male and a non-diabetic female. The overall incidence of overt diabetes is consistently over 80%. As these rats are strictly insulin-dependent they received daily subcutaneous injections of protamine zinc insulin (1.0 u per 100 g body weight) until the day before their sacrifice. At the time the rats were killed (approximately 18 h after the last insulin injection) they were weighed and venous blood collected. Plasma glucose concentration was measured by the glucose-oxidase method (Sigma). Animals used in this study were diabetic for approximately 10 weeks. Age-matched littermates which did not develop diabetes served as controls.

Isolated atrial preparations

Animals were anaesthetized with ether and injected with heparin (1.5 mg kg^{-1}, i.v.). The heart was quickly excised and perfused via the aorta for 15 min at 37°C with freshly oxygenated (95% O_2: 5% CO_2) modified Krebs-Henseleit bicarbonate buffer solution of the following composition (mM): NaCl 117.56, $NaHCO_3$ 25.00, KCl 5.36, NaH_2PO_4 1.17, $CaCl_2$ 1.25, $MgSO_4$ 1.16, dextrose 11.10, glycerol 9.90 and sodium lactate 5.30 (pH 7.4). After visible blood had been removed from the tissue, left and right atria were excised and suspended vertically in a chamber containing 25 ml of the above solution. Atria were attached on one end to a stimulating electrode and the other end was attached by silk thread to a Grass FTO3C force transducer. Right atria were allowed to beat spontaneously while left atria were electrically stimulated (Grass Stimulator SD9) at a frequency of 1 Hz with rectangular wave pulses of 4 ms duration at a voltage approximately 20% above the threshold. The tissues were allowed to equilibrate for 60 min under a resting tension of 1 g. Based on preliminary resting tension/developed tension curves, this level of resting tension produced approximately 90% maximal force development in atria from both control and diabetic animals. The tension developed was recorded on a Grass Model 7 oscillograph. The force signal arising from the right atria was used to trigger a cardiotachometer (Grass 7P4) from which contraction rate could be recorded.

After a 60 min equilibration period, concentration-response curves were obtained for isoprenaline, phenylephrine, and adenosine. Inotropic (left atria) and chronotropic (right atria) responses to increasing agonist concentrations were obtained by a cumulative increase in the total concentration of the drug in the organ chamber. Each atria was allowed to remain in contact with the drug for 3 min before each successive concentration of the agonist was added. In order to prevent any β-adrenoceptor mediated effects, the concentration-response curves for phenylephrine were obtained in the presence of a β-adrenoceptor antagonist (Benfey, 1982). Propranolol (2 μM) was added to the bathing solution and the atria were allowed to equilibrate for 30 min before phenylephrine was added. When the maximum inotropic or chronotropic effect to an agonist was attained, the atria were washed twice and allowed to equilibrate for 30 min before another concentration-response curve was obtained. No more than three concentration-response curves were performed in each atria in order to maintain maximum tissue responsiveness.

At the end of each experiment the tissues were blotted dry, weighed, frozen with liquid nitrogen and stored at −70°C.

Radioligand binding assays

Binding studies were performed on crude atrial membrane fractions. Frozen atrial tissue from 5 animals was thawed and added to 10 ml of ice-cold 50 mM Tris-HCl buffer containing 250 mM sucrose and the following protease inhibitors: 5 mM EDTA, 0.1 mM benzamidine, 1 mM iodoacetamide, 1 μM pepstatin A, and 1 mM phenylmethylsulphonyl fluoride (pH 7.4). The homogenate was initially prepared by ten passes of a motor-driven glass-Teflon homogenizer. Further homogenization was effected with a Brinkman Polytron by three 5 s bursts at half-maximal setting. The resulting homogenate was passed through 4 layers of surgical gauze under vacuum and centrifuged at 105,000 *g* for 90 min at 4°C using an International B-60 Ultracentrifuge. The resulting membrane pellet was resuspended and washed twice in 8 ml of buffer containing 50 mM Tris-HCl, 1 mM EDTA, 5 mM KCl, 1.5 mM $CaCl_2$, 4 mM $MgCl_2$ and 120 mM NaCl (pH 7.4). Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard while sialic acid content of atrial membranes was determined by the enzymatic method of Araki & Yamada (1983).

Radioligand binding experiments were performed as outlined by Williams & Lefkowitz, 1976. Membrane fractions (200 μl, 100–150 μg protein) were incubated with varying concentrations of [^{3}H]-dihydroalprenolol (0.5–20 nM) or [^{3}H]-prazosin (0.02–5 nM) in a final volume of 600 μl. All assays were performed in duplicate. Binding was initiated by placing the tubes in a shaking water bath at 32°C for 30 min. These conditions permitted the complete equilibration of the radioligand with its receptor.

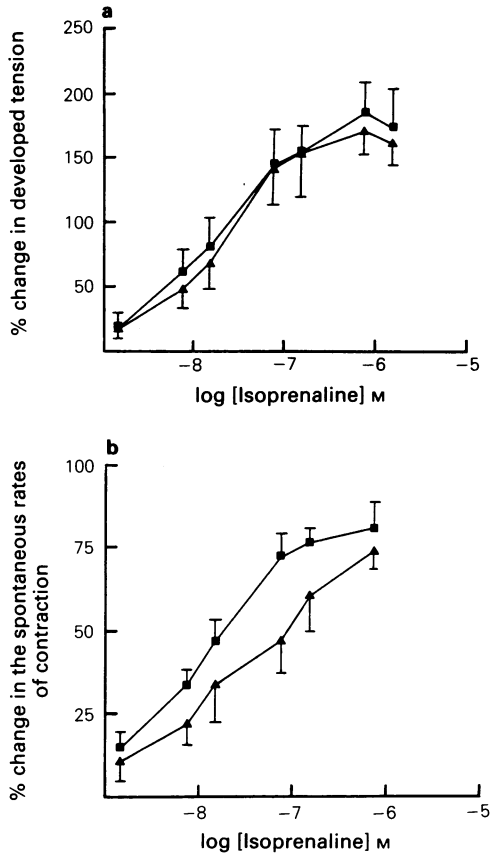

Figure 1 Cumulative concentration-response curves showing the inotropic (a) and chronotropic (b) responses to isoprenaline in atria from control (■) and diabetic (▲) animals. Means (n = 5–8) are shown with vertical bars indicating s.e.mean.

The reaction was terminated by the rapid filtration through a Titertek Cell harvester (Skatron Inc.) using glass fibre 'receptor binding' filter mats (Skatron). Filters were washed with 5 ml of 50 mM Tris-HCl buffer (pH 7.4), placed in plastic mini-scintillation vials along with 4 ml of scintillant and monitored for tritium 16 h later in a Packard 4660 liquid scintillation spectrometer at approximately 50% efficiency. Specific binding was defined as that radioligand displaced by either 10 μM (±)-propranolol or 10 μM phentolamine for [^{3}H]-dihydroalprenolol or [^{3}H]-prazosin, respectively. Radioligand binding from saturation isotherms was analyzed by the non-linear least squares, curve fitting programme LIGAND (Munson & Rodbard, 1980).

Drugs

The following drugs were used in the present study: (−)-phenylephrine hydrochloride (Sigma), (±)-isoprenaline (Sigma), adenosine (Sigma), (±)-propranolol hydrochloride (Sigma), phentolamine hydrochloride (Ciba-Geigy), and protamine zinc insulin (Eli Lilly and Company). [^{3}H]-dihydroalprenolol (95 Ci mmol^{-1}) and [^{3}H]-prazosin (82 Ci mmol^{-1}) were obtained from New England Nuclear. All other chemicals were of analytical grade. Drugs were prepared in Krebs-Henseleit buffer containing ascorbic acid (0.1 μM). All concentrations are expressed as final molar concentrations of the base in the organ bath.

Statistical analysis

The inotropic and chronotropic responses were expressed as % change from baseline. The EC_{50} values were calculated by computer fitting concentration-response curves to a logistic equation:

$$E = 100 - M \times A^p/(A^p + K^p)$$

Where E is normalized response, M is the maximum response to each drug, A is drug concentration, K is the EC_{50} value of each drug and p is the slope parameter (Parker & Waud, 1971). The EC_{50} values are presented as pD_2 values where $pD_2 = -\log EC_{50}$. Experimental values are given as mean ± s.e.mean. Statistical significance was tested by unpaired Student's t test and considered significant if $P < 0.05$.

Results

Animal model

Approximately 10 weeks after the onset of diabetes, body weights were significantly less in diabetic rats relative to age-matched controls ($P < 0.01$). Body weights were 386 ± 8 g and 307 ± 15 g in control and diabetic rats, respectively. Both left and right atrial weight also tended to be lower in diabetic animals but this was not statistically significant ($P > 0.05$). Left atrial weights were 14.8 ± 1.1 mg and 13.1 ± 0.9 mg; while right atrial weights were 12.9 ± 1.4 mg and 11.8 ± 1.2 mg in control and diabetic rats, respectively. Finally, diabetic animals exhibited significantly elevated ($P < 0.01$) plasma glucose levels (22.3 ± 1.3 mM) in comparison to control rats (8.0 ± 0.6 mM).

Inotropic and chronotropic studies

Mechanical properties of atria isolated from diabetic animals were similar to the atria obtained from age-

Table 1 pD_2 values and maximum response of various agonists in atria from control and diabetic rats

	pD_2		*Maximum response (%)*	
	Control	*Diabetic*	*Control*	*Diabetic*
Inotropy (left atria)				
Isoprenaline	7.70 ± 0.12	7.59 ± 0.11	174.8 ± 29.8	162.7 ± 14.3
Phenylephrine	5.60 ± 0.06	5.70 ± 0.06	117.2 ± 15.3	153.0 ± 11.3*
Adenosine	4.19 ± 0.09	4.37 ± 0.11	84.5 ± 2.6	85.7 ± 2.3
Chronotropy (right atria)				
Isoprenaline	7.82 ± 0.09	7.55 ± 0.11*	81.3 ± 5.1	74.3 ± 8.6
Phenylephrine	5.49 ± 0.13	5.61 ± 0.15	18.3 ± 1.9	41.8 ± 4.6*
Adenosine	4.42 ± 0.05	4.35 ± 0.15	80.5 ± 15.8	86.6 ± 8.3

Values are means ± s.e.mean of 4–7 animals.
* Statistically different from control ($P < 0.05$).

matched control rats. Both basal developed tension and spontaneous contraction rate did not differ significantly ($P > 0.05$) between control and diabetic animals. Basal developed tension of left atria was $40.4 \pm 5.1\,mg\,mg^{-1}$ tissue for control animals while it was $38.8 \pm 4.0\,mg\,mg^{-1}$ tissue for diabetic animals. The spontaneous contraction rates of right atria were $214 \pm 10\,beats\,min^{-1}$ and 198 ± 14 $beats\,min^{-1}$ for control and diabetic preparations, respectively.

Cumulative additions of isoprenaline (1.5 nM–1500 nM) produced concentration-dependent increases in developed tension in tissues from both control and diabetic animals (Figure 1a). There was no significant difference in either the sensitivity (Table 1) or in the maximum inotropic responses between the two groups of atria. Along with producing changes in inotropy, isoprenaline also produced a concentration-dependent increase in the spontaneous rate of contraction (Figure 1b). Although the maximum chronotropic response was unchanged, diabetic atria exhibited a decreased sensitivity to the isoprenaline-induced chronotropy (Table 1).

In the presence of β-adrenoceptor antagonism (propranolol; 2 μM), phenylephrine (0.2 μM–100 μM) also produced cumulative increases in both developed tension and contraction rate. However, atria from diabetic animals exhibited a significantly greater maximum response to both the inotropic and chronotropic effects of phenylephrine than corresponding atria from control rats (Figure 2a,b). There was no shift in the concentration-response curves to either the inotropic or chronotropic effects in atria from diabetic rats as reflected by the pD_2 values (Table 1).

In contrast to α- and β-adrenoceptor agonists, cumulative additions of adenosine (0.15 μM–300 μM) produced concentration-dependent decreases in both developed tension and spontaneous contraction rate. The maximum responses to both the inotropic and

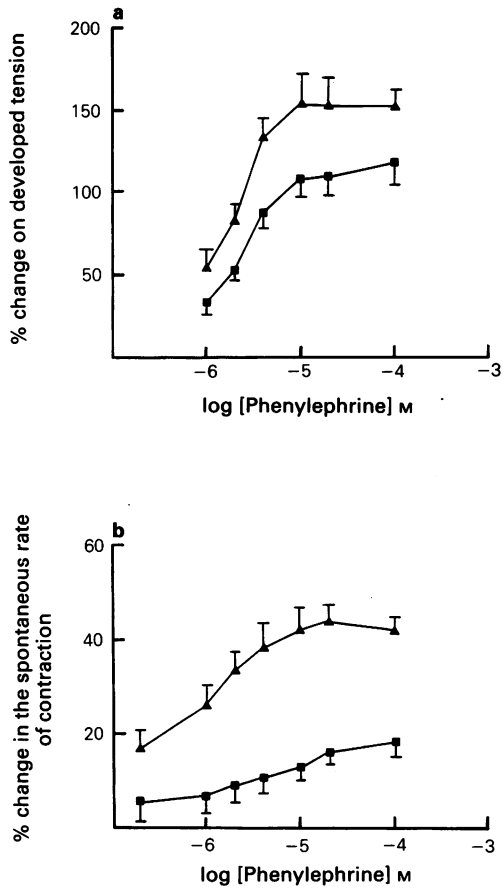

Figure 2 Cumulative concentration-response curves showing the inotropic (a) and chronotropic (b) responses to phenylephrine in the presence of propranolol (2 μM) in atria from control (■) and diabetic (▲) animals. Means ($n = 4$) are shown with vertical bars indicating s.e.mean.

chronotropic effects of adenosine were similar in diabetic and control preparations (Table 1). Furthermore, the diabetic state did not significantly alter the pD_2 value for adenosine-induced changes in contraction rate and developed tension (Table 1).

Receptor binding study

As adrenoceptor stimulation resulted in altered responses in atria from diabetic animals further experiments were undertaken to characterize the adrenoceptors. Previous studies have shown that [^{3}H]-dihydroalprenolol and [^{3}H]-prazosin bind specifically and with high affinity to β- and α_1-adrenoceptors, respectively (U'Prichard *et al.*, 1978; Sastre *et al.*, 1984). In our study, specific binding for [^{3}H]-dihydralprenolol ranged from 40–60% while specific binding for [^{3}H]-prazosin was between 70–90%.

Results from saturation experiments with [^{3}H]-dihydroalprenolol binding to atrial membranes of control and diabetic membranes are shown in Table 2. Membranes from diabetic rats demonstrated a significant decrease in the total number (B_{max}) of β-adrenoceptors. The density of β-adrenoceptors in membranes from diabetic animals was reduced by 64% and 45% in left and right atria, respectively, compared to corresponding control values. Equilibrium K_d values, on the other hand, were similar in both groups. Saturation isotherms for [^{3}H]-prazosin also yielded a reduction in receptor density that was comparable to the decline seen in β-adrenoceptors. The α_1-adrenoceptor population, decreased by 66% and 56% in left and right atria, respectively, in tissues from diabetic animals (Table 2). The K_d for [^{3}H]-prazosin was similar in both groups of membranes.

Finally, diabetes did not alter the yield of membrane protein per mg of initial atrial mass produced by our homogenization procedure nor did it alter the sialic acid content of the membranes relative to the protein concentrations (Table 2).

Discussion

Myocardial reactivity in experimental diabetes has been studied, but no consensus, especially concerning adrenoceptor-mediated stimulation has been reached. In the present study, spontaneously diabetic BB rats were used. These rats have now become an important animal model of type I insulin-dependent diabetes mellitus (Marliss *et al.*, 1982). Although there are numerous investigations relating to various metabolic and biochemical abnormalities in these animals, studies examining cardiovascular function are just beginning (Agrawal & McNeill, 1987; Durante *et al.*, 1988).

This study examined the ability of atria obtained from BB rats to respond to various drugs. It was observed that only the response to adrenoceptor agonists was changed in diabetic animals. Both the inotropic and chronotropic effects of adenosine

Table 2 Membrane properties of atria obtained from control and diabetic rats

	Control	*Diabetic*
Membrane protein yield ($\mu g\,mg^{-1}$ atrial wet weight)	54.0 ± 2.6	52.6 ± 2.0
Sialic acid content (nmol mg^{-1} protein)	47.0 ± 3.1	44.9 ± 1.9
Left atria		
[^{3}H]-dihydroalprenolol binding		
B_{max} (fmol mg^{-1} protein)	6.68 ± 1.06	2.41 ± 0.83*
K_d (nM)	4.52 ± 1.51	4.84 ± 1.41
[^{3}H]-prazosin binding		
B_{max} (fmol mg^{-1} protein)	12.08 ± 2.05	4.16 ± 1.47*
K_d (nM)	0.38 ± 0.06	0.30 ± 0.09
Right atria		
[^{3}H]-dihydroalprenolol binding		
B_{max} (fmol mg^{-1} protein)	8.29 ± 0.76	4.55 ± 1.71*
K_d (nM)	4.73 ± 1.62	4.60 ± 1.33
[^{3}H]-prazosin binding		
B_{max} (fmol mg^{-1} protein)	22.03 ± 2.85	9.66 ± 2.96*
K_d (nM)	0.46 ± 0.06	0.49 ± 0.15

Values are means ± s.e.mean of 4 membrane preparations. Each membrane preparation utilized 5 atria.
* Statistically different from control ($P < 0.05$).

remained unaltered in diabetic preparations. Compared to atria from control animals atria from diabetic rats demonstrated a hyperresponsiveness to both the inotropic and chronotropic effects of phenylephrine while exhibiting no change in sensitivity to α-adrenoceptor stimulation. On the other hand, atria obtained from diabetic rats demonstrated a subsensitivity to the chronotropic effects of the β-adrenoceptor agonist isoprenaline. In contrast, the inotropic effects of isoprenaline were unchanged by the diabetic state.

Recent work seems to support our finding of an augmented myocardial response following α-adrenoceptor stimulation in diabetic animals. Downing *et al.* (1983), utilizing diabetic lambs, observed that changes in contractility as reflected by dP/dt_{max} were twice as great in diabetics as in controls. More relevant to our work, Canga & Sterin-Borda (1986) were able to find an enhanced efficacy in tension development of left atria from streptozotocin-treated rats to α_1-receptor stimulation. In addition, a study by Jackson *et al.* (1986) showed that right atria from streptozotocin-treated rats produced a significantly greater increase in contraction rate in response to phenylephrine.

A variety of alterations in heart rate have been described in human diabetics. These include a resting tachycardia, reduced heart rate response to postural change, and in some studies a blunted heart rate response to exercise (Rubler & Arvan, 1976; Hilsted *et al.*, 1980; Sundkvist *et al.*, 1980). These abnormalities may result from a change in output of the autonomic nervous system and/or from an altered responsiveness of the sinus node to autonomic stimuli. The decrease in pD_2 value for isoprenaline-induced chronotropy in diabetic atria suggests that there may be a diminished sinus node sensitivity to catecholamines in diabetes. The fact that the inotropic response to isoprenaline was unchanged in diabetic preparations further suggests that diabetes may lead to a very selective change in cardiac β-adrenoceptor function.

Alteration of the hormonal status induced by pathological or pharmacological manipulations is known to cause diverse effects on various receptor-mediated events. These effects are often attributed to changes in the number or function of receptors. In the current study diabetes resulted in a significant decrease in both β- and α_1-receptors with no significant effect on the equilibrium dissociation constants of the receptors. This confirms previous work which demonstrated a decline in adrenoceptor density in left ventricular tissue from streptozotocin-treated rats (Savarese & Berkowitz, 1979; Williams *et al.*, 1983). Our study extends this observation to atrial tissue of spontaneously diabetic rats. In addition, our results also indicate that changes in receptor density were not the result of differential membrane purification since the yields of membrane protein and the content of the sarcolemmal membrane marker sialic acid were similar in both control and diabetic groups.

It has been suggested that changes in adrenoceptors may be responsible for altered cardiac responses to β- and α-agonists in diabetic tissue (Heyliger *et al.*, 1982). Data obtained in our study do not support this notion. Despite the fact that β-adrenoceptor density was reduced by 64% in left atria from diabetic rats there was no significant change in maximum β-adrenoceptor-mediated inotropic response or in sensitivity for isoprenaline-mediated contractility. In addition, a comparable loss of cardiac α_1-adrenoceptors in atria from diabetic animals was not associated with a corresponding decrease in inotropic or chronotropic responses but rather with an enhanced responsiveness to phenylephrine. Clearly, changes in adrenoceptor density in diabetes are not responsible for the altered myocardial response to adrenoceptor agonists. It is possible that steps following drug-receptor coupling may be altered in diabetes.

The importance of endogenous adenosine in biological functions is being increasingly recognized. In the heart, adenosine is a potent extracellular messenger. Aside from causing coronary vascular smooth muscle relaxation (Schnarr & Sparks, 1972), adenosine inhibits noradrenaline release from sympathetic nerve endings (Verhaeghe & Vanhoutte, 1977), reduces atrioventricular conduction velocity (Belardinelli *et al.*, 1983) and has negative inotropic (Endoh & Yamashita, 1980) and chronotropic effects (Belardinelli *et al.*, 1983). Recent studies have reported diminished coronary vascular responses to exogenous adenosine in diabetic animals (Downing *et al.*, 1982; Durante *et al.*, 1989). As such, we wanted to examine whether other cardiac effects of adenosine were also changed in diabetic animals. Our atrial experiments showed that the cumulative addition of adenosine produced negative inotropic and chronotropic effects which were unaltered in diabetic preparations. These results suggest that diabetes leads to a selective alteration in adenosine action in cardiac tissue.

In conclusion, this study demonstrated that isolated atria from spontaneously diabetic rats exhibited an altered response to adrenoceptor agonists. Atria from diabetic animals displayed a marked hyperresponsiveness to both the inotropic and chronotropic effects of selective α_1-adrenoceptor activation by phenylephrine. In addition, the sensitivity to the chronotropic effect of the β-adrenoceptor agonist isoprenaline is reduced in diabetic preparations. Radioligand binding studies revealed that both α_1- and β-adrenoceptor populations were substantially

reduced in atria from diabetic rats. However, there was no change in receptor affinity for either adrenoceptor. Future studies examining post receptor events may provide further insight as to the nature of the defect in these diabetic animals.

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Superoxide anions enhance platelet adhesion and aggregation

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1 Superoxide dismutase (SOD, 60 u ml^{-1}) or ferricytochrome c (70 μM) significantly inhibited thrombin-stimulated platelet adhesion to gelatin-coated plastic, whereas catalase (1000 u ml^{-1}) or mannitol (1 mM) had no effect.

2 The platelet aggregation induced by low concentrations of thrombin (causing less than 45% maximal change in light transmission) was inhibited by SOD. Catalase or mannitol had no effect on platelet aggregation.

3 Pyrogallol (an O_2^- generator) enhanced both platelet adhesion to gelatin-coated plastic and platelet aggregation induced by thrombin; this enhancement was neutralized by SOD.

4 These results indicate that O_2^- increase both platelet adhesion and aggregation, whereas other free radicals such as hydrogen peroxide or hydroxyl radicals are not involved.

Introduction

Stimulated and unstimulated platelets generate superoxide anions (O_2^-; Marcus, 1979; Joseph *et al.*, 1987), which may contribute to the killing of bacteria and parasites (Cesbron *et al.*, 1987). However, the role of O_2^- in platelet function is unclear. Although exogenously added superoxide dismutase (SOD) had no effect on thrombin-induced platelet aggregation *in vitro* (Marcus, 1979), generation of O_2^- in the external milieu of platelets can either induce aggregation or lower the threshold for the action of thrombin (Handin *et al.*, 1977). Since O_2^- destroy endothelium-derived relaxing factor (EDRF; Gryglewski *et al.*, 1986; Rubanyi *et al.*, 1986), and EDRF is a potent inhibitor of platelet adhesion (Radomski *et al.*, 1987a; Sneddon & Vane, 1988) and aggregation (Azuma *et al.*, 1986; Radomski *et al.*, 1987b), it is possible that the production of O_2^- by platelets modulates platelet function in haemostasis.

We show here that O_2^- significantly enhanced adhesion of thrombin-stimulated platelets to gelatin-coated plastic, whereas SOD inhibited it. In addition, we also present evidence that O_2^- promotes platelet aggregation. Some of these results have been presented to the British Pharmacological Society (Salvemini *et al.*, 1988).

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Methods

Isolation and labelling of human platelets

Blood from healthy volunteers who had not taken drugs for at least 15 days was collected by venopuncture into a plastic flask containing 3.15% sodium citrate (1:9 v/v). Platelet rich plasma (PRP) was prepared by centrifugation (800 *g* × 8 min). Platelets were labelled with [^{3}H]-adenine (1 μCi ml^{-1} PRP; Curwen *et al.*, 1982) at room temperature for 30–40 min. To prepare washed platelets, prostacyclin (PGI_2, 300 ng ml^{-1}) was then added to the PRP and the mixture centrifuged for 18 min. The supernatant was removed and the platelet pellet resuspended in 10–15 ml of calcium-free oxygenated (95% O_2/5% CO_2) Krebs buffer and diluted to 50 ml with the same buffer. Prostacyclin (PGI_2, 300 ng ml^{-1}) was added and the platelets were centrifuged (800 *g*) for 18 min. The supernatant was aspirated and the pellet was resuspended in calcium-free Krebs solution (Radomski & Moncada, 1983). Indomethacin (10 μM) and calcium (1 mM) were added to the final platelet suspension.

Platelet adhesion to gelatin-coated plastic

Multiwell tissue culture plates (24 well, Flow Laboratories) were coated with 0.1% (w/v) gelatin dissolved in phosphate-buffered saline by incubation

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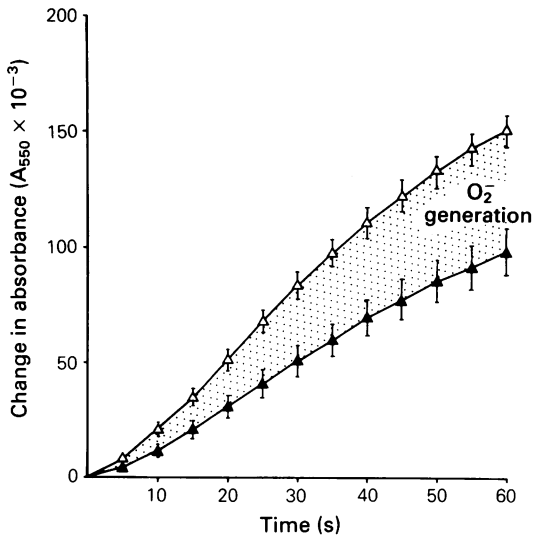

Figure 1 Ferricytochrome c reduction by pyrogallol. Superoxide anion (O_2^-) generation was determined by the increase in light absorbance at 550 nm. Specificity of the reduction reaction was measured by including superoxide dismutase in the reaction mixture. The reduction of ferricytochrome c (70 μM) by pyrogallol was nearly complete within 1 min. That part of the reduction inhibited by superoxide dismutase (SOD, 60 u ml^{-1}) represented the generation of superoxide anions. Graphs represent ferricytochrome c reduction with (▲) and without (△) SOD. Data are presented as means of 6 observations; vertical lines indicate s.e.mean.

at 37°C for 1 h. Immediately before use, the gelatin was aspirated and the wells washed with 2 × 1 ml Krebs solution containing calcium (1 mM) and indomethacin (10 μM).

To the appropriate wells were added aliquots (25 μl) of the free radical scavengers (SOD 60 u ml^{-1}, catalase 1000 u ml^{-1} or mannitol 1 mM) or Krebs buffer, followed by 450 μl of platelet suspension. Platelet adhesion was stimulated with thrombin (20–320 mu ml^{-1}) and the plates incubated for 20 min at 37°C with gentle shaking. After incubation, the platelet suspension was aspirated and each well gently washed with 3 × 1 ml Krebs buffer to remove any non-adherent platelets. Distilled water (500 μl) was added to each well to lyse the remaining platelets and the residual radioactivity in the lysate was counted and expressed as d.p.m.

Platelet aggregation

A suspension of washed human platelets (500 μl) was incubated at 37°C for 2 min in a Payton Dual channel aggregometer (Born & Cross, 1963) with continuous stirring at 1000 r.p.m. and then stimulated with thrombin (20–80 mu ml^{-1}). Changes in light transmission were recorded for 3 min after thrombin stimulation. Test compounds were added to the platelet suspension 1 min before addition of thrombin.

Measurement of superoxide anion production by pyrogallol

Superoxide anions were measured by the increase in absorbance due to the reduction of ferricytochrome c (McCord & Fridovich, 1969) with a Shimadzu V-160 spectrophotometer at 550 nm. Specificity of the reduction reaction was demonstrated by the use of SOD (60 u ml^{-1}), which competitively inhibits O_2^- oxidation by ferricytochrome c (McCord & Fridovich, 1969). The levels of O_2^- produced were calculated by use of $\Delta_\varepsilon = 21.1$ mM^{-1} cm^{-1} (Van Gelder & Slater, 1962). Stock solutions of pyrogallol (930 μM) were prepared in helium-deoxygenated water.

Materials

The composition of the Krebs solution 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. Human thrombin, superoxide dismutase (from bovine erythrocytes), catalase, mannitol, ferricytochrome c, formyl-methionine-leucine-phenylalaline (FMLP) and indomethacin were obtained from Sigma (Poole, UK). Pyrogallol was obtained from Aldrich (Dorset, UK). [^{3}H]-adenine (25 Ci nmol^{-1}; 1 μCi = 37 kBq) was obtained from Amersham (UK). Prostacyclin was a gift from the Wellcome Research Laboratories, Beckenham, UK.

Statistics

Platelet adhesion was expressed as the ratio of platelet adhesion induced by thrombin divided by control adhesion in the absence of thrombin. Results are expressed as the mean ± s.e.mean for (n) experiments. Student's unpaired t test was used to determine the significance of differences between means and a P value of <0.05 was taken as significant.

Results

Production of superoxide anions by pyrogallol

Pyrogallol (5.6 μM) caused a reduction of ferricytochrome c (70 μM) over a period of 1 min. The part of the reduction of the ferricytochrome c inhibited by incubation with SOD (60 u ml^{-1}), represented the generation of O_2^-. At the end of 1 min 3.55 nmol O_2^- ml^{-1} were generated. The part unaf-

fected by SOD was due to direct reduction of ferricytochrome c by pyrogallol (Figure 1).

Incorporation of [^3H]-adenine

Routine high speed centrifugation (10,000 *g* for 3 min) of the final platelet suspension showed that 85–90% of the radioactivity was associated with the platelets.

Non-specific binding of [^3H]-adenine to wells

Less than 1% of the [^{3}H]-adenine diluted with Krebs buffer (1 μCi ml^{-1}) remained associated with the gelatin coating in the absence of platelets. Thus, the radioactivity associated with the gelatin after the standard washing procedure was a reflection of the number of adhering platelets.

Platelet adhesion

Thrombin (20–320 mu ml^{-1}) induced a dose-dependent increase in platelet adhesion to gelatin-coated plastic, reaching a maximum at 80–160 mu ml^{-1} of thrombin (Figure 2). Time-course studies indicated that adhesion was maximal 15 min after addition of thrombin and was constant for at least 30 min (results not shown). For convenience, platelet binding was determined 20 min after addition of thrombin. At concentrations of thrombin higher than 160 mu ml^{-1}, adhesion did not increase, but platelet aggregates were formed, as observed through a phase contrast microscope. When platelets were aggregated by thrombin in an aggregometer and then transferred to the wells, no adhesion was observed.

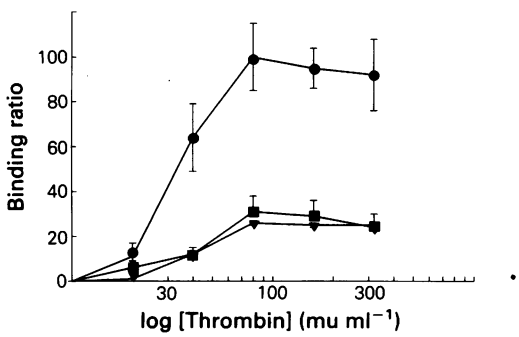

Figure 2 Superoxide dismutase (SOD; 60 u ml^{-1}) and ferricytochrome c (70 μM) inhibit thrombin-induced platelet adhesion to gelatin-coated plastic. Results are expressed as the ratio of the number of thrombin-stimulated platelets adhering to the gelatin-coated plastic divided by the number of platelets adhering in the absence of thrombin. SOD and ferricytochrome c significantly reduced platelet adhesion at all concentrations of thrombin tested (20–320 mu ml^{-1}). (●) Represents control adhesion ($n = 13$), (■) adhesion in the presence of SOD ($n = 6$) and (▼) adhesion in the presence of ferricytochrome c ($n = 4$). Means with s.e.mean shown by vertical bars.

Table 1 The effects of superoxide dismutase, catalase and mannitol on thrombin-induced platelet adhesion

Thrombin (mu ml^{-1})	*Controls*	*SOD*	*Cat*	*Man*
2.5	3 ± 0	2 ± 0	3 ± 0	3 ± 0
5	8 ± 1	5 ± 1	8 ± 1	8 ± 1
10	14 ± 2	8 ± 1*	13 ± 2	14 ± 2
20	22 ± 4	11 ± 2*	21 ± 4	22 ± 4
40	26 ± 3	15 ± 3*	25 ± 4	26 ± 4

Results are expressed as the binding ratio. In contrast to superoxide dismutase (SOD, 60 u ml^{-1}), neither catalase (Cat, 1000 u ml^{-1}) nor mannitol (Man, 1 mM) reduced platelet adhesion induced by different concentrations of thrombin (2.5–40 mu ml^{-1}). Each result is expressed as the mean ± s.e.mean of 6 experiments.
*Indicates $P < 0.05$.

SOD (60 u ml^{-1}) significantly reduced thrombin-induced platelet adhesion to gelatin-coated plastic (Figure 2). The inhibitory action of SOD was abolished when the enzyme was boiled for 1 min (results not shown). Ferricytochrome c (70 μM) also reduced adhesion (Figure 2). Catalase (1000 u ml^{-1}) or mannitol (1 mM) had no effect on the thrombin-induced adhesion (Table 1). FMLP (10^{-8} to 10^{-4} M) did not increase platelet adhesion to gelatin-coated plastic (results not shown).

Comparison of the experiments summarised in Figure 2 with those presented in Table 1 shows a substantial variation in the binding ratio to thrombin alone. Apart from the fact that these experiments were done several months apart, we have no explanation for this variation. However, in both series of experiments, SOD strongly inhibited the adhesion of platelets.

Platelet aggregation

SOD (60 u ml^{-1}) reduced platelet aggregation induced by concentrations of thrombin that caused a change in light transmission of less than 45% of maximum (from 43 ± 5% to 10 ± 3%; $n = 7$,

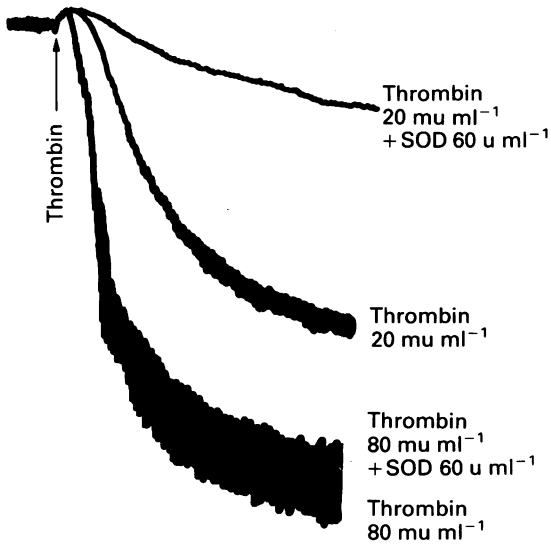

Figure 3 The effect of superoxide dismutase (SOD; 60 u ml^{-1}) on thrombin-induced platelet aggregation. SOD reduced platelet aggregation induced by a low concentration of thrombin (20 mu ml^{-1}) whereas it had no effect when a higher concentration of thrombin was used (80 mu ml^{-1}). Tracings are representative of 7 similar experiments.

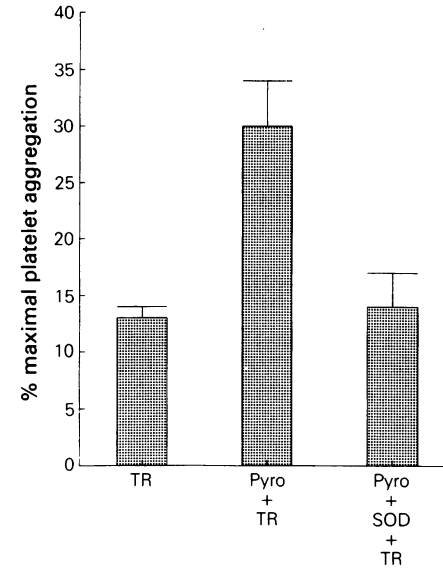

Figure 4 The effect of pyrogallol on thrombin-induced platelet aggregation. Pyrogallol (Pyro; 5.6 μM) significantly ($P < 0.01$) increased platelet aggregation when threshold concentrations of thrombin (TR; 12–16 mu ml^{-1}) were used. This potentiation was prevented by superoxide dismutase (SOD; 60 u ml^{-1}). Platelet aggregation was expressed as % of the maximal aggregation. The vertical bars represent the s.e.mean of 4 experiments.

$P < 0.0005$), but when aggregation was greater than this SOD had no significant effect (from 85 ± 3% to 74 ± 4%; $n = 7$, $P < 0.1$). A typical tracing of the aggregatory response is shown in Figure 3. The anti-aggregatory effect of SOD was lost when the enzyme was boiled for 1 min. Catalase (1000 u ml^{-1}) or mannitol (1 mM) did not affect thrombin-induced platelet aggregation (results not shown).

Pyrogallol (5.6 μM) potentiated thrombin-induced aggregation when threshold concentrations (12–16 mu ml^{-1}) of thrombin were used. This potentiation was neutralized by SOD (60 u ml^{-1}) (Figure 4).

Discussion

Our results demonstrate that SOD inhibited thrombin-induced platelet adhesion and aggregation. Since this enzyme has a high specificity for O_2^- (McCord & Fridovich, 1969), these findings indicate the involvement of O_2^- in platelet activation. Superoxide anions could originate from contaminating neutrophils or platelets themselves or from the oxygenated Krebs solution. Neutrophils were not a source for O_2^- in these experiments, since no neutrophils were present in the platelet suspension when examined by light microscopy. In addition, the specific neutrophil chemotactic agent, FMLP (Snyderman & Goetzl, 1981), given at concentrations known to stimulate O_2^- production by neutrophils (Truett *et al.*, 1988) had no effect on platelet adhesion (results not shown).

Platelets produce O_2^- by an as yet unidentified mechanism (Marcus *et al.*, 1979). Interestingly, platelets also contain SOD (Marcus *et al.*, 1979), an enzyme which increases the stability of EDRF by preventing its destruction by O_2^- (Gryglewski *et al.*, 1986). EDRF (NO) is a potent inhibitor of platelet adhesion (Radomski *et al.*, 1987a; Sneddon & Vane, 1988). Thus, the balance between O_2^- production and SOD activity may be an important mechanism by which platelets influence their adherence to endothelial cells.

Superoxide anions are present in Krebs solution (de Nucci *et al.*, 1988), but the amounts were below our level of detection. Since at 10 s pyrogallol gener-

ated 0.42 nmol O_2^- ml^{-1}, the concentration of O_2^- in the Krebs solution was below this level. Furthermore, the potentiation of aggregation seen in Figure 4 was produced by 3.55 nmol O_2^- ml^{-1}, so that it is unlikely that the source of O_2^- in these experiments was simply the Krebs solution.

Any system producing O_2^- might also be expected to generate hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH•; Weiss 1983; Fridovich, 1986; Halliwell & Gutteridge, 1986). There is a membrane-bound H_2O_2-generating system in platelets that is activated when they are stimulated with either membrane perturbing agents or immune complexes (Finazzi-Agro *et al.*, 1982). Some authors found that H_2O_2 inhibited the platelet release reaction (Stuart & Holmsen, 1977) and aggregation (Levine *et al.*, 1976; Koufos & Sagone, 1980; Krinsky *et al.*, 1981), while others showed that H_2O_2 caused platelet aggregation (Canoso *et al.*, 1974; del Principe *et al.*, 1985). We have found that, in contrast to SOD (an O_2^- scavenger), neither catalase (an H_2O_2 scavenger) nor mannitol (an OH• scavenger) affected platelet adhesion or aggregation. Thus, if H_2O_2 or OH• are present, they do not influence the platelet reaction to thrombin.

SOD inhibited platelet aggregation when low concentrations of thrombin were used. This indicates that O_2^- potentiated platelet aggregation, a finding further supported by the increase in platelet aggregation induced by pyrogallol (an O_2^- generator) when threshold concentrations of thrombin were used.

A possible mechanism by which O_2^- can increase both platelet adhesion and aggregation is through inhibition of guanylate cyclase. *In vitro* preparations of hepatic guanylate cyclase contain O_2^- and SOD stimulates this enzyme (Mittal & Murad, 1977), indicating that O_2^- may be inhibiting guanylate cyclase.

The anti-adhesive and anti-aggregatory effects of SOD described here may contribute to the beneficial effects observed during administration of SOD in animal models of shock (Bitterman *et al.*, 1988) and post-ischaemic injury (Tamura *et al.*, 1988). They also indicate a therapeutic potential for scavengers of O_2^- in the prevention of atherosclerosis.

There are several criteria which are used to help identify EDRF-like substances. These include inhibition of activity by haemoglobin or methylene blue (Martin *et al.*, 1985) and potentiation of activity by SOD (Gryglewski *et al.*, 1986; Rubanyi *et al.*, 1986). Another important conclusion from our results is that potentiation by SOD of the effects of EDRF-like substances on platelets can no longer be regarded as evidence for the release of EDRF, since the simple removal of O_2^- by SOD gives similar results.

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The effect of EU4093 (azumolene sodium) on the contraction of intrafusal muscle in the soleus muscle of the anaesthetized rat

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1 EU4093 (azumolene sodium) is a direct acting, skeletal muscle relaxant with structural similarities to dantrolene sodium in that the *para*-nitro phenyl group of dantrolene sodium is replaced by a *para*-bromo phenyl group.

2 The effect of EU4093 on the twitch of the intact rat soleus preparation is nearly maximal at a dose of $20\,mg\,kg^{-1}$. This dose of EU4093 reduces the amplitude of the twitch to 31.9% of the control value. By comparison, dantrolene sodium reduced the twitch amplitude to 31.3% of the control value at a dose of $5\,mg\,kg^{-1}$.

3 The contraction of intrafusal muscle as measured by the response of spindle afferent discharge was also reduced by EU4093.

4 The most significant difference between the effect of EU4093 and dantrolene sodium on the contraction of intrafusal muscle was that EU4093 had an appreciable depressant action at high stimulation frequencies at which dantrolene sodium had only a minimal relaxant effect on the intrafusal muscle contraction.

5 The significance of this difference in the actions of EU4093 and dantrolene sodium is discussed in terms of the possible effect of the drug in the intact animal and human patient. It is concluded that, because the discharge frequency is not likely to move into the high frequency range at which dantrolene sodium and EU4093 have significantly different effects, the overall effect of these two drugs is likely to be similar.

Introduction

Abnormal skeletal muscle contraction may be controlled pharmacologically by drugs that act either directly on muscle or indirectly on the central nervous control of muscle. Dantrolene sodium is an example of a drug which acts directly upon skeletal muscle at the level of excitation-contraction coupling (Ellis & Bryant, 1972). Recently derivatives of dantrolene sodium have been synthesized (White *et al.*, 1987). Several of these compounds have also been shown to have a direct muscle relaxant effect (White *et al.*, 1987; Pong *et al.*, 1987). We describe here the effect of one of these compounds (EU4093, azumolene sodium, see Figure 1) on the contraction of intrafusal muscle in anaesthetized rats.

Muscle spindle afferents have an excitatory action upon synergist motoneurones. Any relaxant effect of a drug on the intrafusal muscle will thus reduce the excitatory input onto the alpha motoneurones. Data on the action of the drug on the contraction of intrafusal muscle are essential therefore in order to understand the mode of action of the drug in the intact patient.

The most striking feature of the action of dantrolene sodium is the relationship between the depression of muscle contraction and stimulation frequency for both extrafusal (Bowman *et al.*, 1979; Leslie &

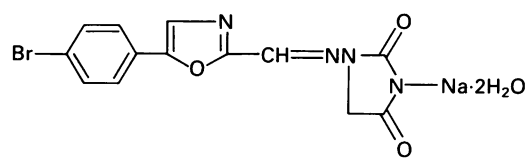

Figure 1 The structure of EU4093 (azumolene sodium). The full chemical name of this compound is 1-[[[5-(4-bromophenyl)-2-oxazolyl]methylene]amino] - 2,4-imidazolidinedione sodium salt dihydrate.

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Part, 1981a) and intrafusal (Leslie & Part, 1981b) muscle. If the stimulation frequency is sufficiently great, the muscle is spared the relaxant effect of the drug. In this present work we have investigated the effect of EU4093 on the contraction of intrafusal muscle at a range of stimulation frequencies. The methods used were similar to those used previously for investigation of the action of dantrolene sodium (Leslie & Part, 1981b).

Methods

The experiments were carried out on a total of 16 female Sprague Dawley rats, 300–400 g body weight. Anaesthesia was induced with trichlorethylene vapour and maintained by intraperitoneal injection of urethane, 1500 mg kg^{-1} body weight. Supplementary injections were given as necessary.

The soleus muscle was used for these experiments. The use of this preparation for spindle experiments has been described previously (Andrew *et al.*, 1978) and is outlined in Figure 2. Briefly, the muscle was prepared for stimulation of the muscle nerve and the distal tendon tied either to a tension transducer

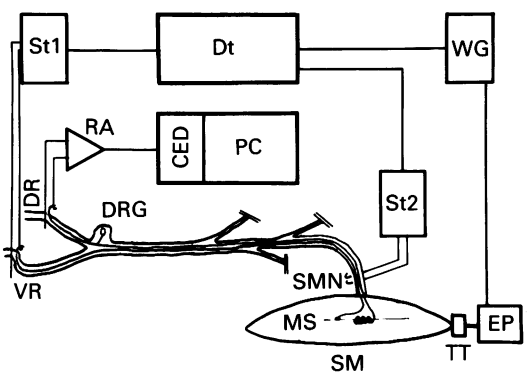

Figure 2 Simplified diagram of the experimental arrangement used for the recording of intrafusal muscle contraction. Stimulation of the ventral root (VR) filament and soleus muscle nerve (SMN) is by means of two stimulators (St1 and St2, respectively) both controlled by a Digitimer (Dt) control system (Digitimer Ltd). The digitimer is also used to control a waveform generator (LF141, Servomex Ltd) which in turn controls an electromagnetic puller (EP). The tension in the soleus muscle (SM) is recorded with a tension transducer (TT). The afferent discharge of a muscle spindle (MS) is recorded in a dorsal root (DR) filament with a recording amplifier (RA) and hence passed via a CED1401 computer interface (CED) to a PC-AT compatible computer (PC). In making this preparation a gamma fibre has been selected that activates the muscle spindle primary afferent previously isolated.

(Devices, 2ST02, 0–1 kg range) or to the arm of an electromagnetic puller with a tension transducer mounted upon it. Single spindle afferent nerve fibres were isolated in dorsal root filaments and single gamma nerve fibres active to the same spindle in ventral root filaments. The response of the spindle afferent to stimulation of the gamma efferent was recorded with the muscle held at constant length. Action potential discharges were displayed as instantaneous frequencies. Permanent records of the frequency displays were stored on BBC microcomputer disc via a video camera (Leslie, 1986). The responses of the spindle afferent to sinusoidal and ramp length changes with and without simultaneous stimulation of the gamma fibre were also recorded. The discharge of the passive, unstretched spindle was displayed as an interspike interval histogram generated with a CED1401 (Cambridge Electronic Design) intelligent interface connected to a Tandon 20 microcomputer. This system was also used for the generation of instantaneous frequency displays.

Twitch contractions of the soleus muscle were elicited by stimulation of the muscle nerve, at 0.1 s^{-1}. The resulting tension records were sampled and analysed by a microcomputer (3D09, Digital, Design & Development) running software under the FLEX operating system.

EU4093 was freshly prepared by dissolving 4 mg ml^{-1} in a carrier solution of sodium hydroxide, pH 10.3, made isotonic with 5% mannitol. This carrier solution has been shown to be without effect on extrafusal (Leslie & Part, 1981a) and intrafusal (Leslie & Part, 1981b) muscle contraction. In most experiments the drug was given in bolus injections via the external jugular vein to achieve concentrations of 5, 10, 15 or 20 mg kg^{-1}.

Results

The effect of EU4093 on extrafusal muscle twitch tension

A preliminary investigation of the effect of EU4093 on the isometric twitch contraction of the soleus muscle was made in three animals. In these experiments the tension transducer was rigidly attached to a micromanipulator whereas in the experiments designed to test the effect of the drug on intrafusal muscle the muscle had to be attached to the more compliant electromagnetic puller. The effects of the drug on twitch amplitude in the three experiments are shown in Figure 3. The aim of these preliminary experiments was to determine the dose of EU4093 to be used for the intrafusal work. It was decided on the basis of the results shown in Figure 3 to use a dose of 20 mg kg^{-1}, which gave a reduction of the

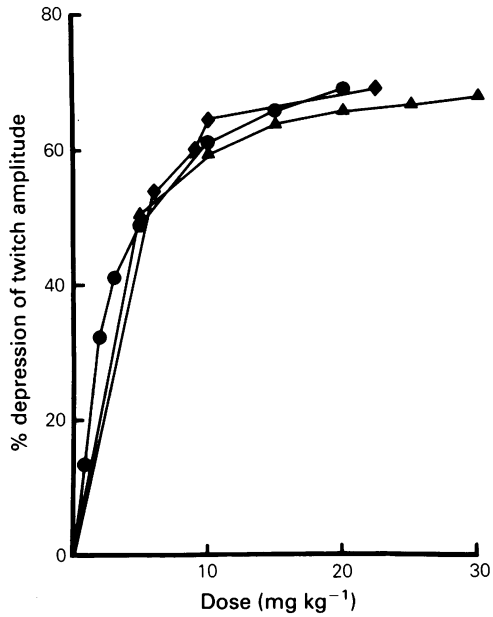

Figure 3 The effect of incrementally increased intravenous dose of EU4093 on the twitch amplitude of the soleus muscle stimulated supra-maximally via the muscle nerve at a frequency of 0.1 s^{-1}. The results of three individual experiments are shown.

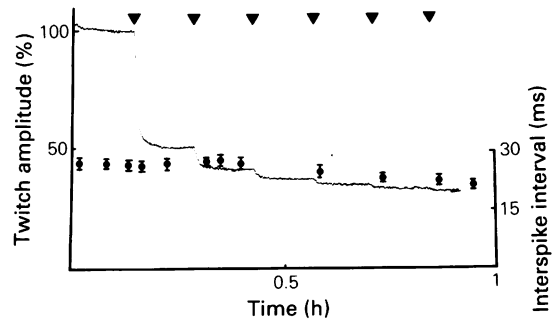

Figure 4 The effect of increased intravenous dose of EU4093 given at the arrows (▼) on the twitch amplitude of the soleus muscle and the interspike interval of a muscle spindle primary ending. The increment at each arrow was 5 mg kg^{-1} body weight, giving a final concentration of 30 mg kg^{-1}. The line shows the twitch amplitude with the muscle being stimulated once every 10 s via the muscle nerve. The interspike intervals are illustrated as the mean value of interval (●) and the range. It can be seen that there is no drug-related change in the interspike interval.

twitch amplitude to 31.9% of the control, a nearly maximal effect.

The time course of the action of EU4093 on extrafusal contraction is shown in the plots of Figure 4.

In ten experiments the effect of EU4093 on twitch amplitude was measured with the tension transducer mounted on the puller, the compliance of which precluded reliable results for the absolute values for the reduction of twitch amplitude. Nevertheless the relative dose-response curves were very similar to those obtained with the Devices transducer and confirmed the near maximum effect of a dose of 20 mg kg^{-1}.

The effect of EU4093 on the discharge of the passive spindle

If the contraction of intrafusal muscle is to be monitored from the discharge of the spindle sensory endings, it is essential to establish that the drug does not have a significant effect upon the discharge of the passive spindle. In three experiments interval histograms of muscle spindle primary discharge before and after the intravenous injection of each dose of EU4093 were recorded. The results of one of these experiments are shown in Figure 4. There is no drug-related change of interspike interval. In all the other spindle experiments additional data were obtained on the effect of EU4093 on the discharge of the passive spindle in the form of the control records of the discharge in response to ramp and sinusoidal stretch. None of these showed any evidence for an effect of the drug on the passive spindle.

The effect of EU4093 on the response of spindle afferents to gamma nerve stimulation

When first isolated a gamma nerve fibre was classified as static or dynamic (Crowe & Matthews, 1964) by means of the effect of stimulation of the gamma on the response to sinusoidal stretch. This test gives a clear demarcation between the two classes of gamma fibre to the rat soleus (Andrew *et al.*, 1978).

The response of the spindle afferent to stimulation of the classified gamma fibre at a range of frequencies with the muscle held at l_0 was recorded. The stimulation protocol was repeated after intravenous injection of EU4093. The effect of the drug on the discharge caused by gamma nerve stimulation varied considerably from preparation to preparation and so it is not appropriate to present mean results. Figure 5 shows graphs of the effect of EU4093 on the spindle discharge of a representative dynamic (Figure 5a) and static (Figure 5b) gamma fibre. It is apparent that the depression of intrafusal contraction as measured by spindle afferent discharge is dependent upon the frequency of stimulation. At low frequencies, at which there is just a slight increase in afferent frequency in the absence of the drug, EU4093 causes a total elimination of the

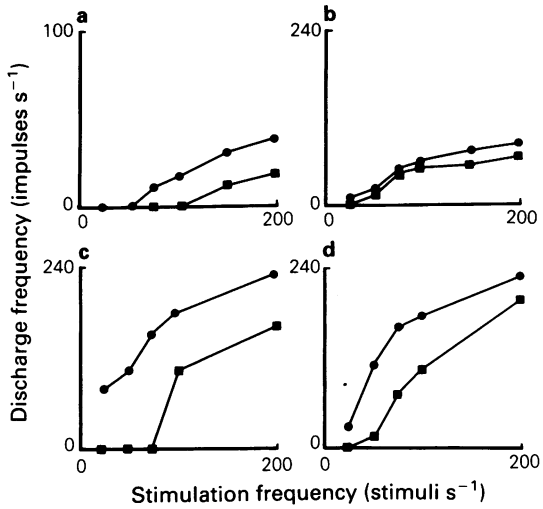

Figure 5 (a and b) Graphs showing the effect of EU4093 at a dose of 20 mg kg^{-1} body weight on the discharge frequency of muscle spindle primary afferents when a dynamic and static gamma fibre respectively are stimulated at frequencies between 25 and 200 stimuli s^{-1}; (c and d) show for comparison the results from similar previous experiments with dantrolene sodium (Leslie & Part, 1981b). Data obtained before (●) and after (■) injection of the drug.

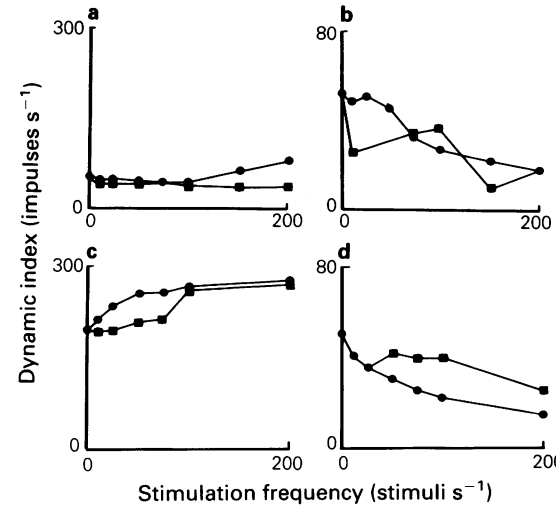

Figure 6 (a and b) Graphs showing the effect of EU4093 at a dose of 20 mg kg^{-1} body weight on the dynamic index of muscle spindle primary afferents when a dynamic and static gamma fibre respectively are stimulated at frequencies between 0 and 200 stimuli s^{-1}; (c and d) show for comparison the results from previous similar experiments with dantrolene sodium (Leslie & Part, 1981b). Data obtained before (●) and after (■) injection of the drug.

increase. At high frequencies of stimulation EU4093 still causes a similar reduction in frequency increase. In contrast the effect of dantrolene sodium was relatively less at high frequencies of stimulation than at intermediate frequencies; this is illustrated with previously published dantrolene sodium results (Leslie & Part, 1981b) plotted in the same manner (Figure 5c and d).

The effect of EU4093 on the dynamic index of the spindle response to ramp stretch was also investigated. The dynamic index is a measure of the dynamic sensitivity of a spindle ending (Crowe & Matthews, 1964). In general, stimulation of a dynamic gamma fibre will produce an increase in the dynamic sensitivity and hence dynamic index whereas stimulation of a static gamma fibre will cause a decrease in dynamic sensitivity.

As with the constant length experiments, the effect of EU4093 on dynamic sensitivity varied considerably from preparation to preparation. Figure 6 shows the effect of EU4093 on the changes of dynamic index brought about by the same static and dynamic gamma fibres as are shown in Figure 5. This figure illustrates the general finding that even at high frequencies (200 s^{-1}) of stimulation EU4093 causes a considerable reduction in the increase of dynamic index brought on by dynamic gamma fibre stimulation. The records of the response to sinusoidal stretch shown in Figure 7 also illustrate how EU4093 can have an appreciable effect on the increased modulation to stretch brought about by dynamic gamma nerve fibre stimulation at high stimulation frequencies. The dynamic gamma fibres investigated in this study happened not to bring about an appreciable increase in dynamic index until stimulated at quite high frequencies and therefore not too much significance should be read into the lack of change of dynamic index seen in Figure 6a.

Figure 6b shows the effect of EU4093 on the dynamic index with static gamma fibre stimulation. The striking feature of these results is the irregularity of the effect of increasing the stimulation frequency on the effectiveness of the drug. Figure 6c and d illustrate for comparison the typically much more regular relationship between stimulation frequency and effect of dantrolene sodium observed in previous experiments (Leslie & Part, 1981b).

Figure 7 illustrates that EU4093 altered the response of the spindle ending to stimulation of the static fibre at 200 s^{-1}; there is a slight decrease in the mean frequency of discharge with a considerable decrease in the reduction of modulation brought about by static gamma fibre stimulation. However with other gamma fibres the depth of the modulation

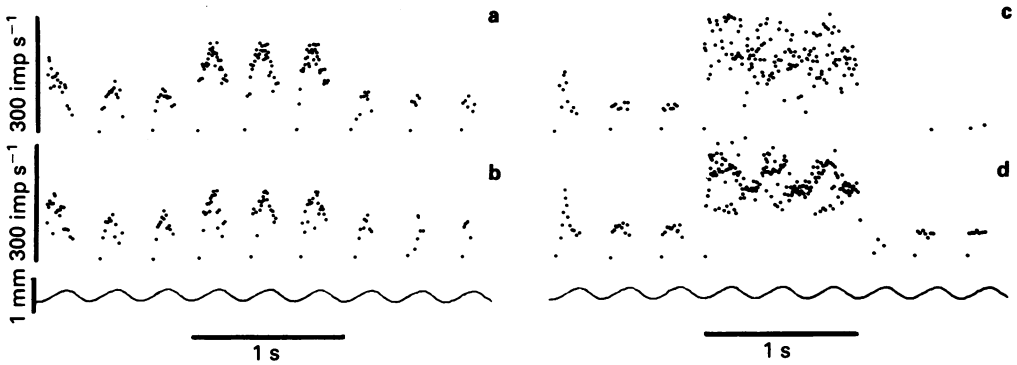

Figure 7 Instantaneous frequency displays of responses of muscle spindle primary endings to sinusoidal stretch before (a and c) and after (b and d) EU4093 at 20 mg kg^{-1}. Records (a) and (b) are with stimulation of a dynamic gamma fibre and records (c) and (d) with a static gamma fibre. Both fibres were stimulated at 200 stimuli s^{-1} for 1 s as shown by the bar.

to the stretch was about the same before and after the drug but the mean frequency of discharge was reduced.

Discussion

In a small number (3) of preliminary experiments we have shown that EU4093 depresses the twitch amplitude of the rat soleus muscle on average to 31.9% of the control at a dose of 20 mg kg^{-1} (body wt). This is a very similar result to that obtained with dantrolene sodium which depressed the soleus twitch to 31.3% of the control value at a dose of 5 mg kg^{-1} (Leslie & Part, 1981a). It is known that dantrolene sodium acts by means of inhibiting the release of calcium ions from the sarcoplasmic reticulum (van Winkle, 1976). Given the similarity of effect of EU4093 to that of dantrolene sodium on the twitch contraction of extrafusal muscle, it is probable that EU4093 acts in like fashion.

EU4093, like dantrolene sodium, has an effect on the contraction of intrafusal muscle. The effect of the drug is dependent upon the frequency of stimulation. At low frequencies the drug may abolish completely the acceleration of the spindle sensory ending. However, this does not necessarily mean that the drug has had the effect of totally preventing intrafusal muscle contraction as it may be that the contraction in the presence of the drug is below the threshold required to cause an acceleration of the afferent discharge. At high frequencies of stimulation the effect of EU4093 was rather different from that of dantrolene sodium in that EU4093 produced a pronounced depression of the acceleration of discharge frequency on stimulation of the gamma nerve fibre with the muscle held at constant length. This was observed in all the gamma fibres investigated, both static (7 fibres) and dynamic (5 fibres).

Dantrolene sodium has an effect on the dynamic index which is dependent upon the frequency of stimulation, with the greatest depression occurring at intermediate frequencies. The present results from EU4093 show that this drug had an unpredictable effect on the dynamic index of muscle spindle primaries as measured by the dynamic index in relation to stimulation frequency. For some gamma fibres EU4093 may cause a considerable reduction in the change of dynamic index brought about by stimulation of the gamma fibre, whereas for others EU4093 causes a minimal change of dynamic index.

The reduction of intrafusal muscle contraction caused by dantrolene sodium may be important in its overall muscle relaxant properties. A decrease in the response of spindle afferents to the gamma nerve activity will result in a decrease in excitatory input onto the motoneurones of the synergist muscles and hence a reduction in the extrafusal muscle contraction. Because of the form of the frequency-dependence of the effect of dantrolene sodium on the intrafusal contraction (Leslie & Part, 1981b), it is possible that the nervous system could overcome the muscle relaxant effect of dantrolene sodium on intrafusal muscle by increasing the frequency of discharge of the gamma nerve fibres. However, this is not a possibility with EU4093 because this drug still has an effect on intrafusal contraction at a stimulation frequency of 200 s^{-1}. In the decerebrate rat, dantrolene sodium does not have any effect upon the discharge of gamma nerve fibres to the de-afferented soleus muscle (Farquhar & Part, 1988). Hence in this preparation, at least, the nervous system is not compensating for the action of dantrolene sodium on intrafusal muscle and so the fact that EU4093 has a

greater effect than dantrolene sodium at high frequencies of discharge will not have any bearing on the relative muscle relaxant effect of the two drugs. If the gamma nerve discharge in the intact animal or human patient is as unresponsive to dantrolene sodium as it is in the decerebrate animal then in these also there should be no significant difference in the action of the two drugs in terms of their action on intrafusal muscle.

The effect of dantrolene sodium on extrafusal muscle is highly dependent upon the frequency of stimulation, with the action of the drug being minimal at high frequencies. At present there are no data on the effect of EU4093 on the tetanic contraction of extrafusal muscle in the anaesthetized rat. However in the anaesthetized and decerebrate rat the nervous system compensates for the effect of dantrolene sodium (Farquhar *et al.*, 1986; Farquhar & Part, 1988) only to a limited extent by increasing the frequency of discharge of the alpha motoneurones; more important are recruitment of additional motor units and modification of the patterns of discharge. It is possible therefore that dantrolene sodium and EU4093 would have a similar muscle relaxant action in the intact animal even if the effect of EU4093 on extrafusal muscle had a similar frequency dependency as that on intrafusal muscle.

In conclusion, it seems probable that EU4093 has a similar muscle relaxant action in the intact animal to dantrolene sodium despite the difference in the relation between the frequency of stimulation and the effect upon intrafusal muscle.

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Angiotensin neuromodulation of adrenergic and purinergic co-transmission in the guinea-pig vas deferens

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1 The effects of angiotensin II (AII) and angiotensin III (AIII) on the isolated vas deferens of the guinea-pig were studied via three parameters: the overflow of adenosine 5'-triphosphate (ATP) and tritiated noradrenaline (NA), the mechanical response to field stimulation and the mechanical response to exogenous NA and α,β-methylene ATP (α,β-mATP).

2 At 2 Hz, AII enhanced the overflow of ATP and NA, whereas at 20 Hz, AII enhanced the overflow of NA but was without significant effect on ATP overflow. AIII, at 2 Hz, inhibited the overflow of ATP, but enhanced NA overflow, whereas at 20 Hz ATP overflow was unaffected, but NA overflow was still enhanced.

3 At 2 Hz, AII enhanced both phases of the response to field stimulation and at 20 Hz the overall response. AIII at 2 Hz enhanced the adrenergic response, but was without effect on the purinergic response to field stimulation. At 20 Hz, AIII was without effect on the overall response.

4 AII enhanced responses to exogenous NA and α,β-mATP, whereas AIII was without effect.

5 These results provide evidence that both ATP and NA release are capable of being modulated by angiotensins. Furthermor , modulation of ATP release is frequency-dependent, whereas [^{3}H]-NA release is not. These results raise questions about the mechanisms of storage and release of the sympathetic co-transmitters NA and ATP; they also show that angiotensin receptors in the guinea-pig vas deferens are not a homogeneous population.

Introduction

The guinea-pig vas deferens has been widely used for the study of the action of co-transmitters. The neurogenic response of the vas deferens is biphasic, consisting of an initial twitch response and a slow second component. This second phase is adrenergic in nature (Swedin, 1971), whereas the twitch response has been shown to be non-adrenergic, non-cholinergic in nature and is due to the release of adenosine 5'-triphosphate (ATP) (Levitt *et al.*, 1984; Sneddon & Burnstock, 1984; Sneddon & Westfall, 1984; Stjärne & Åstrand, 1985; Kirkpatrick & Burnstock, 1987; Kasakov *et al.*, 1988). ATP and noradrenaline (NA) are stored together in storage vesicles of sympathetic nerve terminals (Lagercrantz & Stjärne, 1974; Lagercrantz, 1976; Fried *et al.*, 1978). ATP release in response to neurogenic stimulation of the guinea-pig vas deferens has been shown in a number of studies (Kirkpatrick & Burnstock, 1987; Lew & White, 1987; Kasakov *et al.*, 1988). ATP which is released originates from sympathetic nerve terminals and its release is blocked by tetrodotoxin (Kirkpatrick & Burnstock, 1987). ATP release from guinea-pig vas deferens has been shown to be of neuronal origin as release is not affected with the nerves intact and with the mechanical response blocked by α,β-methylene ATP (α,β-mATP) and prazosin (Kasakov *et al.*, 1988), nor is ATP release affected following depletion of NA by reserpine pretreatment, suggesting that ATP release is not secondary to NA release (Kirkpatrick & Burnstock, 1987).

In the early 1960s, angiotensin II (AII) was shown to facilitate adrenergic neurotransmission (McCubbin & Page, 1963; Benelli *et al.*, 1964). This was shown in the guinea-pig vas deferens to be a direct effect, only facilitating NA release when the nerves were actively firing (Bell, 1972). More recently, the pharmacological effects of AII, and its active

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metabolite, angiotensin III (des-Asp^1-angiotensin II) (AIII), have been studied on purinergic and adrenergic neurotransmission in the rabbit vas deferens (Trachte *et al.*, 1984; Trachte, 1988). The latter study drew conclusions on ATP and NA release by analysis of the mechanical response of the vas deferens to field stimulation.

The purpose of this study was to examine the action of AII and AIII on neurotransmission in the guinea-pig vas deferens by directly measuring the release of endogenous ATP and the release of radiolabelled NA. The actions of these substances on the mechanical response to field stimulation and the pharmacological response to exogenously added α,β-mATP and NA were also studied.

Methods

General

Male guinea-pigs (250–400 g) were stunned and exsanguinated. The prostatic portions of the vasa deferentia were excised and stripped of connective tissue. Following incubation (60 min) with [^{3}H]-NA, the isolated preparations (10–20 mg wet weight) were placed between pairs of platinum ring electrodes (15 mm apart) and were allowed to equilibrate, with one end tied to the holder and the other attached to a Grass force-displacement transducer (FTO3 C). Contractions were measured as changes in isometric tension by a Washington data recording system. The preparations, at an initial tension of 0.5–1 g, were superfused at a flow rate of 2.5 ml min^{-1} by use of a Watson-Marlow peristaltic pump with a modified Krebs solution (MKS) (mM: NaCl 133, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 2.5, NaH_2PO_4 1.4, $NaHCO_3$ 16.3, glucose 7.7 and ascorbic acid 0.1 and gassed continuously with 5% CO_2 in O_2 at 36°C). After a 90 min equilibration period the superfusate was collected, before, during and after stimulation, in polypropylene vials for subsequent determination of [^{3}H]-NA and ATP levels. The preparations were stimulated electrically twice, at 9 and 45 min for 240 pulses with trains of rectangular pulses of 0.1 ms duration, 50 V, and either at 2 or 20 Hz delivered by Grass SD9 stimulators. Perfusate was collected for periods corresponding to the length of the stimulus train. Therefore at 2 Hz the collection period was 120 s, whereas at 20 Hz the collection period was 12 s. Results are expressed as the ratio of responses to the second stimulation (S_2) versus responses to the first stimulation (S_1), that is the S_2/S_1 ratio. Control values for S_2/S_1 ratios were obtained when the second stimulation was carried out in the absence of any drug. Experimental data were obtained by the superfusion of the preparation with MKS containing the angiotensins 15 min before the second stimulation. This superfusion continued until the end of the experiment. Tissues from different animals were used to assess the effects of the angiotensins.

ATP overflow

ATP overflow from the sympathetic nerves supplying the vas deferens was assayed with the luciferin-luciferase technique described by Stanley & Williams (1969). Briefly, 50 mg of luciferin-luciferase was dissolved in 12.5 ml of sterile water and left in the dark for 2 h to equilibrate. Under these conditions the assay was capable of measuring ATP levels as low as 30 fmol. Prior to the experiment, a calibration curve for ATP was prepared to be used as a standard. Automatic injection of 200 μl of luciferin-luciferase solution to 100 μl of superfusate or standards produced bioluminescence which was measured by a Packard Picolite Luminometer. The MKS was assayed for ATP content. This was used as a blank and subtracted from each sample.

[^{3}H]-noradrenaline overflow

After preparation (described above), the tissues were incubated for 60 min in continuously gassed MKS containing 0.1 nM tritium-labelled noradrenaline ([^{3}H]-noradrenaline, New England Nuclear; specific activity 1624.3 GBq mmol^{-1}; two preparations in 5 ml medium) at 36°C. After rinsing five times with MKS, the preparations were placed between the stimulating electrodes and superfused to wash out the excess [^{3}H]-NA during the 90 min equilibration period. Samples (2.5 ml for 2 Hz and 0.25 ml for 20 Hz) were added in a ratio of 1:3 to Optiphase mp (LKB) scintillation cocktail and ^{3}H-radioactivity in the samples was counted in a Beckman 7500 liquid scintillation system. The superfusion was stopped immediately after the end of the experiment and the preparations were weighed and dissolved in 0.5 ml Optisolv (LKB) tissue solubilizer. The total tissue [^{3}H]-NA radioactivity was counted as described above. The results were calculated as the percentage fractional rate (FR%; mean $\pm$ s.e.mean) of the basal release and stimulation-evoked release of [^{3}H]-NA which were calculated according to Alberts *et al.* (1981). Previous investigations in rodent vas deferens have shown that [^{3}H]-NA is the major constituent of the total radioactivity released upon stimulation of the tissue (Beattie *et al.*, 1986; Kapocsi *et al.*, 1987). Hence the evoked efflux of [^{3}H]-NA reflected a quasi-physiological release of NA (Reichenbacher *et al.*, 1982).

Pharmacological study

Prostatic portions of the vas deferens were tied to tissue holders and placed in 5 ml organ baths containing MKS at 36°C and equilibrated for 90 min. After the equilibration period, either NA or α,β-mATP at bath concentrations of 30 μM and 1 μM respectively were added for short periods every 15 min. The tissues were washed as soon as a maximum response was reached to prevent desensitization. α,β-mATP was used as it is a stable analogue of ATP. After establishing control responses to agonists, the angiotensins were added 10 min before a further addition of NA or α,β-mATP. Results were calculated as the percentage response to each agonist relative to the previous response when no angiotensins were present.

Analysis of results

The effects of the angiotensins were assessed by the comparison of S_2/S_1 ratios for the experimental data versus S_2/S_1 ratios for the control data. The results for ATP overflow, [^{3}H]-NA overflow and mechanical responses were evaluated by Student's t test for unpaired observations. Results from the pharmacological study were evaluated by Student's t test for paired observations. In both cases, probability less than 0.05 was considered statistically significant.

Drugs

Angiotensin II, angiotensin III, noradrenaline, α,β-methylene ATP and luciferin-luciferase were obtained from Sigma. AII, AIII and α,β-mATP were first prepared as stock solutions in distilled water. NA was prepared in a 0.4% ascorbic acid solution to prevent oxidation. Luciferin-luciferase was prepared in sterile water.

Results

Field stimulation

Control responses The contractile response of the vas deferens to electrical field stimulation (EFS) at 2 Hz (stimulation period lasting 2 min) exhibited two distinct phases: an initial twitch response which reached its maximum in 2–3 s and then faded rapidly and a second slow phase which reached its maximum in 10–15 s and whose amplitude slowly decreased, but which lasted for the duration of the stimulation period. The second stimulation period, 30 min later, exhibited similar contractions but both phases were slightly reduced in amplitude (S_2/S_1 ratio for 1st phase was 0.81 ± 0.09, 2nd phase was 0.84 ± 0.06, $n = 6$) (Figure 3). During both stimulation periods there was a marked rise in [^{3}H]-NA and ATP overflow over basal levels. [^{3}H]-NA overflow was little affected during the second stimulation (S_2/S_1 ratio was 1.05 ± 0.06, $n = 6$) (Figure 2). However, ATP overflow during the second phase was reduced (S_2/S_1 ratio was 0.50 ± 0.06, $n = 6$) (Figure 1).

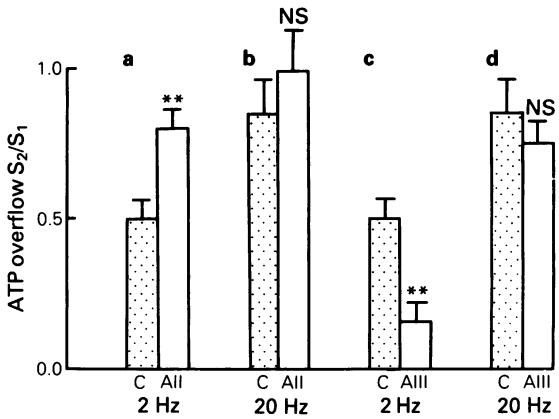

Figure 1 Effects of angiotensin II (AII) and angiotensin III (AIII) on the overflow of ATP from the guinea-pig vas deferens induced by electrical field stimulation. Alterations in the overflow of ATP are shown by comparing the control S_2/S_1 ratio when no drug is present throughout the experiment to the S_2/S_1 ratio obtained when the drug is added to the superfusate 15 min before the second stimulation. (a) Overflow at 2 Hz in the presence of AII (100 nM) compared to control (C, stippled columns). (b) Overflow at 20 Hz in the presence of AII (100 nM) compared to control. (c) Overflow at 2 Hz in the presence of AIII (100 nM) compared to control. (d) Overflow at 20 Hz in the presence of AIII (100 nM) compared to control. Each column is the mean of six observations and vertical lines indicate s.e.mean. Significance of drug effects were assessed by using Student's t test for unpaired observations (NS = not significant, $^{**}P < 0.01$).

At 20 Hz (stimulation period lasting 12 s) the distinction between the two phases was not clear. Therefore mechanical responses are given as the overall magnitude of the response and not divided into two distinct phases. The magnitude of the response elicited by the second stimulation was unchanged (S_2/S_1 ratio was 1.02 ± 0.03, $n = 6$) (Figure 3). Again during both stimulation periods there was a marked rise in [^{3}H]-NA and ATP overflow over basal levels. [^{3}H]-NA release elicited by stimulation was unchanged during the second stimulation (S_2/S_1 ratio was 0.95 ± 0.06, $n = 6$) (Figure 2). ATP overflow was only slightly diminished during the second stimulation (S_2/S_1 ratio was 0.85 ± 0.11, $n = 6$) (Figure 1).

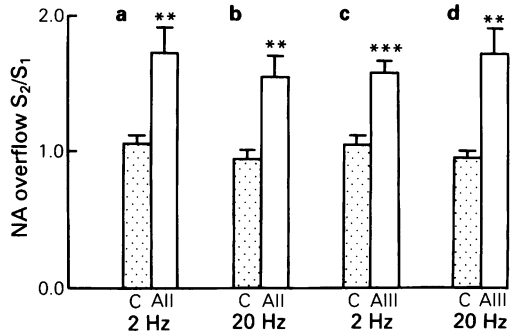

Figure 2 Effects of angiotensin II (AII) and angiotensin III (AIII) on the overflow of [^{3}H]-noradrenaline ([^{3}H]-NA) from the guinea-pig vas deferens induced by electrical field stimulation. Alterations in the overflow of [^{3}H]-NA are shown by comparing the control S_2/S_1 ratio when no drug is present throughout the experiment to the S_2/S_1 ratio obtained when the drug is added to the superfusate 15 min before the second stimulation. (a) Overflow at 2 Hz in the presence of AII (100 nM) compared to control (C, stippled columns). (b) Overflow at 20 Hz in the presence of AII (100 nM) compared to control. (c) Overflow at 2 Hz in the presence of AIII (100 nM) compared to control. (d) Overflow at 20 Hz in the presence of AIII (100 nM) compared to control. Each column is the mean of six observations and vertical lines indicate s.e.mean. Significance of drug effects were assessed by using Student's t test for unpaired observations (**$P < 0.01$, ***$P < 0.001$).

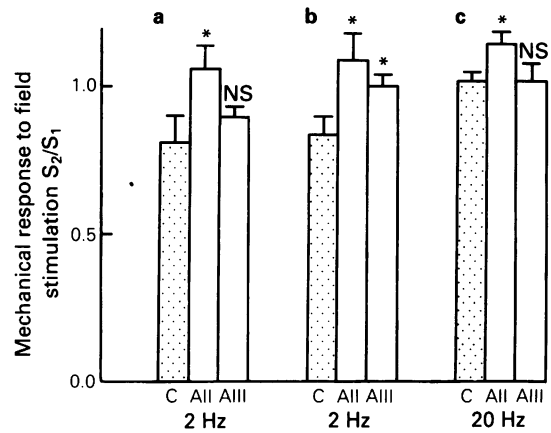

Figure 3 Effects of angiotensin II (AII) and angiotensin III (AIII) on the mechanical response of the guinea-pig vas deferens to electrical field stimulation. Alterations in the mechanical responses are shown by comparing the control S_2/S_1 ratio when no drug is present throughout the experiment to the S_2/S_1 ratio obtained when the drug is added to the superfusate 15 min before the second stimulation. (a) The purinergic response at 2 Hz in the presence of AII (100 nM) and AIII (100 nM) compared to control (C, stippled columns). (b) The adrenergic response at 2 Hz in the presence of AII (100 nM) and AIII (100 nM) compared to control. (c) The total response at 20 Hz in the presence of AII (100 nM) and AIII (100 nM) compared to control. Each column is the mean of at least six observations and vertical lines indicate s.e.mean. Significance of drug effects were assessed by Student's t test for unpaired observations (NS = not significant, *$P < 0.05$).

Angiotensin II The addition of angiotensin II (AII) (100 nM) to the superfusate 15 min before the second stimulation had no effect on the baseline tension of the preparations. At 2 Hz, when compared to the control values, AII enhanced both phases of the response. The S_2/S_1 ratio for the purinergic phase of the response was 1.06 ± 0.07 ($n = 8$) (Figure 3a) and for the adrenergic phase it was 1.09 ± 0.09 ($n = 6$) (Figure 3b). AII at this frequency also enhanced, relative to the second control stimulation, the stimulus-evoked overflow of both [^{3}H]-NA (S_2/S_1 ratio was 1.72 ± 0.19, $n = 6$) (Figure 2a) and of ATP (S_2/S_1 ratio was 0.80 ± 0.06, $n = 6$) (Figure 1a). At 20 Hz, AII enhanced the magnitude of the response (S_2/S_1 ratio was 1.15 ± 0.04, $n = 6$) (Figure 3c). At this frequency, AII enhanced the overflow of [^{3}H]-NA (S_2/S_1 ratio was 1.54 ± 0.16, $n = 6$) (Figure 2b); however, compared to controls it was without significant effect on ATP overflow (S_2/S_1 ratio was 0.99 ± 0.13, $n = 6$) (Figure 1b).

Angiotensin III Angiotensin III (AIII) (100 nM) did not affect the baseline tension of the preparations. At 2 Hz, compared to controls, AIII significantly enhanced the adrenergic phase of the response (S_2/S_1 ratio was 1.00 ± 0.04, $n = 6$) (Figure 3b); however, it was without significant effect on the fast initial purinergic phase (S_2/S_1 ratio was 0.90 ± 0.03, $n = 6$) (Figure 3a). At this frequency, AIII enhanced the stimulus-evoked overflow of [^{3}H]-NA (S_2/S_1 ratio was 1.58 ± 0.09, $n = 6$) (Figure 2c), whereas it decreased the overflow of ATP in response to EFS (S_2/S_1 ratio was 0.16 ± 0.06, $n = 6$) (Figure 1c). At 20 Hz, relative to control values AIII did not alter the mechanical response of the tissue to EFS (S_2/S_1 ratio was 1.02 ± 0.06, $n = 6$) (Figure 3c). At this frequency AIII enhanced the stimulus-evoked overflow of [^{3}H]-NA to EFS (S_2/S_1 ratio was 1.72 ± 0.19, $n = 6$) (Figure 2d). It was however, compared to controls, without significant effect on ATP overflow (S_2/S_1 ratio was 0.75 ± 0.07, $n = 6$) (Figure 1d).

Pharmacological responses

Responses to exogenously added NA (30 μM) and α,β-mATP (1 μM) did not show tachyphylaxis provided the tissues were washed immediately the

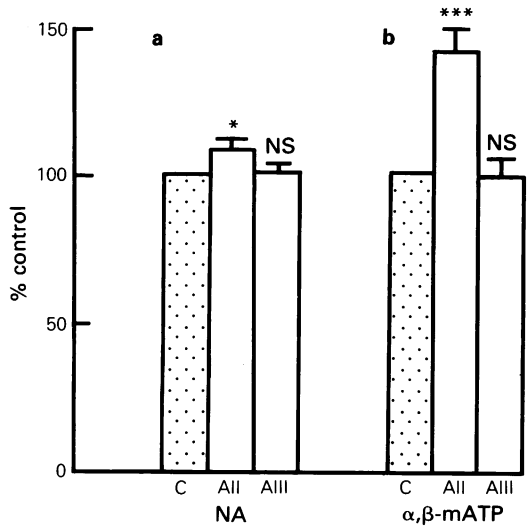

Figure 4 Effects of angiotensin II (AII) and angiotensin III (AIII) on the response of guinea-pig isolated vas deferens to noradrenaline (NA) and exogenous α,β-methylene ATP (α,β-mATP). Responses to drugs are compared to the control value (C, stippled columns). (a) Responses to NA in the presence of AII (100 nM) and AIII (100 nM). (b) Responses to α,β-mATP in the presence of AII (100 nM) and AIII (100 nM). Each column is the mean of six observations and vertical lines indicate s.e.mean. Significance of drug effects were assessed by Student's *t* test for paired observations (NS = not significant, $*P < 0.05$, $***P < 0.001$).

responses reached their maximum. AII (100 nM) added 10 min before the addition of NA produced a slight increase in the response to a value 109 ± 3% of the control level. It produced a large increase in the response to α,β-mATP (141 ± 7% of the control value). AIII (100 nM) did not significantly affect responses to either NA or α,β-mATP, respective values being 102 ± 4 and 98 ± 6% of the control responses (Figure 4).

Discussion

Interpretation of these results should take into account pre- and postjunctional interactions of NA, ATP and adenosine, resulting from ecto-enzymatic breakdown of ATP. There is also the possibility of an interaction with the neuromodulator neuropeptide Y (NPY). NPY is known to coexist with NA and ATP (Fried *et al.*, 1985; Stjärne *et al.*, 1986) and to be released along with NA and ATP upon stimulation of sympathetic nerves of the guinea-pig vas deferens (Kasakov *et al.*, 1988). NPY has a dual function; postjunctionally at low concentrations potentiating NA- and ATP-mediated responses and at higher concentrations producing prejunctional inhibition of release of NA and ATP (Stjärne & Lundberg, 1986). It is therefore possible that the angiotensins, by affecting the release of NPY, may have subsequent effects on NA and ATP release and their postjunctional effects. Confirmation of any involvement of NPY will have to await a study on the effect of angiotensins on NPY release. There is also a possible interaction with the autoinhibition by adenosine and NA itself. Adenosine is known to inhibit the release of both ATP and NA (Sneddon *et al.*, 1984), whereas NA inhibits its own release (see Starke, 1987).

The biphasic nature of the response and the concomitant release of both [^{3}H]-NA and endogenous ATP confirms previous studies that the response of this tissue to field stimulation is due to NA and ATP acting as co-transmitters (see Burnstock, 1982; Burnstock, 1985). Many investigations into the action of angiotensins on sympathetic nerve stimulation in the vas deferens (Hughes & Roth, 1971; Bell, 1972; Johnson *et al.*, 1974) were carried out before the concept of co-transmission was raised (Burnstock, 1976). These investigations therefore were only concerned with the enhancement/inhibition of adrenergic transmission. More recently some experiments have been carried out on the effects of angiotensins on both purinergic and adrenergic components of the rabbit vas deferens to sympathetic nerve stimulation (Trachte *et al.*, 1984). However, these studies deduced angiotensin effects on release by the indirect method of analysing pharmacological studies which have the inherent problems of pre- and postjunctional interactions. Neither in this paper nor in a later study (Trachte, 1988) was ATP release measured, either by direct measurement of endogenous ATP by the luciferin-luciferase technique (Stanley & Williams, 1969) or by the indirect method of labelling with [^{3}H]-adenosine. This latter method however, cannot exclude uptake and release from non-neuronal as well as neuronal sources. By directly measuring endogenous ATP release in the present study, this problem has been overcome.

Purinergic transmission

Against low-frequency stimulation (2 Hz), angiotensins differed in their effects on ATP overflow. They also differed in that AII enhanced responses of the agonists postjunctionally, whereas AIII was without effect. However, at high frequency (20 Hz) the angiotensins were without significant effect on ATP overflow. The purinergic component of the mechanical response was enhanced by AII but unaffected by AIII at 2 Hz. This enhancement of the

mechanical response by AII is to be expected, as AII enhances both the overflow of ATP and the action of ATP on its postjunctional receptors. Surprisingly, AIII does not reduce the mechanical response, although it inhibits the overflow of ATP and is without postjunctional effects. At present this observation is difficult to explain. Our results show some similarity to recent studies carried out in the rabbit vas deferens (Saye *et al.*, 1986; Trachte, 1988). The latter study, as in the present study, showed that at 2 Hz the angiotensins enhanced the adrenergic phase of the response. However the reported inhibition of the purinergic phase by both of the angiotensins was not repeated in this study, although AIII has been shown to inhibit the overflow of ATP. Saye *et al.* (1986) reported that at low frequencies AII potentiated both phases of the response as is seen in the present study, whereas AIII inhibited both phases of the response. Interestingly, Saye *et al.* (1986) found that the inhibition by AIII was turned into a potentiation in the presence of indomethacin. As both studies mentioned used the same tissue and stimulation parameters it is difficult to explain the discrepancy. We conclude that AII and AIII have opposing actions on ATP release at low frequency. This is probably due to an action at different angiotensin receptors situated prejunctionally (Trachte *et al.*, 1984; Trachte, 1988). Moreover, angiotensin neuromodulation is frequency-dependent, as at high frequencies the angiotensins do not affect ATP release. These results also provide evidence for receptors located postjunctionally for the angiotensins. At these receptors AII markedly enhances the responses to α,β-mATP and slightly to NA, whereas AIII would seem to have no affinity for these receptors.

Adrenergic transmission

Both angiotensins enhance [^{3}H]-NA overflow at both low and high frequencies. AII enhances slightly the response to exogenous NA, whereas AIII has no effect. Mechanical responses to field stimulation at 2 Hz bear out this enhancement. However, the response at 20 Hz, which is due to both purinergic and adrenergic components, shows that AII enhances the mechanical response to field stimulation, whereas AIII has no effect. As neither angiotensin affects ATP overflow, at this frequency, the mechanical response will be due to the actions of the angiotensins on NA overflow and their postjunctional effects if any. At 20 Hz, it is likely that more NA is being released than is required to produce the maximal response. Therefore the enhancement seen by AII is probably due to the postjunctional enhancement of ATP and NA action, whereas AIII does not have any effects postjunctionally. The angiotensins have been shown to facilitate adrenergic transmission in a variety of tissues and species (Magnan & Regoli, 1979; Boke & Malik, 1983; Trachte, 1988) and at low and high frequencies (Zimmerman *et al.*, 1972). By measuring [^{3}H]-NA release, our study shows that facilitation of adrenergic transmission by angiotensins can be attributed to an increase in the amount of NA released and is not due to a postjunctional action.

The lack of effect of the angiotensins on the resting tension suggests that the angiotensins, at least at the concentrations used, are without direct effect on the smooth muscle of the vas deferens.

The reason for the disparate effects of angiotensins on ATP and NA release is unclear. It is possible that in the vas deferens there are nerves that are predominantly purinergic and those that are predominantly adrenergic. This hypothesis could also be used to explain the observation that adrenergic transmission is predominant at the epidymal end and purinergic transmission is predominant at the prostatic end of the rodent vas deferens (McGrath, 1978; Rohde *et al.*, 1986). On the other hand, NA and ATP are known to be stored in small dense-cored vesicles and in large vesicles (Lagercrantz & Stjärne, 1974; Lagercrantz, 1976; Fried *et al.*, 1978), and so a neuromodulator may alter release from one type of vesicle in one way and the release from another type in another. Also the ratio of small vesicles to large vesicles may vary, as may the ratio of catecholamine to ATP in the vesicles within different nerves or even within the same nerve terminal. Large vesicles may contain both ATP and NA or there may be vesicles containing only NA and vesicles containing only ATP, as has been suggested for NPY and NA storage (Fried *et al.*, 1985).

In summary, angiotensins were found to have differing actions at low frequency and no effect at high frequency on purinergic transmission in the guinea-pig vas deferens. In contrast, both angiotensins enhance adrenergic transmission at low and high frequency. This action is predominantly prejunctional as shown by the direct method of measurement of release of transmitters, although AII also facilitates transmission postjunctionally. The different actions of the angiotensins suggests that they are acting prejunctionally on different receptor subtypes, with different affinities for AII and AIII. The opposing effects on purinergic and adrenergic transmission by the angiotensins suggest that ATP and NA storage and/or release are not similar in the guinea-pig vas deferens. This is borne out by the finding that ATP neuromodulation by angiotensins is frequency-dependent, whereas NA modulation is not. Our results provide the first direct evidence of the effects of angiotensins on both ATP and NA release and the role of frequency of stimulation on neuromodulation.

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Analysis of the relaxant effects of AH 21–132 in guinea-pig isolated trachealis

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1 Experiments have been performed with the dual intent of analysing the mechanism by which AH 21–132 relaxes airways smooth muscle and determining whether the effects of this compound can be distinguished from those of theophylline.

2 AH 21–132 (0.25–8 μM) and theophylline (1–1000 μM) each caused concentration-dependent suppression of the spontaneous tone of guinea-pig isolated trachealis. The maximal effect of AH 21–132 was equivalent to that of theophylline. No evidence was obtained that the tissue became sensitized or desensitized to the action of AH 21–132.

3 Propranolol (1 μM) profoundly antagonized the tracheal relaxant action of isoprenaline but not that of AH 21–132.

4 In indomethacin (2.8 μM)-treated tissues, tone was induced by K^+-rich (120 mM) Krebs solution, acetylcholine (ACh, 1 mM) or histamine (200 μM). Log concentration-relaxation curves for AH 21–132, isoprenaline and theophylline were all moved to the right in the presence of the spasmogens, the smallest rightward shift being induced by histamine and the greatest by ACh. While maximal effects of AH 21–132 and theophylline were unaffected by the spasmogens, that of isoprenaline was reduced by KCl and ACh.

5 In tissues treated with indomethacin (2.8 μM), AH 21–132 (0.1–100 μM) inhibited the spasmogenic effects of potassium chloride (KCl), ACh and histamine in a concentration-dependent manner. The inhibition was characterized by rightward shifts in the spasmogen concentration-effect curves with depression of their maxima.

6 In tissues treated with both indomethacin (2.8 μM) and ACh (1 mM), the removal of tracheal epithelium caused a small but significant leftward shift in the log concentration-relaxation curve for AH 21–132 but did not alter that for theophylline.

7 In tissues treated with indomethacin (2.8 μM) and maintained at 12°C, theophylline (0.1–3.2 mM) caused concentration-dependent spasm. This effect was not shared by AH 21–132.

8 AH 21–132 (0.1–1000 μM) more potently inhibited the activity of cyclic AMP-dependent than of cyclic GMP-dependent phosphodiesterase derived from homogenates of guinea-pig trachealis. Theophylline, too, inhibited these enzymes but was less potent in each case than AH 21–132 and did not exhibit selectivity for the cyclic AMP-dependent enzyme.

9 It is concluded that AH 21–132 exerts a non-specific (i.e. effective no matter what agent is used to support tone) relaxant effect on the trachealis muscle which does not involve the activation of β-adrenoceptors. The profile of the relaxant action of AH 21–132 more closely resembles that of theophylline than that of isoprenaline. However, AH 21–132 can be differentiated from theophylline in that: (a) its relaxant potency is increased by epithelial removal; (b) it does not cause tracheal spasm; (c) it exhibits selectivity as an inhibitor of cyclic AMP-dependent as opposed to cyclic GMP-dependent phosphodiesterase. It is possible that the relaxant effects of AH 21–132 are related to its ability to inhibit cyclic nucleotide phosphodiesterases.

Introduction

The pharmacological properties of (±)-*cis*-6-(*p*-acetamidophenyl)-1,2,3,4,4a,10b-hexahydro-8,9-dimethoxy-2-methylbenzo-[c] [1,6]naphthyridine (AH 21–132) have, as yet, been the subject only of brief reports. However, this agent has been shown to relax both human and guinea-pig airways smooth muscle

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in vitro by a mechanism not involving the activation of β-adrenoceptors (Bewley & Chapman, 1988). In anaesthetized guinea-pigs AH 21–132 has also been shown to reduce the airways hyper-reactivity to bombesin which follows the intravenous infusion either of platelet activating factor (Paf) or isoprenaline (Kristersson *et al.*, 1988). Furthermore, AH 21–132 decreases Paf-induced migration of eosinophils into the lumen of guinea-pig airways (Aoki *et al.*, 1988).

Inhibition of Paf or isoprenaline-induced airways hyper-reactivity and inhibition of Paf-induced eosinophil migration may indicate that AH 21–132 should exert prophylactic activity in asthma. Accordingly it has been proposed that AH 21–132 might prove useful in the therapy of asthma both by virtue of its bronchodilator activity and of its ability to inhibit inflammatory processes in the lung which lead to the development of airways hyper-reactivity (Aoki *et al.*, 1988; Bewley & Chapman, 1988; Kristersson *et al.*, 1988).

The experiments of the present study were designed to analyse the mechanism by which AH 21–132 evokes relaxation of isolated airways smooth muscle and to determine whether the pharmacological profile of AH 21–132 acting on airways smooth muscle could be differentiated from that of methylxanthines, such as theophylline.

Methods

Guinea-pigs (350–700 g) of either sex were killed by stunning and bleeding. Tracheae were excised, cleaned of adhering adipose and connective tissue and opened by cutting longitudinally through the cartilage rings diametrically opposite the trachealis. Small segments of trachea were set up for the isometric recording of tension changes as previously described (Foster *et al.*, 1983).

Effects of relaxant agents against the spontaneous tone of the trachea

At the outset of each experiment, tissues were subjected to an imposed tension of 1 g. After 20 min, aminophylline (1 mM) was added in order to establish the recorder pen position at zero tone. The aminophylline was subsequently washed from the tissue and, when tone was fully regained, study of the relaxant drugs commenced.

Relaxant drugs were studied by the construction of cumulative concentration-effect curves. Two fold (AH 21–132) or ten fold (isoprenaline, theophylline) concentration increments were made at intervals of 4 (isoprenaline), 5 (theophylline) or 8 (AH 21–132) min. Each tissue was challenged with AH 21–132 and either isoprenaline or theophylline. When propranolol (1 μM) was used as an antagonist it was added to the Krebs solution 15 min before the construction of the second concentration-effect curves of AH 21–132 and isoprenaline. Control tissues were run concurrently with test tissues but were not exposed to propranolol.

Effects of relaxants against tone induced by ACh, K^+-rich Krebs solution or histamine

In these experiments the tissue was challenged with only one of AH 21–132, isoprenaline or theophylline. An initial log concentration-effect curve for the relaxant was constructed essentially as described above. Indomethacin (2.8 μM) was then added to the Krebs solution to suppress spontaneous tone. Tone was subsequently induced by tissue exposure to ACh (1 mM), K^+-rich (120 mM) Krebs solution or histamine (200 μM). The log concentration-effect curve for the relaxant drug was then reconstructed in presence both of indomethacin and the tone-inducing agent.

Effects of AH 21–132 on the log concentration-effect curves of spasmogens

These experiments were carried out in the presence of indomethacin (2.8 μM). In each tissue a cumulative concentration-effect curve for one of ACh, KCl or histamine was constructed. Two fold (KCl) or ten fold (ACh, histamine) concentration increments were made at intervals of 4 (ACh), 6 (histamine) or 12 (KCl) min. Following construction of the initial spasmogen concentration-effect curve, AH 21–132 (1 μM) was added to the Krebs solution bathing test tissues, 15 min later, the spasmogen concentration-effect curve was reconstructed in the presence of AH 21–132. Subsequent curves were constructed in the presence of AH 21–132 (10, 100 and 1000 μM). Control tissues were run concurrently but were not exposed to AH 21–132.

Effects of epithelial removal on the relaxant actions of AH 21–132 and theophylline

In these experiments a log concentration-relaxation curve was constructed either for AH 21–132, or for theophylline, in the presence of both indomethacin (2.8 μM) and ACh (1 mM). Such curves were constructed both in tissues where the epithelium was intact and in tissues where epithelium had been removed (confirmed by histological examination) by rubbing with a wooden stick.

Assessment of the ability of theophylline and AH 21–132 to cause tracheal spasm

These experiments were carried out on tracheae treated with indomethacin (2.8 μM) and maintained

at 12°C, essentially as described by Small *et al.* (1988). In each tissue the spasmogenic activity of AH 21-132 (0.1-1000 μM) or theophylline (0.1-3.2 mM) was assessed by constructing a sequential concentration-effect curve. Two fold (theophylline) or ten fold (AH 21-132) concentration increments were used and 15 min tissue contact was allowed for each concentration. Each tissue was finally challenged with 10 mM caffeine and spasmogenic responses were expressed in terms of the response to the caffeine standard.

Assessment of cyclic nucleotide phosphodiesterase inhibitory activity

Muscle-rich strips of trachea were prepared as previously described (Foster *et al.*, 1983). Tracheal strips from two animals were combined and homogenized with an Ilado laboratory disperser and 10 volumes of cold medium containing 100 mM Tris-HCl, 2 mM $MgCl_2$ and 1 mM dithiothreitol (pH 7.5). The homogenate was used within 30 min of preparation.

Cyclic AMP- and cyclic GMP-phosphodiesterase activity in the homogenate was measured by a modification of the methods of Thompson & Appleman (1971) and Rutten *et al.* (1973). The homogenate was diluted 1 in 20 and 25 μl was assayed for enzyme activity in a final volume of 100 μl containing 40 mM Tris-HCl (pH 8.0), 2.5 mM $MgCl_2$, 3.75 mM β-mercaptoethanol, 0.1 unit calmodulin (phosphodiesterase activator), 10 μM $CaCl_2$ and either 1 μM cyclic AMP with 0.2μCi [^{3}H]-cyclic AMP or 1 μM cyclic GMP with 0.2μCi [^{3}H]-cyclic GMP. In tests of enzyme inhibition the reaction mixture contained various concentrations of AH 21-132 or theophylline as required.

The reagents and homogenate were mixed on ice and the reaction was initiated by transferring the mixture to a water bath at 37°C. Following 30 min incubation the reaction was stopped by transferring the reaction vessel to a bath of boiling water for 3 min. After cooling, 20 μl of a 1 mg ml^{-1} solution of *Ophiophagus hannah* venom was added to the reaction mixture and the mixture was incubated for 10 min. Unreacted [^{3}H]-cyclic AMP or [^{3}H]-cyclic GMP was removed by passing the reaction mixture through a 0.5 ml column (Bio-Rad Econocolumn) of Dowex 1 x 8 (Cl^-) equilibrated with 1 M HCl. The column was washed with 5 ml 0.1 M $NaHCO_3$ and the radioactivity in the total effluent was determined by liquid scintillation counting. Less than 10% of the tritiated cyclic nucleotide was hydrolysed in any assay.

Drugs, solutions and statistical analysis of results

Drug concentrations are expressed in terms of the molar concentration of the active species. The following substances were used: acetylcholine chloride (Sigma), AH 21-132 (Sandoz AG), aminophylline (BDH), caffeine (Sigma), histamine acid phosphate (Sigma), indomethacin (Sigma), (-)-isoprenaline hydrochloride (Sigma), potassium chloride (Hopkin & Williams), (±)-propranolol (ICI), theophylline (Sigma).

Stock solutions of ACh and indomethacin were prepared in absolute ethanol. A stock solution of (-)-isoprenaline was prepared in 0.1 M HCl and dilutions prepared from this stock contained ascorbic acid (0.57 mM) as an antioxidant. Stock solutions of all other drugs were prepared in twice-distilled water.

The Krebs solution used in all the experiments had the following composition (mM): Na^+ 143.5, K^+ 5.9, Ca^{2+} 2.6, Mg^{2+} 1.2, Cl^- 127.6, HCO_3^- 25, SO_4^{2-} 1.2, $H_2PO_4^-$ 1.2 and glucose 11.1. The K^+-rich Krebs solution was of identical osmolality to Krebs solution and had the following composition (mM): Na^+ 26, K^+ 120, Ca^{2+} 2.6, Mg^{2+} 1.2, Cl^- 125, HCO_3^- 25, SO_4^{2-} 1.2, $H_2PO_4^-$ 1.2 and glucose 11.1.

The significance of differences between means was assessed by use of either a one-tailed or a two-tailed unpaired t test. The null hypothesis was rejected when $P < 0.05$.

Results

Effects of relaxant agents against the spontaneous tone of the trachea

AH 21-132 (0.25-8 μM) and theophylline (1-1000 μM) each caused concentration-dependent suppression of the spontaneous tone of guinea-pig isolated trachea. AH 21-132 was the more potent relaxant agent (Table 1) but shared the same maximum effect as theophylline. Repeated log concentration-effect curves of AH 21-132 and theophylline remained constant in shape and position (Figure 1) suggesting that tachyphylaxis developed to neither agent nor did either agent sensitize the tissue to its own action.

Isoprenaline (0.001-1 μM), too, caused concentration-dependent suppression of tracheal tone. While isoprenaline was profoundly antagonized by propranolol (1 μM) the action of AH 21-132 was unaffected by blockade of β-adrenoceptors (Figure 2).

Effects of relaxant agents against tone induced by ACh, K^+-rich Krebs solution or histamine

In the presence of indomethacin (2.8 μM) and a tone-inducing agent such as ACh (1 mM), K^+-rich (120 mM) Krebs solution or histamine 200 μM, the log concentration-relaxation curves of AH 21-132, iso-

Table 1 The influence of various tone-inducing agents on the relaxant potency of isoprenaline, theophylline and AH 21–132 in guinea-pig isolated trachealis

	Spontaneous tone	*Histamine* (200 μM)	*KCl* (120 mM)	*ACh* (1 mM)
Isoprenaline	7.69 ± 0.05	7.66 ± 0.06	6.64 ± 0.07*	6.37 ± 0.08*
Theophylline	4.15 ± 0.05	3.48 ± 0.03*	3.49 ± 0.04*	2.93 ± 0.07*
AH 21–132	5.55 ± 0.07	5.39 ± 0.09	4.64 ± 0.05*	4.09 ± 0.04*

Data are mean pD_2 values ± s.e.mean derived from at least 6 observations. * indicates a significant difference from the corresponding value under conditions of spontaneous tone. Where histamine, KCl or ACh were used to induce the tone against which the relaxant drugs were tested, the Krebs solution also contained indomethacin (2.8 μM).

prenaline and theophylline were all moved to the right (Figures 3, 4 and 5). However, the extent of the rightward shift was dependent on the tone-inducing agent. In general the smallest rightward shift was induced by histamine and the greatest by ACh. In the case of isoprenaline and AH 21–132, histamine failed to decrease significantly the mean pD_2 value of the relaxant agent (Table 1).

While the maximal effects of AH 21–132 and theophylline were unaffected by the various tone inducing agents, the maximal relaxant effect of isoprenaline was depressed when either ACh or the K^+-rich solution was used as the tone-inducing agent (Figure 5).

Effects of AH 21–132 on the log concentration-effect curves of spasmogens

In tissues treated with indomethacin (2.8 μM), AH 21–132 (0.1–100 μM) caused concentration-dependent depression of the log concentration-effect curves of KCl, ACh and histamine (Figure 6).

Effects of epithelial removal on the relaxant actions of AH 21–132 and theophylline

AH 21–132 evoked concentration-dependent relaxation of trachealis treated both with indomethacin (2.8 μM) and with ACh (1 mM). This held true both for

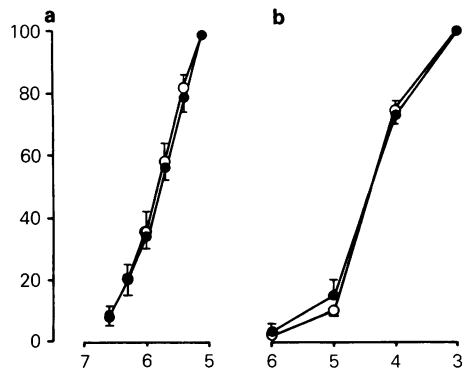

Figure 1 Suppression of the spontaneous tone of guinea-pig isolated trachealis by AH 21–132 (a) and by theophylline (b). Abscissae: −log molar concentration of AH 21–132 (a) or theophylline (b). Ordinate scales: relaxation expressed as a percentage of the maximal relaxation induced by theophylline. (●) indicates the initial and (○) the second concentration-effect curve for each agonist, the two curves being constructed in the same tissues. Each data point indicates the mean value derived from at least 6 experiments, vertical bars show s.e.mean.

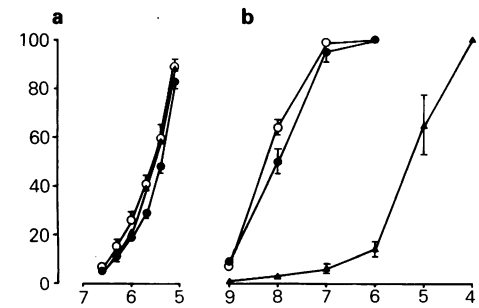

Figure 2 Suppression of the spontaneous tone of guinea-pig isolated trachealis by AH 21–132 (a) and by isoprenaline (b): the influence of propranolol. Abscissae: −log molar concentration of AH 21–132 (a) or isoprenaline (b). Ordinate scale: relaxation expressed as a percentage of the maximal relaxation induced by isoprenaline. (●) pooled initial log concentration-effect curve; (○) subsequent log concentration-effect curve obtained in control tissues; (▲) subsequent log concentration-effect curve obtained in test tissues treated with 1 μM propranolol. Each data point indicates the mean of values derived from at least 6 experiments; vertical bars show s.e.mean.

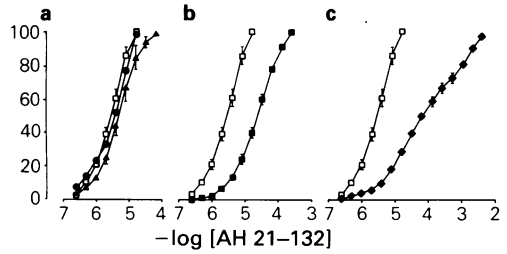

Figure 3 Guinea-pig isolated trachealis: the effects of some tone-inducing agents on the relaxant action of AH 21-132. Abscissae: −log molar concentration of AH 21-132. Ordinate scales: relaxation expressed as a percentage of the maximal relaxation induced by aminophylline. The pooled initial log concentration-effect curve (●) and the subsequent control log concentration-effect curve (□) were constructed against spontaneous tone. In test tissues subsequent log concentration-effect curves were constructed in the presence of 2.8 μM indomethacin and either 200 μM histamine (▲) (a), 120 mM KCl (■) (b) or 1 mM ACh (◆) (c). Each data point indicates the mean of values derived from at least 6 experiments; vertical bars show s.e.mean.

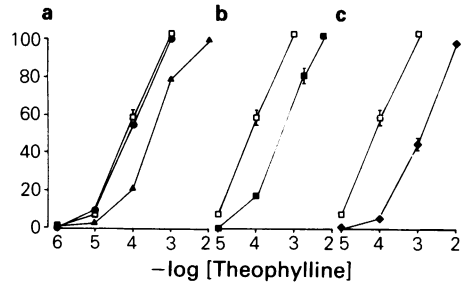

Figure 4 Guinea-pig isolated trachealis: the effects of some tone-inducing agents on the relaxant action of theophylline. Abscissae: −log molar concentration of theophylline. Ordinate scales: relaxation expressed as a percentage of the maximal relaxation induced by aminophylline. The pooled initial log concentration-effect curve (●) and subsequent control log concentration-effect curve (□) were constructed against spontaneous tone. In test tissues, subsequent log concentration-effect curves were constructed in the presence of 2.8 μM indomethacin and 200 μM histamine (▲) (a), 120 mM KCl (■) (b) or 1 mM ACh (◆) (c). Each data point indicates the mean ± s.e.mean of values derived from at least 6 experiments.

tissue with intact epithelium and for tissue where the epithelium had been rubbed away. Removal of the epithelium, however, caused a small but significant leftward shift of the concentration-effect curve of AH 21-132 (Figure 7). The mean (±s.e.mean) pD_2 values for AH 21-132 in rubbed and intact tissues were 4.34 ± 0.05 and 4.07 ± 0.05 respectively. Theophylline, too, relaxed trachealis treated both with indomethacin (2.8 μM) and with ACh (1 mM). However, removal of the tracheal epithelium did not cause sig-

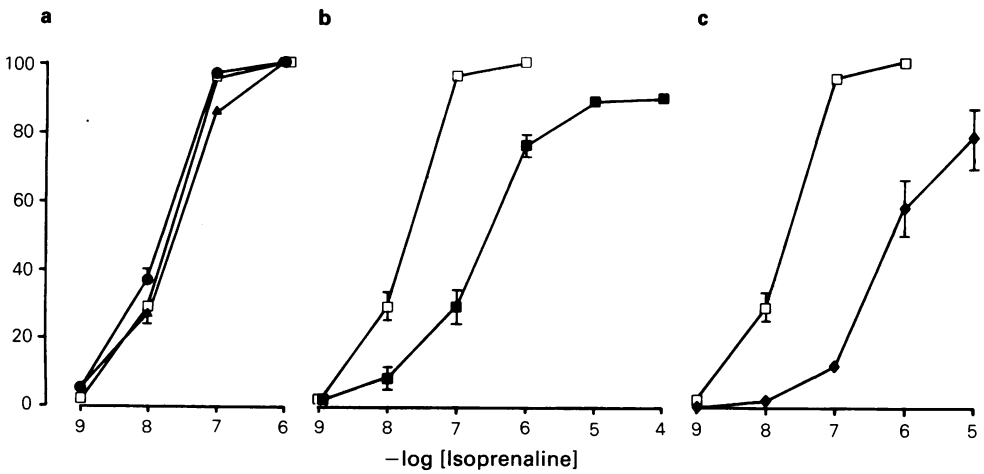

Figure 5 Guinea-pig isolated trachealis: the effects of some tone-inducing agents on the relaxant action of isoprenaline. Abscissae: −log molar concentration of isoprenaline. Ordinate scales: relaxation expressed as a percentage of the maximal relaxation induced by aminophylline. The pooled initial log concentration-effect curve (●) and subsequent control log concentration-effect curves (□) were constructed against spontaneous tone. In test tissues subsequent log concentration-effect curves were constructed in the presence of 2.8 μM indomethacin and 200 μM histamine (▲) (a), 120 mM KCl (■) (b) or 1 mM ACh (◆) (c). Each data point indicates the mean of values derived from at least 6 experiments; vertical bars show s.e.mean.

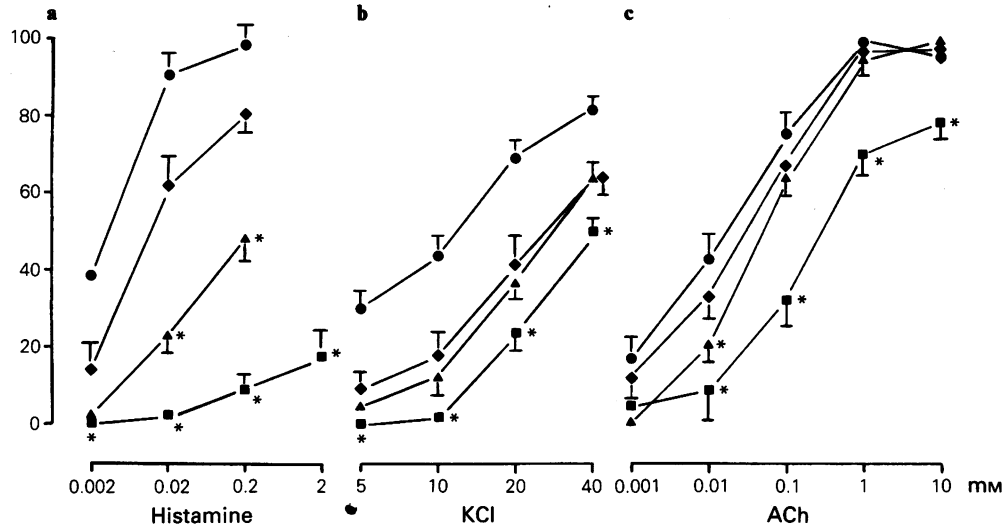

Figure 6 Guinea-pig isolated trachealis: the effects of AH 21–132 against log concentration-spasm curves for histamine (a), KCl (b) and ACh (c). Abscissae: concentration (mM) of spasmogen (log scale). Ordinate scales: spasm evoked expressed as a percentage of the maximal spasm evoked by ACh. (●) indicates initial control log concentration-effect curve for the spasmogen; (◆), (▲) and (■) represent curves obtained after tissue equilibration with AH 21–132 0.1, 1 and 10 μM respectively. Indomethacin (2.8 μM) was present throughout. Each data point indicates the mean of values derived from at least 6 experiments. * indicates a significant ($P < 0.05$) reduction in response compared with the corresponding response in time-matched control tissues.

nificant shift in the log concentration-effect curve of theophylline (Figure 7). The pD_2 values for theophylline in rubbed and intact tissues were 2.82 ± 0.03 and 2.81 ± 0.03 respectively.

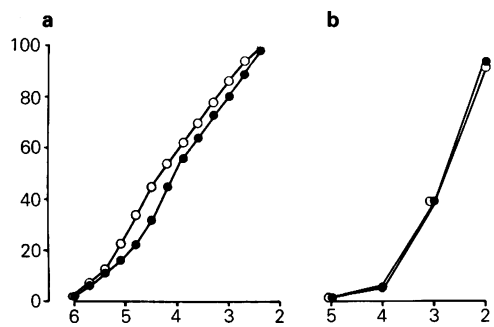

Figure 7 Guinea-pig trachealis treated with indomethacin (2.8 μM) and ACh (1 mM): the influence of epithelial removal on the relaxant actions of AH 21–132 and theophylline. Abscissae: −log molar concentration of AH 21–132 (a) or theophylline (b). Filled symbols (●) indicate tissues with intact epithelium, open symbols (○) indicate tissues denuded of epithelium. Each data point indicates the mean of values derived from at least 6 experiments. Standard error bars largely fall within the plotted symbol size and hence are not included.

Assessment of the ability of theophylline and AH 21–132 to cause tracheal spasm

In tissue treated with indomethacin (2.8 μM) and maintained at 12°C, theophylline produced concentration-dependent spasm as previously reported (Small *et al.*, 1988). The maximal effect of theophylline closely approached the (maximal) effect of the caffeine (10 mM) standard. In contrast AH 21–132 (0.1–1000 μM) was devoid of spasmogenic activity under these conditions (Figure 8).

Assessment of cyclic nucleotide phosphodiesterase inhibitory activity

Measured with 1 μM cyclic nucleotide as substrate in the presence of Ca^{2+} and calmodulin, the muscle-rich strips of trachea contained phosphodiesterase activity equivalent to the hydrolysis of 1.8 ± 0.2 nmol cyclic AMP g^{-1} min^{-1} or 2.8 ± 0.2 nmol cyclic GMP g^{-1} min^{-1}.

AH 21–132 and theophylline each caused concentration-dependent inhibition of the hydrolysis of cyclic AMP and cyclic GMP. AH 21–132 was the more potent PDE inhibitor in each case and, in contrast to theophylline, exhibited selectivity as an inhibitor of cyclic AMP-PDE as opposed to cyclic GMP-PDE (Table 2 and Figure 9).

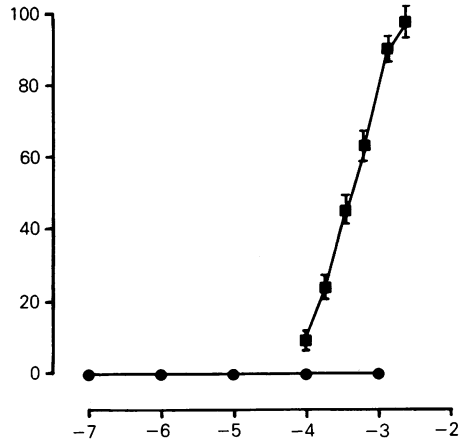

Figure 8 The spasmogenic activity of theophylline and AH 21-132 in guinea-pig isolated trachealis treated with indomethacin (2.8 μM) and maintained at 12°C. Abscissa scale: molar concentration of theophylline (■) or AH 21-132 (●) (log scale). Ordinate scale: spasm expressed as a percentage of the spasm evoked by caffeine (10 mM). Each data point indicates the mean of values from at least 6 experiments; vertical bars show s.e.mean.

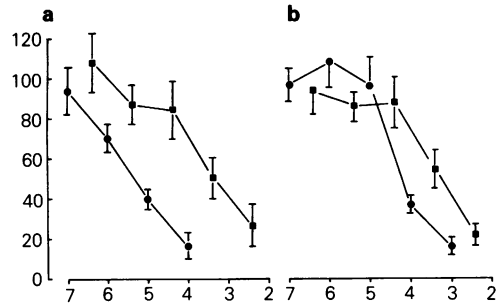

Figure 9 Inhibition by AH 21-132 (●) and by theophylline (■) of cyclic AMP-dependent (a) and cyclic GMP-dependent (b) phosphodiesterase of muscle rich strips of guinea-pig trachea. Abscissae: −log molar concentration of inhibitor. Ordinate scale: enzyme activity (cyclic AMP-PDE in a; cyclic GMP-PDE in b) expressed as a percentage of control activity. Each data point indicates the mean of values from 6 experiments; vertical bars indicate s.e.mean.

Discussion

The tracheal relaxant action of AH 21-132

We have observed that AH 21-132 suppresses the spontaneous tone of the trachea and suppresses tone induced by histamine, KCl or ACh. The relaxant action of AH 21-132 is not antagonized by propranolol. We conclude that AH 21-132 exerts a non-specific relaxant effect on the trachealis which is not mediated by the activation of β-adrenoceptors. Our findings in this respect both confirm and extend the recent report of Bewley & Chapman (1988).

Since AH 21-132 suppressed the spontaneous tone of the trachea and similarly depressed the log concentration-spasm curves for KCl, histamine and ACh, it is unlikely that AH 21-132 acts as an inhibitor of Ca^{2+} influx through voltage-operated channels. Agents that do this include nifedipine and verapamil. They have relatively little effect against the spontaneous tone of the trachea and depress the log concentration-spasm curve for KCl at concentrations that do not modify the corresponding curves for histamine and ACh (Foster *et al.*, 1984; Ahmed *et al.*, 1985; Small & Foster, 1986).

The K^+-channel opener, cromakalim, suppresses the spontaneous tone of guinea-pig trachea but is ineffective against tone induced by K^+-rich (120 mM) Krebs solution (Allen *et al.*, 1986a). Since AH 21-132 retained its relaxant activity in K^+-rich (120 mM) Krebs solution, it is unlikely that AH 21-132 shares the same mechanism of action as cromakalim. The present experiments showed, instead, that the relaxant action of AH 21-132 resembled that of theophylline. Nevertheless, the effects of AH 21-132 in the trachea can be distinguished from those of theophylline (see below).

Phosphodiesterase inhibition by AH 21-132: its possible role in the relaxant action

Tested on the soluble PDE of rat brain and with cyclic AMP as a substrate, AH 21-132 has been

Table 2 Inhibition of phosphodiesterase activity of homogenates of muscle-rich strips of guinea-pig trachea by AH 21-132 and theophylline

	Cyclic AMP-PDE	*Cyclic GMP-PDE*
AH 21-132	5.4 ± 0.20	4.16 ± 0.11*
Theophylline	3.44 ± 0.09	3.35 ± 0.24

Data are mean (±s.e.mean) values of −log molar IC_{50} derived from 6 tissue samples. * indicates a significant ($P < 0.001$) difference between mean −log molar IC_{50} values for cyclic AMP-PDE and cyclic GMP-PDE.

reported to act as a competitive PDE inhibitor with a K_i value of 6 μM (Markstein *et al.*, 1984). The present experiments have confirmed that AH 21–132 has PDE inhibitory activity and suggest that AH 21–132 exerts some selectivity as an inhibitor of cyclic AMP-PDE as opposed to cyclic GMP-PDE unlike theophylline which appeared to be approximately equipotent in this respect.

Our enzyme inhibition studies showed that AH 21–132 is effective in inhibiting PDE over the concentration range 0.1–1000 μM. Since this encompasses the effective concentration range of AH 21–132 as a tracheal relaxant, it is possible that PDE inhibition plays an important role in the process by which relaxation is achieved.

Airways smooth muscle contains PDE which exists as several different isozymes. Amongst these the type Ic isozyme (which is Ca^{2+}- and calmodulin-dependent) accounts for the majority (85%) of the total cyclic AMP hydrolytic activity of tissue homogenates. However, this isozyme may not be important for regulating the cyclic AMP content and mechanical activity of intact tissue. In contrast the type III and type IV isozymes, while accounting for only 5% and 10% respectively of the total cyclic AMP hydrolytic activity of tissue homogenates, may nevertheless be important regulators of cyclic AMP content and mechanical activity in intact tissue (Torphy, 1988). The present enzyme inhibition studies suggest that AH 21–132 can inhibit the type Ic isozyme but whether this agent inhibits (with greater or lesser potency) the type III and type IV isozymes remains to be determined.

Differences between the pharmacological profiles of AH 21–132 and theophylline acting on the trachealis

Results of the present study have confirmed an earlier report (Bewley & Chapman, 1988) that epithelial removal potentiates AH 21–132 in relaxing trachealis muscle but does not modify the actions of methylxanthines such as aminophylline or theophylline.

There are numerous reports (reviewed by Goldie *et al.*, 1988) that the airways epithelium releases a relaxant substance (epithelium-derived relaxant factor, EpDRF) which can modulate the activity of the airways smooth muscle. It is difficult to envisage a mechanism by which AH 21–132 could interfere with the release or action of EpDRF and where epithelial removal would consequently potentiate AH 21–132. It is perhaps more likely that the airways epithelium represents a site of loss of AH 21–132 (by binding or by metabolism) or presents a barrier to the diffusion of the drug into the tracheal tissue. Removal of a site of loss or a diffusion barrier could certainly explain the potentiation of AH 21–132 which occurs on epithelial removal. Presumably, the epithelium represents no site of loss or barrier to diffusion for the methylxanthines. In any event, the effects of epithelial removal serve to discriminate between the tracheal relaxant actions of AH 21–132 and the methylxanthines.

The discrimination between AH 21–132 and methylxanthines provided by the differing effects of epithelial removal is reinforced by the present experiments where AH 21–132 and theophylline were tested in trachea treated with indomethacin (2.8 μM) and maintained at 12°C. Theophylline was spasmogenic but AH 21–132 was devoid of spasmogenic activity.

In trachea treated with indomethacin and maintained at 12°C, xanthine and some of its alkylated derivatives evoke spasm by releasing Ca^{2+} from intracellular sites of sequestration. This action does not depend on the intracellular accumulation of cyclic nucleotides (Small *et al.*, 1988). The present failure of AH 21–132 to mimic the spasmogenic effects of theophylline is consistent with the fact that the AH 21–132 molecule does not contain a xanthine nucleus.

In summary, while the tracheal relaxant action of AH 21–132 in many ways resembles that of the methylxanthines, the actions of AH 21–132 on the trachea could be distinguished from those of methylxanthines in three ways. Firstly, epithelial removal potentiated AH 21–132 in relaxing the trachea but did not potentiate methylxanthines. Secondly, AH 21–132 failed to mimic the tracheal spasmogenic effects of the methylxanthines. Thirdly, AH 21–132 exhibited selectivity as an inhibitor of tracheal cyclic AMP-PDE while theophylline inhibits cyclic AMP-PDE and cyclic GMP-PDE with equal potency.

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Inhibitory effects of AH 21–132 in guinea-pig isolated ileum and taenia caeci

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1 AH 21–132 is being investigated as a potential chemotherapeutic agent for bronchial asthma. The present experiments were designed to determine whether AH 21–132 shares the activity of theophylline as an antagonist at adenosine A_1 receptors and to assess its potency as a relaxant in intestinal smooth muscle.

2 In the transmurally-stimulated guinea-pig ileum, theophylline (1 mM), but not AH 21–132 (1 and 10 μM), antagonized twitch depression induced by adenosine. Higher concentrations (100 μM and 1 mM) of AH 21–132 themselves had a depressant effect. Neither theophylline (1 mM) nor AH 21–132 (1 and 10 μM) antagonized twitch depression induced by noradrenaline.

3 AH 21–132 (100 μM and 1 mM) depressed maximum contractions of ileum induced by both acetylcholine (ACh) and histamine.

4 In ileum treated with hyoscine (1 μM), AH 21–132 (> 10 μM) caused a concentration-dependent depression of the log concentration-effect curve for potassium chloride.

5 Simultaneous extracellular electrophysiological and mechanical recording from taenia caeci showed that AH 21–132 (100 μM–1 mM) inhibited spontaneous tension waves and their associated bursts of electrical spike activity.

6 Intracellular electrophysiological recording from taenia caeci showed that the mechano-inhibitory effect of 1 mM AH 21–132 was accompanied by abolition of spontaneous spike activity. Following spike abolition, the membrane potential assumed a value very close to that observed during periods of electrical quiescence prior to drug exposure.

7 AH 21–132 inhibited the activity of cyclic AMP-dependent and cyclic GMP-dependent phosphodiesterases derived from homogenates of ileal smooth muscle. The effective concentration ranges were 0.1–1000 μM and 1–1000 μM, respectively. Theophylline, too, inhibited these enzymes but in each case was less potent than AH 21–132.

8 It is concluded that AH 21–132 is devoid of antagonist activity at adenosine A_1 receptors which modulate ACh release from intramural cholinergic nerves in the ileum. At concentrations greater than 10 μM, AH 21–132 has a relaxant effect on intestinal smooth muscle characterized by suppression of spontaneous action potentials but by minor change in resting membrane potential. AH 21–132 previously has been reported to depress the spontaneous tone of trachealis muscle with an EC_{50} value of less than 10 μM and the present experiments therefore show that this agent is much less potent in inhibiting intestinal muscle. This potency difference cannot be attributed to a tissue-related difference in the potency of AH 21–132 as an inhibitor of cyclic AMP- or cyclic GMP-dependent phosphodiesterases.

Introduction

(±)-*cis*-6-(*p*-Acetamidophenyl)-1,2,3,4,4,4a,10b-hexahydro-8,9-dimethoxy-2-methylbenzo-[C][1,6]naphthyridine (AH 21–132) exerts a non-specific relaxant effect in airways smooth muscle *in vitro* (Bewley & Chapman, 1988; Small *et al.*, 1989). In many ways the tracheal relaxant action of AH 21–132 resembles that of methylxanthines but, whereas removal of the tracheal epithelium increases the relaxant potency of AH 21–132, it does not alter the relaxant potency of aminophylline or theophylline (Bewley & Chapman,

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1988; Small *et al.*, 1989). AH 21-132 can also be distinguished from theophylline in that it does not share the latter's ability to cause tracheal spasm (Small *et al.*, 1988) by releasing Ca^{2+} from intracellular sites of sequestration (Small *et al.*, 1989).

The present experiments were performed in order to determine (a) whether AH 21-132 shares the activity of theophylline as an antagonist at adenosine A_1 receptors and (b) whether the non-specific relaxant effects of AH 21-132 observed in airways smooth muscle could also be found in intestinal smooth muscle. Our findings suggest that AH 21-132 is not an antagonist at adenosine A_1 receptors and that its potency as a relaxant of gastrointestinal smooth muscle is less than its potency as a relaxant of airways smooth muscle.

Methods

Guinea-pigs (350-700 g) of either sex were killed by stunning and bleeding. Segments of ileum and taenia caeci were removed from the animals and incubated in Krebs solution gassed with 95% O_2 and 5% CO_2 and maintained at 37°C.

Experiments with ileum

Segments of ileum 1-2 cm long were set up for isometric recording of mechanical activity under a resting tension of 1 g. The effects of acetylcholine (ACh) and histamine were studied by the construction of sequential concentration-effect curves. For each concentration of these drugs, the contact time was 45 s in a 4 min cycle; four fold increments in agonist concentration were used. Each tissue was challenged with only one agonist.

Following the construction of the initial agonist concentration-effect curve, test tissues were pretreated for 15 min with AH 21-132 before the construction of a subsequent concentration-effect curve for the agonist in its presence. The concentration of AH 21-132 was then increased before the next concentration-effect curve for the contractile agonist was constructed. Control tissues were not exposed to AH 21-132 and were run concurrently with test preparations.

A further group of experiments was performed on ileum treated with 1 μM hyoscine. KCl (5-80 mM; see below) was used as the agonist, each concentration being allowed 20 s tissue contact. The effects of AH 21-132 (1 μM-1 mM) on the KCl log concentration-effect curve were investigated in a manner similar to that described for the experiments with ACh and histamine.

Experiments with transmurally stimulated guinea-pig ileum

Segments of ileum, 1 to 2 cm long, were set up for transmural stimulation (Paton, 1955) with a resting tension of 1 g and isometric contractions of the longitudinal muscle were recorded. Transmural, supramaximal stimulation was carried out using constant voltage (60 V) pulses of 0.5 ms duration, at a frequency of 0.2 Hz. The effects of adenosine and noradrenaline on the twitches evoked by transmural stimulation were studied by the construction of cumulative concentration-effect curves, ten fold increments in agonist concentration being made at 45 s intervals. In test tissues, agonist action was studied both in the absence and presence (30 min preincubation) of AH 21-132 or theophylline. Control tissues were exposed to neither AH 21-132 nor theophylline and were run concurrently with test preparations.

Extracellular electrophysiological recording from the taenia caeci

Segments of taenia caeci 1-2 cm in length were set up for the simultaneous extracellular recording of electrical activity and tension changes by the perfused capillary method of Golenhofen & V. Loh (1970). Electrical and mechanical changes were displayed on a Grass polygraph and the time constant of the a.c. amplifier used to record the electrical activity was set at 100 ms. This enabled the recording of electrical spike activity in the absence of slower changes in membrane potential. Mechanical activity was recorded isometrically with a resting tension of 1 g.

Following a 45 min preincubation period, the effects of AH 21-132 on spontaneous electrical and mechanical activity were examined by the construction of a cumulative log concentration-effect curve. AH 21-132 was added to the Krebs solution superfusing the tissue. The initial concentration of AH 21-132 studied was 1 μM and the concentration of AH 21-132 was raised in ten fold steps with a tissue contact time of 15 min for each concentration step. The maximal concentration of AH 21-132 applied to the tissue was 1 mM. Thereafter the tissue was washed with drug-free Krebs solution so that recovery from the effect of AH 21-132 could be observed.

Intracellular electrophysiological recording from the taenia caeci

Simultaneous intracellular recordings of membrane potential changes and mechanical activity were made by the method of Small & Weston (1977; 1980). Segments of tissue approximately 2.5 cm in length were used. Mechanical activity was recorded isometrically

at an initial resting tension of 1 g. Following the microelectrode impalement of a smooth muscle cell, several minutes were allowed to elapse both to check the stability of the electrical recording and to monitor activity under control conditions. AH 21–132 (1 mM) was then added to the Krebs solution superfusing the tissue. This concentration of AH 21–132 was chosen on the basis that, in the extracellular electrophysiological studies, it clearly abolished spontaneous spike activity.

The effects of AH 21–132 on electrical and mechanical activity were monitored, where possible, over a 15 min period. For many recordings the electrode became dislodged from the cell before the 15 min drug equilibration period was complete.

Assessment of cyclic nucleotide phosphodiesterase inhibitory activity

Strips of smooth muscle were prepared from guinea-pig ileum by a method similar to that described for rabbit small intestine (Ambache, 1954). Histological examination revealed that these tissue strips contained serosa, longitudinal smooth muscle and elements of the myenteric plexus; some circular smooth muscle but no mucosal tissue was included in the tissue strips. The tissue strips were processed and the effects of AH 21–132 and theophylline on cyclic nucleotide phosphodiesterase activity were assessed as previously described (Small *et al.*, 1989).

Drugs, solutions and statistical analysis of results

Drug concentrations are expressed in terms of the molar concentration of the active species. The following substances were used: acetylcholine chloride (Sigma), adenosine (Sigma), AH 21–132 (Sandoz, AG), ascorbic acid (Sigma), histamine acid phosphate (Sigma), hyoscine hydrobromide (Sigma), (−)-noradrenaline bitartrate (Sigma), potassium chloride (Analar: Hopkin and Williams), theophylline (Sigma). Where KCl was used as a contractile agonist, the stated bath concentration indicates the concentration of KCl in excess of the concentration of K^+ and Cl^- provided by the Krebs solution.

Dilutions of noradrenaline were prepared from a stock solution in 0.1 N HCl on the day of use. Each noradrenaline dilution contained ascorbic acid (0.57 mM) as an antioxidant. Dilutions of adenosine were prepared by suspending or dissolving the solid in a 0.9% (w/v) solution of NaCl immediately before use. Stock solutions of ACh were prepared in absolute ethanol, those of other drugs in twice-distilled water.

The Krebs solution used in all the experiments had the following composition (mM): Na^+ 143.5, K^+ 5.9, Ca^{2+} 2.6, Mg^{2+} 1.2, Cl^- 127.6, HCO_3^- 25, SO_4^{2-} 1.2, $H_2PO_4^-$ 1.2 and glucose 11.1.

The significance of differences between means was assessed by a two-tailed unpaired t test. The null hypothesis was rejected when $P < 0.05$.

Results

Experiments with ileum

ACh (0.004–4 μM) and histamine (0.004–4 μM) each caused concentration-dependent contraction of the longitudinal muscle of the isolated ileum. Control experiments revealed no time-dependent changes in the shape or position of the log concentration-effect curves for either ACh or histamine.

AH 21–132 (0.1–10 μM) caused no changes in ileal tone and affected neither the slope nor the position of the log concentration-effect curves of ACh or histamine. In contrast, at high concentrations (100 μM) AH 21–132 depressed the log concentration-effect curves for both ACh and histamine, significantly reducing maximum responses to each of these agonists (Figure 1).

In preparations treated with hyoscine (1 μM), KCl (5–80 mM) caused concentration-dependent contraction. AH 21–132 (1–1000 μM) caused concentration-

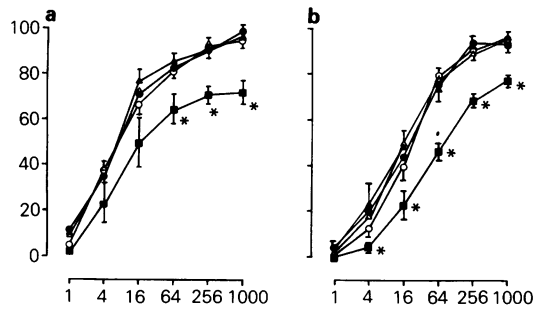

Figure 1 Effects of AH 21–132 on contractions induced by acetylcholine (ACh) (a) and histamine (b) in guinea-pig isolated ileum. Abscissae: concentrations (μM) of ACh (a) or histamine (b) on a log scale. Ordinates: contraction expressed as a percentage of control maximum. Concentration-effect curves are shown prior to tissue treatment with AH 21–132 (○) and after 15 min incubation with, and in the presence of 0.1 (●), 1 (△), 10 (▲) and 100 (■) μM AH 21–132. Data indicate means of values from at least 6 tissues; s.e.mean shown by vertical bars. (*) indicates a significant ($P < 0.05$) reduction in response compared with the corresponding point for control tissues.

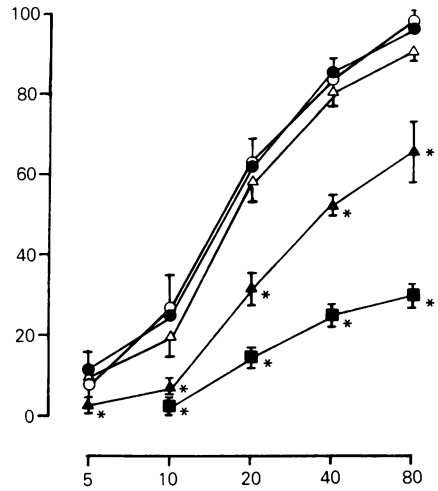

Figure 2 Effects of AH 21-132 on contractions induced by KCl in guinea-pig isolated ileum. Hyoscine (1 μM) was present throughout. Abscissa scale: concentration (mM) of KCl on a log scale. Ordinate scale: contraction expressed as a percentage of maximum. Concentration-effect curves are shown prior to tissue treatment with AH 21-132 (○) and after 15 min incubation with, and in the presence of 1 (●), 10 (△), 100 (▲) and 1000 (■) μM AH 21-132. Data represent the means of values from at least 6 tissues; s.e.mean shown by vertical bars. (*) indicates a significant ($P < 0.05$) reduction in response compared with the corresponding point for control tissues.

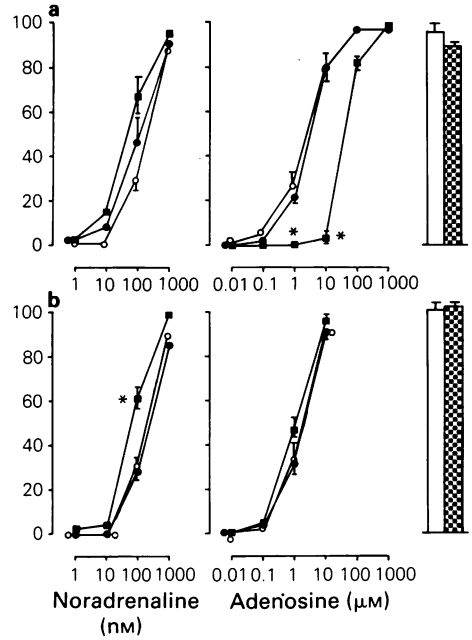

Figure 3 Inhibition of twitches of transmurally stimulated guinea-pig ileum produced by noradrenaline (left-hand panels) or adenosine (right-hand panels): (○) pooled, initial log concentration-inhibition curves for control and test tissues; (●) log concentration-inhibition curves obtained in control tissues after treatment with vehicle; (■) log concentration-inhibition curves obtained in test tissues treated with 1 mM theophylline (row a) or with 10 μM AH 21-132 (row b). Each data point is the mean of at least six experiments; vertical bars show s.e.mean.

The columns show twitch height expressed as a % of the twitch height observed prior to tissue exposure to vehicle (control tissues; open columns) or to 1 mM theophylline (stippled columns; row a) or to 10 μM AH 21-132 (stippled columns; row b).

* Indicates a significant ($P < 0.05$) difference from the corresponding data point for the time-matched control tissues.

dependent depression of the log concentration-effect curve for KCl, significant depression of the curve occurring at AH 21-132 concentrations of 100 and 1000 μM (Figure 2).

Experiments with transmurally-stimulated guinea-pig ileum

Noradrenaline (1 nM-1 μM) and adenosine (10 nM-10 μM) each produced concentration-dependent suppression of ileal twitches evoked by transmural stimulation (Figure 3). Theophylline (1 mM) itself had no significant effect on twitch amplitude and did not reduce the ability of noradrenaline to evoke twitch suppression. However, in the presence of theophylline, the inhibitory log concentration-effect curve for adenosine was moved (approximately ten fold) to the right (Figure 3). Concurrent control experiments revealed little or no changes in the slopes or positions of the log concentration-effect curves of noradrenaline or adenosine. Hence the reduced potency of adenosine (but not of noradrenaline) observed in the presence of theophylline was indicative of selective antagonism by theophylline.

AH 21-132 (1 and 10 μM) itself had no significant effect on twitch amplitude and did not reduce the ability of either noradrenaline or adenosine to cause twitch suppression. Indeed, in the presence of 10 μM AH 21-132, the inhibitory log concentration-effect curves of both noradrenaline and adenosine were moved (approximately four fold and two fold, respectively) to the left (Figure 3). Higher concentrations (100 μM and 1 mM) of AH 21-132 themselves caused suppression of ileal twitches.

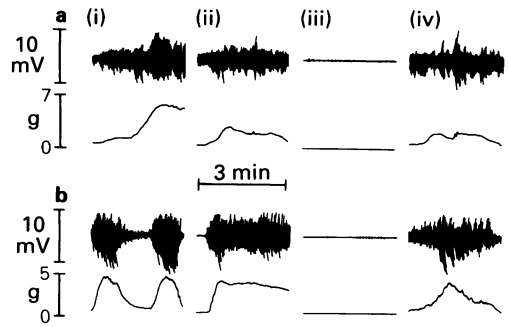

Figure 4 Effects of AH 21–132 on the extracellularly-recorded electrical (upper trace) and mechanical (lower trace) activity of guinea-pig taenia caeci. Recordings from two separate (a and b) preparations are shown. Panel (i) shows control activity. Panels (ii) and (iii) show activity recorded 12 min after the cumulative administration of AH 21–132 0.1 and 1 mM respectively. Panel (iv) shows activity recorded 20 min after drug washout.

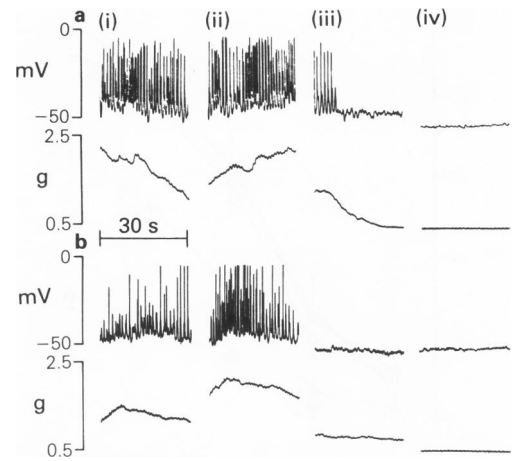

Figure 5 Effects of AH 21–132 on the intracellularly-recorded electrical (upper trace) and mechanical (lower trace) activity of guinea-pig taenia caeci. Recordings from two separate (a and b) preparations are shown. Panel (i) shows control activity. Panels (ii), (iii) and (iv) show activity recorded 1, 2 and 8 min, respectively, after the addition of 1 mM AH 21–132. In each row the electrical traces are taken from a continuous recording from a single cell.

Extracellular electrophysiological recording from taenia caeci

The spontaneous activity of the taenia caeci comprised phasic tension waves and associated bursts of electrical spike activity. The frequency and duration of the tension waves and spike bursts were somewhat variable (Figure 4a and b).

AH 21–132 (1–10 μM) had little or no effect on the ongoing electrical and mechanical activity of the taenia caeci. AH 21–132 (100 μM), in some preparations, reduced the magnitude and/or duration of the tension waves and spike bursts (e.g. Figure 4a). In all preparations, AH 21–132 (1 mM) abolished both the spontaneous tension waves and their associated spike bursts (Figure 4a and b).

Intracellular electrophysiological recording from the taenia caeci

In the extracellular electrophysiological experiments, AH 21–132 consistently abolished spontaneous spike activity only at a concentration of 1 mM. Accordingly, this concentration of AH 21–132 was chosen for use in the intracellular electrophysiological experiments.

In all tissues examined, AH 21–132 (1 mM) abolished intracellularly-recorded spike potentials and phasic tension waves. Spike abolition generally became manifest 2–3 min after tissue exposure to AH 21–132 (Figure 5). During AH 21–132-induced spike suppression the membrane potential assumed a level close to, or marginally more negative than, the level of membrane potential measured between successive spike bursts during the pre-drug control period (Table 1). In other words, AH 21–132 caused

Table 1 Effects of AH 21–132 (1 mM) on resting membrane potential of guinea-pig taenia caeci

Time after administration of AH 21–132 (min)	*Increase in membrane potential over the level assumed between spike bursts during the pre-drug control period* (mV)
1	0.11 ± 0.6 (9)
2	1.22 ± 0.6 (9)
4	0.38 ± 1.7 (8)
8	0.00 ± 1.2 (4)

Data are mean (± s.e.mean) increases in membrane potential. A single recording was obtained from each tissue which was subjected to a single exposure to AH 21–132. The figures in parentheses indicate the number of tissues contributing to the mean. The decrease in group size with time reflects the duration of individual recordings.

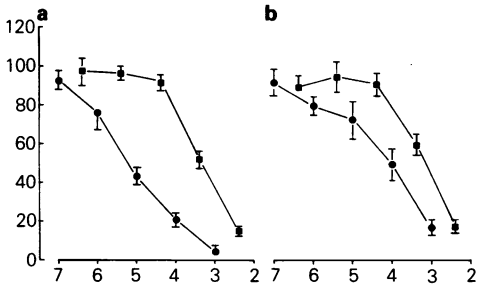

Figure 6 Inhibition by AH 21-132 (●) and by theophylline (■) of cyclic AMP-dependent (a) and cyclic GMP-dependent (b) phosphodiesterase of smooth muscle from guinea-pig ileum. The abscissa scale indicates −log molar concentration of inhibitor. The ordinate scale indicates enzyme activity expressed as a percentage of control activity. Data points represent means of values from 6 experiments; vertical bars indicate s.e.mean.

very little, if any, hyperpolarization of the smooth muscle cells of the taenia caeci.

Inhibition of ileal cyclic nucleotide phosphodiesterase by AH 21-132 and theophylline

Measured with 1 μM cyclic nucleotide as substrate, in the presence of Ca^{2+} and calmodulin, the strips of ileal smooth muscle contained phosphodiesterase activity equivalent to the hydrolysis of 5.66 ± 0.87 ($n = 6$) nmol cyclic AMP $g^{-1} min^{-1}$ or 5.15 ± 0.34 ($n = 6$) nmol cyclic GMP $g^{-1} min^{-1}$.

AH 21-132 (1-1000 μM) and theophylline (0.4-4000 μM) each caused concentration-dependent inhibition of the hydrolysis of cyclic AMP. AH 21-132 was the more potent inhibitor of cyclic AMP-dependent phosphodiesterase (Figure 6 and Table 2). AH 21-132 (0.1-1000 μM) and theophylline (0.4-4000 μM) each caused concentration-dependent inhibition of the hydrolysis of cyclic GMP. AH 21-132 was, again, the more potent inhibitor of cyclic GMP-dependent phosphodiesterase (Figure 6 and Table 2). Furthermore, in contrast to theophylline, AH 21-132 was significantly more potent in inhibiting cyclic AMP-dependent PDE than cyclic GMP-dependent PDE.

Table 2 Inhibition of phosphodiesterase (PDE) activity in homogenates of the smooth muscle of guinea-pig ileum

	Cyclic AMP-PDE	*Cyclic GMP-PDE*
AH 21-132	5.08 ± 0.17	4.19 ± 0.30*
Theophylline	3.41 ± 0.09	3.21 ± 0.11

Data are mean values of −log molar IC_{50} ± s.e.mean derived from 6 experiments. * indicates a significant ($P < 0.05$) difference between mean values of −log molar IC_{50} for cyclic AMP- and cyclic GMP-dependent phosphodiesterase.

Discussion

Effects of AH 21-132 on intestinal smooth muscle

The present experiments with guinea-pig ileum showed that, in concentrations of 100 μM or greater, AH 21-132 was able to depress log concentration-effect curves for ACh, histamine and KCl. Since the experiments with KCl were carried out in the presence of hyoscine (1 μM), we ascribe the spasm induced by KCl to a direct effect of KCl in depolarizing the smooth muscle cells rather than to the release of ACh from intramural nerves. Accordingly, the ability of AH 21-132 to suppress concentration-effect curves for ACh, histamine and KCl suggests that AH 21-132 exerts a non-specific relaxant effect on ileal smooth muscle. Such an effect would explain why, in the experiments with transmurally-stimulated ileum, ileal twitches were suppressed when the concentration of AH 21-132 was raised from 10 to 100 μM. Non-specific relaxation of intestinal smooth muscle would also explain the ability of AH 21-132 (100 μM-1 mM) to suppress the spontaneous tension waves and electrical spike activity of the taenia caeci.

In airways smooth muscle AH 21-132 exerts non-specific relaxant effects in the concentration-range 0.1-10 μM (Bewley & Chapman, 1988; Small *et al.*, 1989). In the present study AH 21-132 exerted relaxant effects on intestinal smooth muscle only in concentration greater than 10 μM. This suggests that AH 21-132 exhibits some selectivity for smooth muscle of the airways as opposed to that of the intestine.

Cyclic nucleotide phosphodiesterase (PDE) inhibition and its relation to the intestinal smooth muscle relaxant action of AH 21-132

AH 21-132 has been reported to act as a competitive inhibitor of cyclic AMP-PDE isolated from rat brain (Markstein *et al.*, 1984) and in guinea-pig trachealis to exhibit selectivity as an inhibitor of cyclic AMP-dependent PDE as opposed to cyclic GMP-

dependent PDE (Small *et al.*, 1989). The present observation that AH 21–132 acts as an inhibitor of PDE derived from the smooth muscle of guinea-pig ileum and exhibits selectivity for cyclic AMP-PDE as opposed to cyclic GMP-PDE is consistent with the findings made in rat brain and guinea-pig trachealis.

The concentrations ($>10\,\mu$M) of AH 21–132 required to exert spasmolytic effects in guinea-pig ileum were found to be very similar to those causing marked inhibition of PDE activity in homogenates of the ileal smooth muscle. This allows the possibility that PDE inhibition may be involved in the relaxant action of AH 21–132.

The $-\log IC_{50}$ values for AH 21–132 as an inhibitor of cyclic AMP-PDE and cyclic GMP-PDE derived from the ileum (Table 2) were almost identical to those described for the corresponding enzymes of guinea-pig trachealis (Small *et al.*, 1989). This suggests that tissue-related differences in the potency of AH 21–132 either as an inhibitor of cyclic AMP-PDE or an inhibitor of cyclic GMP-PDE (as measured with 1 μM cyclic nucleotide as substrate) do not account for the difference (see above) in the potency of AH 21–132 in suppressing the spontaneous tone of guinea-pig trachea compared with its potency as a relaxant of intestinal smooth muscle. Of course, the present measurements of phosphodiesterase activity were performed using crude tissue extracts and a single nucleotide concentration. Accordingly they do not discriminate between the various isozymes of PDE (Torphy, 1988) now known to exist. It may be that AH 21–132 exhibits selectivity as an inhibitor of a PDE isozyme that plays an important role in the mechanism by which relaxation is achieved and which exhibits differing distribution or activity between airways and gastrointestinal smooth muscle.

An alternative explanation of the low potency of AH 21–132 in relaxing intestinal smooth muscle may be that (in contrast to its action on airways smooth muscle) such relaxant activity is not founded on its ability to inhibit cyclic nucleotide-dependent PDE. In any event, were AH 21–132 to receive clinical use as a bronchodilator, its low potency in relaxing intestinal muscle *in vitro* may be predictive that inhibition of intestinal motility would not feature as an unwanted effect.

Antagonism at prejunctional receptors for adenosine

Sawynok & Jhamandas (1976) showed that adenosine could suppress the twitches of transmurally stimulated guinea-pig ileum by preventing ACh release and that this action could be antagonized by theophylline. Using a range of agonists at adenosine receptors, Paton (1981; 1988) has obtained evidence which indicates that the adenosine receptor which modulates ACh release in the ileum is of the A_1 subtype. Accordingly it may be suggested that theophylline prevents adenosine-induced ileal twitch suppression by acting as an antagonist at A_1 receptors for adenosine.

The ability of theophylline to inhibit adenosine-induced ileal twitch suppression was confirmed in the present study. However, since AH 21–132 did not share this action of theophylline, we conclude that AH 21–132 does not act as an antagonist at A_1 receptors for adenosine. Our findings are consistent with the observation (Markstein *et al.*, 1984) that AH 21–132 did not inhibit the binding of [^{3}H]-N^6-cyclohexyladenosine to synaptosomes of rat brain. It is also of relevance to the possible therapeutic use of AH 21–132 in the therapy of asthma. A number of the unwanted effects of theophylline observed during its use for the treatment of asthma have been ascribed to its ability to act as an antagonist at adenosine receptors (Persson, 1985; Jenne, 1987). The results of the present experiments suggest that AH 21–132 might lack many of the unwanted effects of theophylline which are attributable to blockade of adenosine receptors.

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Characterization of adenosine receptors in guinea-pig isolated left atria

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1 The effects of purinergic stimulation on action potential, force of contraction, ^{86}Rb efflux and ^{45}Ca uptake were investigated in guinea-pig left atria.

2 Adenosine exerted a negative inotropic effect which was antagonized by adenosine deaminase but enhanced by dipyridamole.

3 The negative inotropic effect of adenosine was mimicked by 5'-(N-ethyl)-carboxamido-adenosine (NECA) and the isomers of N^6-(phenyl-isopropyl)-adenosine, **R**-PIA and **S**-PIA. NECA and **R**-PIA were about 1000 times more potent than adenosine, whereas **R**-PIA was about 100 times more potent than **S**-PIA.

4 The inotropic effects of adenosine (in the presence of dipyridamole), NECA, **R**-PIA and **S**-PIA were competitively antagonized either by theophylline (pA_2 about 4.5) or 8-phenyltheophylline (pA_2 about 6.3).

5 NECA and **R**-PIA shortened the action potential duration and increased the rate constant of the efflux of ^{86}Rb in a concentration-dependent manner with no differences in potency; the effects were competitively antagonized by 8-phenyltheophylline.

6 Barium ions reduced the efflux of ^{86}Rb under control conditions and antagonized the increase induced by NECA and **R**-PIA.

7 NECA and **R**-PIA significantly reduced ^{45}Ca uptake in beating preparations.

8 It is concluded that adenosine, NECA and **R**-PIA activate a common receptor population (P_1 or A_3) on the outside of the cell membrane of atrial heart muscle to increase the potassium conductance and to reduce the action potential and, thereby, calcium influx and force of contraction.

Introduction

The negative inotropic effect of adenosine in atrial heart muscle is accompanied by a decrease in the action potential duration (Johnson & McKinnon, 1956; Hollander & Webb, 1957; de Gubareff & Sleator, 1965). This effect of adenosine has been ascribed, mainly, to an increase in the potassium conductance of the myocardial cell membrane (Belardinelli & Isenberg, 1983; Jochem & Nawrath, 1983; Hutter & Rankin, 1984; Kurachi *et al.*, 1986). Characteristically, the myocardial effects of adenosine in multicellular atrial preparations are observed only at very high concentrations (in the millimolar range). This has raised the question as to whether or not the effects of adenosine are due to the stimulation of specific receptors or represent non-specific membrane effects.

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Two functionally and pharmacologically distinct adenosine receptors have been identified in the central nervous system, both being associated with membrane bound adenylate cyclase. One type of receptor mediates stimulation of activity; the other type mediates inhibition. Both types of receptor are located at the outer site of the membrane, seem to require an intact ribose moiety and have been termed A_1 and A_2 (van Calker *et al.*, 1979) or R_i and R_a (Londos *et al.*, 1980). In addition to these receptors, adenylate cyclases contain another site for adenosine action, termed the P-site, which mediates inhibition of activity (Londos & Wolff, 1977).

Burnstock & Meghji (1981) ascribed the effects of adenosine in guinea-pig atria to the stimulation of P_1-receptors. The effects of adenosine at P_1-receptors are blocked competitively by methylxanthines. Methylxanthines have also been shown to

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block the effects at the external R-site receptors but not those on the internal P-site (Londos & Wolff, 1977; Londos *et al.*, 1978; Schwabe, 1981; Daly, 1982).

Whereas the adenosine derivative (−)-N^6-(R-phenyl-isopropyl)-adenosine (R-PIA) has been shown to be more potent at A_1-receptors, 5'-(N-ethyl)-carboxamido-adenosine (NECA) is thought to be more potent at A_2-receptors (Londos *et al.*, 1980; Hüttemann *et al.*, 1984; Ukena *et al.*, 1984; 1987). Collis (1983) and Kurahashi & Paton (1986) interpreted the effects of adenosine in guinea-pig and rat atria, respectively, as mediated by A_1-receptors. Recently, Martens *et al.* (1987) described the existence of A_1-receptors in rat isolated ventricular myocytes. In radioligand binding studies, selective antagonists for A_1-receptors have been described (Lohse *et al.*, 1987; Stiles & Jacobson, 1987). Nevertheless, the distinction of A_1- and A_2-receptors in cardiac cells remains unclear because of the lack of, firstly, differences in the potency of R-PIA and NECA (Brückner *et al.*, 1985) and, secondly, the existence of selective antagonists in physiological experiments (Collis *et al.*, 1985; 1987).

In the present study, the functional effects of adenosine, R-PIA and NECA were studied quantitatively, in the absence and presence of inhibitors, in guinea-pig left atria. In addition to the effects on the force of contraction, the effects on action potential duration, ^{86}Rb efflux and ^{45}Ca uptake were studied. Preliminary accounts of this work have been published (Nawrath, 1986; Jahnel & Nawrath, 1986).

Methods

Preparations

Guinea-pigs of weight 250–400 g were killed by a blow to the head and bled from the carotid arteries. The hearts were quickly removed and transferred to a dissection chamber containing oxygenated warm Tyrode solution. Whole hearts were pinned down on Sylgard so that the left atria could be cut off. For flux studies and for the measurement of the force of contraction, the whole left atrial appendage was used. For electrophysiological recordings, left atria were opened and fine trabeculae were prepared by ligating both ends with a silk suture.

Solutions

Tyrode solution was prepared from stock solutions in distilled deionized water, and was of the following composition (in mmol l^{-1}): NaCl 136.9, KCl 5.4, $MgCl_2$ 1.05, NaH_2PO_4 0.42, $NaHCO_3$ 11.9, $CaCl_2$ 1.8, glucose 5.6. The solution was equilibrated with 95% O_2 and 5% CO_2 at 37°C (pH 7.4). For removal of extracellular Ca^{2+}, physiological salt solution (PSS) was prepared according to Leijten & van Breemen (1984) (composition in mmol l^{-1}: NaCl 140, KCl 4.6, EGTA 2.0, $MgCl_2$ 1.0, glucose 10.0, HEPES 5.0). The pH was adjusted at 0°C to 7.4 by addition of 0.1 mol l^{-1} NaOH and the solution was bubbled with O_2.

Measurement of the action potential and the force of contraction

For the measurement of the action potential, atrial trabeculae were mounted horizontally in a 2 ml organ bath which was built into a perspex block that also contained a main reservoir of 100 ml Tyrode solution. Communication between both compartments was provided by connecting pores through which the fluids were driven by gas (95% O_2 and 5% CO_2). The preparations were fixed in the organ bath to keep the muscle length as constant as possible. One end of the preparation was positioned between two platinum electrodes and the other end connected to an inductive force displacement transducer via a stainless steel wire.

The preparations were electrically driven at 1 Hz by rectangular pulses of 0.1–1 ms duration at 10% above threshold intensity using a Grass stimulator (model S4) and isolation unit. Force of contraction (F_c) was recorded at the apex of the preload active tension curve via the inductive force displacement transducer in conjunction with a Hellige frequency carrier preamplifier.

The preparations were allowed to stabilize for at least 30 min. The effects of drugs were investigated by exposure to either single or to cumulatively increasing concentrations, achieved by adding drugs to the main Tyrode reservoir, and increasing the concentration after the establishment of a stable response.

The transmembrane potential was detected intracellularly by the use of 10–20 MΩ glass microelectrodes filled with KCl 3 mol l^{-1}. The signals were led off by means of a voltage follower with input capacity compensation (built by H. Ehrler, Homburg, Saar). Both transmembrane potential and tension were displayed on a cathode ray oscilloscope (Tektronix 5103N) and recorded on magnetic tape (Lyrec FM tape recorder, bandwidth 0–10 kHz). During the course of an experiment, all parameters could be observed on a digital scope (Nicolet Explorer I). For quantitative evaluation, all data were stored, amplified and edited by means of a transient recorder (Physical Data, 512A). The analogue output of the transient recorder was fed to an XY – pen recorder (MFE 815) for amplification. All evaluations were done from records read off the pen recordings.

For the measurement of the F_c only, the left atria were attached to a stainless steel hook built in a perspex rod and positioned next to two platinum electrodes. The preparations were then placed with the muscle holder in organ baths containing 5 ml Tyrode solution and electrically driven at 3 Hz. The muscles were connected via stainless steel wires to the transducer, the output of which was recorded on a Hellige pen recorder.

Measurement of ^{86}Rb efflux

Guinea-pig whole left atria were first exposed to about 10 MBq ^{86}Rb (specific activity: 79.2 GBq g^{-1}) for 90 min in Tyrode solution and then transferred to the test baths. The release of ^{86}Rb into nonradioactive Tyrode solution was then measured (a) for 30 min under control conditions and (b) for 15 min in the presence of a test substance. All preparations were kept at rest since changes in ^{86}Rb efflux are much more complicated in beating preparations, due to voltage- and time-dependent changes in various potassium conductances during the action potential. The bath solution was changed every 5 min and collected in scintillation vials for later determination of radioactivity. At the end of the experiment, the tissues were blotted, weighed and solubilized by the addition of 1 ml TS-1 (Zinsser, Frankfurt) and incubation at 65°C for three hours. Six ml of Minisolve (Zinsser, Frankfurt) was added to each sample in the counting vials. Radioactivity was determined by liquid scintillation counting in a Tricarb 3380 (Packard Instruments, Frankfurt).

Measurement of ^{45}Ca influx

The preparations were equilibrated and exposed for 30 min to control or test solutions. Thereafter, tissues were exposed to corresponding solutions containing 150 kBq ^{45}Ca (specific activity: 1.295 TBq g^{-1}) for 5 min. The experiments were carried out at a driving frequency of 3 Hz. Tissues were then washed three times for 5 min in ice-cold PSS containing 2 mmol l^{-1} EGTA gassed with O_2, blotted and dried at 60°C for three hours. Finally, the tissues were treated as described for the experiments with ^{86}Rb, and the ^{45}Ca content of each tissue was measured by liquid scintillation counting in a Tricarb 3380 (Packard Instruments, Frankfurt).

Chemicals

The following drugs were used (sources in parentheses): adenosine; adenosine deaminase (ADA): (−)-N^6-(**R**-phenyl-isopropyl)-adenosine (**R**-PIA); (+)-N^6-(S-phenyl-isopropyl)-adenosine (S-PIA) (Boehringer, Mannheim); 5′-(N-ethyl)-carboxamidoadenosine (NECA) (Byk Gulden, Konstanz); dipyridamole (Thomae, Biberach an der Riss); theophylline (Merck, Darmstadt); 8-phenyltheophylline (8-PT) (Sigma, München); barium chloride (Merck, Darmstadt); ^{45}Ca chloride and ^{86}Rb chloride (NEN, Dreieich). Tissue solubilizer TS-1; scintillation cocktail Minisolve (Zinsser, Frankfurt). All other chemicals were obtained from Merck, Darmstadt.

Evaluation of results and statistical analyses

Results are either demonstrated as original figures or expressed as means ± s.e.mean. EC_{50} values were determined by regression analysis and the two points on the steep portion of each individual concentration-response curve were taken into account. Confidence limits were calculated according to Documenta Geigy (1968). pA_2 values were evaluated according to Arunlakshana & Schild (1959). Peak levels of phasic contractions were evaluated and given as % of control values. Action potential recordings were analysed for duration (APD) at 20% and 90% of repolarization, APD_{20} and APD_{90}, respectively.

Intracellular potassium is kinetically homogeneous (Langer & Brady, 1966) and given the fact that Rb in tracer amounts passes through potassium channels, albeit to a smaller extent than potassium itself (Hille, 1973; Henquin *et al.*, 1979; Clay & Shlesinger, 1983; Plant, 1986), a single rate constant λ of ^{86}Rb efflux could be determined according to $\lambda = (\ln A_0 - \ln A)/t$ derived from $A = A_0 e^{-\lambda t}$. When appropriate, statistically significant differences were assessed by Student's t test or by analysis of variance (repeated measurements design according to Wallenstein *et al.*, 1980) followed by modified t statistics according to Dunnett (1964). Significant differences are marked by asterisks ($P < 0.01$).

Results

Adenosine produced a negative inotropic effect in guinea-pig left atria. The original tracing in Figure 1 shows a rapid decline of F_c upon the addition of adenosine 10 μmol l^{-1}, followed by a partial recovery of F_c to a new steady-state value. In the presence of adenosine deaminase (ADA) 1 u μmol^{-1}, the recovery of F_c was complete, whereas in the presence of dipyridamole 10 μmol l^{-1} the maximal effect of adenosine was strongly enhanced and the recovery phenomenon virtually abolished. This concentration of dipyridamole has been shown to inhibit the uptake of adenosine by about 80% in guinea-pig left atria (Hopkins, 1973; Nawrath *et al.*, 1985). The EC_{50} of the effects of adenosine in the steady-state was 3.9×10^{-5} mol l^{-1} (Table 1). The concentration-

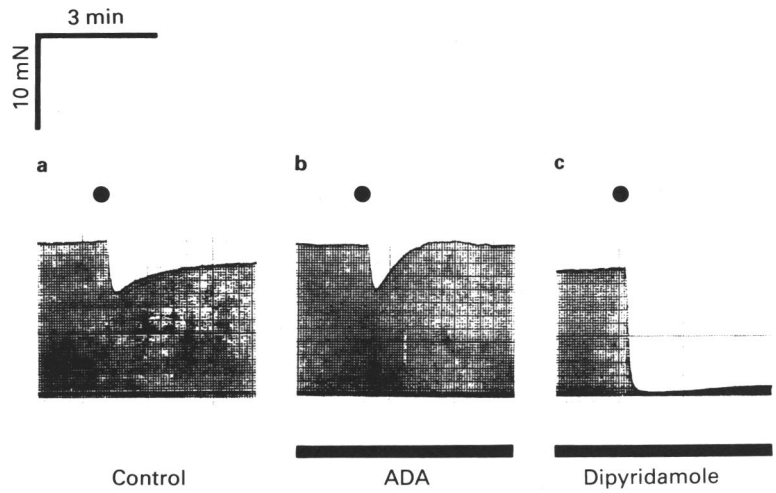

Figure 1 Effects of adenosine 10 μmol l^{-1} (●) on force of contraction (F_c) in a guinea-pig left atrium (a) under control conditions, (b) in the presence of adenosine deaminase (ADA, 1 u μmol^{-1} and (c) in the presence of dipyridamole 10 μmol l^{-1}. Original records from the same preparation which was exposed successively to all three conditions after wash periods of 15 min in drug-free Tyrode solution.

response relationships of adenosine were shifted to the left in the presence of dipyridamole 10 μmol l^{-1} about 100 fold (not shown). Under these conditions, the EC_{50} of adenosine was reduced to 2.9×10^{-7} mol l^{-1} (Table 1).

The fading of the effects of adenosine is probably related to the rapid uptake of adenosine and its deamination to inosine (Schrader *et al.*, 1972); inosine itself is completely ineffective (Nawrath *et al.*, 1985). It was therefore of interest to compare the effects of adenosine analogues which are not substrates for adenosine deaminase. NECA and **R**-PIA exerted negative inotropic effects at much lower concentrations. No fading of the effects of NECA and **R**-PIA as observed with adenosine, was seen (not shown). The EC_{50} of NECA was 3.8×10^{-8} mol l^{-1} and the EC_{50}s of **R**-PIA and **S**-PIA were 3.3×10^{-8} mol l^{-1} and 2.3×10^{-6} mol l^{-1}, respectively. The EC_{50} values, including 95% confidence limits, of all the agonists investigated are summarized in Table 1.

Table 1 Potencies of different adenosine agonists in guinea-pig left atria as measured by changes in force of contraction

	EC_{50} (95% confidence limits)	n
Adenosine	$3.9\ (3.3–4.8) \times 10^{-5}$ mol l^{-1}	66
Adenosine + dipyridamole 10^{-5} mol l^{-1}	$2.9\ (1.9–4.3) \times 10^{-7}$ mol l^{-1}	16
R-PIA	$3.3\ (1.9–5.7) \times 10^{-8}$ mol l^{-1}	12
S-PIA	$2.3\ (1.6–3.3) \times 10^{-6}$ mol l^{-1}	8
NECA	$3.8\ (3.2–4.6) \times 10^{-8}$ mol l^{-1}	12

We next investigated the effects of the adenosine agonists described so far in the presence of theophylline and/or 8-phenyltheophylline which have both been described as competitive antagonists for the effects of adenosine (Griffith *et al.*, 1981; Collis, 1983; Collis *et al.*, 1985). The evaluation of the interaction of adenosine agonists and antagonists on F_c in guinea-pig left atria was carried out according to Arunlakshana & Schild (1959). In our experiments, the interaction of adenosine and theophylline was described by a slope of 0.51 which is not compatible with a simple competitive interaction, since a 100 fold increase in the theophylline concentration produced only a 10 fold decrease in the inotropic response to adenosine.

It is possible that the removal of adenosine from the extracellular into the intracellular space may have strongly influenced the actual concentration of adenosine at the receptor sites. The experiments were therefore repeated in the presence of the uptake inhibitor dipyridamole 10 μmol l^{-1}. Under these conditions, the Schild plot yielded a slope of 1.04 and a pA_2 value of 4.48 was determined. The interaction of theophylline and **R**-PIA (without dipyridamole) was described by a slope of 1 and a pA_2 value of 4.65. In the presence of 8-phenyltheophylline, the inhibition of the effects of adenosine was almost 100 times greater than with theophylline. Furthermore, almost identical pA_2

Table 2 Evaluation of the interaction of adenosine agonists and antagonists on force of contraction in guinea-pig left atria

Agonist	*Antagonist*	*Slope*	*pA_2 (95% confidence limits)*	*n*
Adenosine	Theophylline	0.51	—	16
Adenosine + dipyridamole 10^{-5} mol l^{-1}	Theophylline	1.04	4.48 (4.19–5.16)	8
R-PIA	Theophylline	1.00	4.65 (4.49–4.86)	32
R-PIA	8-PT	0.99	6.43 (6.31–6.67)	25
S-PIA	8-PT	1.02	6.28 (5.99–7.03)	20
NECA	8-PT	1.04	6.25 (6.03–6.81)	12

The interaction of the agonists and antagonists was evaluated according to Arunlakshana & Schild (1959). 8-PT, 8-phenyltheophylline.

values of 6.43, 6.28 and 6.25 were found with **R**-PIA, S-PIA and NECA, respectively. The slopes and pA_2 values (including 95% confidence limits) of all Schild plots are summarized in Table 2.

The effects of NECA and **R**-PIA on action potential configuration, ^{86}Rb efflux and ^{45}Ca uptake were investigated, alone and in the presence of the antagonist 8-phenyltheophylline. Figure 2 shows that both substances, at maximally effective concentrations of 1 μmol l^{-1}, shorten the duration of the action potential to a similar extent. The effects of both NECA and **R**-PIA on APD_{20} and APD_{90} were concentration-dependent. The concentration-response relationships of both substances were shifted to the right about 10 fold in the presence of 8-phenyltheophylline 10 μmol l^{-1} (Figure 3). Figure 4 demonstrates that both NECA and **R**-PIA increased the efflux of ^{86}Rb. In this series of experiments, the atria were first exposed to ^{86}Rb and then washed repeatedly so that the efflux of the previously gained isotope could be measured. Under control conditions, a fairly stable rate constant of about 0.01 min^{-1} was calculated. After the addition of NECA 1 μmol l^{-1}, the rate constant was almost doubled within 15 min and returned virtually to control values after 30 min washout. Finally, **R**-PIA 1 μmol l^{-1} was added and, again, the rate constant of ^{86}Rb was significantly increased. The same experiments with NECA and **R**-PIA were also carried out in the presence of $BaCl_2$ 0.1 mmol l^{-1}. Ba ions seem to occupy potassium channels by electrostatic forces (Armstrong & Taylor, 1980; Armstrong *et al.*, 1982) and to reduce the conductance of potassium channels in various heart muscle preparations (Sperelakis *et al.*, 1967; Osterrieder *et al.*, 1982; Cohen *et al.*, 1983). Ba *per se* decreased the rate constant of ^{86}Rb

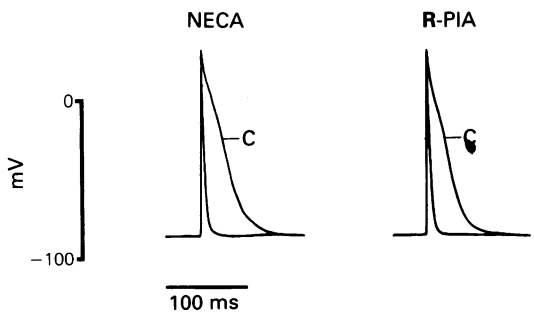

Figure 2 Effects of 5'-(N-ethyl)-carboxamido-adenosine (NECA) and (−)-N^6-(**R**-phenyl-isopropyl)-adenosine (**R**-PIA) (1 μmol l^{-1} each) on the configuration of the action potential in a guinea-pig left atrial trabecula. The same preparation was first exposed to NECA for 15 min, then washed for 45 min and finally exposed to **R**-PIA for 15 min. Original records under control conditions (C) and 15 min after the addition of the respective substance were graphically superimposed.

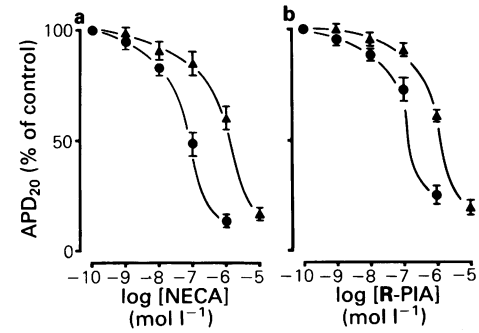

Figure 3 Effects of 5'-(N-ethyl)-carboxamido-adenosine (NECA) (a) and (−)-N^6-(**R**-phenyl-isopropyl)-adenosine (**R**-PIA) (b) on action potential duration at 20% repolarization (APD_{20}) in guinea-pig left atrial trabeculae. Cumulative concentration-response relationships under control conditions (●) and in the presence of 8-phenyltheophylline 10 μmol l^{-1} (▲). The effects of each concentration were observed for 15 min. Symbols represent means (n = 6 in each group) and vertical lines show s.e.mean.

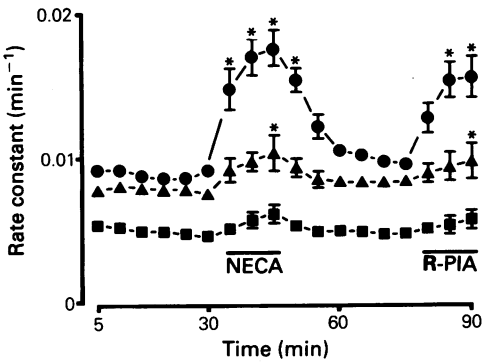

Figure 4 Time course of the effects of 5'-(N-ethyl)-carboxamido-adenosine (NECA) and (−)-N^6-(R-phenyl-isopropyl)-adenosine (R-PIA) (1 μmol l^{-1} each) on the rate constant of ^{86}Rb efflux in guinea-pig left atria under control conditions (●), in the presence of 8-phenyltheophylline 10 μmol l^{-1} (▲), and in the presence of $BaCl_2$ 0.1 mmol l^{-1} (■). Symbols represent means ($n = 6$ in each of the three groups); vertical lines show s.e.mean except when it is smaller than the size of the symbols. Significance level $P < 0.01$ is marked by an asterisk (control vs test values; analysis of variance).

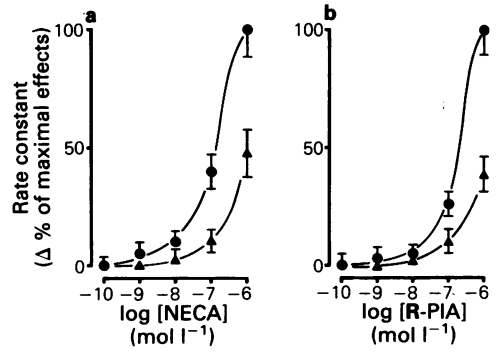

Figure 5 Effects of (a) 5'-(N-ethyl)-carboxamido-adenosine (NECA) and (b) (−)-N^6-(R-phenyl-isopropyl)-adenosine (R-PIA) on the rate constant of ^{86}Rb efflux under control conditions (●) and in the presence of 8-phenyltheophylline 10 μmol l^{-1} (▲). Cumulative concentration-response relationships are shown. Symbols represent means ($n = 6$ in each of the four groups); vertical lines show s.e.mean except when it is smaller than the size of the symbols used.

efflux by about 40%. In the presence of Ba, the effects of NECA and R-PIA on the efflux of ^{86}Rb were almost eliminated. 8-Phenyltheophylline 10 μmol l^{-1} also antagonized the increase in ^{86}Rb efflux by NECA or R-PIA, without significantly changing the control values.

The increase in ^{86}Rb efflux by NECA or R-PIA was concentration-dependent and the concentration-response relationships were shifted to the right by 8-phenyltheophylline (Figure 5).

The shortening of the action potential by adenosine analogues may be responsible for the negative inotropic effects of the substances by impairment of calcium influx during excitation (as already discussed by Grossman & Furchgott, 1964; Schrader *et al.*, 1975). Influx studies with ^{45}Ca generally reveal only relatively small changes (Grossman & Furchgott, 1964; Guthrie & Naylor, 1967; Meinertz *et al.*, 1973). Figure 6 demonstrates a small reduction of ^{45}Ca uptake (about 20%) in the presence of NECA or R-PIA (1 μmol l^{-1} each) which was statistically significant (comparison of control values versus all test values).

Discussion

We have confirmed and extended earlier results that adenosine has a negative inotropic effect in atrial heart muscle at relatively high concentrations. The effects of adenosine were antagonized in the presence of adenosine deaminase but enhanced in the presence of dipyridamole. This indicates that, firstly, receptors located outside on the cell surface probably mediate the inotropic effects and, secondly, an efficient uptake system for adenosine rapidly eliminates the effects of extracellularly applied adenosine. This may explain why maximal and stable effects of adenosine are established only at 1 mmol l^{-1}.

The effects of adenosine on F_c were equally well mimicked by NECA and R-PIA at about thousand times lower concentrations, R-PIA being about 100 times more potent than S-PIA. The observed large

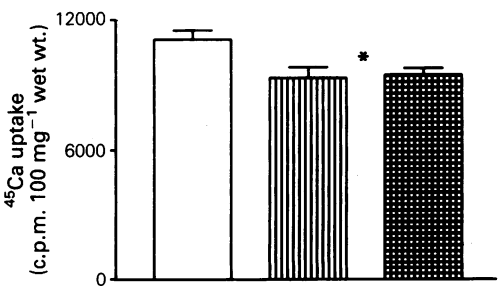

Figure 6 Effects of 5'-(N-ethyl)-carboxamido-adenosine (column with vertical lines) and (−)-N^6-(R-phenyl-isopropyl)-adenosine (cross-hatched column) (1 μmol l^{-1} each) on ^{45}Ca uptake (5 min) in beating (3 Hz) guinea-pig left atria; the open column shows the control value. Columns represent means (7 preparations in each group); vertical lines show s.e.mean. Significance level $P < 0.01$ is marked by an asterisk (control vs test values; analysis of variance).

difference in potency between R-PIA and S-PIA is in accord with an earlier study (Collis, 1983) and in agreement with the assumption that the inhibition of F_c by adenosine is mediated by an interaction with A_1-receptors. However, it would be surprising if R-PIA and NECA are equally effective at A_1-receptors. In addition, it is debatable whether or not any changes of the cyclic AMP content are responsible for the changes in F_c (Huang & Drummond, 1976; Anand-Srivastava & Cantin, 1983; Brückner *et al.*, 1985; Böhm *et al.*, 1988). Our results suggest that, in guinea-pig left atria, adenosine and both R-PIA and NECA stimulate a common receptor population which mediates the observed inotropic and electrophysiological changes. This hypothesis is in line with the physiological importance of specific adenosine receptors on the cell surface, but calls into question the significance of the receptor classification, A_1 and A_2 in atrial heart muscle. In the present study, two competitive inhibitors, theophylline and 8-phenyltheophylline, did not distinguish between the effects of adenosine, R-PIA, S-PIA or NECA. Other xanthine or non-xanthine derivatives, selective for A_1-receptors, have been described (Lohse *et al.*, 1987; Stiles & Jacobson, 1987; Daly *et al.*, 1988; Shamim *et al.*, 1988). Attempts to show selective inhibition of the effects of PIA and NECA on F_c in atrial heart muscle, however, have failed (Collis *et al.*, 1987). It is, therefore, possible that the inotropic effects of adenosine or its derivatives in the atrium are due to the activation of a receptor population which is unrelated to A_1- or A_2-receptors, and that these receptors may be better described as P_1 (Burnstock, 1972; 1978) or A_3 (Ribeiro *et al.*, 1986; Williams, 1987).

It has been suggested earlier that the effects of adenosine in the atrium may be ascribed to an increase in potassium conductance (Hartzell, 1979). This effect shortens the action potential and may, indirectly, diminish the entry of calcium during excitation. In the present study, it was shown that the effects of adenosine on action potential, potassium conductance and calcium entry are mimicked by NECA and R-PIA at micromolar concentrations. Again, no rank order of potency could be found for the effects of NECA and R-PIA which could serve as an indication for the separation of effects mediated by either A_1- or A_2-receptors.

An increase in the potassium conductance prevents the calcium slow inward current from running its normal time course (Ten Eick *et al.*, 1976). Therefore, from the present experiments, we cannot deduce whether NECA and R-PIA not only increase the potassium conductance but also decrease the calcium conductance of the myocardial cell membrane. Voltage clamp experiments are needed to provide this information.

The effects of purinergic stimulation in the atrium, including the effects on F_c, APD and potassium conductance (Nawrath *et al.*, 1985) are, therefore, virtually identical to those of cholinergic stimulation. It seems plausible from the results of many studies that the activation of either adenosine or acetylcholine receptors in atrial heart muscle produces an identical chain of cellular events via the same post receptor pathways. As such, adenosine may either help mediate the effects of cholinergic stimulation or serve as a reserve neurotransmitter in atrial heart muscle (Burnstock, 1986).

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Pronounced cholinergic but only moderate purinergic effects in isolated atrial and ventricular heart muscle from cats

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1 The effects of cholinergic and purinergic stimulation on action potential, force of contraction and ^{86}Rb efflux were investigated in cat atrial and/or ventricular heart muscle.

2 Acetylcholine and carbachol exerted a concentration-dependent negative inotropic effect in cat atrial heart muscle. Carbachol $10\,\mu mol\,l^{-1}$ completely abolished the force of contraction and increased the rate constant of ^{86}Rb efflux 2–3 fold, whereas the action potential duration was shortened to about $\frac{1}{10}$ of its length under control conditions.

3 The effects of acetylcholine and carbachol in cat atrial heart muscle were mimicked, qualitatively, by adenosine and its analogues 5'-(N-ethyl)-carboxamido-adenosine (NECA) and (−)-N^6-(**R**-phenyl-isopropyl)-adenosine (**R**-PIA). Maximal purinergic effects, however, amounted to about 15–50% in comparison to those of cholinergic stimulation.

4 In cat ventricular heart muscle, cholinergic or purinergic stimulation had no significant effects on the force of contraction in the absence of a cyclic AMP-dependent positive inotropic effect. Carbachol antagonized the positive inotropic effect elicited by either 3-isobutyl-1-methylxanthine, isoprenaline or cyclic 8-(4-chlorphenylthio)adenosine-3':5'-monophosphate; NECA and **R**-PIA were less effective. The inhibition by carbachol of the effects of isoprenaline was not related to a change in the rate constant of ^{86}Rb efflux.

5 It is concluded that the effects of cholinoceptor and purinoceptor agonists in the cat heart involve a change in the potassium conductance in the atrium, whereas the effects in the ventricle may be related to changes of intracellular cyclic AMP levels. It seems reasonable to assume that, in comparison to cholinergic stimulation, a low density of purinoceptors in the cat heart is responsible for the relatively weak effects of adenosine agonists in this species.

Introduction

The negative inotropic effect of cholinergic stimulation in atrial heart muscle is accompanied by a hyperpolarization of the myocardial cell membrane and a decrease in the action potential duration (Burgen & Terroux, 1953; Del Castillo & Katz, 1955; Trautwein & Dudel, 1958; for review see Löffelholz & Pappano, 1985). Maximally effective concentrations of acetylcholine or carbachol, which virtually abolish the force of contraction, shorten the action potential to about $\frac{1}{10}$ of its normal length in preparations from various species. The electrophysiological effects in the atrium are thought to be mediated by an increase in the potassium conductance of the myocardial cell membrane (Harris & Hutter, 1956; Hutter, 1961; Ten Eick *et al.*, 1976; Nawrath, 1977; Lipsius, 1982; Baumgarten *et al.*, 1984). In guinea-pig left atria, there is a striking similarity between the actions of acetylcholine and adenosine, including the effects of force on contraction, action potential configuration and ion fluxes (Johnson & McKinnon, 1956; de Gubareff & Sleator, 1965; Belardinelli & Isenberg, 1983a; Jochem & Nawrath, 1983).

In ventricular heart muscle, the negative inotropic effects of cholinergic stimulation are detectable only in the presence of agents which are known to increase adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels (Watanabe & Besch, 1975; Endoh & Honma, 1979; Nawrath *et al.*, 1981; Belardinelli & Isenberg, 1983b; for review see Endoh, 1987). Adenosine acts, also in this respect, in a similar manner (Schrader *et al.*, 1977; Rockoff &

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Dobson, 1980; Böhm et al., 1984; for review see Endoh, 1987). The effects of either acetylcholine or adenosine in the ventricle are not linked to changes in action potential configuration or alterations of the potassium conductance (Nawrath et al., 1981; 1985; Josephson & Sperelakis, 1982; Inoue et al., 1983; Isenberg & Belardinelli, 1984; Böhm et al., 1985), but are related to an interaction of the substances with the adenylate cyclase system (Schrader et al., 1977; Dobson, 1978; 1983; Watanabe et al., 1978; Biegon & Pappano, 1980; Baumann et al., 1981; Leung et al., 1983; Hosey et al., 1984).

Earlier studies on the effects of adenosine have revealed distinct species differences in the sensitivity to adenosine; intravenous infusion of adenosine markedly reduced the heart rate in dogs and guinea-pigs but only to a minor extent in rabbits and cats (see Drury, 1936; de Gubareff & Sleator, 1965). Hopwood et al. (1987) observed an antiadrenergic effect of adenosine in guinea-pig but not rabbit ventricles. In the isolated perfused heart of the cat, both an increase and a decrease in heart function, depending on the experimental conditions, were observed in response to bolus injections of adenosine (Weissel et al., 1974). We have shown that the effects of adenosine in cat isolated atrium are much less pronounced than those of acetylcholine, indicative of either a reduced number of adenosine receptors in this tissue or a coupling defect between receptor activation and changes of ionic conductances (Jakob et al., 1987).

In the present paper, we have investigated the effects of carbachol and 5'-(N-ethyl)-carboxamido-adenosine (NECA) and (−)-N^6-(**R**-phenyl-isopropyl) -adenosine (**R**-PIA) on action potential, force of contraction and ^{86}Rb efflux in cat atrial heart muscle. For comparison, the effects of the same agonists on force of contraction and ^{86}Rb efflux were also investigated in cat ventricular heart muscle. A preliminary account of this work has been published (Jakob et al., 1987).

Methods

Hearts of young cats (either sex; body weight 800–1500 g) were rapidly excised under ether anaesthesia and immediately rinsed in warm oxygenated Tyrode solution (composition in mmol l^{-1}: NaCl 136.9, KCl 5.4, $MgCl_2$ 1.05, NaH_2PO_4 0.42, $NaHCO_3$ 11.9, $CaCl_2$ 1.8, glucose 5.6). The solution was gassed with 95% O_2 and 5% CO_2 at 37°C (pH 7.4). For the measurement of force of contraction, both atria were dissected from the heart and split in half to produce four preparations; the region of the sinus node was cut off, and no spontaneous contractions were observed. Ventricular preparations were obtained from the right ventricle (thin papillary muscles or trabeculae). For electrophysiological or flux studies, atrial and ventricular trabeculae or papillary muscles (diameter 0.4–0.9 mm, length 3.5–8.5 mm) were isolated by ligating both ends with a fine silk suture and dissecting them from the heart. All preparations were driven at 1 Hz. The procedures for measuring and evaluating the force of contraction, the action potential duration and the rate constant of ^{86}Rb efflux have been described in detail in the preceding paper (Jahnel & Nawrath, 1989). The uptake of adenosine was measured in left atria from guinea-pigs and cats by exposure of the preparations to adenosine 10 μmol l^{-1} containing [^{14}C]-adenosine (specific activity: 74 GBq mol^{-1}) for 30 min. The preparations were then blotted, weighed and dissolved in 1 ml of TS-1 and radioactivity determined in a liquid scintillation counter as described for the determination of ^{45}Ca (Jahnel & Nawrath, 1989). The uptake of adenosine was calculated by determining the ratio of adenosine concentrations in the organ bath and the tissue preparations after 30 min and expressed as nmol adenosine 100 mg^{-1} wet weight.

Results are shown as original records or given as means ± s.e.mean. When appropriate, statistically significant differences were assessed by Student's t test for paired data or analysis of variance followed by modified t statistics according to Wallenstein et al. (1980).

Chemicals

The following drugs were used (sources in parentheses): carbachol (E. Merck, Darmstadt); adenosine; adenosine deaminase (ADA); (−)-N^6-(**R**-phenyl-isopropyl)-adenosine (**R**-PIA) (Boehringer, Mannheim); 5'-N-(ethyl)-carboxamido-adenosine (NECA) (Byk Gulden, Konstanz); dipyridamole (Thomae, Biberach); 3-isobutyl-1-methylxanthine (IBMX) (Serva, Heidelberg); cyclic 8-(4-chlorphenylthio)adenosine-3':5'-monophosphate (Boehringer, Mannheim); isoprenaline sulphate (Boehringer, Ingelheim). ^{86}Rubidium chloride and [^{14}C]-adenosine (NEN, Dreieich); tissue solubilizer TS-1 and scintillation cocktail Minisolve (Zinsser, Frankfurt). All other chemicals were obtained from Merck, Darmstadt.

Results

Atrial preparations

Adenosine exerted a concentration-dependent negative inotropic effect in cat atrial heart muscle (Figure 1). Maximally effective concentrations of adenosine

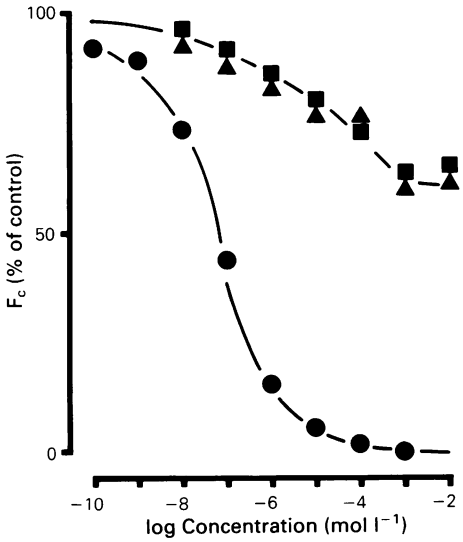

Figure 1 Effects of adenosine (▲, $n = 23$), of adenosine in the presence of dipyridamole 10 μmol l^{-1} (■, $n = 8$) and of acetylcholine (●, $n = 110$; summarizes all our control data) on the force of contraction (F_c) in cat atrial preparations. Cumulative concentration-response relationships are shown. The effect of each concentration was observed for 15 min. Each point represents the mean and the s.e.mean (within the size of the symbols).

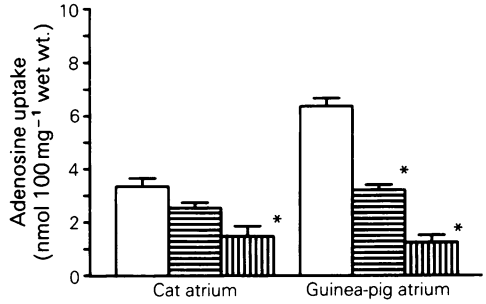

Figure 2 Effects of dipyridamole, 1 (columns with horizontal lines) and 10 (columns with vertical lines) μmol l^{-1}, on adenosine uptake in cat and guinea-pig atrial heart muscle. Each column represents the mean of 3–8 preparations; vertical lines show s.e.mean. Open columns represent control uptake. Significant differences ($P < 0.01$) are marked by asterisks.

(1 mmol l^{-1}) reduced the force of contraction (F_c) by about 30%. Comparatively, the negative inotropic effect of acetylcholine was achieved at much lower concentrations, and full depression of F_c was observed at 1 μmol l^{-1} (Nawrath *et al.*, 1984). In the presence of dipyridamole 10 μmol l^{-1}, the concentration-response curve for adenosine was not significantly affected. This indicates that the failure of adenosine to abolish F_c completely is probably not due to a removal of the drug from the receptor sites into the intracellular space. Figure 2 shows that a nucleoside transporter exists in cat atrial heart muscle. The uptake of adenosine was inhibited by dipyridamole in a concentration-dependent manner. The transport capacity for adenosine in preparations from cats was, however, only about half of that seen in left atria from guinea-pigs.

Qualitatively, the effects of acetylcholine and adenosine on cat atrial heart muscle can be compared to those of carbachol and both NECA and R-PIA, respectively. Quantitatively, the maximal effects of NECA and R-PIA, which were observed at 10 μmol l^{-1}, were somewhat more pronounced than those of adenosine 1 mmol l^{-1}, although a striking difference in magnitude persisted between the effects of cholinergic and purinergic stimulation (Figure 3).

To exclude any influence of endogenously released adenosine, the effects of NECA and R-PIA were also tested in the presence of ADA. In the presence of ADA 2000 u l^{-1}, NECA and R-PIA (10 μmol l^{-1} each) depressed F_c to 61.8 ± 6.2% and to

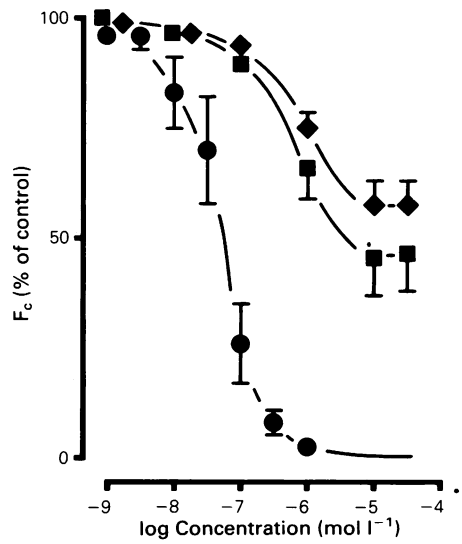

Figure 3 Effects of 5'-(N-ethyl)-carboxamido-adenosine (NECA) (■, $n = 14$), (−)-N^6-(R-phenyl-isopropyl)-adenosine (R-PIA) (◆, $n = 16$) and carbachol (●, $n = 4$) on the force of contraction (F_c) of cat atrial preparations. Cumulative concentration-response relationships are shown. The effect of each concentration was observed for 15 min. Each point represents the mean and vertical lines show s.e.mean (error bars are sometimes within the size of the symbols).

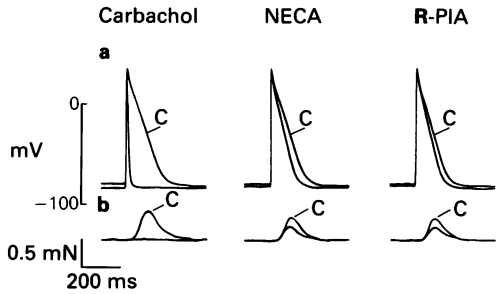

Figure 4 Effects of carbachol 10 μmol l^{-1}, 5'-(N-ethyl)-carboxamido-adenosine (NECA) 10 μmol l^{-1} and (−)-N^6-(R-phenyl-isopropyl)-adenosine (R-PIA) 10 μmol l^{-1} on (a) action potential configuration and (b) force of contraction in cat right atrial preparations. Original records under control conditions (C) and 15 min after the addition of the respective drug were superimposed. Each drug was tested in a different preparation.

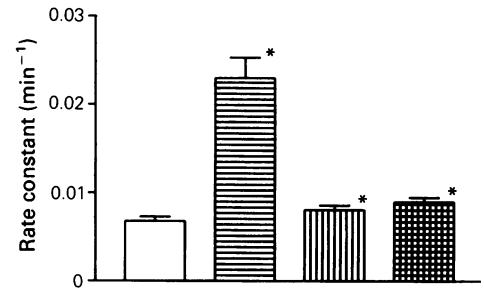

Figure 5 Effects of carbachol 10 μmol l^{-1} (column with horizontal lines; $n = 15$), 5'-(N-ethyl)-carboxamido-adenosine (NECA) 10 μmol l^{-1} (column with vertical lines; $n = 14$) and (−)-N^6-(R-phenyl-isopropyl)-adenosine (R-PIA) 10 μmol l^{-1} (cross-hatched column; $n = 15$) on the rate constant of ^{86}Rb efflux in resting cat right and left atrial preparations. Each column represents the mean and vertical lines show s.e.mean. Control and test values were determined in the same preparations and observed for 30 min each. Control values were virtually identical in all three groups and were pooled (open column; $n = 44$). Statistically significant differences ($P < 0.01$) are marked by asterisks (test vs control).

75.1 ± 3.8% of controls, respectively (means ± s.e.mean of 4 preparations each).

Carbachol 10 μmol l^{-1} shortened the action potential duration to about $\frac{1}{10}$ of its length under control conditions; F_c was completely abolished (Figure 4). In contrast, APD was shortened by only about 25% in the presence of either NECA or R-PIA (10 μmol l^{-1} each); F_c was reduced to about 50% of control values (Figure 4). A summary of all action potential measurements is given in Table 1.

The shortening of the action potential by activation of either cholinoceptors or purinoceptors in atrial heart tissue is thought to be mediated by an increase in the potassium conductance of the myocardial cell membrane (see Introduction). A relatively direct measure of the potassium conductance is given by the rate constant of ^{86}Rb efflux (see Jahnel & Nawrath, 1989). Figure 5 shows that the rate constant of ^{86}Rb efflux in resting preparations was increased by 243 ± 13% in the presence of carbachol 10 μmol l^{-1}. In contrast, NECA and R-PIA 10 μmol l^{-1} each increased the rate constants of ^{86}Rb efflux by only 32 ± 11% and 48 ± 20%, respectively.

Ventricular preparations

Carbachol 10 μmol l^{-1} ($n = 4$), adenosine 1 mmol l^{-1} ($n = 21$), NECA 10 μmol l^{-1} ($n = 11$) and R-PIA 10 μmol l^{-1} ($n = 12$) did not increase F_c in the absence of a cyclic AMP-dependent positive inotropic effect (not shown). In contrast, adenosine in concentrations between 1 mmol l^{-1} and 10 mmol l^{-1} induced a slight positive inotropic effect (not shown, $n = 21$) which was, however, eliminated in the presence of atenolol 10 μmol l^{-1} (not shown, $n = 4$).

A negative inotropic effect of cholinergic or purinergic stimulation in the ventricle was revealed in the presence of inotropic interventions which are known

Table 1 Effect of carbachol, 5'-(N-ethyl)-carboxamido-adenosine (NECA) and (−)-N^6-(R-phenyl-isopropyl)-adenosine (R-PIA) on action potential parameters in cat atrial heart muscle

	RP (mV)	APD_{20} (ms)	APD_{90} (ms)
Control	−76 ± 3	26 ± 5	140 ± 16
Carbachol 10 μmol l^{-1}	−81 ± 2*	5 ± 1*	16 ± 2*
Control	−78 ± 2	27 ± 4	153 ± 6
NECA 10 μmol l^{-1}	−81 ± 2*	20 ± 3	118 ± 9*
Control	−77 ± 2	23 ± 3	144 ± 7
R-PIA 10 μmol l^{-1}	−81 ± 2	15 ± 2	105 ± 8

Values are mean ± s.e.mean, $n = 6$. * $P < 0.01$. RP, resting potential; APD_{20} and APD_{90}, action potential duration at 20% and 90% of repolarization, respectively.

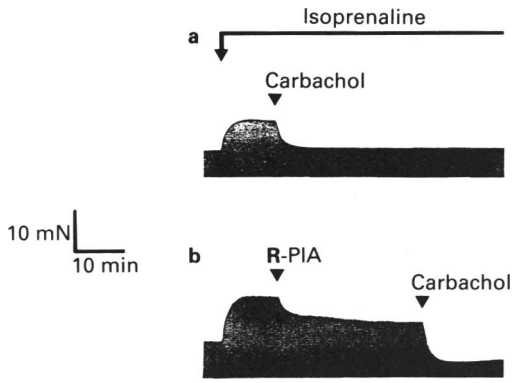

Figure 6 Effects of (a) carbachol 10 μmol l^{-1} and (b) (−)-N^6-(R-phenyl-isopropyl)-adenosine (R-PIA) on force of contraction (F_c) in a cat papillary muscle after stimulation with isoprenaline 0.03 μmol l^{-1}. In (b) the further addition of carbachol 10 μmol l^{-1} decreased F_c by about 60%. Original records.

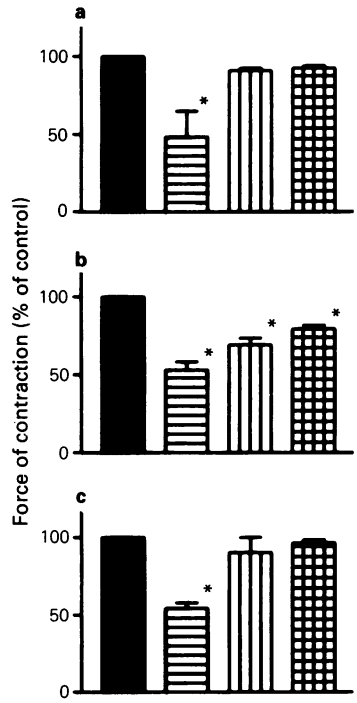

Figure 7 Effects of carbachol 10 μmol l^{-1} (columns with horizontal lines), 5'-(N-ethyl)-carboxamido-adenosine (NECA) 10 μmol l^{-1} (columns with vertical lines) and (−)-N^6-(R-phenyl-isopropyl)-adenosine (R-PIA) 10 μmol l^{-1} (cross-hatched columns) on force of contraction (F_c) previously enhanced by isobutyl methylxanthine (IBMX) 0.1 mmol l^{-1} (a, $n = 3$), isoprenaline 0.03 μmol l^{-1} (b, $n = 8$), or cyclic 8-(4-chlorphenylthio)-adenosine-3':5'-monophosphate 0.1 mmol l^{-1} (c, $n = 3$) in cat right ventricular preparations. Each column represents the mean and vertical lines show s.e.mean. The values before addition of carbachol, NECA or R-PIA were regarded as new control values and set as 100% (solid columns). The effects of drugs were observed for 15 min under each condition. In each preparation only one drug was tested and no further washing procedures were performed. Significant differences ($P < 0.01$) are marked by an asterisk.

to produce an elevation of cyclic AMP levels. In the following experiments, maximally effective concentrations of carbachol, NECA and R-PIA were compared. In the experiment depicted in Figure 6, a papillary muscle was first exposed to isoprenaline 0.03 μmol l^{-1}. The addition of carbachol 10 μmol l^{-1} completely antagonized the positive inotropic response to isoprenaline (Figure 6a). R-PIA 10 μmol l^{-1} exerted a relatively weak effect (Figure 6b). It is interesting to note that, after the exposure to both isoprenaline 0.03 μmol l^{-1} and R-PIA 10 μmol l^{-1}, carbachol 10 μmol l^{-1} exerted an additional negative inotropic effect (Figure 6b).

When the preparations were first exposed to IBMX 0.1 mmol l^{-1}, the addition of carbachol 10 μmol l^{-1} reduced F_c by about 50%, whereas NECA and R-PIA 10 μmol l^{-1} only reduced F_c by about 10% (Figure 7a). IBMX *per se* increased F_c to 165 ± 15% of control values ($n = 9$). However, IBMX is not only an inhibitor of phosphodiesterase activity but also acts as an adenosine antagonist (Kenakin & Beek, 1987) which may have partially masked the inhibitory effects of NECA and R-PIA. Figure 7b shows that all three agonists were able to antagonize the positive inotropic response to isoprenaline 0.03 μmol l^{-1}, although the inhibition by NECA and R-PIA of the effects of isoprenaline was still somewhat smaller than that observed with carbachol. Isoprenaline *per se* increased F_c to 171 ± 7% of control values ($n = 24$). Finally, we stimulated the cat papillary muscles with the cyclic AMP derivative cyclic 8-(4-chlorphenylthio)adenosine-3':5'-monophosphate 0.1 mmol l^{-1} which *per se* increased F_c to 174 ± 21% of control values ($n = 9$). Carbachol also induced a marked decline of F_c, under these conditions, whereas NECA and R-PIA were less effective (Figure 7c).

We were also interested to find out whether or not the cholinergic or purinergic effects in the ventricle were related to changes in the potassium conductance. Isoprenaline 0.03 μmol l^{-1} slightly increased the rate constant of ^{86}Rb efflux in beating preparations, whereas the further addition of either carbachol, NECA or R-PIA (10 μmol l^{-1} each) did not reveal any significant effects. Almost identical results were obtained in resting preparations not stimulated by isoprenaline ($n = 6$ in each group; not shown).

Therefore, the inhibition of the effects of isoprenaline by carbachol, NECA and R-PIA does not seem to be related to a change in the potassium conductance of the myocardial cell membrane.

Discussion

We have shown in the present paper that the effects of cholinergic stimulation in the cat atrium are more pronounced than those of purinergic stimulation. This holds true for the effects of F_c, action potential duration and increase in the rate constant of ^{86}Rb fluxes. It seems reasonable to assume that a low density of purinoceptors in the cat atrium is responsible for the relatively weak effects of adenosine agonists in this tissue. Alternatively, the important link between adenosine receptors and potassium channels in the atrium might somehow be disturbed in this species.

In the ventricle, the overall effects of both cholinergic and purinergic stimulation are generally weaker than those in the atrium, and quantitative differences between the activation of either acetylcholine or adenosine receptors are more difficult to assess. In our experiments in ventricular heart muscle, the indirect effects of adenosine derivatives, either in the absence or presence of IBMX, isoprenaline or cyclic 8-(4-clorphenylthio)adenosine-3':5'-monophosphate, were also weaker than those of carbachol. If carbachol antagonizes cyclic AMP-mediated positive inotropic effects by an inhibitory action at the enzyme adenylate cyclase, it is difficult to understand why this agonist has also inhibited the effect of the cyclic AMP derivative. There are two possibilities to explain this. Either the cyclic AMP derivative inhibits the phosphodiesterase which would indirectly increase the cyclic AMP content of the cell, as with isoprenaline or IBMX, or carbachol exerts effects other than those on the adenylate cyclase system; for instance, it may increase cyclic GMP levels which may counteract the effects of cyclic AMP (Hartzell & Fischmeister, 1986).

Heart muscle from different species obviously varies in its response to adenosine. Goto et al. (1978) have shown that the bullfrog atrium is very insensitive to adenosine. Martens et al. (1987) demonstrated that ventricular myocytes from rats, a species which shows strong adenosine effects in the atrium (Meinertz et al., 1973), have a more than 60 fold lower density of binding sites for adenosine than those in brain and fat cell membranes.

Several studies have suggested that the effects of adenosine in the heart are mediated by the receptor subclass A_1 (Evans et al., 1982; Collis, 1983; Leung et al., 1983; Kurahashi & Paton, 1986; Hamilton et al., 1987). Henrich et al. (1987) and Martens et al. (1987) demonstrated an inhibition of catecholamine-stimulated cyclic AMP levels in rat ventricular myocytes with the rank order of potency: R-PIA > NECA, which also suggests the presence of A_1-receptors. The preceding paper (Jahnel & Nawrath, 1989) and this paper, however, did not reveal any significant differences in the potency of NECA and R-PIA. Thus, we have demonstrated in two species, in accord with other studies (Hughes & Stone, 1983; Collis et al., 1985; Böhm et al., 1985), that the identification of the adenosine receptor subtypes in heart muscle remains problematic as the potency of R-PIA and NECA are similar. It seems unlikely that differences in the hydrophilicity or lipophilicity of the drugs can influence the rank order of potency under steady-state conditions. Therefore, the subclassification of the P_1-adenosine receptor in heart muscle (Burnstock, 1972; 1978) as an A_1- or an A_3-subtype (Ribeiro & Sebastiao, 1986) awaits further clarification.

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Functional role of cholinoceptors and purinoceptors in human isolated atrial and ventricular heart muscle

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1 The effects of cholinergic and purinergic stimulation on action potential, force of contraction and ^{86}Rb efflux were investigated in human atrial and ventricular heart muscle.

2 In atrial heart muscle, carbachol and (−)-N^6-(R-phenyl-isopropyl)-adenosine (R-PIA) and 5'-(N-ethyl)-carboxamido-adenosine (NECA) evoked transient decreases of action potential duration and force of contraction; the steady-state effects on force of contraction were virtually identical to control values. In the presence of propranolol, steady-state values after carbachol, R-PIA or NECA amounted to about 50% of control values.

3 In ventricular heart muscle, carbachol, NECA and R-PIA did not significantly affect the action potential configuration or force of contraction.

4 Carbachol, NECA and R-PIA induced a maintained depression of the positive inotropic response to isoprenaline in both atrial and ventricular heart muscle.

5 The rate constant of ^{86}Rb efflux was slightly increased by carbachol, NECA and R-PIA in atrial (10–20%) but not in ventricular heart muscle.

6 In the presence of isoprenaline, carbachol, NECA and R-PIA did not significantly affect the rate constant of ^{86}Rb efflux in both atrial and ventricular heart muscle. Isoprenaline alone increased the rate constant of ^{86}Rb by about 25% in both tissues.

Introduction

We have characterized, in the preceding paper (Kemmer *et al.*, 1989), the effects of cholinergic and purinergic stimulation in isolated heart muscle preparations from cats. The results have revealed marked differences in the sensitivity to cholinoceptor and purinoceptor agonists, the effects of adenosine and its analogues on force of contraction being much less pronounced than those of acetylcholine and carbachol. Correspondingly, the action potential configuration was less affected in the presence of purinoceptor agonists than in the presence of carbachol. The most obvious difference between cholinergic and purinergic stimulation in cat atria was the finding that the efflux of ^{86}Rb was ten times less affected by purinoceptor agonists than by carbachol. In contrast, cholinergic and purinergic stimulation were equieffective in guinea-pig left atria (Jochem & Nawrath, 1983). The observed species differences were tentatively explained by a reduced number of receptors for adenosine in the cat heart. The discrepancy between results obtained in different species prompted us to investigate in detail the effects of cholinoceptor and purinoceptor agonists in isolated atrial and ventricular heart muscle from man. A preliminary account of this work has been published (Jakob *et al.*, 1987).

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Methods

Preparations

Human papillary muscle samples and atrial trabeculae were obtained from 83 patients undergoing open heart surgery. In the present study, 102 ventricular preparations from 39 patients (31 female, 8 male) were investigated. The age ranged from 50 to 79 years. The patient material, the premedication and anaesthesia and the procedures for isolating and further handling of human ventricular heart muscle *in vitro* have been described in detail (Eckel *et al.*, 1982; Jakob *et al.*, 1988).

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In comparison to the experiments in ventricular heart muscle, it turned out to be much more difficult to perform electrophysiological experiments in atrial heart muscle preparations. Several authors have related the great variability of results obtained in human atrial heart muscle (shape of the action potential, partial depolarization) to disease (Hordof *et al.*, 1976; Ten Eick & Singer, 1979; Mary-Rabine *et al.*, 1983; Escande *et al.*, 1986; 1987; Lee, 1986) or age (Escande *et al.*, 1985) of the patients. In our hands, however, the quality of electrophysiological experiments seemed to be mainly determined by the procedure of isolation and further handling of the preparations. Independent of that, the success rate was much higher in preparations from young than from older patients. In the present study, human right atrial muscle strips were obtained from 44 patients (11 female, 33 male). The age ranged from 12 to 74 years. The children (a girl of 14 and a boy of 12 years) suffered from congenital mitral stenosis and aortic insufficiency, respectively, whereas the adult patients usually suffered from coronary heart disease and rheumatic aortic or mitral valve lesions.

At surgery, approximately $1\,cm^2$ of atrial myocardium was removed from the right atrial appendage as a part of the routine cannulation procedure for cardiopulmonary bypass. After removal, the tissue was immersed in preoxygenated Tyrode solution maintained at 4°C. The time between excision and the beginning of laboratory processing was 20–90 min. In the laboratory, the sample was gently pinned down on Sylgard in oxygenated Tyrode solution at room temperature and cut into 3–4 pieces; bundle orientation was respected and macroscopically damaged tissue discarded. Trabeculae were left without further injury in the surrounding tissue. In the organ bath, the preparations were warmed up gradually over a period of 60 min up to 35°C. Initially, a stimulus rate of 0.5 Hz at two times threshold voltage was applied (pulse duration 1–4 ms). After 1–2 h the frequency was set to 1 Hz and voltage to 10–15% above threshold (pulse duration <0.5 ms). Under these conditions, about 15% of the preparations recovered and displayed stable values for both the force of contraction and electrophysiological parameters. Whereas, in our laboratory, the success rate of electrophysiological experiments is over 95% in human ventricular heart muscle, independent of disease, age or sex of the patients (Jakob *et al.*, 1988), the overall success rate with atrial heart muscle was surprisingly low. Summarizing our experiments in atrial preparations from 174 patients during the last three years, we obtained successful electrophysiological experiments from 26 patients with the following control data (mean ± s.d.): action potential amplitude, 87 ± 9 mV; resting potential, -73 ± 12 mV; overshoot, 12 ± 7 mV; action potential duration at 90% of repolarization, 345 ± 92 ms; maximal upstroke velocity, $193 \pm 77\,Vs^{-1}$. Since atrial and ventricular heart muscle has been obtained for years from the same patient material we conclude that, after isolation, human atrial heart muscle is much more susceptible to damage than ventricular heart muscle. Correspondingly, in our experiments, a clearcut relationship between *in vivo* and *in vitro* data, with respect to disease, could not be demonstrated. Paradoxically, we obtained action potentials with fast upstroke and high amplitude in a preparation from a 61 year old man who suffered from atrial dilatation and fibrillation (depicted in the left panel of Figure 1). On the other hand, there were many cases with no signs of electrical aberrations *in vivo*, whereas our attempts to measure electrical activity in the corresponding preparations after isolation, were completely unsuccessful.

Experimental procedures

The procedures for mounting specimens from human heart muscle and the techniques for measuring both force of contraction and action potential parameters have been described in detail (Eckel *et al.*, 1982; Jakob *et al.*, 1988).

For efflux studies with ^{86}Rb, atrial and ventricular preparations were mounted and treated as described in the first of the two preceding papers (Jahnel & Nawrath, 1989).

Chemicals and solutions

The Tyrode solution used in all experiments had the following composition ($mmol\,l^{-1}$): NaCl 136.9, KCl 5.4, $MgCl_2$ 1.05, NaH_2PO_4 0.42, $NaHCO_3$ 11.9, $CaCl_2$ 1.8, glucose 5.6 and was equilibrated with 95% O_2 and 5% CO_2 at 37°C (ventricle) or 35°C (atrium) (pH 7.4). Carbachol (E. Merck, Darmstadt); (−)-N^6-(R-phenyl-isopropyl)-adenosine (R-PIA, Boehringer, Mannheim); 5'-(N-ethyl)-carboxamido-adenosine (NECA, Byk Gulden, Konstanz); isoprenaline sulphate (Boehringer, Ingelheim); propranolol hydrochloride (ICI-Pharma, Plankstadt). ^{86}Rb chloride (NEN, Dreieich). Tissue solubilizer TS-1, scintillation cocktail Minisolve (Zinsser, Frankfurt). All other chemicals (Merck, Darmstadt).

Evaluation of results

Force of contraction, action potential parameters and rate constants of ^{86}Rb efflux were evaluated as described and are shown as original records or means ± s.e. mean. When appropriate, statistical analysis was performed by means of Student's *t* test for paired data. A *P* value of less than 0.05 was considered significant.

Figure 1 Action potential configurations in human atrial heart muscle. Original records.

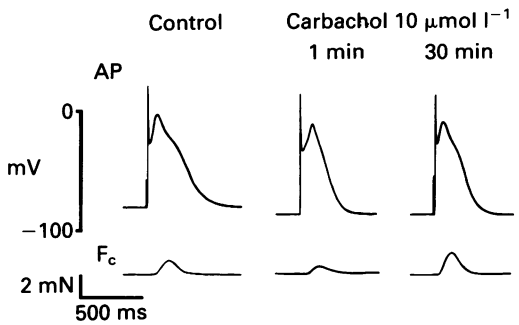

Figure 2 Time-dependent effects of carbachol 10 μmol l^{-1} on action potential (AP) and force of contraction (F_c) in human atrial heart muscle. Original records.

Results

Atrial preparations

Characteristic action potential recordings from human atrial heart muscle are shown in Figure 1. Essentially, we have observed three different types of atrial action potential. Type I is characterized by the lack of a significant phase 1 and a steady repolarization phase yielding action potentials of triangular shape which are also seen in atrial heart muscle from different laboratory animals such as cat, rat or guinea-pig. Type II and III are characterized by a pronounced phase 1 and a second slow depolarization phase which, roughly, forms a plateau with its maximal height near 0 mV and −40 mV, respectively. These observations are in agreement with previous data on action potential parameters in human atrial myocardium (Trautwein *et al.*, 1962; Fabiato & Fabiato, 1971; Gelband *et al.*, 1972; Tuganowski & Tendera, 1973).

Figure 2 shows that maximally effective concentrations of carbachol exerted transient effects on both action potential (AP) and force of contraction (F_c). After 1 min, F_c and action potential duration at 90% repolarization (APD_{90}) were decreased, while action potential amplitude (APA) and resting potential (RP) were slightly increased. Steady state effects after 10 or 30 min, however, were much less pronounced than initial peak effects. Similar effects were

Table 1 Effect of carbachol 10 μmol l^{-1} on action potential parameters in human heart muscle

			Carbachol (10 μmol l^{-1})	
		Control	*1 min*	*30 min*
APA	(mV)	87 ± 4	94 ± 3	92 ± 3
RP	(mV)	−75 ± 1	−80 ± 1	−79 ± 1
OS	(mV)	12 ± 3	14 ± 2	12 ± 2
APD_{90}	(ms)	381 ± 34	130 ± 33*	236 ± 29*
dV/dt_{max}	(V s^{-1})	190 ± 27	259 ± 24*	228 ± 28*

Values are mean ± s.e.mean, $n = 7$. * $P < 0.05$. APA, action potential amplitude; RP, resting potential; OS, overshoot; APD_{90}, action potential duration at 90% repolarization; dV/dt_{max}, maximal upstroke velocity.

Table 2 Effect of 5'-(N-ethyl)-carboxamido-adenosine (NECA, 10 μmol l^{-1}) on action potential parameters in human atrial heart muscle

			NECA (10 μmol l^{-1})	
		Control	*1 min*	*30 min*
APA	(mV)	90 ± 4	96 ± 3	95 ± 3
RP	(mV)	−74 ± 2	−77 ± 2	−76 ± 2
OS	(mV)	16 ± 3	18 ± 2	19 ± 2
APD_{90}	(ms)	384 ± 35	177 ± 25*	256 ± 33*
dV/dt_{max}	(V s^{-1})	237 ± 31	269 ± 36*	264 ± 29*

Values are mean ± s.e.mean, $n = 6$. * $P < 0.05$. For key to abbreviations used see legend of Table 1.

Table 3 Effect of (−)-N⁶-(R-phenyl-isopropyl)-adenosine (R-PIA, 10 μmol l⁻¹) on action potential parameters in human atrial heart muscle

		Control	R-PIA (10 μmol l⁻¹) 1 min	R-PIA (10 μmol l⁻¹) 30 min
APA	(mV)	90 ± 4	94 ± 4	91 ± 5
RP	(mV)	−76 ± 2	−78 ± 1	−77 ± 1
OS	(mV)	14 ± 3	16 ± 3	14 ± 4
APD_{90}	(ms)	365 ± 46	283 ± 23*	288 ± 24*
dV/dt_{max}	($V s^{-1}$)	208 ± 35	285 ± 25*	238 ± 41*

Values are mean ± s.e.mean, $n = 6$. * $P < 0.05$. For key to abbreviations used see legend of Table 1.

seen with maximally effective concentrations of either NECA or R-PIA. Tables 1–3 summarize the results from all electrophysiological experiments obtained with carbachol, NECA and R-PIA, 10 μmol l⁻¹ each.

Continuous recordings of F_c, as depicted in Figure 3, show the time course of both the decline of F_c after the addition of the drugs and the rapid recovery to a new steady-state level. The responses to all three agonists were quite different in preparations which were first exposed to isoprenaline 0.03 μmol l⁻¹ (Figure 4). Isoprenaline *per se* increased F_c to 253%, 307% and 341%. Upon the addition of either carbachol, NECA or R-PIA (10 μmol l⁻¹ each), F_c was persistently decreased to 163, 173 and 200% of the predrug values, respectively. To exclude an interference between the release of endogenously stored noradrenaline and the effects of carbachol, NECA and R-PIA, we repeated the experiments with all agonists in the presence of propranolol 1 μmol l⁻¹. Figure 5 shows that F_c under control conditions and after the addition of either carbachol, NECA or R-PIA (10 μmol l⁻¹ each) (a), was significantly different in the presence of propranolol 1 μmol l⁻¹ (b) indicating that the release of endogenously stored noradrenaline significantly

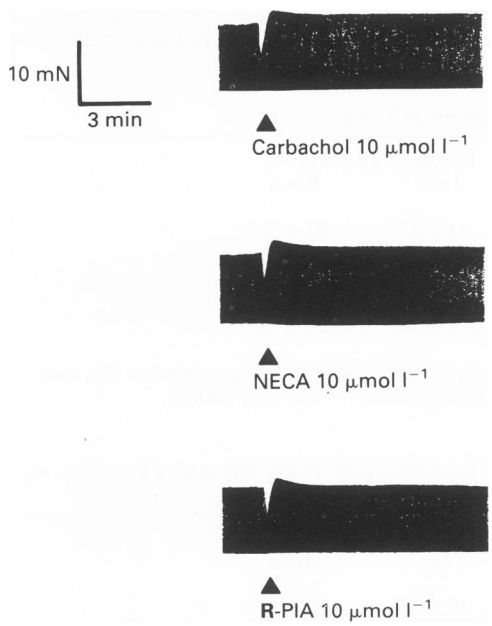

Figure 3 Time-dependent effects of carbachol, 5′-(N-ethyl)-carboxamido-adenosine (NECA) and (−)-N⁶-(R-phenyl-isopropyl)-adenosine (R-PIA) 10 μmol l⁻¹ each on force of contraction in human atrial heart muscle. Original records.

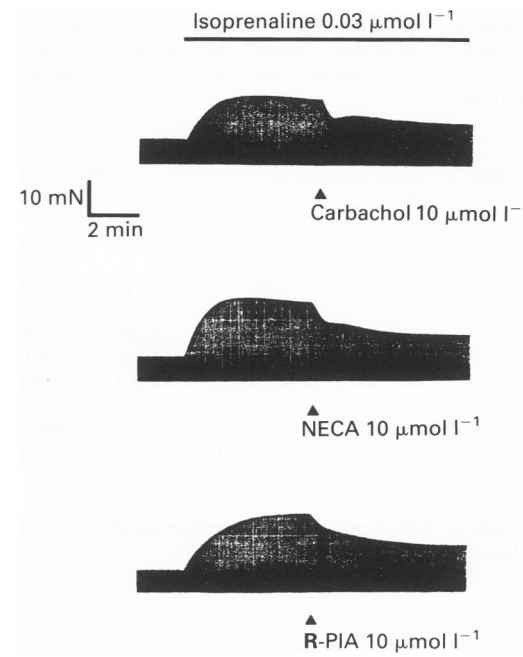

Figure 4 Time-dependent effects of carbachol, 5′-(N-ethyl)-carboxamido-adenosine (NECA) and (−)-N⁶-(R-phenyl-isopropyl)-adenosine (R-PIA) (10 μmol l⁻¹ each), in the presence of isoprenaline 0.03 μmol l⁻¹, on the force of contraction in human atrial heart muscle. Original records.

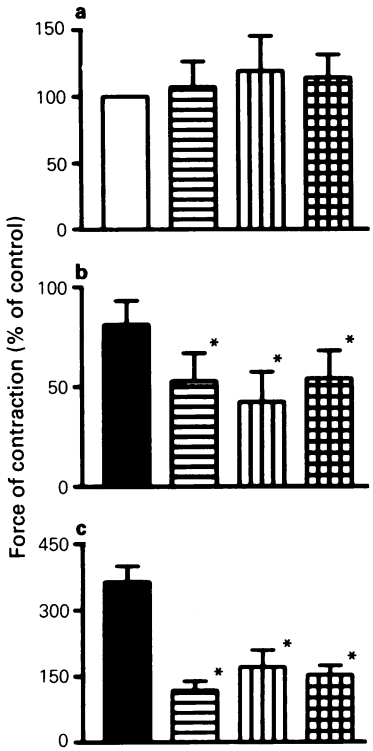

Figure 5 Effect of carbachol (columns with horizontal lines), 5'-(N-ethyl)-carboxamido-adenosine (NECA) (columns with vertical lines) and (−)-N^6-(R-phenyl-isopropyl)-adenosine (R-PIA) (cross-hatched columns), each at 10 μmol l^{-1}, on the force of contraction under normal conditions (a), 30 min after the addition of propranolol 1 μmol l^{-1} (b), and 15 min after the addition of isoprenaline 0.03 μmol l^{-1} (c) in human atrial heart muscle. Open (a) or solid (b,c) columns refer to the respective controls. The effects of carbachol, NECA and R-PIA were observed for 15 min each. Each column represents the mean of 6–8 observations from a total of 67 preparations; vertical lines show s.e.mean. Significant differences are marked by asterisks ($P < 0.01$).

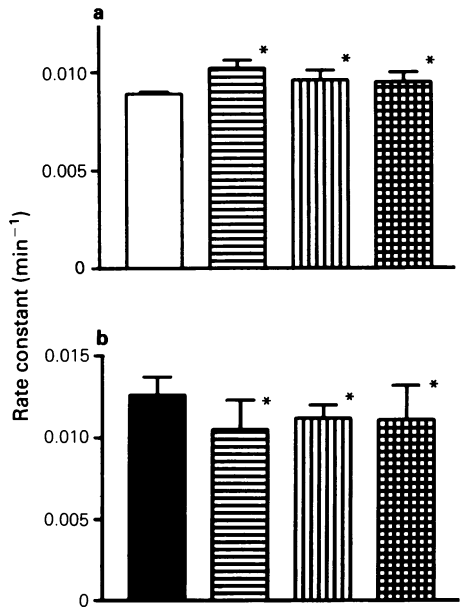

Figure 6 Effect of carbachol (columns with horizontal lines), 5'-(N-ethyl)-carboxamido-adenosine (NECA) (columns with vertical lines) and (−)-N^6-(R-phenyl-isopropyl)-adenosine (R-PIA) (cross-hatched columns), each at 10 μmol l^{-1}, on the rate constant of ^{86}Rb efflux under normal conditions (a) and 15 min after the addition of isoprenaline 0.03 μmol l^{-1} (b) in atrial heart muscle. Open (a) or solid (b) column refers to the respective control. The effects of carbachol, NECA and R-PIA were observed for 30 min each. Each column represents the mean of 6–12 observations from a total of 90 preparations; vertical lines show s.e.mean. Significant differences vs control are marked by asterisks ($P < 0.01$).

contributes to both control and test values. Propranolol *per se* decreased F_c to 81 ± 12% of control values. In the presence of propranolol, the steady-state reduction of F_c in response to carbachol, NECA or R-PIA 10 μmol l^{-1} each amounted to about 53 ± 14%, 42 ± 15% and 54 ± 14% of the respective control values. In the presence of isoprenaline 0.03 μmol l^{-1}, F_c was reduced from 364 ± 36% to 118 ± 21%, 171 ± 38%, and to 153 ± 21%, respectively (Figure 5c).

We were also interested to determine whether or not the described changes of F_c in the steady-state could be ascribed to a change in the potassium conductance, as described for guinea-pig atrial preparations (Jahnel & Nawrath, 1989). To this end, we followed the efflux of ^{86}Rb in previously loaded atrial strips and determined the rate constants of the washout process at rest which was fairly monoexponential under control conditions. There was a small but statistically significant increase (20%) in the rate constant after the addition of carbachol 10 μmol l^{-1}; this increase was mimicked, to a minor degree, by NECA and R-PIA (10 μmol l^{-1} each) (Figure 6a). In electrically driven preparations, stimulated by isoprenaline 0.03 μmol l^{-1}, the rate constant was increased, first, from 0.0089 ± 0.0001 to 0.0104 ± 0.0010 min^{-1} ($P < 0.01$) by driving, and, second, from this value to 0.0126 ± 0.0011 min^{-1} ($P < 0.01$) by isoprenaline; under these conditions, the negative inotropic response to carbachol, NECA or R-PIA was related to a decrease rather than an increase of the rate constant of ^{86}Rb efflux (Figure 6b).

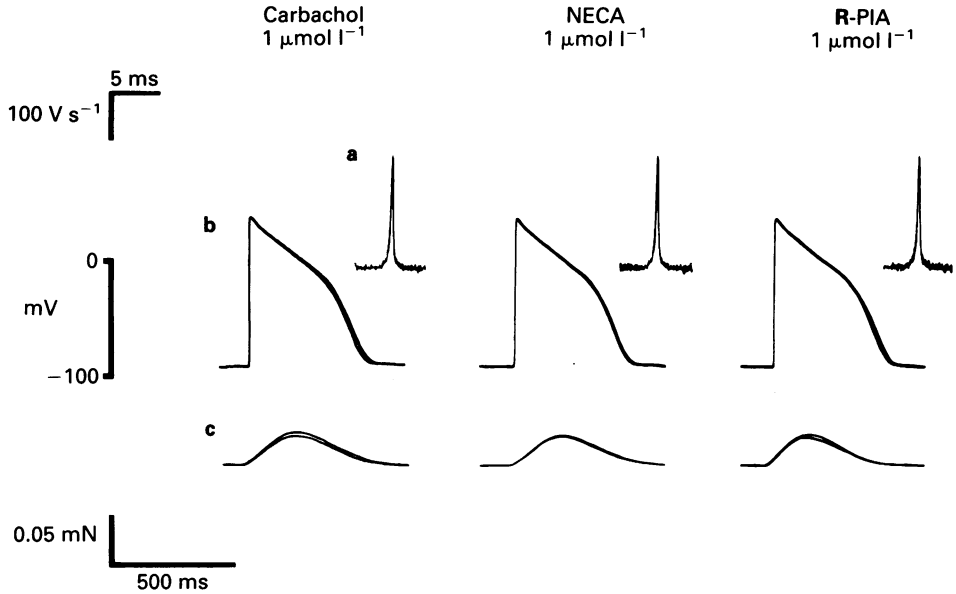

Figure 7 Effects of carbachol, 5'-(N-ethyl)-carboxamido-adenosine (NECA) and (−)-N^6-(**R**-phenyl-isopropyl)-adenosine (**R**-PIA) (1 μmol l^{-1} each) on (a) action potential, (b) maximal upstroke velocity and (c) force of contraction in human ventricular heart muscle. Original records under control conditions and 15 min after the addition of the respective drug were graphically superimposed.

Ventricular preparations

As in ventricular heart muscle from cats (Kemmer *et al.*, 1989), carbachol, NECA and **R**-PIA were completely ineffective in preparations of papillary muscle from man obtained mainly from the left ventricle. Figure 7 demonstrates that the recordings under control conditions and 15 min after the addition of 1 μmol l^{-1} of either drug were virtually identical. Preliminary experiments had revealed that acetylcholine 10 μmol l^{-1} and adenosine 1 mmol l^{-1} also had virtually no effect in human ventricular heart muscle ($n = 4$; paired experiments; results not shown).

When the preparations were first treated with isoprenaline 0.03 μmol l^{-1}, carbachol, NECA and **R**-PIA, each at 10 μmol l^{-1}, completely antagonized the positive inotropic response elicited by isoprenaline (Figure 8). A summary of the results, obtained with carbachol, NECA and **R**-PIA in human ventricular preparations both under normal conditions (a) and after stimulation with isoprenaline (b), is shown in Figure 9. The configuration of the action potential, in the absence and presence of isoprenaline 0.03 μmol l^{-1}, was not significantly affected by carbachol, NECA or **R**-PIA (10 μmol l^{-1} each, data not shown).

Efflux experiments with ^{86}Rb revealed no significant effect of carbachol, NECA or **R**-PIA (10 μmol l^{-1} each) on the rate constant of the isotope exchange either in resting (Figure 10a) or in beating preparations, the latter being stimulated with isoprenaline (Figure 10b). Irrespective of the presence of cholinoceptor or purinoceptor agents, the rate constant of ^{86}Rb efflux was slighly higher in beating than in resting preparations.

Discussion

The basic mechanisms underlying the action potential in the heart, derived from investigations in various laboratory animals, may be basically applicable to the human heart (Trautwein *et al.*, 1962). Several observations, however, indicate to us that such extrapolations may be wrong in the case of receptor-mediated changes in cardiac function. For example, the application of α_1-adrenoceptor agonists induces a strong positive inotropic effect in the rabbit ventricle (Schümann *et al.*, 1974), whereas in human ventricular heart muscle it has almost no effect (Jakob *et al.*, 1988). Conversely, histamine

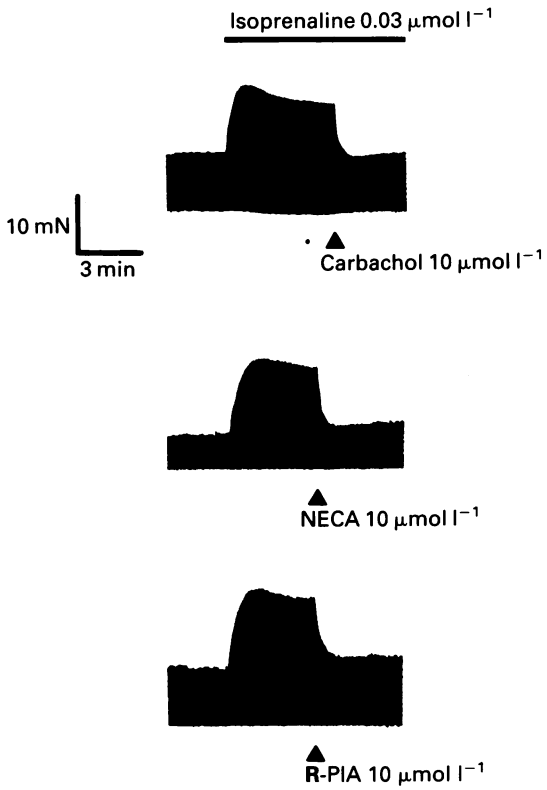

Figure 8 Time-dependent effects of carbachol, 5'-(N-ethyl)-carboxamido-adenosine (NECA) and (−)-N^6-(R-phenyl-isopropyl)-adenosine (R-PIA), 10 μmol l^{-1} each, in the presence of isoprenaline 0.03 μmol l^{-1}, on the force of contraction in human ventricular heart muscle. Original records.

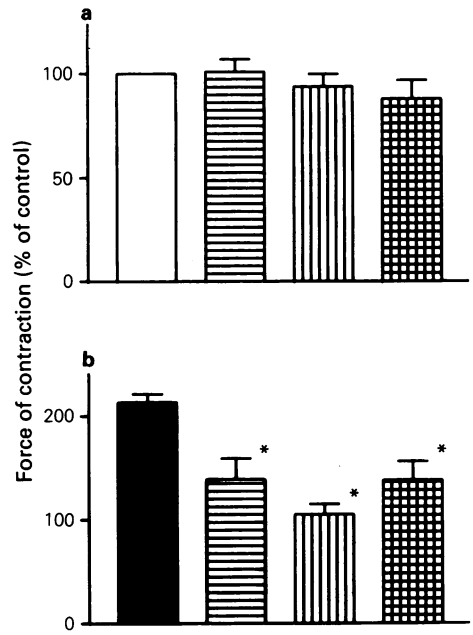

Figure 9 Effect of carbachol (columns with horizontal lines), 5'-(N-ethyl)-carboxamido-adenosine (NECA) (columns with vertical lines) and (−)-N^6-(R-phenyl-isopropyl)-adenosine (R-PIA) (cross-hatched columns), each at 10 μmol l^{-1}, on the force of contraction under normal conditions (a) and 15 min after the addition of isoprenaline 0.03 μmol l^{-1} (b) in human ventricular heart muscle. Open (a) or solid (b) column refers to the respective control. The effects of carbachol, NECA and R-PIA were observed for 15 min each. Each column represents the mean of 3–4 observations from a total of 10 preparations; vertical lines show s.e.mean. Significant differences vs control are marked by asterisks ($P < 0.01$).

markedly increases the force of contraction in human ventricular heart muscle (Eckel *et al.*, 1982) but is devoid of any effects in the cat ventricle (Flynn *et al.*, 1979). With respect to cholinergic and purinergic effects in the heart, there is circumstantial evidence from several earlier studies that both acetylcholine (Trautwein *et al.*, 1962; Sleator & de Gubareff, 1964; Ten Eick & Singer, 1979) and adenosine (de Gubareff & Sleator, 1965; Böhm *et al.*, 1985; Lee & Chang, 1985; Marmo *et al.*, 1986; Genovese & Levi, 1987) can alter cardiac function in the human heart.

In the present paper, we have described systematically the concentration-dependent effects of cholinoceptor and purinoceptor agonists on F_c, AP and ^{86}Rb fluxes in human atrial and ventricular heart muscle. A novel observation in our study is that the potassium conductance and F_c of human atrial heart muscle were affected only transiently and to a lesser extent, by the analogues of acetylcholine and adenosine, in comparison to the effects in atrial heart muscle from other species, e.g., guinea-pig (Jahnel & Nawrath, 1989).

The time course of inotropic effects was obviously influenced by the release of endogenously stored noradrenaline, since propranolol revealed a depression of F_c in response to all agonists under steady-state conditions (Figure 5b). On the other hand, carbachol, NECA and R-PIA also attenuated the positive inotropic effect of isoprenaline (Figure 5c). The overall effects of carbachol, NECA and R-PIA on F_c in the human atrium may therefore be determined by a balance of at least three different mechanisms including (1) direct membrane effects, (2) inhibition

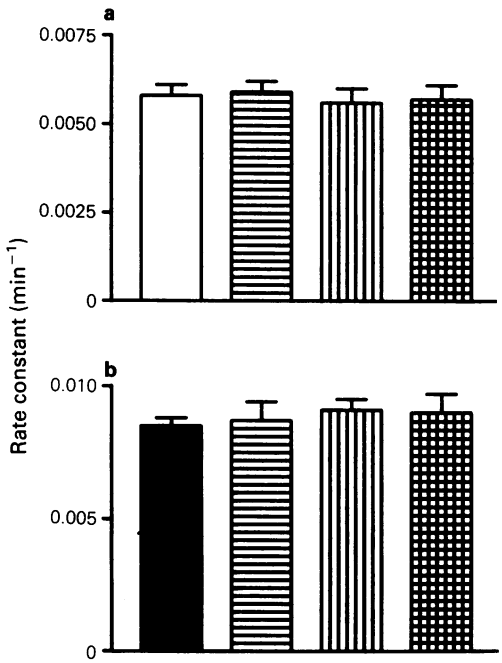

Figure 10 Effect of carbachol (columns with horizontal lines), 5'-(N-ethyl)-carboxamido-adenosine (NECA) (columns with vertical lines) and (−)-N^6-(R-phenyl-isopropyl)-adenosine (R-PIA) (cross-hatched columns), each at 10 μmol l^{-1}, on the rate constant of ^{86}Rb efflux under normal conditions (a) and 15 min after the addition of isoprenaline 0.03 μmol l^{-1} (b) in ventricular heart muscle. Open (a) or solid (b) column refers to the respective control. The effects of carbachol, NECA and R-PIA were observed for 30 min each. Each column represents the mean of 6–12 observations from a total of 55 preparations; vertical lines show s.e.mean.

of the stimulating effects of adenylate cyclase, and (3) release of noradrenaline from the nerve terminals. Whereas mechanisms (1) and (2) will decrease F_c, mechanism (3) will increase it.

In the presence of propranolol, the influence of mechanisms (2) and (3) is probably less important, and mechanism (1) can decrease F_c to values below 100% (Figure 5b). In the presence of isoprenaline, F_c is increased, and mechanisms (1) and (2) can determine the overall response to a greater extent (Figure 5c).

Direct membrane effects of cholinoceptor or purinoceptor agents on excitation and contraction were virtually absent in human ventricular heart muscle. After stimulation of the preparations with isoprenaline both cholinergic and purinergic stimulation resulted in a negative inotropic effect, the latter result has been demonstrated previously by Böhm *et al.* (1985). These indirect negative inotropic effects in the ventricle were not accompanied by any significant changes of action potential configuration or ^{86}Rb fluxes.

The desensitization of the electromechanical effects of cholinergic stimulation in the human atrium occurs within 2–5 min and is in line with two previously published papers (Ten Eick & Singer, 1979; Heidbüchel *et al.*, 1987). Ten Eick & Singer (1979) published the results of one voltage clamp experiment in a partially depolarized trabecula of a diseased right atrial appendage and noticed that, at least in this instance, the preparation did not respond to acetylcholine with as large an increase in the outward current as had been previously noted in normal guinea-pig atrial heart muscle (Ten Eick *et al.*, 1976). In the more systematic voltage clamp study of Heidbüchel *et al.* (1987) in human single atrial cells, the acetylcholine-induced outward current was also considerably smaller than in preparations from other mammalian atria and decreased with time due to desensitization. This may explain why, in our study, only minor changes in the rate constant of ^{86}Rb efflux in response to carbachol were detected and that the configuration of the action potential returned almost to control values within 5 min. Our results are in line with those from an earlier study on the effects of acetylcholine on the isolated heart of human embryos (Garrey & Townsend, 1948). The latter authors found no effects of acetylcholine on the ventricles, even in high concentrations, and only some inhibitory effects in sinoatrial preparations. The authors concluded from these results that the full inhibitory function of the vagus nerve is a later foetal development. Our results are also in agreement with those from the study by Lin *et al.* (1984) in human isolated atrial fibres. These authors found, that in 3 of 12 preparations, acetylcholine induced a substantial increase in F_c following a brief initial decrease. Atropine diminished both the initial negative and the later positive inotropic effects induced by acetylcholine, while propranolol abolished only the later effect.

De Gubareff & Sleator (1965) were the first to describe the effects of adenosine in human atrial heart muscle (see their Figure 6). The effects of adenosine on action potential and force of contraction in human atrial heart muscle were qualitatively similar to those seen with acetylcholine in this tissue (Sleator & de Gubareff, 1964), but further details, such as time course or desensitization phenomena, were not presented. Lee & Chang (1985) found that adenosine in concentrations up to 1 mmol l^{-1} exerted only minor effects on the configuration of the AP of human atrial heart muscle.

In contrast to the direct effects of carbachol, NECA and R-PIA, the indirect effects (antagonism

of the positive inotropic response to isoprenaline) did not show desensitization either in the atrium or the ventricle. The antagonism between cholinoceptor or purinoceptor agonists and isoprenaline was not related to a change in the potassium conductance in either tissue, based on measurements of action potential and ^{86}Rb fluxes. It is therefore conceivable to assume that, in the human heart, direct and indirect effects of cholinoceptor and purinoceptor agonists are also mediated by different biochemical pathways, as demonstrated in numerous investigations in other mammalian hearts (for reviews see Löffelholz & Pappano, 1985; Endoh, 1987).

Our study has shown that activation of either acetylcholine or adenosine receptors in the human heart is, under the same conditions, equally effective in changing myocardial contractility. Whereas the direct effects on potassium channels in the atrium are transient in nature, the antagonistic effects on cyclic AMP-dependent increases in force of contraction are rather stable. Therefore, both cholinoceptors and purinoceptors in the human heart seem to be equally well suited to regulate myocardial contractility under equivalent conditions. The possible implications *in vivo*, in physiological or pathological conditions, have been reviewed recently (Sollevi, 1986) and will not be discussed here.

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Effect of age and methacholine on the rate and coronary flow of isolated hearts of diabetic rats

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1 Isolated hearts perfused by the method of Langendorff from 6, 12 and 24 week streptozotocin (STZ) diabetic rats displayed a significant bradycardia following 60 min equilibration. The rate of hearts from 12-week diabetic rats (164 ± 17) displayed the greatest bradycardia compared to age-matched controls (268 ± 15; $P < 0.001$), and diabetics treated with insulin (232 ± 17; $P < 0.01$), but by 52 weeks the heart rate of the 3 groups was similar. With advancing age the effect of STZ diabetes on the rate of rat isolated perfused hearts remained unchanged but the rate of the control and diabetic + insulin groups declined.

2 Hearts from 6–52 week STZ-treated rats were found to be more sensitive to the negative chronotropic effect of methacholine, the greatest difference occurring in hearts from the 12 week animals. Atropine (10^{-7} M) did not affect the resting heart rate of age-matched controls or diabetics but blocked methacholine (2.6×10^{-6} M)-induced bradycardia in both, suggesting that the site of action of diabetic bradycardia is not the muscarinic receptors.

3 At the end of equilibration there was a significant decrease in coronary flow in hearts from 12 week diabetic animals. In spontaneously beating diabetic rat hearts administration of methacholine (2.6×10^{-6} M) produced a significantly greater decrease in coronary flow in the 12, 24 and 52 week diabetic hearts. When electrically paced (5 Hz) however, there was no difference in response to methacholine between the three groups except at 52 weeks between the age-matched control and diabetic groups. This suggests that the more pronounced reduction induced by methacholine on the coronary flow of diabetic hearts is secondary to its negative chronotropic effect.

4 In general, hearts from diabetic animals treated with insulin respond similarly to their age-matched controls in the presence and absence of methacholine.

Introduction

Coronary artery disease as well as cardiac failure are often sequelae of diabetes mellitus. Even in the absence of atherosclerosis, blood vessel disease and cardiomyopathy can occur in diabetes (Hamby *et al.*, 1974; Kannel *et al.*, 1974; Ledet, 1976; Regan *et al.*, 1977; Sanderson *et al.*, 1978). Autonomic dysfunction is considered to be one of the major secondary complications responsible for diabetic heart disease (Clarke *et al.*, 1979), but the underlying mechanism(s) remain unknown. Stuesse *et al.* (1982) reported that enhanced sensitivity to vagal stimulation was an early detectable feature of diabetic cardiac autonomic neuropathy. Bradycardia and cholinergic supersensitivity in streptozotocin-induced diabetic *in vitro* rat myocardial preparations have been reported (Foy & Lucas, 1976; Vadlamudi & McNeill, 1983; Carrier *et al.*, 1984; Carrier & Aronstam, 1987), as well as an increase in baroreflex sensitivity in conscious diabetic rats (up to 24 weeks) and then a decrease as diabetic duration progressed (48 weeks) (Chang & Lund, 1986). Thus, an alteration in parasympathetic function appears to be dependent on the duration of diabetes in the rat, but a clear picture characterizing the progression of impaired function in STZ-induced diabetes remains to be established.

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Alterations in vascular sensitivity to certain agonists have been described in experimental diabetic animals (Savarese & Berkowitz, 1979; Cavaliere *et al.*, 1980; Pfaffman, 1980; Jackson & Carrier, 1983; Mueller, 1984). However, to our knowledge, no study has been performed correlating the chronotropic and coronary blood flow responses elicited by cholinoceptor agonists *in vitro* beyond diabetes of 8 weeks' duration. Moreover, 'the status of coronary flow in experimental diabetes is somewhat unclear' (Dhalla *et al.*, 1985). A number of studies utilizing streptozotocin have now been published attesting to the validity of the diabetic state it produces in animals. Accordingly, the present investigation sought to characterize some of the effects of methacholine on heart rate and coronary flow of isolated hearts from diabetic rats, 6 to 52 weeks following treatment with streptozotocin.

Methods

Male Sprague-Dawley rats (from Simonsen Laboratories, Gilroy, CA) weighing 200–250 g on arrival were randomly divided into three groups: control (C), diabetic (D) and diabetic treated with insulin (D + I). Diabetes was induced by a single injection of streptozotocin (STZ) (55 mg kg^{-1}, i.v.) in the tail vein (Rerup, 1970; Chang & Lund, 1986). Age-matched control rats received vehicle alone (0.01 M citrate buffer in saline). Diabetics treated with insulin were injected with two units of protamine zinc insulin subcutaneously daily, after streptozotocin. Blood samples were obtained just before the rats were killed and non-fasting plasma glucose determined with a Beckman glucose analyzer. The diabetic state was verified by plasma glucose levels exceeding 300 mg dl^{-1}. These animals exhibited excessive daily food and water consumption and a decline in body weight. All animals received water and standard chow *ad libitum*.

Results were obtained on hearts from 150 rats. Six, 12, 24 and 52 weeks after induction of diabetes, hearts were excised under ether anaesthesia, arrested by plunging them into ice cold perfusion media, mounted and perfused by the method of Langendorff in a non-recirculating system at 75 cmH_2O pressure (Tanz *et al.*, 1982). The perfusion medium was a modified Krebs-Hensleit (K-H) bicarbonate solution having the following composition (mM): KCl 4.75, KH_2PO_4 1.19, $MgSO_4 \cdot 7H_2O$ 1.19, $CaCl_2 \cdot 2H_2O$ 2.54, NaCl 119, $NaHCO_3$ 25 and glucose 11.12. When equilibrated with 95% O_2 and 5% CO_2 at 37.5°C the pH was 7.35 and Po_2 598 mmHg. Three fine wire electrodes were affixed to the right and left atrial appendages and the epicardial surface of the left ventricle for the recording of heart rate, and the atrial and ventricular electrocardiogram (ECG) on a direct-writing polygraph. Coronary flow was measured and expressed as ml min^{-1} g^{-1} of wet heart weight. The first 60 min following mounting was allowed for equilibration. Hearts were then exposed to methacholine, 5.1×10^{-7}, 2.6 and 5.1×10^{-6} M for 10 min at each concentration followed by 10 min for washout with normal K-H solution to permit recovery before the next concentration. Additional experiments were performed on hearts from 12-week animals exposed to atropine (10^{-7} M) alone, methacholine (2.6×10^{-6} M) alone, and atropine followed 10 min later by methacholine. All drugs were added to the perfusate reservoir containing oxygenated medium. Since peak effects occurred 10 min after drug addition, heart rate, and coronary flow were determined at that time. The degree of the responses elicited were expressed as the percentage change from the pre-drug value so as to avoid variation between individual hearts. In order to obviate the influence of heart rate on coronary flow, hearts were also paced electrically at 5 Hz. This was accomplished by placing stimulating electrodes on the myocardium, one on the left atrium the other on the apex. Stimulating pulses were 5 ms in duration and 30–50% above threshold voltage. Hearts were then exposed to methacholine (2.6×10^{-6} M). At the conclusion of the experiment, wet weights were determined by weighing hearts dried on blotting paper.

Acetyl-β-methylcholine Cl, atropine and streptozotocin were obtained from the Sigma Chemical Co. and solutions prepared just before use; protamine zinc insulin was from Eli Lilly. Data are presented as means and the standard error of the means. Statistical analysis was by one-way analysis of variance when comparing three groups, followed by Newman-Keuls test and Student's unpaired t test when comparing two groups. A P value of <0.05 was taken as indicating statistical significance.

Results

Mortality following STZ was high with 27.2% of rats succumbing within the first 5 weeks, and a total of 42.4% within the first year. In contrast, there were no deaths in the untreated control group and a mortality of 11.8% in the diabetic + insulin group.

Animals injected with STZ had statistically significantly elevated blood glucose levels compared to the untreated controls and those treated with STZ and then insulin (Table 1). Diabetic animals killed after 6 weeks had the highest blood glucose level

Table 1 Mean blood glucose, body and heart weights after induction of diabetes in rats

Weeks	*Blood glucose* ($mg\,dl^{-1}$)	*Body wt.* (g)	*Heart wt.* (mg)†	*Heart:Body wt.*
6 Controls	96.3 (7) ±8.9	401.7 (9) ±11.2	1190 (9) ±41.0	2.96
	***	***	***	***
Diabetic	500.7 (11) ±30.0	213.8 (11) ±16.0	825 (11) ±48.3	3.95
	** ***	***	***	*
Diabetic + insulin	211.5 (7) ±32.0	396.2 (9) ±8.3	1272 (9) ±54.5	3.21
12 Controls	105.0 (8) ±17.3	429.2 (11) ±14.6	1335 (11) ±38.4	3.13
	***	***	***	***
Diabetic	491.5 (8) ±42.0	214.1 (9) ±16.4	1025 (9) ±44.7	4.88
	* ***	***	***	***
Diabetic + insulin	185.3 (6) ±39.9	444.7 (9) ±7.7	1446 (9) ±44.7	3.26
24 Controls	109.9 (11) ±14.9	512.5 (12) ±9.2	1430 (12) ±35.7	2.80
	***	***	***	***
Diabetic	410.8 (12) ±19.1	229.6 (17) ±10	1004 (17) ±41.1	4.41
	***	***	***	***
Diabetic + insulin	128.7 (13) ±16.1	525.8 (13) ±11.1	1466 (13) ±36.0	2.80
52 Controls	96.2 (6) ±16.0	580.1 (9) ±13.4	1617 (9) ±43.7	2.79
	***	***	***	***
Diabetic	347.0 (10) ±36.3	306.5 (9) ±17.7	1226 (9) ±53.6	4.04
	***	***	***	***
Diabetic + insulin	112.9 (7) ±21.5	553 (7) ±15.6	1648 (7) ±42.6	2.98

† Wet weight: number of individual observations in parentheses. Values are ± standard error of the mean.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

($500.7\,mg\,dl^{-1}$), which slowly declined with time to $347\,mg\,dl^{-1}$ at 52 weeks.

A tabulation of body weight, heart weight and the ratio of heart to body weight is also shown in Table 1. The body weight of diabetic rats was significantly less than both the untreated control group and those injected with STZ + insulin. This also obtained for heart weight. However, since body weight declined to a greater extent than did heart weight, the ratio of heart to body weight ($mg\,g^{-1}$) was consistently greater for the diabetic group than the other groups.

Heart rate

Actual heart rate at the end of equilibration (+60 min) was invariably less in the diabetic group than for either the untreated control or the diabetic + insulin groups, with the exception of the 52 week animals which, although less, was not significantly different (Figure 1).

When preparations were first exposed to 5.1×10^{-7} M methacholine for 10 min the decrease in heart rate from pre-drug levels was significantly greater in hearts from 12 week diabetic rats than in the untreated control and diabetic + insulin groups, but not in the 6, 24 or 52 week groups (Figure 2). However, following exposure to 2.6×10^{-6} M methacholine, the decrease in heart rate was significantly different between all diabetic and untreated control groups. This difference was also present at 12 and 52 weeks between the diabetic and the diabetic + insulin groups. Following exposure to 5.1×10^{-6} M

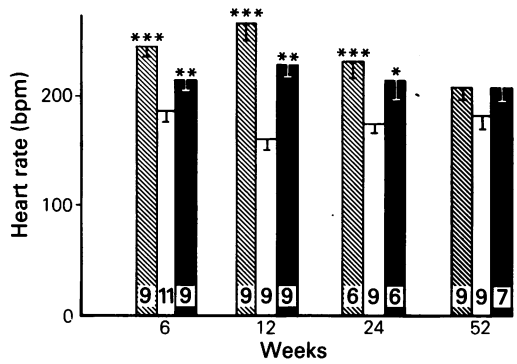

Figure 1 Mean heart rates of the three groups of rat Langendorff hearts at the end of 60 min equilibration 6, 12, 24 and 52 weeks after streptozotocin. Number of individual preparations indicated within the column. Hatched = controls; open = diabetic; solid = diabetic + insulin. Vertical bars show s.e.mean.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

there was a significantly greater decrease in the rate of hearts from the 12 and 24 week diabetic animals and their controls, but not from the 6 or 52 week animals. In the 12 week group, a significant difference in rate between hearts from the diabetic + insulin and diabetic groups was also observed. Analysis of the electrocardiograms revealed no discernible rhythmic alterations.

Effect of atropine on heart rate

In an attempt to ascertain whether increased cholinergic sensitivity of the S-A node is the basis for diabetic bradycardia, additional studies were performed in the presence of atropine (10^{-7} M). In the presence of atropine exposure to methacholine (2.6×10^{-6} M) 10 min later did not raise the rates of either control or diabetic hearts above pre-treatment levels (Table 2), though atropine very effectively blocked methacholine-induced bradycardia. This suggests that the bradycardia of isolated hearts from diabetic rats is not due to a supersensitivity of muscarinic receptors, but that the site of action of enhanced cholinergic sensitivity in diabetics lies elsewhere.

Coronary flow

Actual coronary flow (ml min^{-1} g^{-1}) at the end of equilibration (60 min) was significantly less only in

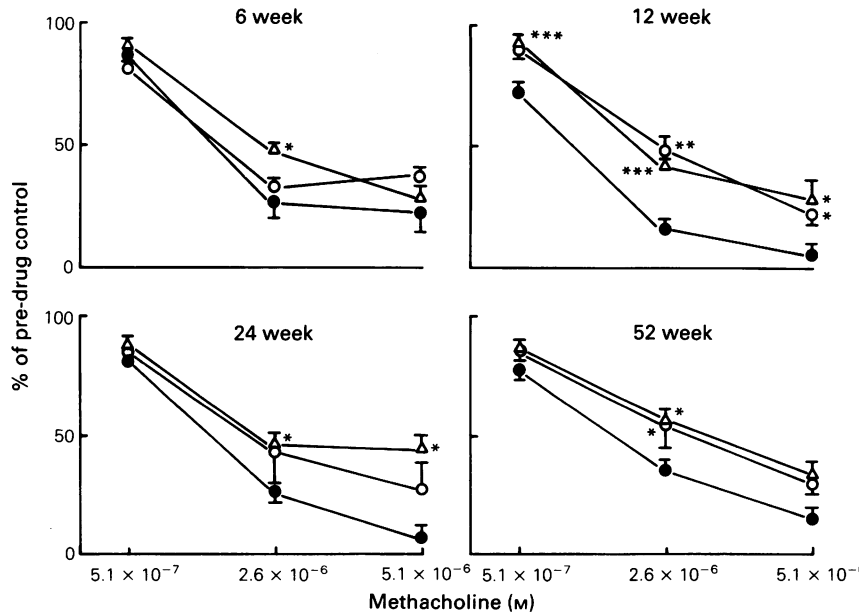

Figure 2 Effect of methacholine on the rate of spontaneously beating rat isolated hearts. The change in heart rate following methacholine was related to the pre-drug rate which was equated to 100%. Age-matched animals were killed 6, 12, 24 and 52 weeks following streptozotocin. (△) = controls; (●) = diabetic; (○) = diabetic + insulin. Vertical bars show s.e.mean.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; compared to diabetic groups.

Table 2 Effect of methacholine (2.6×10^{-6} M) and atropine (10^{-7} M) on the rate of isolated 12-week diabetic and control rat hearts

	Pre-drug	*Methacholine*	*Atropine*	*Atropine + methacholine*
Control	237.0 (15) ±8.8 ***	135.5 (9) ±7.1 ***	247.5 (5) ±10.5 ***	236.0 (7) ±7.5 ***
Diabetic	183.0 (15) ±9.7	34.4 (8) ±6.1	202.0 (5) ±12.5	196.0 (5) ±6.8

Number of individual hearts in parentheses. Values are ± standard error of the mean.
** $P < 0.01$; *** $P < 0.001$.

hearts from 12 week diabetic animals (Figure 3). Figure 4 illustrates the coronary flow observed for the three groups in response to methacholine. Regardless of age, when exposed to methacholine coronary flow was invariably lower in hearts from diabetic rats than either those treated with insulin or in hearts from control animals. Moreover, it was significantly less following exposure to 2.6×10^{-6} M methacholine in hearts from 12, 24 and 52 week animals as well as following the 5.1×10^{-6} M concentration in 52 week old animal hearts. However, when hearts were paced at 5 Hz the only statistically significant difference in coronary flow occurred at 52 weeks between control and diabetic hearts (Figure 5).

Discussion

These results demonstrate that Langendorff perfused hearts from rats made diabetic with streptozotocin

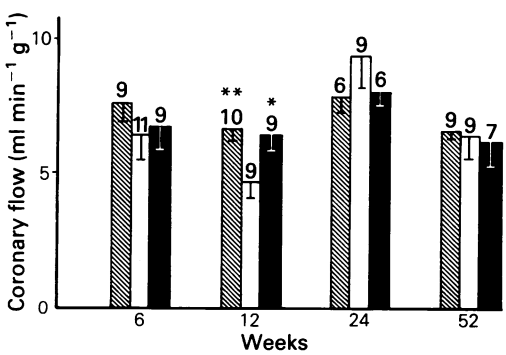

Figure 3 Mean coronary flows of spontaneously beating rat isolated hearts following 60 min equilibration 6, 12, 24 and 52 weeks after streptozotocin. Coronary flow = ml min^{-1} g^{-1} (wet wt.). Number of individual preparations indicated within the column. Hatched = controls; open = diabetic; solid = diabetic + insulin. Vertical bars show s.e.mean.
* $P < 0.05$; ** $P < 0.01$; compared to diabetic groups.

have significantly lower rates *per se* and in response to methacholine than their age-matched and insulin-treated diabetic controls. But is the defect due to streptozotocin or the diabetic state? At the end of 60 min equilibration the rate of hearts from STZ-injected animals was significantly less than the controls as well as those from diabetics treated with insulin (Figure 1). At 12 weeks the rate of diabetic hearts was lower than the rate of hearts from 6, 24 and 52 week control and insulin-treated diabetic animals. The same qualitative trend occurred at 0-time when hearts were initially mounted and perfusion begun (data not presented). By 52 weeks however, the rate of hearts from all three groups was not significantly different. These facts suggest that: (1) STZ did not destroy all the β-cells, but rather there was a partial regeneration as evidenced by the fact that the blood glucose level was declining with the passage of time (Table 1) and (2), STZ is not a direct myocardial depressant *per se*. This conclusion is in agreement with that reached by Fein *et al.* (1981) who found that papillary function returned to normal the longer insulin was given, and concluded that STZ-induced cardiomyopathy is not the result of STZ-induced cardiac toxicity. Thus, age and the duration of STZ-induced diabetes appear to be important determinants affecting chronotropic activity.

In experimental models diabetes-induced bradycardia has been found to occur both *in vivo* and *in vitro* (Foy & Lucas, 1976; Pfaffman, 1980; Bielefeld *et al.*, 1983; Jackson & Carrier, 1983; Chang & Lund, 1986), but the mechanism remains obscure. For example, Senges *et al.* (1980) utilizing isolated atria from rabbits treated with alloxan observed a statistically significant lower sinus rate, A-V block and inhomogeneity of atrial conduction compared to controls. Diabetic-induced bradycardia has been attributed to decreased glucose metabolism (Foy & Lucas, 1976), autonomic dysfunction (Tomlinson & Yusof, 1981), and a decrease in β-adrenoceptors

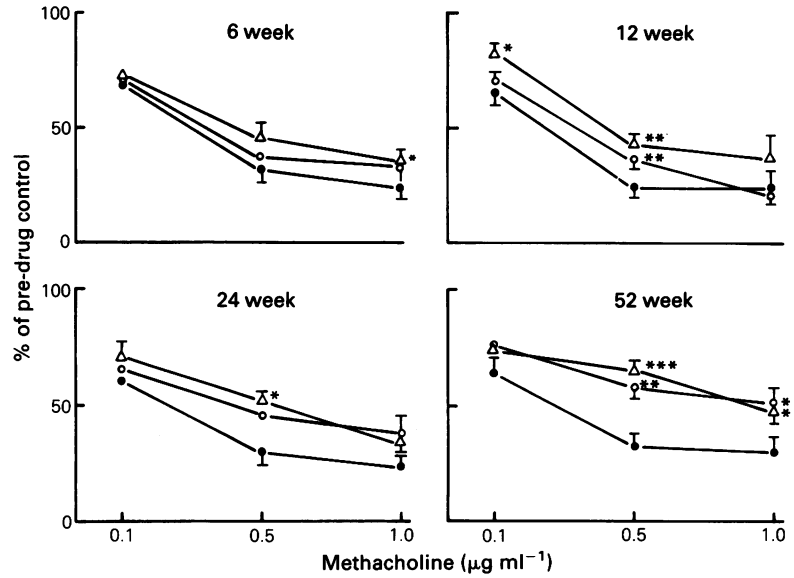

Figure 4 Effect of methacholine on the coronary flow of spontaneously beating rat isolated hearts 6, 12, 24 and 52 weeks following streptozotocin. The change in coronary flow following methacholine was related to the pre-drug flow which was equated to 100%. (△) = controls; (●) = diabetic; (○) = diabetic + insulin. Vertical bars show s.e.mean.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; compared to diabetic groups.

(Savarese & Berkowitz, 1979; Williams *et al.*, 1983; Latifpour & McNeill, 1984; Atkins *et al.*, 1985; Bitar *et al.*, 1987). Autonomic neuropathy is a clinical problem in diabetes (Wheeler & Watkins, 1973; Duchen *et al.*, 1980), as well as in experimental settings (Monckton & Pehowich, 1980; Schmidt *et al.*, 1981). But in the presence of diabetic autonomic neuropathy, tachycardia occurs apparently as a result of diminished vagal tone since hearts fail to accelerate after atropine and there is an absence of reflex bradycardia following a Valsalva manoeuvre (Wheeler & Watkins, 1973).

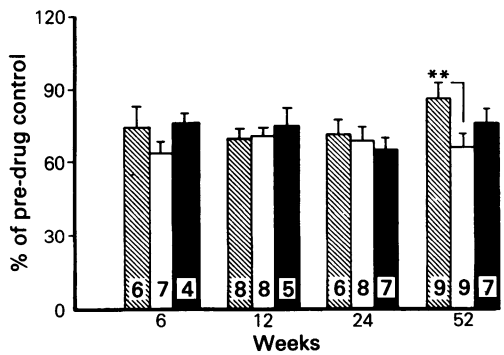

Figure 5 Effect of methacholine (2.6×10^{-6} M) on coronary flow of isolated perfused rat hearts paced at 5 Hz 6, 12, 24 and 52 weeks following streptozotocin. The change in coronary flow following methacholine was related to the pre-drug value which was equated to 100%. Number of individual preparations indicated within the column. Hatched = controls; open = diabetic; solid = diabetic + insulin. Vertical bars show s.e.mean.
** $P < 0.01$; compared to diabetic group.

The results shown in Figure 1 suggested the possibility that hearts from 12 week old animals might show the greatest disparity in response to pharmacological intervention. And indeed this proved to be the case. Figure 2 attests to the fact that methacholine-induced bradycardia produced by larger doses resulted in a significant difference between diabetic preparations and the controls as well as those treated with insulin. This disparity was barely evident at 6 weeks, became greatest at 12 weeks, and then less and less at 24 and 52 weeks. Moreover, the negative chronotropic effect elicited by 2.6 and 5.1×10^{-6} M methacholine was invariably greater in hearts from all diabetic rats, regardless of their age. This enhanced chronotropic sensitivity to a cholinoceptor agonist in diabetes has been previously reported by other investigators (Tomlinson & Yusof, 1981; Carrier *et al.*, 1984), especially in hearts from 8–10 week diabetic rats (Carrier *et al.*, 1984; Carrier & Aronstam, 1987). Vagal stimulation

in 7–9 week alloxan rats has been reported to produce a more pronounced bradycardia compared to controls (Stuesse *et al.*, 1982). Increased baroreflex sensitivity in conscious STZ diabetic rats to methacholine up to 24 weeks, with a progressive decline occurring thereafter, has also been reported (Chang & Lund, 1986). But the mechanism still remains unknown. It has been reported that acetylcholinesterase is significantly lower in right atria from 8–10 week STZ diabetic rats (Carrier & Aronstam, 1987) which could explain the presence of cholinergic supersensitivity and the resulting bradycardia. In an attempt to determine the mechanism responsible for diabetic bradycardia, the chronotropic responses elicited by methacholine in the presence and absence of atropine (Table 2) were determined. The results obtained suggest that cholinergic supersensitivity is not due to an increase in muscarinic receptor activity. Moreover cholinergic supersensitivity in diabetic hearts has been found in the presence of decreased affinity and density of muscarinic receptors (Carrier & Aronstam, 1987). This suggests that the mechanism and site of action of diabetic-induced bradycardia and chronotropic cholinergic supersensitivity lies elsewhere. That the diabetic state was the result of insulin deficiency rather than streptozotocin toxicity is attested to by the fact that the responses observed both in the controls and the insulin-treated diabetics were similar throughout.

Coronary flow at the end of 60 min equilibration was not significantly different for the three groups except in hearts from the 12 week diabetic animals (Figure 3). This correlates with the fact that 12 week diabetic hearts also displayed the greatest reduction in rate at that point in time (Figure 1). This reduction of coronary flow in response to methacholine in hearts from diabetic animals 12 weeks and older was invariably greater then in either the untreated controls or those from diabetics treated with insulin (Figure 4). In hearts from the 12, 24 and 52 week animals, the response elicited by 2.6×10^{-6} M methacholine in diabetic hearts was significantly more, as was that following exposure to 5.1×10^{-6} M in hearts from 52 week diabetics. The difference between the control and diabetic groups at 52 weeks might be due to greater cholinergic sensitivity. Yet when paced at 5 Hz, hearts from 6, 12, 24 and 52 week animals all displayed the same coronary flow in response to methacholine. However, since pacing eliminated the negative chronotropic effect of methacholine between all other groups at 6, 12, 24 and 52 weeks, we conclude that the reduction in coronary flow is secondary to the decline in heart rate in this preparation. It is well known that by autoregulatory mechanisms, myocardial oxygen consumption and demand are the major determinants of coronary blood flow (Berne, 1964). Since the rate of isolated diabetic rat hearts was lower when exposed to methacholine (Figure 2), their requirement for oxygen was also less and therefore coronary flow declined (Figure 4). Thus, hearts from 12 week diabetic rats displayed the greatest bradycardia as well as the greatest reduction in coronary flow in response to methacholine. Although muscarinic stimulation constricts coronary smooth muscle in the rat (Sakai, 1980), the pacing studies suggest that autoregulatory mechanisms predominate in determining coronary flow.

Coronary dilatation in response to cholinoceptor agonists occurs in some species (Blumenthal *et al.*, 1968), but constriction occurs *in vitro* in man, rat, pig, sheep, rabbit and cow (Smith, 1950; Nakayama *et al.*, 1978; Sakai, 1980; Ginsburg, 1983; Kalsner, 1985; Nuutinen *et al.*, 1985). In response to methacholine the coronary flow of hearts from paced STZ-treated animals was similar at all ages. But at 52 weeks control paced hearts displayed a significantly greater coronary flow when exposed to methacholine. This suggests that with advancing age hearts from normal rats become less sensitive to muscarinic agonists while those from diabetic animals retain an enhanced sensitivity, and agrees with the conclusion by Efellah *et al.* (1986) who demonstrated decreased cholinergic myocardial responsiveness in rats with advancing age. One question remaining is whether autonomic receptor density and/or affinity changes in the presence of diabetes and, if so, is there a progressive change that can be correlated with diabetic duration. Chang *et al.* (1984) have shown that 4–12 week diabetic rats have a significant decrease in choline acetyltransferase, indicative of defective parasympathetic innervation that might result in an increase in muscarinic density or affinity. But the few studies that have been reported suggest that there is a decrease in muscarinic receptor density in 12 week diabetic rat atria (Carrier *et al.*, 1984), and 6 month diabetic rat ventricular tissue (Latifpour & McNeill, 1984).

These studies suggest that of the four different diabetic groups (i.e. 6, 12, 24 and 52 weeks), isolated perfused hearts from 12 week animals exhibit the greatest sensitivity to methacholine. This sensitivity is especially evident as a negative chronotropic response and appears to be, at least in part, responsible for the accompanying fall in coronary flow. The changes in muscarinic sensitivity to methacholine in diabetic rats appears not to be due to streptozotocin toxicity *per se*. Moreover, insulin administration tends to correct the defects.

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Actions of tachykinins on the rabbit mesenteric artery: substance P and [Glp^6,L-Pro^9]SP$_{6-11}$ are potent agonists for endothelial neurokinin-1 receptors

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1 Identification of the subtype of neurokinin receptor on the endothelium of the rabbit mesenteric artery was demonstrated by comparing the relative potencies of the naturally occurring tachykinins, substance P, neurokinin A and neurokinin B and the highly selective agonists [Glp^6,L-Pro^9]SP$_{6-11}$ (L-Pro), [Glp^6,D-Pro^9]SP$_{6-11}$ (D-Pro) and N-succinyl-[Asp^6,MePhe^8]SP$_{6-11}$ (senktide).

2 Relaxations of the rabbit mesenteric artery to substance P, neurokinin A and neurokinin B were concentration-dependent and were abolished by the removal of the endothelium.

3 Substance P was more potent than neurokinin A or neurokinin B and L-Pro was more potent than D-Pro or senktide.

4 Substance P, neurokinin A and neurokinin B all significantly reduced the nerve-mediated contractile response in the presence of the endothelium at a concentration of 0.1 μM, with a rank order of potency substance P > neurokinin A > neurokinin B.

5 At a concentration of 0.1 μM, L-Pro also significantly reduced the nerve-mediated contractile response, unlike D-Pro and senktide.

6 It is concluded that the relaxation of the rabbit mesenteric artery produced by substance P is mediated by neurokinin-1 receptors (NK-1) located on the endothelium. Furthermore, of the analogues, L-Pro was particularly potent for these receptors.

Introduction

The tachykinins are a family of biologically active peptides that share the common C-terminal sequence Phe-X-Gly-Leu-Met-NH_2 (Erspamer, 1981). Substance P was initially identified in 1931 (von Euler & Gaddum, 1931) and fairly recently two additional mammalian tachykinins were isolated, these being neurokinin A and neurokinin B (Kangawa *et al.*, 1983; Kimura *et al.*, 1983; Nawa *et al.*, 1984). The discovery of neurokinin A and neurokinin B led to further studies aimed at identifying pharmacological preparations in which substance P was not necessarily the most potent tachykinin. For example, the rabbit pulmonary artery has been found to be particularly sensitive to neurokinin A (D'Orléans-Juste *et al.*, 1985) and the rat portal vein to neurokinin B (Mastrangelo *et al.*, 1986).

It has been proposed that the actions of the different tachykinins are mediated by multiple receptors which have been classified as neurokinin-1 (NK-1) (SP-P), NK-2 (SP-E) and NK-3 (SP-N) subtypes (Watson *et al.*, 1983; Laufer *et al.*, 1985; Lee *et al.*, 1986; Henry, 1987). These receptors have been defined according to the rank orders of potencies of the various tachykinins. At the NK-1 receptor, substance P is the most potent tachykinin (for example, dog carotid artery: Regoli *et al.*, 1984; 1987) while at the NK-2 receptor, neurokinin A is more potent than substance P or neurokinin B (for example rabbit pulmonary artery: Regoli *et al.*, 1987) and at the NK-3 receptor, neurokinin B is the most potent (for example, rat portal vein: Regoli *et al.*, 1987).

In the rabbit mesenteric artery, substance P elicits vasodilatation which is dependent on the presence of an intact endothelium (Zawadzki *et al.*, 1981). In the present study, an attempt has been made to classify the subtype of receptor responsible for eliciting this endothelium-dependent vasodilatation by use of not only the naturally occurring tachykinins substance P, neurokinin A and neurokinin B, but also some recently developed tachykinin analogues that have been shown to exert a degree of selectivity for the receptor subtypes (Lee *et al.*, 1986; Wormser *et al.*, 1986; Fletcher *et al.*, 1987; Iversen *et al.*, 1987).

Until recently, there has been a lack of potent and specific antagonists available to characterize the

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tachykinin receptors, meaning that the only method available was to compare the rank order of potency of the tachykinins. However, preliminary work has shown that the recently developed tachykinin analogues used in this study display a specific pattern of agonist potencies characteristic to the tachykinin receptors: [Glp^6,L-Pro^9]SP$_{6-11}$ (L-Pro) shows a degree of selectivity for the NK-1 receptor on the smooth muscle of the guinea-pig ileum and guinea-pig urinary bladder (Iversen *et al.*, 1987; Lee *et al.*, 1986); [Glp^6,D-Pro^9]SP$_{6-11}$ (D-Pro) shows selectivity for the NK-2 receptor on the rat vas deferens and hamster urinary bladder (Iversen *et al.*, 1987; Lee *et al.*, 1986), while N-succinyl-[Asp^6,MePhe^8]-SP$_{6-11}$ (senktide) has been found to show a degree of selectivity for the neuronal NK-3 receptor in the myenteric plexus of the guinea-pig ileum (Wormser *et al.*, 1986). It has been proposed that the comparison of the relative potencies of these agonists may be useful in characterizing the specific tachykinin receptors; thus, their relative specificities in the rabbit mesenteric artery were ascertained in this study.

Methods

Preparation of material

Adult male New Zealand white rabbits (2.5–3.4 kg) were killed by a blow to the back of the head and exsanguination. Sections of the mesenteric artery (first or second branch) were excised and cleared of surrounding fatty tissue under a dissecting microscope. Care was taken to avoid excessive trauma to the vessels so as to preserve the endothelium (Furchgott & Zawadzki, 1980). Sections of isolated artery were cut into rings, approximately 5 mm in length. Some of these rings deliberately had the endothelium removed by pulling a silk thread through the lumen of the vessels.

Each ring was mounted horizontally under isometric conditions in 20 ml organ baths by inserting two stainless steel wires through the lumen of the vessel, according to the method of Bevan & Osher (1972).

The tissues were bathed in Krebs solution of the following composition (mM): NaCl 133, KCl 4.7, $NaHPO_4$ 1.35, $NaHCO_3$ 16.3, $MgSO_4$ 0.61, glucose 7.8, $CaCl_2$ 2.52 (Bülbring, 1953). The Krebs solution was maintained at 37°C and bubbled with 95% O_2 and 5% CO_2. Bovine serum albumin (0.005%) and bacitracin (30 mg l^{-1}) were added to the Krebs solution in order to prevent peptides adhering to the surfaces of the glassware and peptide degradation. Preparations were allowed to equilibrate for at least 1 h under a resting tension of approximately 0.75 g. Contractions of the circular smooth muscle were recorded by use of a Grass FT03C transducer and displayed on a Grass ink-writing oscillograph (Model 79).

Pharmacology

A concentration-response curve was determined for noradrenaline (0.1–30 μM) on each segment in order to find the maximum contractile response. Relaxation to acetylcholine in segments in which tone was raised by noradrenaline (10 μM) confirmed whether the endothelium was present or absent (Furchgott & Zawadzki, 1980).

The vasodilatator effect of the tachykinins: substance P (0.1 μM), neurokinin A (0.1 μM) and neurokinin B (0.1 μM) was tested in the presence and absence of the endothelium on preparations in which tone had been raised by noradrenaline (10 μM). Due to tachyphylaxis, the concentration of tachykinins could not be raised cumulatively, so separate doses were tested on segments. The tachykinins were washed out of the Krebs solution either once a maximum relaxant response had been reached or after the drug had been in contact with the vessel for approximately 2 min in the case of subthreshold doses. A non-cumulative dose-response curve could then be constructed.

In a second series of experiments, the specific agonists, L-Pro (0.1 μM), D-Pro (0.1 μM) and senktide (0.1 μM) were tested in a similar manner with the endothelium present and again a non-cumulative dose-response curve was constructed.

Nerve stimulation

The intramural nerves were electrically stimulated by two platinum wire electrodes placed parallel to and on either side of the vessel segment approximately 5 mm apart with a Grass S11 stimulator. Supramaximal voltage (60 V) and pulse duration (0.2 ms) were established and kept constant throughout the experiment. The vessel segments were stimulated by trains of pulses with a pulse interval of 32 ms for a period of 5 s which produced a measurable and reproducible response. With these parameters, the contractile response to nerve stimulation was completely blocked by tetrodotoxin (3 μM) and therefore appeared to be due to nerve stimulation and not to direct stimulation of the muscle. Stimulation was repeated every 30 s in order that the transient responses to the tachykinins and tachykinin analogues could be measured.

On each preparation, stimulations were repeated to ensure consistency of the response before a single concentration of substance P (0.1 μM), neurokinin A (0.1 μM) or neurokinin B (0.1 μM) was tested. Shortly after the maximum response had been attained (or approximately 2 min after addition of the drug to the Krebs solution in the case of subthreshold doses), the

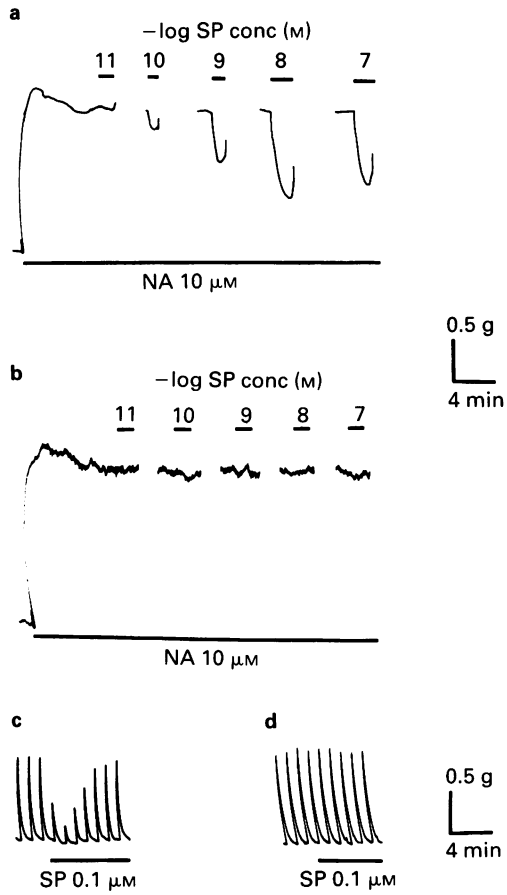

Figure 1 Isolated transverse ring preparation of the rabbit mesenteric artery. (a, b) Non-cumulative concentration-response to substance P (SP) (0.01–100 nM) with tone raised and maintained by noradrenaline (NA) (10 μM), (a) in the presence of endothelium and (b) with endothelium removed. (c, d) Effect of SP on contractions produced by neurogenic transmural stimulation (0.2 ms; supramaximal voltage) for 5 s at 32 Hz in the presence (c) and absence (d) of endothelium.

Krebs solution was changed in order to minimize the tachyphylaxis of the tachykinins on the vessel. Similar experiments were carried out on sections of vessel with the endothelium absent.

The effect of the three tachykinin analogues on neurally-evoked contractions were then tested in a similar manner in a further series of experiments.

Drugs

Substance P, neurokinin A, neurokinin B, acetylcholine chloride and noradrenaline bitartrate were all obtained from Sigma Chemical Company. [Glp^6, L-Pro^9]SP$_{6-11}$, [Glp^6,D-Pro^9]SP$_{6-11}$ and N-succinyl-[Asp^6,MePhe^8]SP$_{6-11}$ were a gift from Merck, Sharp & Dohme. Neurokinin B was dissolved in dimethyl sulphoxide (DMSO). All the other tachykinins and analogues were dissolved in 0.1% bovine serum albumin in distilled water. Noradrenaline was dissolved in 0.1 mM ascorbic acid. The noradrenaline was made up freshly each day. All tachykinins and analogues were stored at −20°C at a concentration of 0.1 mM in aliquots containing approximately 50 μl which were only defrosted once and used immediately.

Treatment of results and statistical methods

Relaxation to tachykinins and analogues were expressed as a percentage of the maximal relaxation to substance P in raised-tone preparations. Contractions to nerve stimulation were expressed as a percentage of the maximal contraction to noradrenaline in each preparation. In the figures, results are given

as a mean value calculated from a number of preparations denoted as n in the figure legends. Vertical bars indicate the standard error of the mean (s.e.mean). With the exception of substance P and L-Pro, the maximal relaxation to a particular tachykinin or analogue was not attained at the concentrations tested; thus, EC_{50} values could not be calculated. Instead, drug potencies were expressed in terms of 40% of the maximal relaxation to substance P. Results from nerve stimulation experiments were analysed by Student's paired t test. A probability of $P < 0.05$ was considered significant.

Results

Rank order of potency of tachykinins and analogues in producing direct responses

Substance P (0.01–100 nM) produced concentration-dependent relaxations in isolated sections of rabbit mesenteric artery when applied in a non-cumulative manner to preparations with tone raised by 10 μM noradrenaline (Figures 1a and 2a). These relaxations did not occur in the absence of the endothelium (Figure 1b). Presence of an intact endothelium was established by testing acetylcholine (0.1–300 μM) on each preparation. A relaxant response of 80% or greater was taken as confirmation of the presence of the endothelium as shown by Furchgott & Zawadzki (1980). When the endothelium had deliberately been removed, the relaxant response to acetylcholine was also abolished.

Neurokinin A and neurokinin B (both 0.1–1000 nM) also produced concentration-dependent relaxations in the presence of the endothelium in raised-tone preparations. The overall rank order of potency was substance P > neurokinin A = neurokinin B (Figure 2a). As neither neurokinin A nor neurokinin B had reached maximal relaxation within the concentration range used (0.1–1000 nM), the EC_{50} values could not be compared. Furthermore, as D-Pro and senktide did not cause relaxations equivalent to 50% of the maximal relaxation to substance P, EC_{40} values were compared, the EC_{40} value being the concentration of a particular tachykinin or analogue required to produce the equivalent of 40% of the maximal relaxation to substance P. The $-\log EC_{40}$ for substance P (9.3 ± 0.13) was significantly different from the values calculated for neurokinin A (7.9 ± 0.09) and neurokinin B (7.7 ± 0.10). Substance P was therefore 20–40 times more potent than neurokinin A or neurokinin B, which were not significantly different from each other. Substance P, neurokinin A and neurokinin B did not produce a response at basal tone.

L-Pro, D-Pro and senktide also produced dose-dependent relaxations in preparations of the rabbit mesenteric artery which were dependent on the presence of the endothelium. Although neither D-Pro nor senktide reached maximal relaxation within the concentration range used (1–1000 nM), it was clear that L-Pro was more potent than either D-Pro or senktide, the rank order of potency thus being L-Pro > D-Pro = senktide (Figure 2b). Again the EC_{40} values were calculated from the equivalent value found for substance P as neither D-Pro nor senktide reached maximal relaxant responses within the concentration-range used. The value calculated for $-\log EC_{40}$ for L-Pro (7.9 ± 0.16) was significantly greater than that for D-Pro (6.3 ± 0.08) or senktide (6.2 ± 0.06). Thus L-Pro is in the order of 40–50 times more potent than D-Pro or senktide, which were not significantly different from each other.

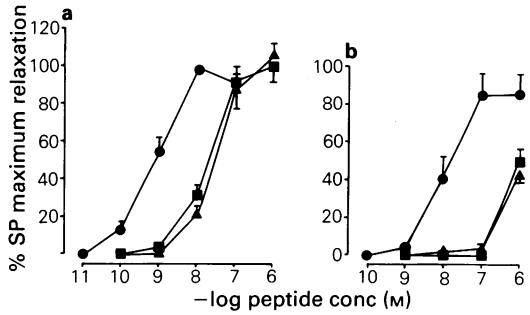

Figure 2 Isolated transverse ring preparation of the rabbit mesenteric artery with tone raised by noradrenaline (10 μM). (a) Non-cumulative concentration-response curves to substance P (SP) (0.01–100 nM) (●) ($n = 6$), neurokinin A (0.1–1000 nM) (■) ($n = 10$) and neurokinin B (0.1–1000 nM) (▲) ($n = 6$). (b) Non-cumulative concentration-response curves to L-Pro (0.1–1000 nM) (●) ($n = 10$), D-Pro (1–1000 nM) (■) ($n = 5$) and senktide (1–1000 nM) (▲) ($n = 6$). In the presence of endothelium. Data points are means with s.e.mean shown by vertical bars.

Effects of tachykinins and analogues on neurogenic contractions of preparations

Substance P (0.1 μM) caused a significant reduction in the contractile response to nerve stimulation in the presence of the endothelium (Figure 1c). There was no reduction in the contractile response to nerve stimulation in the absence of the endothelium (Figure 1d). Neurokinin A (0.1 μM) and neurokinin B (0.1 μM) also caused a significant reduction in the nerve-mediated contractile response in the presence of the endothelium (Figures 3a and 4a). The order of potency at the concentrations used was substance P > neurokinin A > neurokinin B (Figure 3a). As with substance P, neither neurokinin A nor neurokinin B produced a significant change in the neurally

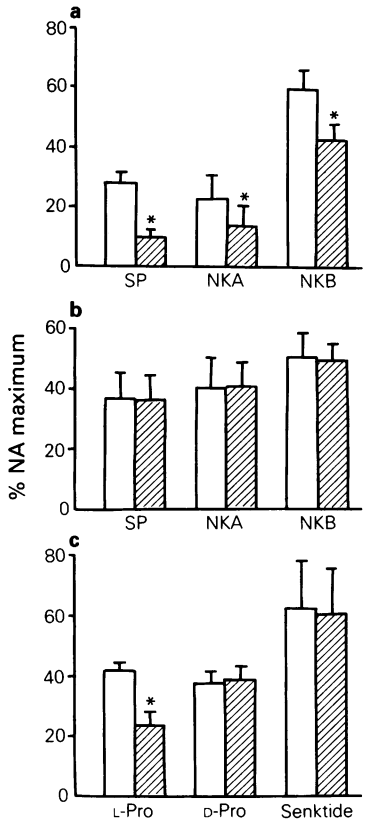

Figure 3 Contractions of isolated transverse ring preparations of the rabbit mesenteric artery to 5 s periods of intramural nerve stimulation (0.2 ms, supramaximal voltage, 32 Hz) expressed as a percentage of the maximal contraction to noradrenaline. Contractions produced in the absence of any drug (control, open columns) are compared with those obtained in the presence of a tachykinin or analogue (hatched columns): 0.1 μM substance P (SP) (n = 8, 6), neurokinin A (NKA) (n = 6, 5) or neurokinin B (NKB) (n = 6, 5) in the presence (a) and absence (b) of endothelium; and (c) in the presence of 0.1 μM L-Pro (n = 7), D-Pro (n = 5) or senktide (n = 5) with endothelium present. Columns show means with s.e.mean shown by vertical bars. Significant differences (* $P < 0.05$) between control and experimental contractions were calculated by Student's paired t test.

induced contractile response when the endothelium was not present (Figure 3b).

At 0.1 μM, L-Pro also caused a significant reduction in the contractile response to nerve stimulation, unlike D-Pro and senktide which had no effect (Figures 3c and 4b). In all three instances, the endothelium was present. In the absence of the endothelium, the inhibitory effect of L-Pro was not observed.

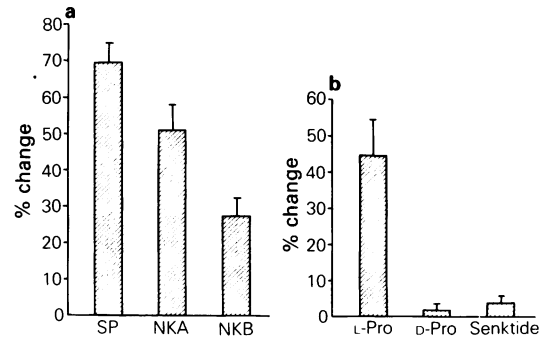

Figure 4 Contractions of isolated transverse ring preparations of the rabbit mesenteric artery to 5 s periods of intramural nerve stimulation (0.2 ms, supramaximal voltage, 32 Hz) expressed as a percentage change in contractile response between control and in the presence of (a) 0.1 μM substance P (SP), neurokinin A (NKA) or neurokinin B (NKB) and (b) 0.1 μM L-Pro, D-Pro or senktide. Endothelium present. Columns show means with s.e.mean indicated by vertical bars.

L-Pro was very selective when compared with D-Pro and senktide in inducing an inhibitory response in nerve-mediated contractions (Figure 4b). Although substance P was more potent than neurokinin A and neurokinin B, the contrast in potencies was not as marked as the contrast between the tachykinin analogues (Figure 4a).

Discussion

The present findings confirm that, in the rabbit mesenteric artery, substance P appears to be acting solely via a substance released from the endothelium, as both direct and nerve-mediated contractile responses were only affected in preparations with an intact endothelium. This is in keeping with previous findings in which it has been reported that substance P acts via the release of endothelium-derived relaxing factor (EDRF) (Zawadzki *et al.*, 1981). Furthermore, the fact that substance P inhibited both the contractile response induced by noradrenaline and nerve stimulation to the same extent, and that the nerve-mediated contractile response was unaffected in the absence of the endothelium, suggests that the effect may be purely due to a change in smooth muscle contractility. This supports previous findings which have shown that, in the rabbit pulmonary artery, cat cerebral artery and the canine blood-perfused gracilis muscle, substance P does not have a prejunctional effect (Endo *et al.*, 1977; Edvinsson & Skärby, 1984; Kahan *et al.*, 1985).

The tachykinin analogues were less potent than substance P, neurokinin A or neurokinin B. It is suggested that the reduction in potency is attributable

to the decrease in the length of the peptide chain from the N-terminal end. Indeed, it has been shown, in the dog carotid artery, that the NK-1 receptors are more sensitive to the tachykinins than to their fragments (Regoli *et al.*, 1987). However, L-Pro was very selective in inhibiting neurally mediated contractions when compared with the analogues, unlike substance P which was not as selective when compared with the other tachykinins.

Substance P was more potent than the other tachykinins in both the direct responses of raised-tone preparations and in reducing the nerve-induced contractile response, which suggests that the NK-1 receptor is responsible for mediating this action. Indeed, in the neurally mediated contractile responses, substance P was found to be more potent than neurokinin A, which was in turn more potent than neurokinin B. This order of potency is in keeping with that proposed for the NK-1 receptor (Regoli *et al.*, 1987). In raised-tone preparations, substance P was again the most potent tachykinin while neurokinin A and neurokinin B appeared to have similar potencies.

The tachykinin agonist L-Pro was found to be significantly more potent than either D-Pro or senktide both in raised-tone preparations and inhibiting the nerve-induced contractile response. This provides further evidence that in the mesenteric artery the tachykinin receptors are the NK-1 type, as in previous studies on established models such as the guinea-pig ileum and rat salivary gland, L-Pro has been shown to be selective for the NK-1 receptor (Iversen *et al.*, 1987). The inactivity of D-Pro and senktide also demonstrates the lack of NK-2 and NK-3 receptors as they are known to be selective for NK-2 and NK-3 receptors respectively (Piercey *et al.*, 1985; Wormser *et al.*, 1986).

One limitation of the present procedure for measuring the relative potencies of the tachykinins and analogues used was that the maximum effect could not be found in all cases as such a high concentration of peptide would be required. This was particularly true for the analogues D-Pro and senktide, which were extremely weak within the concentration-range used. In the absence of maximum effects to neurokinin A, neurokinin B, D-Pro and senktide, the pD_2 values could not be calculated from the incomplete concentration-response curves. Thus the method used to compare drug potencies had to be by comparison of the EC_{40} values, this being the concentration of drug required to produce the equivalent of 40% of the maximum relaxant response to substance P. Nevertheless, it can still be said that the comparison of the potencies of the tachykinins and the analogues clearly demonstrates that the analogues provide a far more selective response.

Table 1 Comparison of agonist potencies on raised-tone preparations of the rabbit isolated mesenteric artery

	$-log\ EC_{40}$	*Relative potencies*
(a) Mammalian tachykinins		
Substance P	9.3 ± 0.13 (6)	1.0
Neurokinin A	7.9 ± 0.09 (10)*	0.04
Neurokinin B	7.7 ± 0.10 (6)*	0.025
(b) Tachykinin analogues		
L-Pro	7.9 ± 0.16 (10)	0.04
D-Pro	6.3 ± 0.08 (5)†	0.001
Senktide	6.2 ± 0.06 (6)†	0.001

All values are given as mean ± s.e.mean with number of observations (n) in parentheses. Note that as the pD_2 value was not attained in all cases, the results are expressed as $-\log$ (40% maximal relaxation to substance P) ($-\log EC_{40}$). Relative potency (substance P = 1) is defined as the ratio of EC_{40} of substance P to the EC_{40} of peptides tested. * Significant difference ($P < 0.05$) between substance P values and other tachykinin values, calculated by Student's unpaired t test. † Significant difference ($P < 0.05$) between L-Pro values and other tachykinin analogue values, calculated by Student's unpaired t test.

Thus it appears that, in the rabbit mesenteric artery, there are NK-1 receptors located on the endothelium. This is in keeping with previous work which has also shown that the endothelial tachykinin receptors are of the NK-1 type, for example, in the guinea-pig basilar artery (Edvinsson & Jansen, 1987), the dog carotid artery (Regoli *et al.*, 1984; 1987) and the pig coronary artery (Gulati *et al.*, 1987). This is in contrast to the tachykinin receptors located on the vascular smooth muscle which are either of the NK-2 type, for example, the rabbit pulmonary artery (Regoli *et al.*, 1987) or the NK-3 type, for example, the rat portal vein (Mastrangelo *et al.*, 1986). The proposition that substance P acts via the endothelium is supported by work carried out by Stephenson *et al.* (1986) who have shown by autoradiographic techniques in the dog carotid artery that there are indeed binding sites for substance P on the endothelium.

The present results thus lead to the proposal that, in the rabbit mesenteric artery, substance P acts via NK-1 receptors on the endothelium to cause vasodilatation.

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Modulation of non-adrenergic, non-cholinergic neural bronchoconstriction in guinea-pig airways via $GABA_B$-receptors

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1 Evidence suggests that γ-aminobutyric acid (GABA) and its receptors are present in the peripheral nervous system. We have now investigated the effect of GABA and related substances on non-adrenergic, non-cholinergic (NANC) neurally-evoked bronchoconstriction in the anaesthetised guinea-pig.

2 Bilateral vagal stimulation (5 V, 5 ms, 3 or 5 Hz) for 30 s, after propranolol (1 mg kg^{-1} i.v.) and atropine (1 mg kg^{-1} i.v.) evoked a NANC bronchoconstrictor response manifest as a mean tracheal pressure rise of 21.9 ± 1.04 cmH_2O (n = 70). The bronchoconstrictor response was reproducible for any given animal.

3 GABA (10 μg–10 mg kg^{-1} i.v.) did not alter basal tracheal pressure but reduced the NANC bronchoconstrictor response to vagal stimulation in a dose-dependent manner (ED_{50} = 186 μg kg^{-1} with a maximal inhibition of 74 ± 3.4% at 10 mg kg^{-1}). Neither the opioid antagonist naloxone (1 mg kg^{-1} i.v.) nor the α-adrenoceptor antagonist phentolamine (2.5 mg kg^{-1} i.v.) had any significant effect on the inhibitory response produced by GABA (500 μg kg^{-1}).

4 GABA-induced inhibition was not antagonised by the $GABA_A$-antagonist bicuculline (2 mg kg^{-1} i.v.).

5 The $GABA_B$-agonist baclofen (10 μg–3 mg kg^{-1} i.v.) caused a dose-dependent inhibition of the NANC response (ED_{50} = 100 μg kg^{-1} with a maximal inhibition of 35.5 ± 2.8% at 3 mg kg^{-1}). The $GABA_A$-agonist, 4,5,6,7-tetrahydroisoxazolo[5,4-C] pyridin-3-ol (THIP), also inhibited the NANC bronchoconstrictor response. However, the dose of THIP required for this effect was high (3 mg kg^{-1}) and the effect (< 10% inhibition) was small.

6 Substance P (SP; 5 μg kg^{-1} or 25 μg kg^{-1}), produced a bronchoconstrictor response equivalent to that produced by NANC vagal stimulation. This response was significantly increased by injection of GABA. Baclofen had no significant effect on responses evoked by exogenous SP.

7 We conclude that GABA inhibits the release of transmitter from NANC nerves via an action at $GABA_B$ receptors and that GABA might play a role in the regulation of neurogenic responses in the airways.

Introduction

γ-Aminobutyric acid (GABA) is a well-characterised inhibitory neurotransmitter in the mammalian central nervous system (Curtis & Johnston, 1974). Recent evidence suggests, however, that GABA and its receptors are present in peripheral neurones (Giotti *et al.*, 1983; Tanaka, 1985; Santicioli *et al.*, 1986). In guinea-pig trachea GABA decreases the contractile response of airway smooth muscle to cholinergic nerve stimulation possibly by inhibiting the evoked release of acetylcholine (Tamaoki *et al.*, 1987).

Non-adrenergic, non-cholinergic (NANC) bronchoconstriction evoked by vagal stimulation *in vivo* may be due to the release from sensory nerves of neuropeptides such as substance P (SP) and neurokinin A (Lundberg *et al.*, 1983). We have now studied the effect of GABA on the NANC bronchoconstrictor response evoked by vagal stimulation to evaluate whether GABA has an inhibitory action on sensory neurones.

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At least two subtypes of GABA receptor have been identified; bicuculline-sensitive and bicuculline-insensitive receptors, which are termed $GABA_A$- and $GABA_B$-receptors, respectively (Bowery *et al.*, 1981; Kaplita *et al.*, 1982). We therefore also investigated the GABA receptor subtype involved by use of selective agonists and an antagonist.

Methods

Measurements

Male Dunkin-Hartley outbred guinea-pigs (Charles River U.K. Ltd., Kent) of 310–470 g weight were housed in a temperature-controlled (21°C) room with food and water freely available. They were anaesthetised with an intraperitoneal injection of urethane (8 ml kg^{-1} of a 25% solution w/v in saline) and placed on a heated blanket (Homeothermic system, Harvard Apparatus Ltd., Edenbridge, Kent) which maintained body temperature at 37°C. The left jugular vein was cannulated for the injection of drugs. The left carotid artery was cannulated for monitoring blood pressure, via an indwelling Portex cannula, filled with heparin-saline (10 u ml^{-1}), linked to a pressure transducer (Druck blood pressure transducer, Druck Ltd., Groby, Leicestershire) and connected to a recorder (Lectromed Multitrace 2, Ormed Ltd., Welwyn Garden City, Hertfordshire). The trachea was cannulated and the animal ventilated with a small animal constant volume respiration pump (Bioscience U.K., Sheerness, Kent), operating at 60 strokes min^{-1} of 1 ml laboratory air per 100 g body weight. Overflow pressure was measured by a modification of the Konzett and Rossler procedure (Konzett & Rossler, 1940) by a differential pressure transducer (Farnell Electronic Components Ltd., Leeds). Both cervical vagus nerves were carefully dissected free, sectioned (to avoid stimulating the CNS) and their peripheral ends placed on platinum electrodes. A Fenton Lewis double-channel stimulator was used to stimulate the nerves using pulses of 5 ms duration 5 V strength for 30 s. The frequency used was either 3 Hz or 5 Hz depending on the size of the NANC bronchoconstrictor response obtained. Only one of these two pulse frequencies was used in a given animal and each animal served as its own control.

Experimental protocol

Atropine (1 mg kg^{-1}) and propranolol (1 mg kg^{-1}) were administered intravenously (i.v.) and the vagi were stimulated after 30 min to give a NANC bronchoconstrictor response as previously described (Belvisi *et al.*, 1988). In some experiments phentolamine (2.5 mg kg^{-1}) was also injected 30 min before vagal stimulation. Vagal stimulation was repeated again 10 min later or when the overflow pressure had returned to its baseline value. Hyperinflation of the lungs was used to return the pressure to control values after a bronchoconstriction. After two identical responses had been elicited the effects of GABA and various GABA receptor agonists were investigated. GABA (10 μg–10 mg kg^{-1}), baclofen (10 μg–3 mg kg^{-1}) or 4,5,6,7-tetrahydroisoxazolo[5,4-c]-pyridin-3-ol (THIP, 3 mg kg^{-1}) were administered i.v. 1 min before vagal stimulation. The time course of the action of GABA (500 μg kg^{-1}) was also studied. Several doses of GABA could be examined in one animal as GABA had a very short duration of action. The action of GABA was also examined in the presence of bicuculline (2 mg kg^{-1}), a $GABA_A$-receptor antagonist, and naloxone (1 mg kg^{-1}), an opioid receptor antagonist, each antagonist being given 10 min before the injection of GABA. In other experiments bicuculline was injected alone to determine whether it had any effect *per se* on NANC bronchoconstriction at these stimulation parameters.

The effects of GABA (500 μg kg^{-1}) and baclofen (2 mg kg^{-1}) on bronchoconstrictor responses to exogenous substance P (SP, 5–25 μg kg^{-1}) (which produced a constrictor response equivalent to that produced by NANC stimulation) were also evaluated. Two bronchoconstrictor responses to SP (5 or 25 μg kg^{-1}) were elicited. A period of 30 min was allowed between successive injections of SP. The effect of GABA on the bronchoconstrictor response evoked by SP was investigated by injecting GABA 1 min before the administration of SP.

Drugs

Drugs and chemicals were obtained from the following sources: urethane, substance P, γ-amino-n-butyric acid, (−)-bicuculline methiodide (Sigma Chemical Co., Poole, Dorset), atropine sulphate BP (Phoenix Pharmaceuticals Ltd., Oxford), propranolol hydrochloride (Imperial Chemical Industries, Cheshire), (±)-baclofen, 4,5,6,7-tetrahydroisoxazolo[5,4,-c]pyridin-3-ol hydrochloride (Research Biochemicals Inc., MA, U.S.A.), naloxone hydrochloride (Du Pont U.K., Hertfordshire), phentolamine mesylate BP (Ciba Laboratories, West Sussex), heparin injection BP (CP Pharmaceuticals Ltd., Wrexham). All drugs were made up in 0.9% saline on the day of experimentation.

Statistical analyses

Mean arterial blood pressure (BP) was calculated from recorded traces as: diastolic pressure + 0.33

(systolic pressure – diastolic pressure). Results are presented as means ± s.e.mean. Differences between means were analysed by Student's t test for paired data. A probability level of $P < 0.05$ was considered statistically significant. The dose of drug required to produce 50% of the maximal inhibition of the NANC bronchoconstrictor response evoked by vagal stimulation (ED_{50}) was read directly from curves constructed using % maximal responses as a function of the dose of GABA or baclofen.

Results

Effect of vagal stimulation

Thirty minutes after atropine and propranolol (each 1 mg kg^{-1}), vagal stimulation produced a significant bronchoconstrictor response that was reproducible within any given animal. The NANC bronchoconstrictor response was manifest as an increase in airways pressure in the range 8–52 cmH_2O. The mean amplitude (±s.e.mean) of the response was 21.9 ± 1.04 cmH_2O, $n = 70$.

Effect of GABA on NANC bronchoconstrictor response

All drugs were dissolved in saline but i.v. saline had no effect on NANC bronchoconstrictor responses. GABA, when injected 1 min before stimulation, caused a dose-dependent decrease in the NANC bronchoconstrictor response evoked by vagal stimulation. The ED_{50} for GABA at inhibiting this response was 186 μg kg^{-1} with a maximum inhibition of 74 ± 3.4% at 10 mg kg^{-1} (Figure 1). The inhibitory effect of GABA was assessed 1, 10 and 20 min after its injection. The inhibitory effect of GABA (500 μg kg^{-1} i.v.) had virtually disappeared by 10 min (Figure 2) and the inhibitory effects of larger doses of GABA followed a similar time course.

The effect of GABA before and after bicuculline was studied in the same animal so that paired data were obtained. The $GABA_A$-antagonist bicuculline (2 mg kg^{-1} i.v.), when injected 10 min before GABA, increased the inhibitory effect of GABA (100 μg kg^{-1}) on the NANC response from 38.3 ± 3.1% to 53.6 ± 6.8% ($n = 5$) ($P < 0.05$). In these experiments the inhibitory effects of bicuculline and GABA in combination were somewhat persistent. The NANC bronchoconstrictor response remained inhibited even at 10 min after the injection of GABA. Bicuculline alone (100, 500 μg kg^{-1} and 2 mg kg^{-1}) also inhibited the NANC bronchoconstrictor response in a persistent manner. Indeed the inhibitory effects of bicuculline increased over a time period up to 30 min after its injection (Figure 3). Phentolamine (2.5 mg kg^{-1}) (Boschetto *et al.*, 1989)

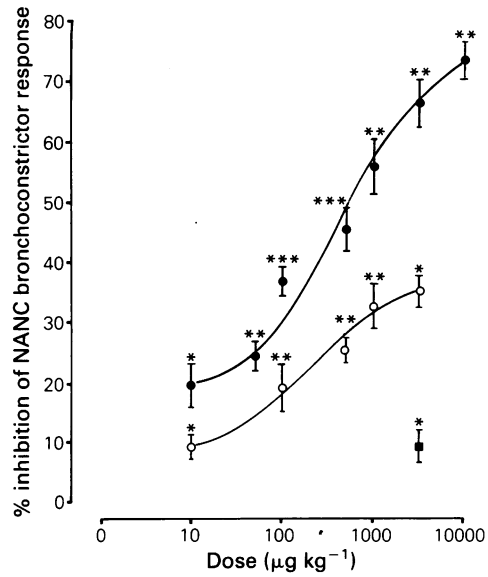

Figure 1 The non-adrenergic, non-cholinergic (NANC) bronchoconstrictor response evoked by vagal stimulation: its inhibition by intravenous GABA (●), baclofen (○) and 4,5,6,7-tetrahydroisoxazolo[5,4,-c] pyridin-3-ol (THIP) (■). Data are means of 4–11 animals. Vertical lines show s.e.mean. Significance of inhibition: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

($n = 4$), injected 30 min before stimulation, did not affect the inhibitory response produced by GABA (500 μg kg^{-1}) nor did naloxone (1 mg kg^{-1}) (Belvisi *et al.*, 1988) ($n = 4$), injected 10 min before GABA (Figure 4).

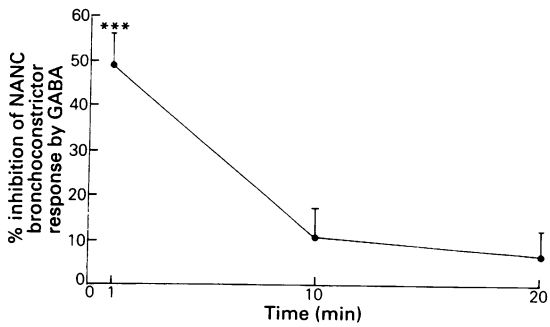

Figure 2 Time course of inhibition of the NANC bronchoconstrictor response by GABA (500 μg kg^{-1}). Bilateral vagal stimulation was carried out at 3 different time points (1, 10 and 20 min) after administration of GABA. Data are means of the same six animals at each time point. Vertical lines show s.e.mean. Significance of inhibition: ***$P < 0.001$.

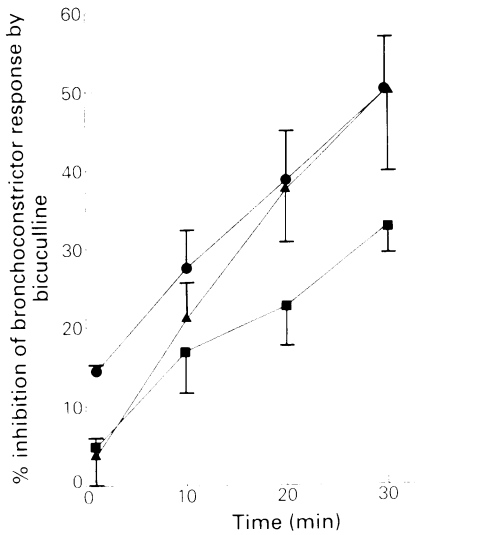

Figure 3 Time course of inhibition of the NANC bronchoconstrictor response by bicuculline 100 μg (■), 500 μg (▲) and 2 mg kg^{-1} (●). Bilateral vagal stimulation was carried out at 4 different time points (1, 10, 20 and 30 min) after administration of bicuculline. Data are means of 3–4 animals at each dose at each time point. Vertical lines show s.e.mean.

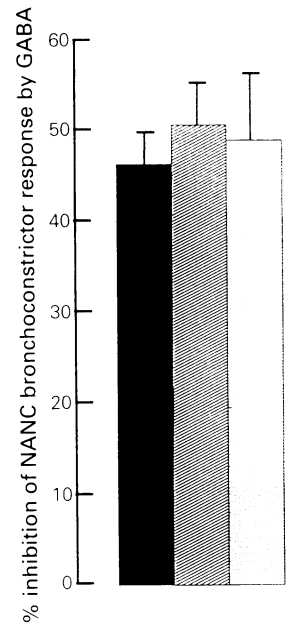

Figure 4 The effect of phentolamine (2.5 mg kg^{-1}) and naloxone (1 mg kg^{-1}) on the inhibitory effect of GABA (500 μg kg^{-1}) on the NANC bronchoconstrictor responses evoked by vagal stimulation. Solid column: inhibitory effect of GABA on NANC bronchoconstrictor response ($n = 8$); hatched column: inhibitory effect of GABA on NANC bronchoconstrictor response in the presence of phentolamine (2.5 mg kg^{-1}) ($n = 4$); stippled column: inhibitory effect of GABA on NANC response in the presence of naloxone (1 mg kg^{-1}) ($n = 4$). Data are presented as means and bars show s.e.mean.

Effect of selective GABA receptor agonists

The $GABA_B$-agonist baclofen, when injected 1 min before stimulation, caused a dose-dependent decrease in the NANC bronchoconstrictor response elicited by vagal stimulation. The ED_{50} for baclofen was 100 μg kg^{-1} with a maximum inhibition of 35 ± 2.8% at 3 mg kg^{-1} (Figure 1). However, the $GABA_A$-selective agonist THIP only inhibited the NANC response at 3 mg kg^{-1} (9.7 ± 2.6%, $n = 6$, $P < 0.05$) (Figure 1).

Effect on substance P responses

Responses to SP were elicited following the injection of atropine and propranolol (each 1 mg kg^{-1}). Doses of SP (5 or 25 μg kg^{-1}) were selected which produced responses equivalent to those evoked by vagal stimulation in each animal.

Increases in tracheal pressure evoked by exogenous SP (5 or 25 μg kg^{-1}) were enhanced by GABA (500 μg kg^{-1}) from 25.8 ± 4.4 cmH_2O to 34 ± 4.7 cmH_2O ($n = 5$, $P < 0.05$) (Figure 5). This dose of GABA inhibited the NANC response by 45.8 ± 3.7%. However, baclofen at a dose (2 mg kg^{-1}) which inhibited the NANC response by approximately 34.5%, did not significantly alter the response to exogenous SP (25 μg kg^{-1}) (Figure 5).

Bicuculline (2 mg kg^{-1}), given alone 10 min before SP (25 μg kg^{-1}) increased the response evoked by SP from 26 ± 2.4 cmH_2O to 32 ± 2.4 cmH_2O ($n = 4$, $P < 0.05$).

Effect of GABA and baclofen on blood pressure

The mean arterial blood pressure (BP) recorded from the left carotid artery before the injection of GABA or the test substance was 40.2 ± 0.96 mmHg ($n = 66$). Flushing the artery, to prevent blood clotting in the indwelling cannula, with heparin-saline before experimentation and i.v. saline had no significant or consistent effect on BP. GABA transiently but significantly decreased the mean arterial BP at every dose used (see Table 1). However, in all but one case the BP was not significantly different from baseline at the time of vagal stimulation. Baclofen (10 μg kg^{-1} to 3 mg kg^{-1}) had a similar effect on BP whereas THIP (3 mg kg^{-1}) had no significant effect.

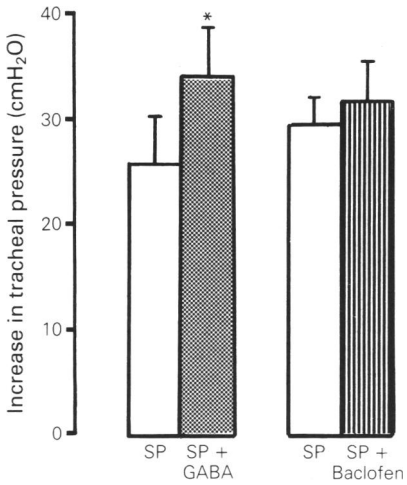

Figure 5 The effect of GABA and baclofen on bronchoconstriction evoked by exogenous substance P (SP). Open columns: SP (5–25 $\mu g\,kg^{-1}$) alone; stippled column: SP (5 $\mu g\,kg^{-1}$) after GABA (500 $\mu g\,kg^{-1}$); lined column: SP (5–25 $\mu g\,kg^{-1}$) after baclofen (2 $mg\,kg^{-1}$). Data are presented as means of 5 or 6 animals. Vertical lines show s.e.mean. $*P < 0.05$.

Discussion

Our study shows that GABA reversibly decreases the neurally-mediated bronchoconstrictor response evoked by stimulation of the peripheral portion of the sectioned vagus in anaesthetised guinea-pigs. This excitatory response resulting from vagal nerve stimulation in the presence of atropine and propranolol is non-adrenergic and non-cholinergic and probably due to the release of neuropeptides, such as SP and neurokinin A, from sensory nerve endings (Andersson & Grundstrom, 1983; Lundberg *et al.*, 1983).

In order to establish whether the inhibitory effect of GABA is due to an effect on neuronal terminals, on tachykinin receptors on airway smooth muscle, or on excitation-contraction coupling in smooth muscle, we compared the effect of GABA on the bronchoconstrictor response to vagal stimulation with its effect on the bronchoconstrictor response to SP. GABA inhibited the NANC response but had no inhibitory effect on the contractile response to SP and even increased the tracheal pressure rise evoked by exogenous SP. This implies that GABA-induced inhibition is not related to an alteration of smooth muscle function but probably represents a prejunctional mechanism, such as inhibition of tachykinin release from sensory nerve endings.

GABA-induced inhibition could be accomplished by enhancing the release of noradrenaline since noradrenaline has been shown to inhibit neural NANC bronchoconstrictor responses in the guinea-pig (Grundstrom *et al.*, 1984). However, the involvement of GABA-induced prejunctional inhibitory α-adrenoceptor activation is unlikely as phentolamine was without effect on the inhibitory action of GABA. GABA transiently decreases blood pressure and this may result in the release of catecholamines which could act on β-adrenoceptors in the airway to inhibit bronchoconstriction. This is probably not the case as the animals had been pretreated with the β-adrenoceptor antagonist, propranolol. The lowering of the blood pressure itself could induce an inhibition of the NANC response to vagal stimulation. However, 1 min after stimulation, which was when GABA was injected, blood pressure had returned to normal, before the effects of nerve stimulation were

Table 1 Effect of GABA and baclofen on mean arterial blood pressure (BP)

Dose GABA ($\mu g\,kg^{-1}$)	n	*Decrease in BP*[a]	*Decrease in BP at stimulation time*[b]
50	5	7.04 ± 2.6*	1.9 ± 1.2 (NS)
100	11	6.9 ± 1.1***	0.6 ± 0.54 (NS)
500	9	13.4 ± 1.2***	2.7 ± 1.8 (NS)
1000	4	14.1 ± 3.1**	7.3 ± 4.4 (NS)
3000	5	10.8 ± 2.9*	7.7 ± 2.2*
Dose baclofen ($\mu g\,kg^{-1}$)			
10	5	15.3 ± 1.6***	1.1 ± 1.5 (NS)
100	5	5.7 ± 1.6*	2.3 ± 1.2 (NS)
500	4	11.5 ± 3.3*	7.8 ± 3.4 (NS)
1000	4	6.2 ± 6.0 (NS)	7.7 ± 3.7 (NS)
3000	3	9.1 ± 2.8 (NS)	7.3 ± 4.1 (NS)

Data are presented as means ± s.e.mean.
[a] Measured at the peak drug effect.
[b] BP 1 min after GABA at the onset of vagal stimulation.
$*P < 0.05$, $**P < 0.01$, $***P < 0.001$, NS = not significant.

studied. In the CNS GABA has been shown to potentiate the release of methionine enkephalin (Sawynok & Labella, 1981; Bourgoin *et al.*, 1985). Opioids of the μ and possibly δ type inhibit release of transmitter from NANC nerves by stimulating opioid receptors (Frossard & Barnes, 1987; Belvisi *et al.*, 1988). However, the ineffectiveness of naloxone suggests that this is not the mode of action of GABA in guinea-pig lung.

The GABA receptor antagonist bicuculline failed to prevent the inhibitory effect of GABA, but paradoxically enhanced the effect of GABA. However, we also found that bicuculline alone inhibited NANC responses in an irreversible manner. It is possible that bicuculline has a toxic effect on sensory nerves as it did not inhibit the responses to exogenous SP. The $GABA_B$-agonist, baclofen, has a dose-related and reversible inhibitory effect on NANC bronchoconstrictor responses. Baclofen was more potent than GABA but did not achieve the same maximum inhibitory effect. An explanation for this relative lack of efficacy is the fact that baclofen is stereoselective. (−)-Baclofen is more potent than the racemic mixture of (±)-baclofen used in our study (Giotti *et al.*, 1983). However, for this explanation to stand (+)-baclofen must be an antagonist or a partial agonist. In fact the (+)- and (−)-isomers of baclofen have been shown to have opposite effects in other systems. (−)-Baclofen (4 μM) inhibits release of preloaded [^{3}H]-D-aspartate from slices of rat cerebral cortex, whereas (+)-baclofen was inactive under these conditions and at 10 μM weakly augmented D-aspartate release (Johnston *et al.*, 1980). THIP, a $GABA_A$-agonist, was only weakly effective (<10% inhibition) at a high dose (3 mg kg^{-1}). All this evidence suggests the involvement of the $GABA_B$-type receptor (Bowery *et al.*, 1981). Confirmation of the involvement of a $GABA_B$-receptor might be obtained with the use of a specific $GABA_B$-antagonist. The only selective antagonist currently available, phaclofen, is not suitable for *in vivo* studies because of its very low potency (Kerr *et al.*, 1987).

In conclusion, the action of GABA in the lung appears to be exerted through a modulatory action on NANC nerves. One site of action is probably the bicuculline-insensitive receptor ($GABA_B$), which may be located on sensory nerve terminals, as GABA and baclofen inhibit the NANC bronchoconstrictor response but THIP is weakly effective. Tamaoki *et al.* (1987) have demonstrated that GABA decreases the contractile response of airway smooth muscle to cholinergic nerve stimulation by inhibiting the evoked release of acetylcholine. However, these authors have suggested that this is a bicuculline-sensitive effect and therefore mediated by $GABA_A$-receptors. Hence, the receptor involved remains to be identified because baclofen, a $GABA_B$-agonist, was found to be equipotent with GABA and muscimol, a $GABA_A$-agonist, was found to be less potent. In the present study GABA enhanced the response to exogenous SP but baclofen was unable to do this. This suggests that a similar system operates here as has been observed in the ileum (Giotti *et al.*, 1983). That is GABA as well as inhibiting SP release also enhances its activity, but via $GABA_A$-receptors.

GABA and its synthetic enzyme glutamic acid decarboxylase appear to be present in the peripheral autonomic nervous system, suggesting that GABA may function as a peripheral neurotransmitter or neuromodulator (Jessen *et al.*, 1979). GABAergic nerves have been found in the myenteric plexus of the gut (Jessen *et al.*, 1986) and since the innervation of the respiratory tract has a common embryological origin with that in the gastrointestinal tract, it seems likely that GABAergic nerves may also be present in the airways. If so this suggests that GABA may have a neuromodulatory role on neurogenic bronchoconstriction. Sensory nerves may play an important role in the neurogenic inflammation in the airways and may be involved in the airway inflammation of asthma (Barnes, 1986). Regulation of the release of sensory neuropeptides from nerves may therefore reduce neurogenic inflammation, as has been previously demonstrated for opioids both *in vivo* (Belvisi *et al.*, 1988) and *in vitro* (Frossard & Barnes, 1987). We have now shown that GABA acting via peripheral $GABA_B$-receptors may have a similar effect and this could suggest a novel approach in the chemotherapy of bronchial asthma.

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NK-1 receptor mediation of neurogenic plasma extravasation in rat skin

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1 Plasma extravasation was induced by electrical nerve stimulation and by perfusion of tachykinins over a vacuum-induced blister base on rat footpad.

2 Stimulation of the sciatic nerve (18 V, 15 Hz, 0.5 ms) for 20 min produced a significant increase in the protein content of the perfusate. The response in capsaicin pretreated rats was only 4% of the control response. This indicates that the electrically-induced plasma extravasation response was mediated by capsaicin-sensitive sensory fibres.

3 Exogenous perfusion of the mammalian tachykinins substance P, neurokinin A and neurokinin B and the non-mammalian tachykinins physalaemin, kassinin and eledoisin was used to determine the tachykinin receptor type mediating the plasma extravasation response. Dose-response curves of the tachykinins (10^{-9} M–10^{-4} M) gave a rank order of potency of substance P = physalaemin > eledoisin ≥ kassinin > neurokinin B = neurokinin A.

4 In addition, specific agonists of neurokinin receptors were perfused. Perfusion of [Glp^6, D-Pro^9]SP_{6-11} and [Glp^6, L-Pro^9]SP_{6-11} demonstrated that the L-Pro isomer was much more potent than the D-Pro isomer.

5 The rank order of potency and the greater potency of [Glp^6, L-Pro^9]SP_{6-11} over its D-isomer indicate an NK-1 neurokinin receptor mediates plasma extravasation in rat footpad skin.

Introduction

Neurogenic inflammation is defined as vasodilatation and plasma extravasation that occurs following electrical nerve stimulation (Jancso *et al.*, 1967). This response is believed to be mediated by peptides such as the tachykinins, which are released from primary afferent nerve terminals (Lembeck & Holzer, 1979). Electrical nerve stimulation releases substance P (SP), the first identified tachykinin, and induces plasma extravasation in a thermal injury blister model (White & Helme, 1985). We have previously used a vacuum-induced blister model of inflammation to demonstrate that neurogenic plasma extravasation can be induced by mechanical, chemical and thermal stimulation (Andrews & Helme, 1986; Helme *et al.*, 1986). In the current study we demonstrate that plasma extravasation can also be induced by electrical nerve stimulation in the vacuum blister model, that this plasma extravasation is neurogenic in nature and that mediation of the response is likely to occur at a specific tachykinin receptor.

Lee *et al.* (1982) were among the first to recognize the nature of multiple receptors for SP, namely, the SP-P receptor where all the tachykinins were essentially equipotent and the SP-E receptor where eledoison (Ele) and kassinin (Kas) were more potent than SP and physalaemin (Phy). The SP-E receptor was subsequently found to be probably the combined effects of two receptors. One of these, the SP-N receptor, was identified in guinea-pig ileum when the effects of SP were inhibited by desensitization (Laufer *et al.*, 1985) and the other receptor, SP-K, was observed after binding studies on rat brain (Buck *et al.*, 1984). Using rank order of potency and currently defined terminology, the receptors can be differentiated into three subtypes as follows:

NK-1: SP > Phy > NKA > NKB > Ele > Kas,

NK-2: NKA > NKB > Kas > Ele > SP > Phy

and

NK-3: NKB ≫ Ele > Kas > NKA = Phy > SP (Lee *et al.*, 1986).

Many of the functional studies with neurokinin receptors have been performed on smooth muscle preparations *in vitro* (Regoli *et al.*, 1984; Mizrahi *et*

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al., 1985) and few have investigated neurogenic plasma extravasation *in vivo*. NKA and SP were almost equipotent in producing plasma extravasation in rat bladder and ureters (Maggi *et al.*, 1986) whereas Gamse & Saria (1985), who investigated Evans blue extravasation to intradermal injection of the tachykinins in abdominal skin of rats, obtained a rank order of potency NKB > SP > NKA. On the dorsal skin of the rat, intradermal injection of the mammalian tachykinins gave similar results, namely NKB > NKA > SP (Couture & Kerouac, 1987) suggesting NK-3 receptor mediation of the response. On the other hand, intradermally injected tachykinins in human skin induce wheal responses with the rank order of potency SP > NKA > NKB (Devillier *et al.*, 1986). These authors also demonstrated that in rat dorsal skin, the rank order for inducing Evans blue extravasation was SP = NKA > NKB which suggests NK-1 mediation.

In the present study, tachykinins (both mammalian and non-mammalian) as well as SP_{1-9}, SP_{5-11}, and the newly described specific agonists D- and L-Pro isomers of [Glp^6, Pro^9]SP_{6-11} (Lee *et al.*, 1986) were perfused over the blister base on rat footpad to determine which neurokinin receptor mediates neurogenic plasma extravasation from dermal vessels. Preliminary data were presented at the Substance P and Neurokinins '86 meeting (Andrews & Helme, 1987).

Methods

Male Sprague-Dawley rats (200–320 g) were used; some had been pretreated as neonates with capsaicin (50 mg kg^{-1}) (Jancso *et al.*, 1977). For all experiments, animals were anaesthetized (sodium pentobarbitone, 65 mg kg^{-1}, i.p.), a femoral vein catheter was inserted (for fluid load [1 ml 50 g^{-1}] and maintenance of anaesthesia), and the footpad placed over a plate heated to 40°C with an aperture opening to a vacuum pump. With a pressure of −40 kPa a blister was induced in 30 min involving separation of epidermis from dermis (Helme *et al.*, 1985). After blister induction, the blister epidermis was removed, replaced by a perfusion chamber (approximately 1 cm^3 capacity) attached to a peristaltic pump (LKB, Sweden) and the chamber perfused with Ringer solution at 4 ml h^{-1}, thus superfusing the blister base. The perfusion chamber was maintained at 37°C with a heating element in the roof of the chamber. Perfusates were collected into an equal volume of cold 2 M acetic acid and stored at −20°C before they were analysed for plasma extravasation by a simple protein assay (Bradford, 1976).

Experiments with electrical nerve stimulation were performed on normal and capsaicin pretreated rats. The sciatic nerve was dissected free, cut proximally and suspended over bipolar platinum electrodes in a paraffin oil bath formed by raising the skin flaps of the wound. The nerve was stimulated at 18 V, 15 Hz, 0.5 ms pulse duration (an intensity sufficient to stimulate 'C' fibres, Yaksh *et al.*, 1980) or 4 V, 15 Hz, 0.5 ms (insufficient to stimulate 'C' fibres maximally) for 20 min. Perfusates were collected from the stimulated footpad and the opposite footpad control. Results are expressed as the mean ± s.e.mean of the increased protein content of the stimulated footpad perfusate over the control footpad perfusate.

Experiments with tachykinin perfusion were performed for two 30 min periods, an initial control period with perfusion of Ringer solution followed by perfusion with a tachykinin solution in both normal and capsaicin pretreated animals. SP, NKA, NKB, Phy, Kas, Ele, SP_{1-9} and SP_{5-11} (Peninsula Labs., San Carlos, U.S.A.) were dissolved in Ringer solution and stored at −20°C until used. NKB was also dissolved in sulpholane (BDH) 1:30 with Ringer solution. The D- and L-Pro isomers of [Glp^6, Pro^9]SP_{6-11} (kindly donated by Dr L.L. Iversen, Merck Sharp and Dohme, U.K.) were dissolved in 10% dimethylsulphoxide (DMSO) and diluted to 1% for perfusion. Plasma extravasation induced by the tachykinins is expressed as the mean ± s.e.mean of the increased amount of protein in the tachykinin perfusion perfusate of each animal compared to its control period. The mean protein concentration of all the control periods was 32.4 μg ml^{-1} ± 1.46, $n = 178$. The pD_2 s for the peptides were calculated by linear regression analysis (Hays, 1973).

Results

Plasma extravasation induced by electrical nerve stimulation

One way analysis of variance (Kirk, 1968) revealed that there were significant differences in the plasma extravasation (increased protein content above control) induced by 18 V (23.6 μg ml^{-1} ± 6.7; $n = 8$) and 4 V stimulation (7.6 μg ml^{-1} ± 2.6; $n = 9$) and 18 V stimulation in capsaicin pretreated animals (0.9 μg ml^{-1} ± 1.2; $n = 8$) ($F_{2,24} = 7.7665$, $P = 0.0028$). Using the Student-Newman-Keuls multiple range test each of the three groups was significantly different from the others at the $P < 0.05$ level.

Plasma extravasation induced by exogenous tachykinins

Exogenous SP induced plasma extravasation at a threshold of 10^{-8} M and produced maximal plasma

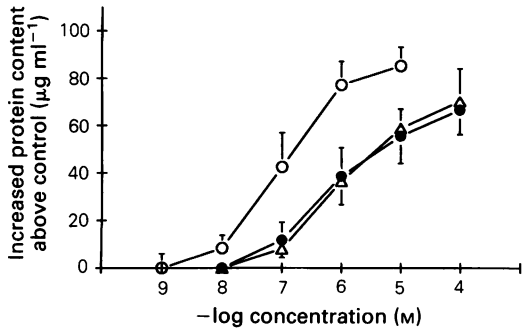

Figure 1 The dose-response curves for plasma extravasation induced by perfusion of the mammalian tachykinins (substance P (SP) ○; neurokinin A (NKA) ●; NKB △). Points are the mean results from 4 animals (s.e.mean shown by vertical bars) except for; SP 8(6) 7(6) 6(10); NKA 6(6) 5(6) 4(10); NKB 4(6). Number of animals in parentheses.

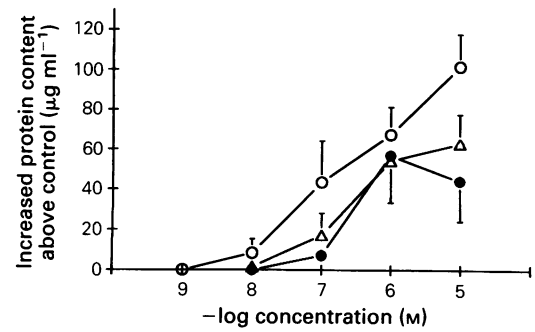

Figure 3 The dose-response curves for plasma extravasation induced by perfusion of the non-mammalian tachykinins (physalaemin ○; kassinin ●; eledoisin △). Points are the mean from 4 animals; vertical bars show s.e.mean.

extravasation ($85\,\mu g\,ml^{-1} \pm 8.5$) at 10^{-5} M (Figure 1). N- and C-terminal analogues of SP were also tested. SP_{1-9} at 10^{-5} M was inactive in inducing plasma extravasation. SP_{5-11} at the same concentration induced plasma extravasation although it was less active than SP ($61.5\,\mu g\,ml^{-1} \pm 6.5$ and $85.5\,\mu g\,ml^{-1} \pm 12.0$ respectively) (Figure 2).

SP (10^{-7} M) was also perfused in rats that had been pretreated with capsaicin. There was no difference between the normal and capsaicin pretreated animals in their response to SP (normal: $73.3\,\mu g\,ml^{-1} \pm 15.45$; capsaicin: $82.0\,\mu g\,ml^{-1} \pm 7.43$; $n = 6$ in both groups).

NKA and NKB were equipotent and more than an order of magnitude less potent than SP. Because NKB is not fully soluble in Ringer solution it was also dissolved in sulpholane in an attempt to increase its solubility, however the ability of NKB (10^{-6} M) to induce plasma extravasation was not altered ($36.5\,\mu g\,ml^{-1} \pm 7.0$ and $48.0\,\mu g\,ml^{-1} \pm 12.0$; $n = 4$ in both groups).

The non-mammalian tachykinins induced plasma extravasation at similar potencies to their mammalian homologues (Figure 3). Phy was the most potent and Kas and Ele were equipotent and about an order of magnitude less potent than Phy. Table 1 is a list of the pD_2s for the peptides obtained from their dose-response curves.

Plasma extravasation induced by the D *and* L*-Pro isomers of* [*Glp*^{6}, *Pro*^{9}]*SP*$_{6-11}$

The L-Pro^9 isomer of [Glp^6, Pro^9]SP_{6-11} induced plasma extravasation into the perfusate with a potency about an order of magnitude less than SP. The D-Pro^9 isomer was much less active with plasma

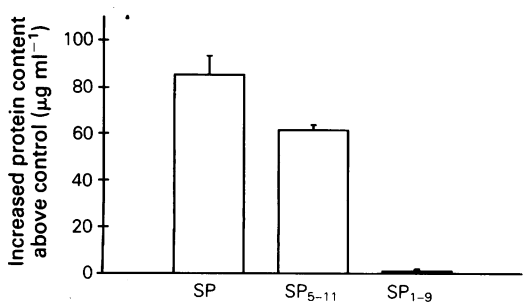

Figure 2 The plasma extravasation response induced by perfusion of substance P (SP), its C-terminal analogue (SP_{5-11}) and its N-terminal analogue (SP_{1-9}), each at a concentration of 10^{-5} M. Columns are the mean from 4 animals; vertical bars show s.e.mean.

Table 1 pD_2s for the tachykinins in inducing a plasma extravasation response in the blister base

Peptide	*pD_2 (95% confidence limits)*
SP	6.93 (7.40 – 6.45)
NKA	5.57 (6.92 – 4.22)
NKB	5.60 (6.55 – 4.65)
Phy	6.90 (7.70 – 6.10)
Ele	6.39 (7.32 – 5.46)
Kas	6.25 (8.79 – 3.71)

SP, substance P; NKA and NKB, neurokinin A and B; Phy, physalaemin; Ele, eledoisin; Kas, kassinin.

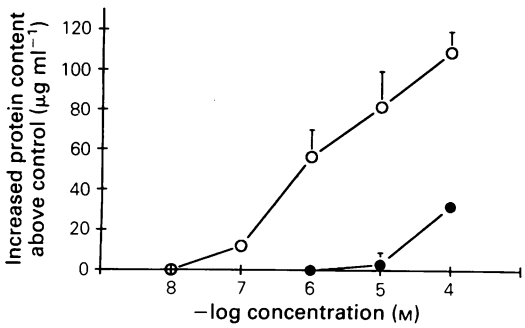

Figure 4 The dose-response curves for plasma extravasation induced by [Glp^6, L-Pro^9]SP$_{6-11}$ (○) and [Glp^6, D-Pro^9]SP$_{6-11}$ (●). Points are the mean from 4 animals; vertical bars show s.e.mean.

extravasation activity only towards the mM concentration range (Figure 4).

Discussion

Neurogenic plasma extravasation in the vacuum blister model

In the past, neurogenic inflammation in the skin was induced by noxious chemical stimulation with mustard oil and xylene (Jancso *et al.*, 1967) and the plasma extravasation response measured as accumulated Evans blue dye in the skin. Neurogenic plasma extravasation has also been quantified following electrical nerve stimulation (Jancso *et al.*, 1967; Lembeck & Holzer, 1979; Morton & Chahl, 1980). In animals that had been treated as neonates with capsaicin, a neurotoxin that selectively destroys unmyelinated nerves in the primary afferent nerve pathway, electrically induced plasma extravasation does not occur thus implicating involvement of small diameter 'C' fibres of the primary afferent nerve in the response.

In the present study neurogenic plasma extravasation was quantified for the first time in an 'on-line' system using the vacuum induced blister model. The neurogenic nature of the plasma extravasation was evident from the lack of a response in capsaicin pretreated animals when the sciatic nerve was stimulated at 18 V, 15 Hz, 0.5 ms, a stimulation intensity that did produce significant plasma extravasation in normal animals and from the small plasma extravasation response when the sciatic nerve of normal animals was stimulated at 4 V, 15 Hz, 0.5 ms. Stimulation at 4 V has been shown to stimulate only A_α and A_β fibres (Yaksh *et al.*, 1980) and no plasma extravasation was observed at this stimulus intensity. However, it is possible that stimulation at 4 V is still able to excite some 'C' fibres and thus account for the small but significant increase in plasma extravasation that was observed.

The vacuum blister model has previously been shown to be appropriate for studying neurogenic inflammation to physiological noxious stimuli (Andrews & Helme, 1986; Helme *et al.*, 1986) and now we have shown that non-physiological electrical nerve stimulation also induces neurogenic plasma extravasation in this model. This neurogenic response is mediated through capsaicin-sensitive primary afferent neurones that have been shown to contain a number of peptides including the tachykinins (Jancso *et al.*, 1981). It is likely that these substances mediate their responses through different receptors. We have therefore attempted to determine which neurokinin receptor is responsible for neurogenic plasma extravasation in rat footpad skin.

Tachykinin-induced plasma extravasation

In the current study the tachykinins, both mammalian and non-mammalian, induced plasma extravasation in a dose-dependent manner. The tachykinins were active in the range of 2 pmol to 200 nmol peptide perfused. This amount of peptide is comparable to studies where plasma extravasation in skin was induced by intra-arterial infusion (Lembeck & Holzer, 1979) and intra-dermal injection (Foreman *et al.*, 1983; Gamse & Saria, 1985; Devillier *et al.*, 1986; Couture & Kerouac, 1987) but is more potent than intravenously administered SP (Saria *et al.*, 1983).

SP was also perfused over the blister base of animals that had been pretreated with capsaicin as neonates. In these animals the plasma extravasation response was no different from that of normal animals. This indicates that the tachykinin-induced plasma extravasation response occurs independently of the primary afferent nerve pathway as had earlier been shown with the wheal response to SP in capsaicin-desensitized human skin (Anand *et al.*, 1983; Foreman *et al.*, 1983) and with local administration to the rat nasal mucosa (Lundblad *et al.*, 1983) and rat tracheal mucosa (Lundberg & Saria, 1983).

The SP-induced plasma extravasation was shown to be mediated by an interaction of the C-terminal of the peptide with its receptor. An N-terminal analogue of SP had no plasma extravasation inducing activity. This verifies previous work in human skin where an N-terminal analogue was inactive in inducing wheal (Foreman *et al.*, 1983). The common C-terminal of the tachykinins would suggest that they

should all induce a plasma extravasation response and this was found to be the case.

The rank order of potency of the tachykinins in inducing plasma extravasation was SP ≥ Phy > Ele ≥ Kas > NKB ≥ NKA. Previously, binding studies have demonstrated rank orders of potency of agonists to inhibit radiolabelled-SP binding in brain tissues of:

SP ≥ Phy ≫ NKA = Kas = Ele > NKB (Buck & Burcher, 1986)

SP > Phy > NKA ≥ NKB ≥ Ele ≥ Kas (Lee *et al.*, 1986)

SP > Phy > NKA > Ele > Kas > NKB (Quirion, 1985).

These orders of potency have been used to classify receptors of the NK-1 type where SP is the most potent agonist. On this basis, it could be suggested that plasma extravasation in rat footpad skin is mediated by an NK-1 receptor.

Studies *in vitro* have demonstrated release of histamine from mast cells by SP and its N-terminal analogues (Devillier *et al.*, 1985) but not with NKA or NKB (Devillier *et al.*, 1986). Thus it is possible that the greater potency of SP over NKA and NKB is due to mast cell-derived histamine. However, the C-terminal dependence of the plasma extravasation in this study suggests that histamine is not involved in the response and antihistamine treatment did not affect the plasma extravasation induced by SP in this model (Andrews & Helme, 1987).

More recent studies from our laboratory have examined SP-induced plasma extravasation in more detail with 10 min perfusate collections (Khalil & Helme, 1989). It was observed that there is histamine-independent plasma extravasation early in the stimulatory period and histamine is involved only in the later stages. Thus a significant proportion of the SP-induced plasma extravasation measured in the current study is histamine-independent and the potency difference between SP, NKA and NKB is due to neurokinin receptor activation. To date, the effect of other mast cell mediators on the response has not been examined in this model.

The order of potency observed in the present study contrasts with two previously published studies (Gamse & Saria, 1985; Couture & Kerouac, 1987) which demonstrated an order of potency NKB > SP > NKA to intradermal injections in rat skin suggesting NK-3 receptor-mediation of the response. Another study (Devillier *et al.*, 1986) observed a rank order of potency of SP = NKA > NKB in rat skin and SP > NKA > NKB in human skin which is more equivalent to the results of the current study.

Solubility was not found to be a factor affecting the different potencies of NKB. A 1:1 solution of sulpholane in water dissolved NKB for *in vitro* experiments (D'Orléans-Juste *et al.*, 1985) however sulpholane induced plasma extravasation from the blister base at this concentration. A 1:30 solution had no activity of its own and, while increasing the solubility of NKB, had no effect on plasma extravasation. NKB dissolved in 12.5 N acetic acid or 0.1% trifluoroacetic acid has the same effect as NKB dissolved in water (Couture & Kerouac, 1987).

Use of selective agonists to differentiate receptors

In the current study, the receptor type mediating plasma extravasation was investigated further using specific neurokinin receptor agonists, the D-Pro and L-Pro isomers of [Glp^6, Pro^9]SP$_{6-11}$ (Lee *et al.*, 1986) which have different D-Pro/L-Pro ratios of their EC_{50}s on the different neurokinin receptors. The ratios on NK-1, NK-2 and NK-3 receptors are about 100, 0.001 and 0.1 respectively. The [Glp^6, Pro^9]SP$_{6-11}$ analogues were used to ascertain which neurokinin receptor mediated inositol phospholipid hydrolysis in hamster bladder (Bristow *et al.*, 1987) and with smooth muscle contraction of the guinea-pig ileum, rat vas deferens and rat portal vein (Iversen *et al.*, 1987).

In the current study the D-Pro/L-Pro ratio of [Glp^6, Pro^9]SP$_{6-11}$ could not be determined as it was not possible to produce a full dose-response curve for the D-Pro isomer due to the high concentrations of the peptide that would have been required. However, the L-Pro isomer was significantly more potent than the D-Pro isomer in inducing plasma extravasation in rat skin. This is highly suggestive that an NK-1 type neurokinin receptor mediates the response in rat footpad vessels. As there are no selective tachykinin antagonists available this is the most conclusive evidence that can be produced to distinguish receptor types. The Gamse & Saria (1985) and Couture & Kerouac (1987) studies did not use selective agonists and thus lack this confirmatory evidence. However, the reason for the different findings remain unproven.

It should also be noted that the major critical difference in receptor differentiation between the studies is the greater potency of NKB over SP. Although NKB has been measured in the spinal cord of rats, its concentrations in the spinal roots and dorsal root ganglia are 35–40 times less than SP (Ogawa *et al.*, 1985). NKB has not yet been demonstrated in nerve fibres in the periphery and consequently it would seem to be unlikely that it has a major role in the peripheral function of the primary afferent nerves. Taken together with the present results, which are suggestive of an NK-1-mediated

response, the involvement of an NK-3 receptor in the plasma extravasation response in rat footpad skin is therefore doubtful.

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Sodium salicylate facilitates calcium-dependent release of transmitter at mouse neuromuscular junctions

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1 The effects of sodium salicylate on the frequency of miniature endplate potentials (m.e.p.ps) and on the quantal content of endplate potentials (e.p.ps) in mouse diaphragm muscles at 36°C and 24°C were studied by conventional microelectrode techniques.

2 At 36°C, salicylate (10 mM) elevated the frequency of m.e.p.ps in a manner which was insensitive to $[Mg^{2+}]_o$ and independent of the presence of $[Ca^{2+}]_o$. Lowering the temperature to 24°C abolished the stimulating effect. 2,4-Dinitrophenol (10 μM) had similar effects at 36°C and these disappeared at 24°C. All subsequent experiments were performed at 24°C.

3 Salicylate (10 mM) further increased the frequency (F) of m.e.p.ps stimulated by $[Ca^{2+}]_o$ in a depolarizing solution. When $[Ca^{2+}]_o$ was varied in the absence of salicylate, a linear relationship between ln(F) and ln($[Ca^{2+}]_o$) was obtained. Salicylate shifted this relationship to the left, with respect to the control, without altering the slope.

4 Salicylate (5 mM) also increased the quantal content (m) of e.p.ps in a solution that contained 5 mM Mg^{2+}, in a concentration-dependent fashion. As $[Ca^{2+}]_o$ was varied in the absence of salicylate, a linear relationship between ln(m) and ln($[Ca^{2+}]_o$) was observed. Salicylate shifted this linear relationship to the left, with respect to the control, without altering the slope. Doubling the concentration of $[Mg^{2+}]_o$ antagonized this effect of salicylate on the quantal content of e.p.ps.

5 These results indicate that salicylate enhances the effect of changing $[Ca^{2+}]_o$. Salicylate probably facilitates the entry of Ca^{2+} into the nerve terminal or sensitizes the process that is regulated by Ca^{2+}, thereby stimulating the release of transmitter. Surface negative charges may have an important role in the effect of $[Ca^{2+}]_o$.

Introduction

$[Ca^{2+}]_o$ determines the frequency of miniature endplate potentials (m.e.p.ps) in depolarizing media (Matthews & Wickelgren, 1977) and the quantal content of e.p.ps (Katz & Miledi, 1965). Such a dependence on $[Ca^{2+}]_o$ probably implies an influx of Ca^{2+} into the nerve ending, and $[Ca^{2+}]_i$ appears to trigger the mechanism for the release of the transmitter (Katz & Miledi, 1967). Before the influx of Ca^{2+}, binding of Ca^{2+} to the surface membrane is an initial and essential step for a variety of cells (Hagiwara & Takahashi, 1967; Sanborn & Langer, 1970; Chevallier & Butow, 1971; Brading & Widdicombe, 1977). Negative charges on the outer surface of most animal cells bind Ca^{2+} ions with low affinity (Hagiwara & Takahashi, 1967; Shlatz & Marinetti, 1972; Bosmann *et al.*, 1972; Jaques *et al.*, 1977). Such negative charges on nerve endings have been postulated to provide a functional site of storage which supplies Ca^{2+} ions to increase $[Ca^{2+}]_i$, thereby causing the release of transmitter (Nishimura *et al.*, 1984). Alternatively, it has been suggested that divalent cations, including Ca^{2+}, neutralize the fixed

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negative surface charges on the outer face of the nerve terminals and reduce the release of transmitter (Madden & van der Kloot, 1978).

It has been shown that sodium salicylate binds to and increases the surface negative potential of phospholipid bilayers (McLaughlin, 1973). This effect has been used to analyse various membrane-related events (Levitan & Barker, 1972; Blood, 1977; Cohen *et al.*, 1979; Spedding, 1984). If such an effect of this agent occurs on the nerve terminal, then salicylate might modify the effect of $[Ca^{2+}]_0$ on the release of transmitter. Experiments have been carried out to test this possibility and the results reveal an enhancement of the effect of $[Ca^{2+}]_0$ on both the asynchronous and phasic modes of release of transmitter quanta in the presence of salicylate.

Methods

Experiments were performed on preparations of isolated left hemidiaphragm from male ddY mice of 11 to 20 weeks of age. The preparation was pinned to a silicone resin which lined the bottom of a plastic chamber of about 30 ml capacity, and was soaked in Krebs-Ringer solution. The solution was constantly recirculated by means of an 'oxygen lift' system. The circulating solution had the following composition (mM): NaCl 136, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, $NaHCO_3$ 15, glucose 11. A Ca^{2+}-deficient solution was prepared with addition of 2 mM EGTA (ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid). To depolarize the presynaptic endings (Liley, 1956), the preparation was soaked in Krebs-Ringer solution in which 10 mM NaCl was replaced by 10 mM KCl (10 mM K^+). The preparation was equilibrated in the 10 mM K^+ solution for at least 30 min before the addition of any agent. Bathing solutions which contained 5 mM Mg^{2+} or 10 mM Mg^{2+} were also prepared, and these solutions contained between 0.2 and 1.0 mM Ca^{2+}, for measurement of e.p.ps. The bathing solution was bubbled with a mixture of 95% O_2 and 5% CO_2 and maintained at pH 7.3 and at 36°C or 24°C. The temperature of the solution in the bath was monitored by a thermister (Shibaura Electric Co, Model MGA-II) and held constant by means of an external water jacket and a thermoregulatory device (Taiyo, Thermominder Mini 80) during each experiment.

Intracellular recordings were made with glass microcapillary electrodes, filled with 3 M KCl, with resistance of 6 to 8 megohms. The electrode was inserted into fibres near endplate regions. The signals were led through a high-impedance, unity-gain preamplifier (Nihon Kohden, Tokyo, MEZ-8201), displayed on an oscilloscope (Nihon Kohden, VC-10) and stored on an FM instrumentation tape recorder (Nihon Kohden, RMG-5204).

M.e.p.ps of 0.1 mV or larger amplitude were counted by a computer (Nihon Kohden, DAB-1100). M.e.p.ps were recorded for successive periods of 1 min after exposure to a given solution; from these data the mean frequency of m.e.p.ps (s^{-1}) was calculated. E.p.ps were evoked by stimulation of the phrenic nerve with 256 square pulses of 0.1 ms duration and supramaximal voltage at 1 Hz by use of a suction electrode in a bathing solution with low concentrations of Ca^{2+} (0.2 to 1.0 mM). The quantal content (m) was estimated by the method of failures from

$$m = \log_e (N/N_0),$$

where N = number of trials and N_0 = number of failures (Crawford, 1974). Student's *t* tests were used for statistical analyses and a probability of less than 0.05 was deemed statistically significant.

Drugs used: sodium salicylate (Wako, Osaka), ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (Sigma, St. Louis, MO). All other chemicals were of analytical grade.

Results

Effect of decreasing temperature

The first series of experiments examined the effects of temperature on the asynchronous release of transmitter at the mouse neuromuscular junction in the presence and absence of salicylate. The results are summarized in Figure 1. At 36°C, 10 mM salicylate elevated the frequency of m.e.p.ps. This effect started from 10 to 15 min after incubation with salicylate. When sodium chloride (10 mM) was added hypertonically to the same bathing solution, no such stimulant effect was observed. The stimulant effect was observed at concentrations of salicylate of 1 mM or higher, and the extent of stimulation varied in a concentration-dependent manner. Raising the concentration of Mg^{2+} from 1 mM to 5 mM did not reduce this effect. Ca^{2+}-free bathing solution, containing 2 mM EGTA, slightly lowered the frequency of m.e.p.ps in the absence of salicylate, but it failed to block the stimulant effect of this agent. However, lowering the temperature to 24°C largely abolished the stimulant effect irrespective of $[Ca^{2+}]_0$. Thus, these results indicate that, at normal physiological temperature, salicylate has the intrinsic ability to stimulate asynchronous release of transmitter. This

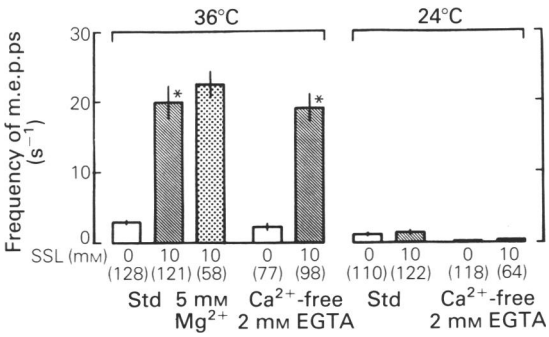

Figure 1 Effects of sodium salicylate (SSL) on the frequency of miniature endplate potentials (m.e.p.ps) at the mouse neuromuscular junction in bathing solutions with different compositions and the effect of lowering the temperature to 24°C. At 36°C, all experiments were performed on 13 preparations each, and at 24°C, 12 preparations each. M.e.p.ps were measured at several junctions for 10 min from 30 min after setting up the preparations in the control solutions. Salicylate was added at the end of the control measurement. Measurements of m.e.p.ps were started 20 min after the treatment and continued for 30 min. Then, Mg^{2+} (4 mM) was given only to those preparations incubated with salicylate in the standard bathing solution at 36°C. M.e.p.ps were measured for 15 min from 5 min after the addition of Mg^{2+}. Ordinate scale: frequency (s^{-1}) of m.e.p.ps. Std: Krebs-Ringer solution with standard composition. 5 mM Mg: standard bathing solution plus 4 mM Mg^{2+}. Ca-free 2 mM EGTA: Ca-free bathing solution containing 2 mM EGTA. * $P < 0.01$. Number of endplates tested are indicated in parentheses.

effect may mask any possible modification, by salicylate, of the effect of $[Ca^{2+}]_0$ on the release of transmitter. Thus, all subsequent experiments were performed at 24°C.

In the other experiments, similar stimulant effects of 2,4-dinitrophenol (10 μM) were observed, at 36°C, on the frequency of m.e.p.ps in the standard bathing solution ($4.98 \pm 0.22\,s^{-1}$, $n = 137$ endplates of 11 muscles exposed to this agent for 30 to 90 min, control; $3.13 \pm 0.09\,s^{-1}$, $n = 256$ endplates of 11 preparations) and also in the Ca^{2+}-free bathing solution ($2.47 \pm 0.09\,s^{-1}$, $n = 280$ endplates of 8 muscles exposed to this agent for 30 to 90 min, control; $1.01 \pm 0.03\,s^{-1}$, $n = 288$ endplates of 16 preparations). Burst-like discharges of potential changes of endplate were frequently observed in the preparations exposed to 2,4-dinitrophenol, but these were not included in the results. At higher concentrations (50 μM or higher), this agent frequently caused muscle contracture at 36°C which dislodged the electrode from endplates and made continuous recording of the potential changes impossible. All of these effects disappeared at 24°C within at least 90 min of incubation at this temperature.

Experiments at 24°C

The effect of $[Ca^{2+}]_0$ on the spontaneous release of transmitter was examined by measuring the frequency of m.e.p.ps in the presence and absence of salicylate at 24°C (Figure 2a). M.e.p.ps were measured at several endplates for about 15 min in the Ca^{2+}-free, 10 mM K^+ bathing solution. The concentration of Ca^{2+} was then increased to 0.2, 0.4 and 0.8 mM by addition of calcium chloride to this solution. M.e.p.ps were measured at 5 endplates for each muscle, 5 min after the addition of Ca^{2+}. In cases of pretreatment with 10 mM salicylate, each addition of Ca^{2+} was made 25 min after the addition of salicylate. Each value in the presence of Ca^{2+}, but in the absence of salicylate, was corrected for the mean value of the frequency of m.e.p.ps ($0.35 \pm 0.04\,s^{-1}$, $n = 35$ endplates of 7 muscles) measured in Ca^{2+}-free, 10 mM K^+ bathing solution, because m.e.p.ps were detectable independently of $[Ca^{2+}]_0$. In experiments that involved pretreatment with salicylate, m.e.p.ps were measured at 5 endplates in each muscle for 15 min, 10 min after the addition of 10 mM salicylate to the Ca^{2+}-free, 10 mM K^+ bathing solution. From these data, a mean value of the frequency of m.e.p.ps in the presence of salicylate and with $[Ca^{2+}]_0$ equal to zero ($0.71 \pm 0.07\,s^{-1}$, $n = 35$ endplates of 7 muscles) was calculated. This value was used to correct for m.e.p.ps that occurred in the presence of salicylate and increasing concentrations of Ca^{2+}. Addition of Ca^{2+} increased the frequency of m.e.p.ps in a concentration-dependent manner. This effect was potentiated by the presence of salicylate but not by the addition of sodium chloride (10 mM).

E.p.ps were elicited in low concentrations of Ca^{2+} (0.2 to 0.8 mM) and with a high concentration of Mg^{2+} (5 mM) in a bathing solution that contained 0 mM or 5 mM salicylate (Figure 3a). Measurement of e.p.ps in the presence of salicylate was begun 10 min after the addition of salicylate to solutions that contained increasing concentrations of Ca^{2+} and was continued for a further 20 min at 5 endplates in each preparation. The quantal content (m) of e.p.ps, as estimated by the method of failures (see Methods), increased as $[Ca^{2+}]_0$ rose. This effect of $[Ca^{2+}]_0$ was augmented in the presence of 5 mM salicylate. Such a facilitating effect was dependent on the concentration of salicylate when the quantal content of e.p.ps was measured at $[Ca^{2+}]_0 = 0.4$ or 0.6 mM (Figure 4). Concentrations of 1.25 mM salicylate or higher were needed to elicit this effect and such an effect was more potent in the presence of higher $[Ca^{2+}]_0$.

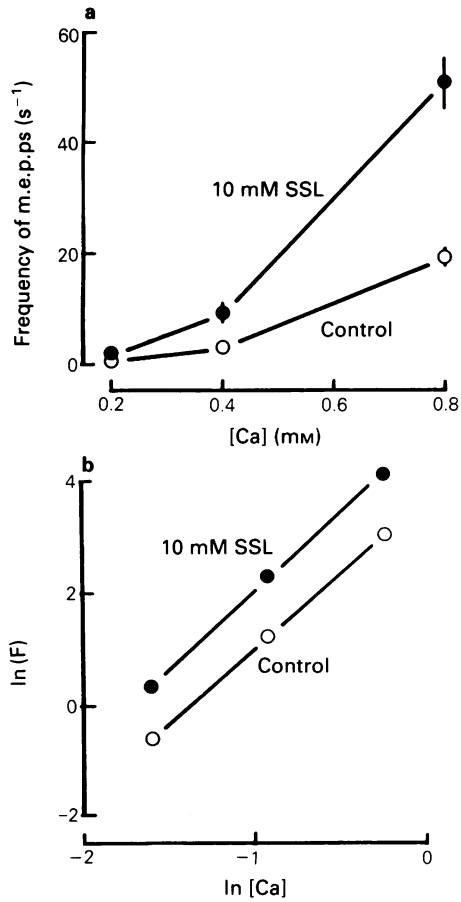

Figure 2 (a) Effect of sodium salicylate (SSL, 10 mM) on the frequency of miniature endplate potentials (m.e.p.ps) at the mouse neuromuscular junction stimulated by Ca^{2+} in a depolarizing bathing solution at 24°C. Ordinate: frequency (s^{-1}) of m.e.p.ps. Abscissa scale: concentration (mM) of external Ca^{2+}. Each value has been corrected for the mean value of the frequency of m.e.p.ps ($0.35 \pm 0.04\,s^{-1}$, $n = 35$ endplates of 7 muscles and $0.71 \pm 0.07\,s^{-1}$, 35 endplates of 7 muscles) in a Ca^{2+}-free, 10 mM K^+ bathing solution (in the absence and of presence of 10 mM salicylate, respectively). Vertical lines show s.e.mean. (b) Double logarithmic plot of averaged frequency of me.p.ps and variations in $[Ca^{2+}]_0$ using the data depicted in (a). Ordinate scale: frequency (F, s^{-1}) of m.e.p.ps as ln(F). Abscissa scale: concentration (mM) of external Ca^{2+} as $\ln([Ca^{2+}]_0)$. Slopes of both lines are 2.8.

To determine whether salicylate exerts its effect on the rate of release of transmitter by accelerating the action of external Ca^{2+} ions, the effects of variations in $[Ca^{2+}]_0$ on the frequency of m.e.p.ps and on the quantal content of e.p.ps were examined in the presence and absence of salicylate. If we assume that Ca^{2+} binds to a site (X) and facilitates the release of transmitter by formation of an active moiety CaX:

$$Ca^{2+} + X \overset{K_{Ca^{2+}}}{\rightleftharpoons} CaX \text{ (active)}$$

with a dissociation constant $K_{Ca^{2+}}$, and if Mg^{2+} competitively reduces the rate of release of transmitter by binding to X:

$$Mg^{2+} + X \overset{K_{Mg^{2+}}}{\rightleftharpoons} MgX \text{ (inactive)}$$

then, the frequency (F) of m.e.p.ps in a solution that contains a high concentration of K^+, as well as the quantal content (m) of e.p.ps, both of which are dependent on $[Ca^{2+}]_0$, is given as:

$$F = K[CaX]^n = K\left(\frac{[Ca^{2+}]_0}{1 + \frac{[Ca^{2+}]_0}{K_{Ca^{2+}}} + \frac{[Mg^{2+}]_0}{K_{Mg^{2+}}}}\right)^n$$

where K is a proportionality constant and n is an integer (Jenkinson, 1957; Dodge & Rahamimoff, 1967). Figure 2b shows the curves which relate ln(F) to $\ln([Ca^{2+}]_0)$ in the presence of 0 mM and 10 mM salicylate; constructed using the data depicted in Figure 2a. In the absence of salicylate, the relationship between ln(F) and $\ln([Ca^{2+}]_0)$ is linear with a slope of 2.8. The only effect of salicylate is to shift the curve to the left (towards lower values of $\ln[Ca^{2+}]_0$), suggesting that salicylate accelerates the action of $[Ca^{2+}]_0$ at some site(s) during the release of transmitter.

From the data used to generate Figure 3a, ln(m) was plotted against $\ln([Ca^{2+}]_0)$ (Cooke *et al.*, 1973) in the presence and absence of 5 mM salicylate (Figure 3b). The relationship between ln(m) and $\ln([Ca^{2+}]_0)$ is linear with a slope of 3.4 in the presence or absence of salicylate. The curve in the presence of salicylate is shifted to the left as was also observed in the case of the frequency of m.e.p.ps. Figures 3a and b show the effect of doubling $[Mg^{2+}]_0$ from 5 mM to 10 mM on the quantal content of e.p.ps in the presence and absence of salicylate. The doubling of $[Mg^{2+}]_0$ reduced the quantal content of e.p.ps irrespective of the presence or absence of salicylate, indicating that the effect of $[Ca^{2+}]_0$ is sensitive to Mg^{2+}.

Discussion

Sodium salicylate increased asynchronous release of transmitter at 36°C, but not at 24°C. This stimulant

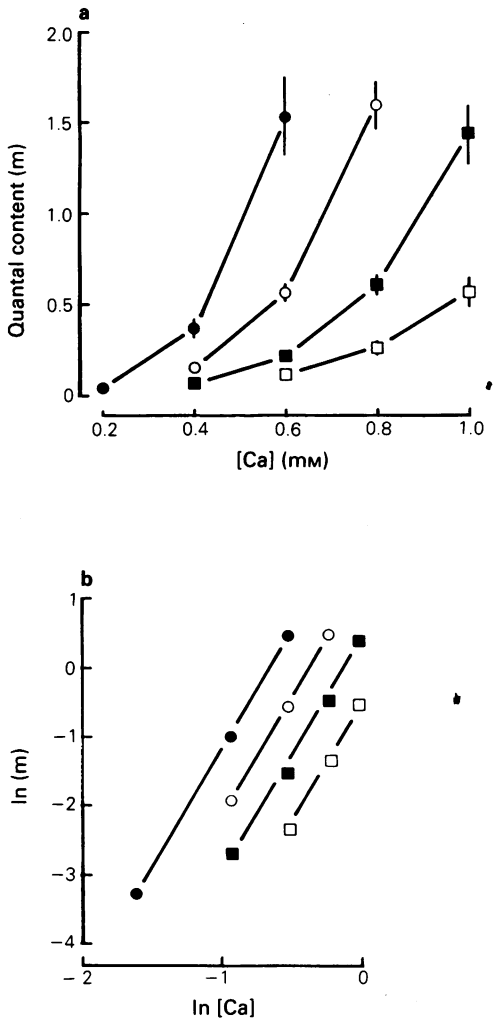

Figure 3 (a) Effect of 5 mM sodium salicylate on the quantal content (m) of endplate potentials (e.p.ps) at the mouse neuromuscular junction in a 5 mM Mg^{2+} (●) or 10 mM Mg^{2+} (■) bathing solution containing different concentrations of external Ca^{2+}. Measurement of e.p.ps started 10 min after the addition of salicylate and was continued for the next 20 min at 5 endplates in each preparation. (○, □) Control measurements in 5 and 10 mM Mg^{2+}, respectively, in the absence of salicylate. Ordinate scale: quantal content (m) of e.p.ps. Abscissa scale: concentration (mM) of external Ca^{2+}. Vertical lines show s.e.mean of 35 endplates of 7 muscles (5 endplates in each preparation). (b) Double logarithmic plot of averaged quantal content of e.p.ps and concentration of Ca^{2+} in mM using the data depicted in (a). Ordinate scale: averaged quantal content (m) of e.p.ps as ln(m). Abscissa scale: concentration (mM) of external Ca^{2+} as $\ln([Ca^{2+}]_0)$. Slopes of both lines are 3.4. Symbols used are the same as in (a).

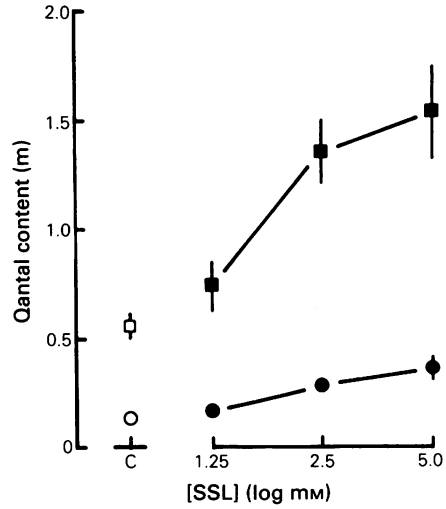

Figure 4 Concentration-response curves demonstrating the potentiating action of sodium salicylate on the effect of $[Ca^{2+}]_0$ on the quantal content of endplate potentials (e.p.ps) at the mouse neuromuscular junction in a 5 mM Mg^{2+} bathing solution that contained 0.4 (●) or 0.6 (■) mM Ca^{2+}. Ordinate scale: quantal content (m) of e.p.ps. Abscissa scale: log mM concentration of sodium salicylate (SSL). Vertical lines show s.e.mean of 20 endplates of 4 muscles (5 endplates in each muscle) in the case of 1.25 and 2.5 mM salicylate, and of 35 endplates of 7 muscles in the case of 5 mM salicylate. (○, □) Control responses in 0.4 and 0.6 mM Ca^{2+}, respectively, in the abseence of salicylate.

effect was insensitive to both $[Ca^{2+}]_0$ and $[Mg^{2+}]_0$. These characteristics suggest that, at 36°C, this agent might affect mitochondria, as salicylates have been shown to uncouple oxidative phosphorylation (Brody, 1956). This possibility was supported by the finding that another well known uncoupler of oxidative phosphorylation, 2,4-dinitrophenol (Brody, 1956), mimicked such effects. At a concentration of 10 μM, 2,4-dinitrophenol significantly increased the asynchronous release of transmitter at 36°C, while the effect was blocked at 24°C, in the presence or absence of $[Ca^{2+}]_0$.

The present experiments indicate that salicylate accelerates the rate of both asynchronous and phasic release of transmitter quanta in a concentration-dependent manner. Salicylate did not alter the power relationship between the frequency (F) or quantal content (m) and $[Ca^{2+}]_0$ but did shift the relationship towards lower concentrations of $[Ca^{2+}]_0$. This suggests that salicylate facilitates the Ca^{2+}-dependent release of transmitter but does not alter the characteristics of the release, as shown by the parallel shifting of curves.

With respect to the mechanism by which the rate of release of quanta of transmitter is increased, the following effects of salicylate are possible: (1) it may accelerate the entry of Ca^{2+} into the presynaptic nerve terminals via voltage-dependent channels; (2) it may potentiate the action of Ca^{2+} ions at the sites where vesicles are released (Cooke *et al.*, 1973); (3) it may reduce the neutralization of intrinsic negative surface charges that attract Ca^{2+} to the external surface of the nerve terminal membrane (Misler & Hurlbut, 1980); or (4) it may be involved in the sensitization of the process that is stimulated by Ca^{2+}. It is known that Mg^{2+} ions block the release of transmitter by diminishing the access by Ca^{2+} to receptors located on the outer side of the presynaptic nerve terminal and, subsequently, the ability of Ca^{2+} to traverse the membrane channels (Jenkinson, 1957). Our results indicate that an elevation of $[Mg^{2+}]_0$ shifted the relationship between quantal content (m) and $[Ca^{2+}]_0$ in the presence or absence of salicylate toward lower values of $[Ca^{2+}]_0$. Thus, it is likely that salicylate facilitates the entry of Ca^{2+} into the presynaptic nerve terminals via a Mg^{2+}-sensitive mechanism. The possibility that salicylate may sensitize the process that is regulated by $[Ca^{2+}]_i$, thereby stimulating the release of transmitter, remains to be investigated.

The dependence of the release of transmitter on $[Ca^{2+}]_0$ implies an influx of Ca^{2+} into the nerve ending, and $[Ca^{2+}]_i$ appears to be the trigger for the mechanism involved in the release of transmitter (Katz & Miledi, 1967). Before the influx of Ca^{2+}, binding of Ca^{2+} to the surface membrane is an initial and essential step in a variety of cells (Hagiwara & Takahashi, 1967; Sanborn & Langer, 1970; Chevallier & Butow, 1971; Brading & Widdicombe, 1977). The outer surfaces of most animal cells are negatively charged (Van der Kloot, 1978). These negative charges bind Ca^{2+} ions with low affinity (Hagiwara & Takahashi, 1967; Shlatz & Marinetti, 1972; Bosmann *et al.*, 1972; Jacques *et al.*, 1977). A site Y has been proposed by Cooke *et al.* (1973), as the site for binding Ca^{2+} on the nerve terminal membrane for the transportation of the Ca^{2+} ion to a site X which regulates the release of transmitter. It has been postulated that negative charges on the nerve ending provide a functional storage site which supplies Ca^{2+} ions to the interior of the cell to cause the release of transmitter (Nishimura *et al.*, 1984). It has been shown that salicylate binds to and increases the negative surface potential of phospholipid bilayers (McLaughlin, 1973). This effect has been confirmed in various preparations (Levitan & Barker, 1972; Blood, 1977; Cohen *et al.*, 1979; Spedding, 1984). Such an increase in the negative surface potential might permit the facilitation of the entry of Ca^{2+} mentioned above. The neutralization of negative charges by the weak binding of Ca^{2+} ions at sites near to calcium channels on the surface membrane would be convenient for the synchronous transport of these ions into the nerve terminal. However, if very large numbers of Ca^{2+} ions screen the negative charges on the surface of the outer face of the nerve terminal, these Ca^{2+} ions might interfere with the influx of Ca^{2+}, thereby reducing the release of transmitter (Madden & van der Kloot, 1978).

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The binding of (+)-isradipine by guinea-pig intact atria

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1 The accumulation of [^{3}H]-(+)-isradipine (PN 200-110) was measured in quiescent guinea-pig left atria with normal (K^+ 2.7 mM) or lowered (K^+ 40 mM) membrane potential.

2 Under control conditions (2.7 mM K^+) a high affinity binding of (+)-isradipine could not be detected. If, however, the atria were partially depolarized to about −30 mV by 40 mM K^+, high affinity binding became evident displaying a dissociation constant of 4.2×10^{-11} M and a capacity of 9.7 nmol kg^{-1} wet wt.

3 The depolarization-induced binding was reversible upon repolarization of the atria although isradipine was still present in the medium. This indicates that the high affinity binding sites disappear as soon as the cell membranes become polarized.

4 Isradipine belongs to the less hydrophobic dihydropyridines, but nevertheless the unsaturable binding led to an accumulation of about 84 fold. At a concentration of 2×10^{-8} M (+)-isradipine, which reduces the contractile force by 50%, the cellular concentration will rise to more than 10^{-6} M.

Introduction

It is generally believed that Ca antagonists of the dihydropyridine type exert their pharmacological effects via binding to a specific binding site, the so-called dihydropyridine receptor localized within the Ca-channel macromolecules (see Tanabe *et al.*, 1987). The affinity of dihydropyridines, e.g. (±)-nitrendipine, has been shown to lie in the subnanomolar range. The presence of these high affinity binding sites has been well documented in homogenates of a variety of tissues, although only smooth muscles show a pharmacological response in this low concentration range. In spite of the presence of high affinity binding sites in membrane preparations of heart muscle, the pharmacological effects, that is depression of contractile force, can only be evoked by nearly micromolar concentrations of nitrendipine, nifedipine and others. This raises the question whether or not high affinity binding sites can be detected in intact cardiac tissue with polarized plasma membranes. Furthermore, it was intended to elucidate whether or not depolarization (by high K^+) increases high affinity binding, as previously shown in freshly dissected myocytes by Green *et al.* (1985) and in cultured embryonic chick heart cells by Lee *et al.* (1987). An augmentation of high affinity binding was also demonstrated by Kokubun *et al.* (1986), Kamp & Miller (1987), and by Kamp *et al.* (1988) on lowering the membrane potential from a partially depolarized state to a nearly fully depolarized state.

As previously shown by Lüllmann & Mohr (1987), high affinity binding of [^{3}H]-(±)-nitrendipine could not be detected in intact beating atria of guinea-pigs (4.5 mM K^+, beat frequency 3 Hz). Since 3 transient depolarizations per second seemed to be insufficient to induce a measurable high affinity binding of (±)-nitrendipine, a sustained partial depolarization was imposed on quiescent atria by raising the potassium concentration to 40 mM. The increment in nitrendipine binding was only small due to the high degree of unsaturable binding and a statistically significant increase was hard to obtain. We, therefore, chose a dihydropyridine derivative with more favourable properties: lower lipid solubility and hence reduced unsaturable binding, and the availability of a labelled enantiomer, i.e. [^{3}H]-(+)-isradipine. In this paper we describe the binding of (+)-isradipine to isolated resting left atria of guinea-pigs with polarized membranes (control conditions) and partially depolarized membranes (40 mM K^+).

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Methods

Left atria, dissected from the hearts of guinea-pigs of either sex (weight ~300 g), were suspended in organ baths containing 500 ml bathing solution. The atria were placed between stimulation electrodes made from platinum wire and were driven at a frequency of 2.5 Hz (rectangular pulse, 5 ms duration, intensity 30% above threshold). Eight to twelve atria could be positioned at any one time within one organ bath. The [^{3}H]-(+)-isradipine binding experiments were carried out in Tyrode solution of the following composition (in mM): NaCl 136.8, KCl 2.7, $CaCl_2$ 1.8, $NaHCO_3$ 11.9, $MgCl_2$ 1.05, NaH_2PO_4 0.21, glucose 5.5; this solution was gassed with carbogen (95% O_2, 5% CO_2), yielding a pH of 7.3. The temperature was kept at 32°C.

After 1 h of adaptation with electrical stimulation, the atria were transferred to organ baths, containing a solution with normal K^+ or elevated (40 mM) K^+ (NaCl iso-osmotically replaced by KCl). Electrical stimulation was no longer imposed on the preparations because the high K^+ concentration of the experimental group prohibited excitation and thus contractions, since the membrane potential was lowered to about −30 mV as determined by a conventional microelectrode technique. [^{3}H]-(+)-isradipine was added 10 min later, in some instances together with an amount of unlabelled dihydropyridine. After different times of incubation, atria were removed from the bath. The muscle pieces were gently blotted, weighed, and dissolved in 2 ml of Soluene 250 (Packard Instr., U.S.A.). After addition of 10 ml of Dimilume 30 (Packard Instr.) the radioactivity was counted by a liquid scintillation counter (Packard Tricarb 460) at an efficiency of about 35%. The data are usually presented as the ratio between the accumulated tritiated dihydropyridine per g tissue (wet wt.), divided by tritium concentration per ml of incubation medium (tissue/medium ratio, T/M ratio).

Even if the atria were incubated in isradipine-containing Tyrode solution for 5 h equilibrium was not completely attained. It was not possible to prolong the experimental period (1 h adaptation plus 5 h incubation) because of the viability of the atria. Therefore, in order to obtain estimates of the equilibrium (T/M values) a non-linear regression analysis according to Marquardt (1963) was applied. This procedure has already been used for the analysis of nitrendipine binding data in a preceding paper (Lüllmann & Mohr, 1987).

Since dihydropyridine derivatives are light-sensitive, all experiments were done in the presence of weak sodium-light. [^{3}H]-(+)-isradipine (specific activity 72.6 Ci mmol^{-1}) was purchased from New England Nuclear (F.R.G.). The enantiomers of unlabelled isradipine were a generous gift from Sandoz, Switzerland.

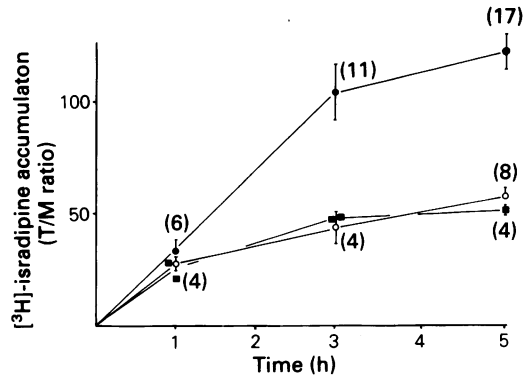

Figure 1 Time course of accumulation of 10^{-10} M [^{3}H]-(+)-isradipine in quiescent left atria suspended in 2.7 mM K^+ (○), 40 mM K^+ (●) and high K^+ plus 3×10^{-7} M nitrendipine (■) containing Tyrode solution. Values denote means of number of atria given in parentheses. For the third group, single values are depicted for 1 and 3 h. Vertical lines show s.e.mean.

Results

Binding of [^{3}*H*]-(+)-*isradipine*

Typical results obtained with one particular concentration of [^{3}H]-(+)-isradipine are depicted in Figure 1: atria partially depolarized by 40 mM K^+ accumulated isradipine within 5 h to about 120 fold but had not attained the final equilibrium at this time. To check whether or not a specific and saturable component was involved in the accumulation curve the dihydropyridine derivative nitrendipine (3×10^{-7} M) was added in a parallel experiment. As clearly shown in Figure 1 the T/M ratio hardly attained a value of 50 thus indicating a saturable component. The difference between the total accumulation and the non-specific component yields an estimate of the amount of [^{3}H]-(+)-isradipine specifically bound. Figure 1 also demonstrates that atria kept in normal Tyrode solution (2.7 mM K^+) accumulate [^{3}H]-(+)-isradipine to the same extent as was found for the non-specific accumulation (at 40 mM K^+). This suggests that atrial muscles with polarized membranes do not exhibit saturable and specific binding ability.

To obtain information on the concentration-dependency of the isradipine accumulation, similar experiments were performed with different concentrations. As shown in Figure 2 the accumulation of [^{3}H]-(+)-isradipine by partially depolarized atria exceeded considerably the accumulation by polarized atria at concentrations up to 3×10^{-10} M.

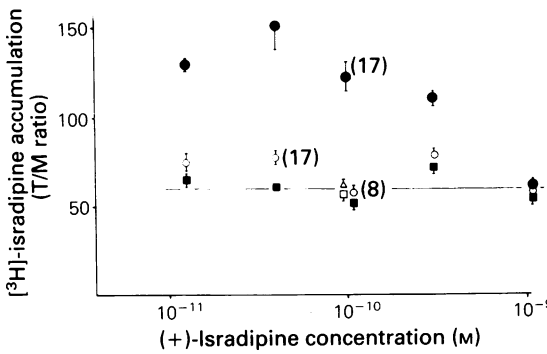

Figure 2 Concentration-dependent accumulation of [^{3}H]-(+)-isradipine after 5 h under various conditions: (○) polarized muscles (2.7 mM K^+); (●) partially depolarized muscle (40 mM K^+); (■) partially depolarized muscles (40 mM K^+) plus 3×10^{-7} M (±)-nitrendipine; (□) polarized muscles (2.7 mM K^+), control to (△) plus 10^{-6} M nifedipine. The horizontal line represents the mean of all values obtained in partially depolarized muscles in the presence of 3×10^{-7} M (±)-nitrendipine (■). Vertical lines show s.e.mean.

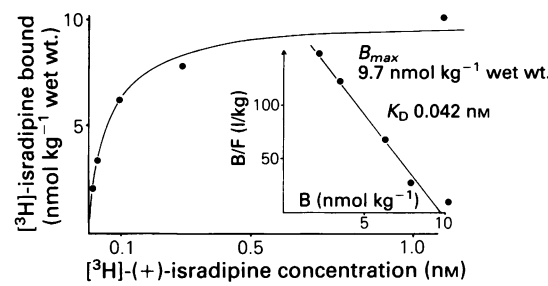

Figure 3 Concentration-dependence of saturable equilibrium binding of [^{3}H]-(+)-isradipine to partially depolarized atria (K^+ 40 mM). The points represent equilibrium values which are calculated from uptake curves. For each concentration at least 8 atria are included. The non-specific binding was likewise determined, by using identical [^{3}H]-(+)-isradipine concentrations together with 3×10^{-7} M (±)-nitrendipine for each condition. The saturable binding was obtained by subtraction of the equilibrium value for non-specific binding from that of total binding. The inset depicts the Scatchard transformation of these data.

Again at 40 mM K^+, unlabelled nitrendipine was able to displace isradipine from saturable specific binding sites. Binding in the presence of excess nitrendipine matched isradipine binding obtained at 2.7 mM K^+. However, at 10^{-9} M isradipine the T/M value for partially depolarized atria had dropped to the T/M values obtained in the presence of 3×10^{-7} M nitrendipine or at 2.7 mM K^+. This again indicates that the accumulation of isradipine by depolarized muscles is saturable. In order to check whether isradipine accumulation at 2.7 mM K^+ contained a saturable component, an accumulation experiment was performed with 10^{-10} M [^{3}H]-(+)-isradipine plus 10^{-6} M nifedipine or 3×10^{-7} M nitrendipine. Figure 2 shows that no displacement could be induced by the addition of an excess of unlabelled dihydropyridine. Accordingly, saturable binding of isradipine was not detectable in polarized atria.

To characterize the saturable component of isradipine binding in partially depolarized atrial muscles more closely, the extrapolated equilibrium values obtained from accumulation curves by means of the Marquardt-procedure were used to calculate the specific binding. This is the difference between the total T/M and the T/M found in the presence of excess of nitrendipine. These 'specific T/M values' were converted into nmol isradipine per kg of tissue and plotted against the isradipine concentration (Figure 3). The resulting curve resembled an adsorption isotherm. Plotting the data according to Scatchard (see inset Figure 3) yielded a straight line indicative of binding of isradipine to a homogeneous population of non-interacting binding sites displaying a K_D of 0.04 nM and a B_{max} of 10 nmol kg^{-1} wet wt.

Reversibility of [^{3}H]-(+)-isradipine binding to partially depolarized atria

To check whether or not the binding of (+)-isradipine was reversible upon repolarization, the following experiment was done: left atria were pre-incubated in high K^+ solution containing [^{3}H]-(+)-isradipine for 3 h and then subjected to different conditions (Figure 4). When the bath solution remained unchanged, the uptake proceeded a little further within the next two hours. In contrast, when the K^+ concentration was reduced to the normal value of 2.7 mM, with [^{3}H]-(+)-isradipine still present in the incubation medium the muscles lost nearly half of their accumulated isradipine. The loss of labelled (+)-isradipine was equally pronounced when, in addition to the normalization of the K^+ concentration, the labelled compound was removed from the incubation medium. The result suggests that the K^+-induced high affinity binding of (+)-isradipine is lost upon repolarization.

Discussion

To prove a certain mode of action which involves receptor binding of a drug, one has to measure directly the drug-receptor interaction. This can be done in binding experiments by use of radiolabelled ligands. Secondly, a parallelism has to be demonstrated to exist between drug receptor binding and the pharmacological effect. In the case of dihydropy-

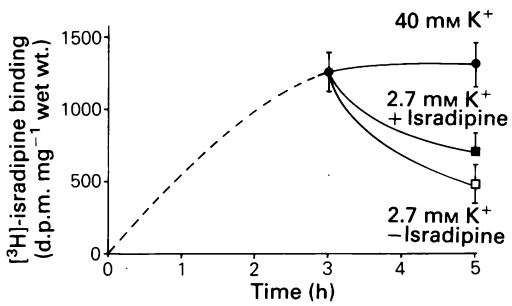

Figure 4 Loss of [^{3}H]-(+)-isradipine from atrial tissue upon repolarization. All preparations were incubated for 3 h with 10^{-10} M [^{3}H]-(+)-isradipine (40 mM K^+). Then, 5 atria were immediately sampled (●), and 5 were further incubated and removed after 5 h (●). Five atria were placed for 2 h in an organ bath containing 10^{-10} M [^{3}H]-(+)-isradipine at a K^+ concentration of 2.7 mM (■). (□) Indicates the value from 5 atria suspended for 2 h in normal K^+ (2.7 mM) solution devoid of [^{3}H]-(+)-isradipine. The latter means do not differ significantly. All values are means and vertical lines show s.e.mean.

ridines, the existence of high affinity binding sites has been firmly established, but the significance of high affinity binding sites in heart muscles for the cardiodepressive action of dihydropyridines is under debate (Marsh *et al.*, 1983; Vaghy *et al.*, 1984; Lüllmann & Mohr, 1987). There is a marked discrepancy between the half saturation constant for the high affinity binding, as determined in membrane preparations, and the concentration causing half-maximal effects, the difference amounting to factors of 100 to 1000. This result does not support the idea that binding to high affinity sites mediates the effects in heart muscle. In contrast, the only study dealing with intact beating atrial tissue failed to detect high affinity binding sites for (±)-nitrendipine (Lüllmann & Mohr, 1987). Instead, in this study and in that of Askholt & Nielsen-Kudsk (1986), the high degree of non-specific cellular accumulation of (±)-nitrendipine (125 fold) and felodipine (500 fold), respectively, became obvious. It is reasonable to assume that the high cellular concentration plays a role in the cardiodepressive action of the drugs.

In the present paper, a high affinity binding site for a dihydropyridine-derivative could be demonstrated as soon as the membranes of the intact tissues were partially depolarized by high K^+. In the case of (+)-isradipine the increment in binding was two fold in the concentration range 10^{-11} M and 10^{-10} M (Figure 2), or 10 nmol kg^{-1} wet wt. (Figure 3), whereas, in the polarized state no high affinity binding could be identified, as previously shown for (±)-nitrendipine. When high affinity binding had been established by raising the K^+ concentration to 40 mM, it was possible to remove (+)-isradipine from the intact tissue by simply repolarizing the atria by lowering the K^+ concentration to normal.

Although (+)-isradipine is less hydrophobic than (±)-nitrendipine, it accumulated to an overall cellular concentration 84 fold that of the incubation medium (average of 5 h T/M values in the presence of excess dihydropyridine, $n = 28$, corrected for 30% extracellular space to yield cell/medium ratios). Since the half-maximum inhibition of contractile force required about 2×10^{-8} M (+)-isradipine (2.5 Hz, not shown), the overall cellular concentration amounted to about 1.6×10^{-6} M under these conditions.

Although a high affinity binding site for dihydropyridines could not be demonstrated in intact tissue with normal membrane potentials, it is of course possible that, during the action potentials, the dihydropyridines bind transiently, exert their effect and then rapidly dissociate. This would formally yield a low affinity binding (Bean, 1984) which is not detectable with radioligands. However, direct proof for a receptor-mediated mode of action of Ca antagonists of the dihydropyridine type in cardiac tissue is still lacking under physiological conditions. When contemplating the mode of action of the dihydropyridines one should also be aware of the non-specific accumulation within the cardiac cells.

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Inhibition of platelet-activating factor- and zymosan-activated serum-induced chemotaxis of human neutrophils by nedocromil sodium, BN 52021 and sodium cromoglycate

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1 Inflammatory cells such as eosinophils and neutrophils are thought to contribute actively to the pathogenesis of asthma since they infiltrate into the lung tissue. These cells are mobilized by lipid-like and protein-like chemotactic factors. As illustrative examples of both groups, platelet-activating-factor (Paf) and zymosan-activated-serum (ZAS) were used in this study. The inhibitory effects of nedocromil sodium, the Paf antagonist BN 52021 and sodium cromoglycate on Paf- and ZAS-induced neutrophil chemotaxis were evaluated.

2 All tested drugs inhibited Paf-induced neutrophil chemotaxis with approximately the same potency ($IC_{50} \approx 1$ nM).

3 Nedocromil sodium and sodium cromoglycate were equally potent in inhibiting ZAS-induced neutrophil chemotaxis ($IC_{50} = 0.1$–$1\ \mu M$), whereas BN 52021 was considerably less potent ($IC_{30} = 10\ \mu M$).

4 To find out whether the drugs tested could inhibit early events in cell activation, their capacity to inhibit Paf- and ZAS-induced cytosolic free Ca^{2+}-mobilization was investigated. BN 52021, at a concentration of $100\ \mu M$, completely inhibited Paf-induced Ca^{2+}-mobilization and inhibited ZAS-induced Ca^{2+}-mobilization by about 50%. Nedocromil sodium and sodium cromoglycate were ineffective.

Introduction

When allergic asthmatic individuals are challenged with allergen, an early phase asthmatic reaction (within 30 min after challenge) and a late phase asthmatic reaction (4–6 h after challenge) may develop (Durham & Kay, 1985; de Monchy, 1986). This late phase asthmatic reaction develops without further exposure to the allergen. The late phase asthmatic reaction has a different pattern compared to the early phase reaction; it has a slower onset of action and it leads to a more prolonged bronchoconstriction. In general the late reaction is clinically more severe (Verhagen & Bruijnzeel, 1985). The cellular events taking place during this late phase asthmatic reaction are considered highly relevant to the pathogenesis of asthma (Durham & Kay, 1985; de Monchy, 1986; Bruijnzeel & Verhagen, 1989). Bronchoalveolar lavage (BAL) studies of the cellular changes during this response have demonstrated increased numbers of inflammatory cells, particularly eosinophils and neutrophils (de Monchy *et al.*, 1985; Metzger *et al.*, 1985; Diaz *et al.*, 1987), indicating active migration of those cells into the bronchial lumen. Inflammatory cells may be mobilized by chemotactic agents of protein- or lipid-nature such as neutrophil chemotactic factor (NCF), eosinophil chemotactic factor (ECF), complement fragments as C5a, leukotriene B_4, and platelet-activating factor (Paf) (Shaw *et al.*, 1981; Atkins & Wasserman, 1983).

Here, we describe the inhibitory effects of nedocromil sodium, the Paf-antagonist BN 52021 and sodium cromoglycate on the chemotactic response of human neutrophils *in vitro*, induced by Paf and ZAS.

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Furthermore, the effects of these drugs on the Paf- and ZAS-induced cytosolic free calcium mobilization have been investigated.

Methods

Purification of neutrophils

Blood of normal volunteers was collected in one-tenth volumes of sterile trisodium citrate (138 mM; pH 7.0). Platelet-rich plasma was removed after centrifugation (15 min, 275 g_{max}, room temperature). The buffy coats were collected, diluted four times (v/v) with buffer A (phosphate-buffered saline/trisodium citrate/human pasteurized plasma protein = 80/10/10, v/v/v) and 16 ml of Ficoll-paque (density 1.077 g ml^{-1}) were syringed carefully under 35 ml of this cell suspension. Tubes were centrifuged (20 min, 1000 g_{max}, room temperature), the upper layers of mononuclear cells removed and granulocytes with contaminating erythrocytes were collected from the bottom of the tube. The remaining erythrocytes were removed by ammonium chloride lysis at 0°C and subsequent centrifugation. Thereafter the cells were washed with buffer A and regenerated at 37°C in minimum essential medium/10% foetal calf serum for 30 min. By this isolation procedure neutrophils were obtained with a recovery over 85%, a purity over 97% and a viability over 99%. The viability was tested with the vital stains fluorescein diacetate and ethidium bromide (Edidin, 1970; Takasugi, 1971). After isolation the neutrophils were resuspended in Geys buffer (Geys balanced salt solution/human serum albumin (5%, w/v)/heparin (10 iu ml^{-1}) and glucose (1 mg ml^{-1})).

Chemotaxis

Chemotaxis was measured with a modified Boyden chamber technique. In the chamber the lower filter had a pore width of 0.45 μm (Millipore) and the upper filter a pore width of 8 μm (Sartorius). The cells as well as the reagents used were suspended in Geys buffer. The cell suspension, 5×10^5 cells in 250 μl, was added to the upper compartment and 220 μl of the chemotactic agent was added to the lower compartment of the chamber. Concentration ranges of the chemotactic agents Paf, lyso-Paf and ZAS were tested with the buffer as a control. At optimal concentrations of these chemotactic agents dose-response inhibition curves for nedocromil sodium, BN 52021 and sodium cromoglycate were constructed. The cells were preincubated for the indicated periods of time with the drugs before the chemotaxis was carried out. The Boyden chambers were incubated for 1.5 h at 37°C. Thereafter the filters were removed, fixed in butanol/ethanol (20/80, v/v) for 10 min and stained with Weigert solution (1% haematoxylin (v/v) in 95% ethanol (v/v) and an acidic $FeCl_3$-solution (70 mM) in a volume ratio of 1:1). The filters were dehydrated with ethanol, made transparent with 100% xylene (v/v) and fixed upside down. The number of cells per high power field (hpf) was determined with light microscopy (magnification 400 ×). In this way, the number of cells that have passed the upper filter was determined.

Measurement of cytosolic free calcium concentration

For cytosolic free calcium measurements indo-1 was used as the fluorescent indicator, described previously (Koenderman *et al.*, 1988). The cells were loaded with the acetoxy-methyl ester of indo-1 (Grynkiewicz *et al.*, 1985); control cells were incubated with 0.1% DMSO. Calibration of indo-1 fluorescence as a function of cytosolic free calcium was determined essentially as described for quin-2 fluorescence (Pozzan *et al.*, 1983), with the following modifications. To saturate all trapped indo-1 with Ca^{2+}, digitonin (5 μM) was added to the cell suspension. Subsequently, the indo-1 signal was quenched by adding 0.5 mM Mn^{2+}. Calculations (Bijsterbosch *et al.*, 1986) were made using a K_d of 250 nM for the indo-1/Ca^{2+} complex (Grynkiewicz *et al.*, 1985). Dye content in the cells was determined by adding indo-1 free acid to the cell suspension. Cells were preincubated for 30 min with the indicated concentrations of the various drugs before they were challenged with Paf or ZAS.

Statistical analysis

Statistical analysis was performed by use of Student's *t* test for paired data. *P* values of <0.05 were considered to be significant.

Materials

Zymosan A was purchased from Sigma (St. Louis, MO, U.S.A.). 1-O-hexadecyl/octadecyl-2-O-acetyl-sn-glycero-3-phosphoryl-choline (Paf) and 1-O-hexadecyl-sn-glycero-3- phosphorylcholine (lyso-Paf) were purchased from Bachem (Bubendorf, Switzerland) and were stored at −20°C in a toluene/ethanol (1:1, v/v) solution under N_2. The acetoxy-methyl ester of indo-1 and indo-1 free acid were purchased from Molecular Probes (Junction City, OR, U.S.A.). Digitonin was obtained from Merck (Darmstadt, F.R.G.). Ficoll-Paque (1.077 g ml^{-1}) was obtained from Pharmacia (Uppsala, Sweden). Nedocromil sodium and sodium cromoglycate were provided by Fisons plc (Loughborough, U.K.). BN 52021 was a kind gift of Dr P. Braquet (Institut

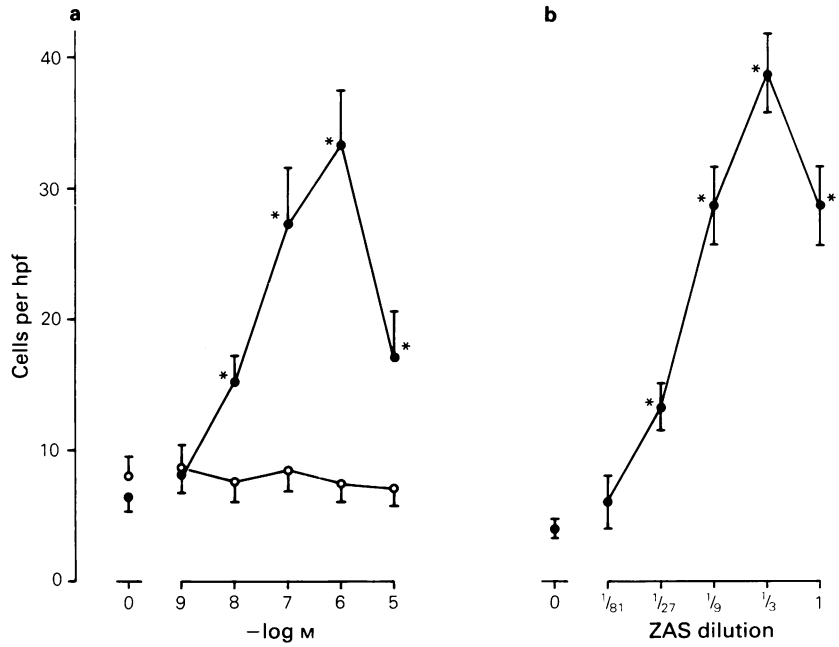

Figure 1 (a) Paf (●), lyso-Paf (○) and (b) zymosan-activated serum (ZAS) (●)-induced chemotaxis of normal human granulocytes using buffer as a control; mean values are shown with vertical bars indicating s.e.mean. ($n = 12$ (Paf, lyso-Paf); $n = 8$ (ZAS); $^*P < 0.05$).

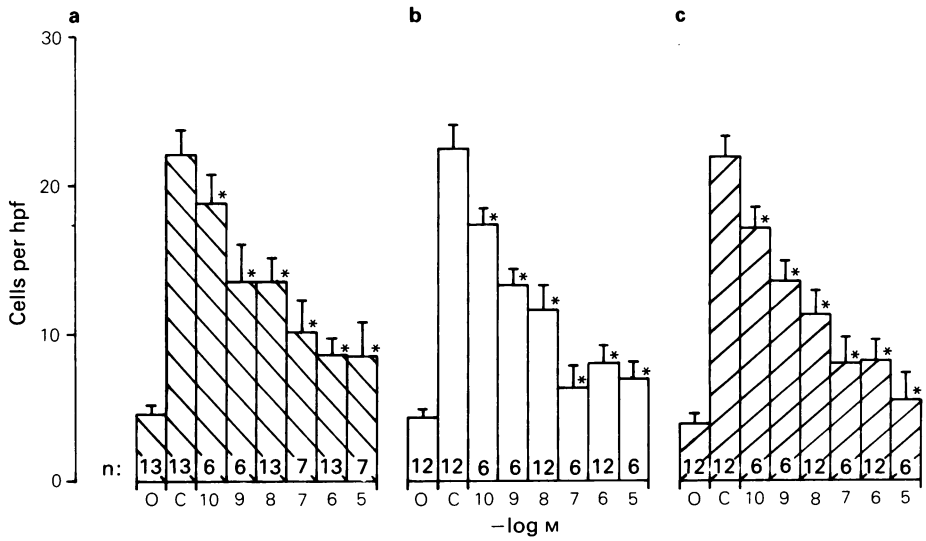

Figure 2 Dose-dependent inhibition of Paf-induced chemotaxis of human granulocytes by (a) nedocromil sodium, (b) BN 52021 and (c) sodium cromoglycate. O: buffer; C: chemotaxis obtained with Paf at a concentration of 1 μM. $^*P < 0.05$, when compared with the control value (C).

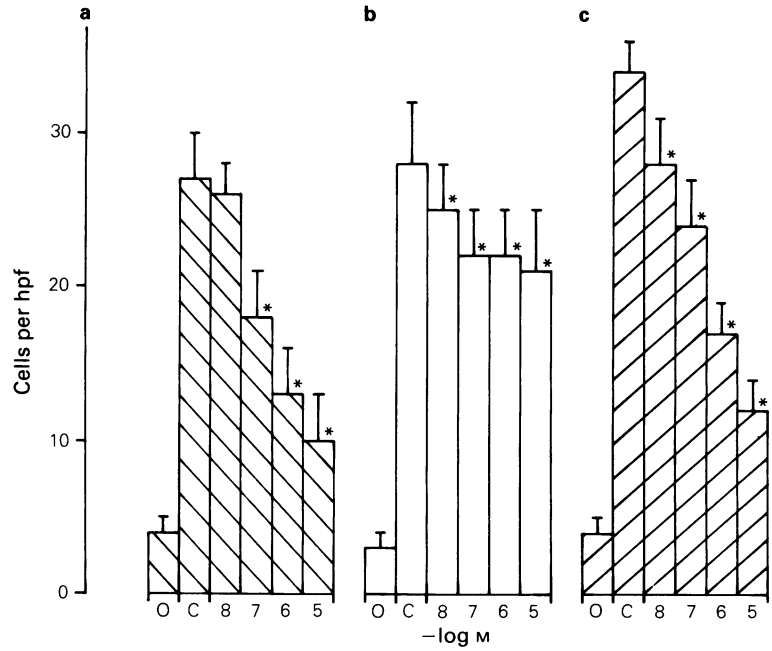

Figure 3 Dose-dependent inhibition of zymosan-activated serum (ZAS)-induced chemotaxis of human granulocytes by (a) nedocromil sodium, (b) BN 52021 and (c) sodium cromoglycate ($n = 6$, mean with s.e.mean shown by vertical bars). O: buffer; C: chemotaxis obtained with ZAS (dilution 1/3). $^*P < 0.05$, when compared with the control value (C).

Henri Beaufour, Le Plessis Robinson, France). All other materials were reagent grade. Zymosan activated serum (ZAS) was prepared as previously described (Goldstein *et al.*, 1975).

Results

Chemotaxis of neutrophils

Paf, lyso-Paf and ZAS concentration ranges The concentration dependency of the Paf and ZAS induced chemotaxis by human neutrophils is shown in Figure 1. Lyso-Paf did not induce chemotaxis. The optimal chemotactic concentration for Paf was 1 μM and for ZAS a serum dilution of 1/3. The inhibitory potencies of nedocromil sodium, BN 52021 and sodium cromoglycate were tested at these optimal chemotactic concentrations.

Inhibition of the Paf- and ZAS-induced chemotaxis of human neutrophils by nedocromil sodium, BN 52021 and sodium cromoglycate The inhibition of Paf-induced chemotaxis of human neutrophils by concentration-ranges of nedocromil sodium, BN 52021 and sodium cromoglycate is shown in Figure 2. All drugs appeared to be equally potent in inhibiting Paf-induced chemotaxis of granulocytes. The IC_{50} was approximately 1 nM.

The inhibition of ZAS-induced chemotaxis of human neutrophils by concentration ranges of nedocromil sodium, BN 52021 and sodium cromoglycate is shown in Figure 3. Nedocromil sodium and sodium cromoglycate were almost equally potent in inhibiting ZAS-induced chemotaxis (IC_{50} = 0.1–1 μM), whereas BN 52021 had a very weak inhibitory potency (IC_{30} = 10 μM).

A series of control experiments was carried out to verify that the drug-induced inhibition of chemotaxis was due to an interaction of the drug with the cells and not with the chemotactic agent. Addition of the various drugs to the compartment of the Boyden chamber in which the chemotactic compound had been inserted did not inhibit the chemotactic response, whereas addition of the various drugs to the compartment in which the cells had been inserted did inhibit the chemotactic response.

Variation of the preincubation time of the cells with the inhibiting drugs To exclude an effect of the duration of the preincubation time of the cells with the various drugs tested, the preincubation time was

varied from 5 min up to 120 min. Nedocromil sodium was used as representative for the other drugs tested. After a preincubation time of 30 min about 70–80% inhibition of Paf- and ZAS-induced chemotaxis was obtained, thereafter reaching a plateau. Therefore a preincubation time of 30 min was considered optimal. This preincubation time was used in all experiments described.

Inhibition of the Paf- and ZAS-induced cytosolic free calcium mobilization by nedocromil sodium, BN 52021 and sodium cromoglycate.

To determine whether the investigated drugs would interfere with early events of cell activation, the possible inhibition of Paf- and ZAS-induced cytosolic free calcium mobilization in neutrophils was investigated.

In the case of the Paf-induced transient rise in cytosolic free Ca^{2+} concentration, only the Paf antagonist BN 52021 at a concentration of 100 μM was capable of completely blocking this transient rise, whereas nedocromil sodium and sodium cromoglycate were ineffective at these concentrations (see Figure 4).

In the case of inhibition of the ZAS-induced transient rise in cytosolic free Ca^{2+} concentration, only the Paf antagonist BN 52021 at a concentration of 100 μM proved effective (50% inhibition), whereas the other drugs at the same concentration were ineffective (see Figure 4).

When in the above-mentioned experiments the drug concentrations were decreased to 10 and 1 μM respectively the inhibitory potency of BN 52021 against Paf stimulation gradually decreased; in the case of ZAS stimulation, the inhibitory effects had already disappeared at a concentration of 10 μM. The other drugs remained ineffective.

Discussion

The mobilization of inflammatory cells, particularly neutrophils and eosinophils, in the lung tissue *in vivo* may be caused by chemotactic factors of lipid or protein nature. As representatives of those groups of chemotactic factors Paf and ZAS were chosen for our studies. Both these factors induced chemotaxis of human neutrophils in a concentration-dependent way. Nedocromil sodium, BN 52021 and sodium cromoglycate all induced concentration-dependent

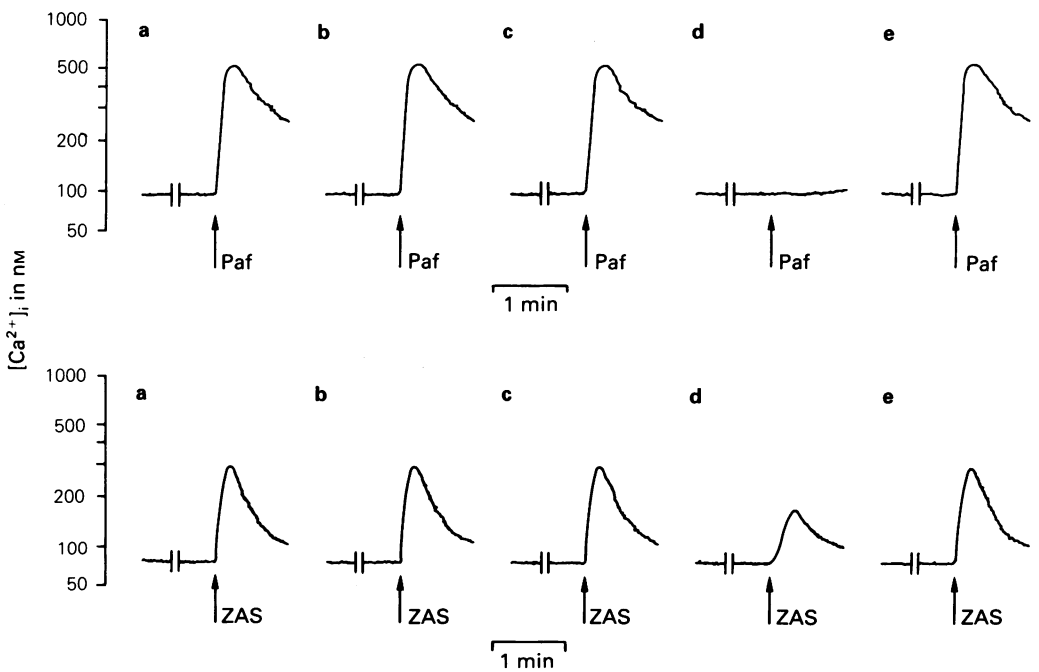

Figure 4 Intracellular free Ca^{2+} concentrations in neutrophils upon stimulation with Paf (1 nM, upper panel) and zymosan-activated serum (ZAS) (1/1000 dilution, lower panel). Effects are shown of (a) buffer, (b) dimethyl sulphoxide (0.2%), (c) nedocromil sodium (100 μM), (d) BN 52021 (100 μM), (e) sodium cromoglycate (100 μM), representative of three experiments. The cells were preincubated for 30 min with the various drugs.

inhibition of Paf-induced chemotaxis of human neutrophils ($IC_{50} \approx 1$ nM), demonstrating that they are very potent inhibitors. Since BN 52021 has been shown (Kurihara *et al.*, 1988) to antagonize specific Paf binding to neutrophils, our results suggest that this drug at least may act by interfering with the cell surface interaction of Paf with the neutrophil. The results described here contrast with those of Kurihara *et al.* (1988), who found that Paf-induced chemotaxis of neutrophils and eosinophils could only be inhibited by BN 52021 and not by nedocromil sodium and sodium cromoglycate. These authors also found a rather high IC_{50} (around 10 μM), which in our opinion does not necessarily support the idea that BN 52021 is a potential anti-allergic agent. When ZAS was used instead of Paf, nedocromil sodium and sodium cromoglycate were capable of inhibiting the chemotaxis to the same extent with an $IC_{50} = 0.1$–1 μM, whereas BN 52021 was hardly capable of inhibiting this chemotaxis ($IC_{30} = 10\,\mu$M). This could indicate that nedocromil sodium and sodium cromoglycate may also interfere with the C5a receptor on neutrophils, although less powerfully than with the Paf receptor on the same cells.

To obtain more evidence of a possible interaction between the tested drugs and the above-mentioned receptor sites on neutrophils, binding studies with labelled Paf and C5a have been performed. So far, these binding studies have not yielded any conclusions, mainly because of high non-specific binding. Therefore, we decided to investigate whether an early step in cell activation, such as the induction of a transient rise in the cytosolic free Ca^{2+} concentration by Paf and ZAS, could be blocked. At low drug concentrations (1 μM) no inhibition was observed. However, BN 52021 at a concentration of 100 μM effectively blocked both Paf- and ZAS-induced Ca^{2+} mobilization (100 and 50% respectively), whereas nedocromil sodium and sodium cromoglycate were ineffective. Our data are in contrast with those of Skedinger *et al.* (1987), who found a 50% inhibition of the ZAS-induced Ca^{2+} mobilization by sodium cromoglycate at a concentration of 100 μM. However, they did not measure the intracellular Ca^{2+} concentration continuously, but sampled every minute after addition of the stimulus and therefore used a ZAS concentration 100 times higher than ours. Because the fast and transient rise of the Ca^{2+} concentration takes place within 20 s of addition of the stimulus, we believe that our method is more reliable.

Taken together, these data support the idea that BN 52021 acts as a Paf-receptor antagonist. Since nedocromil sodium and sodium cromoglycate were ineffective in inhibiting the Paf- and ZAS-induced Ca^{2+} mobilization, the effect of these drugs for inhibition of Paf- and ZAS-induced chemotaxis is most likely to be at a later event in the activation mechanism, for example, inhibition of protein kinase C as suggested for rabbit peritoneal neutrophils (Bradford & Rubin, 1986), rat peritoneal mast cells (Kay, 1987) and reptilian pigment cells (Lucas & Shuster, 1987).

Although the effects described here were obtained *in vitro*, they might be relevant to the *in vivo* situation. Since inflammatory cells such as neutrophils are actively involved *in vivo* in the pathogenesis of asthma, then our *in vitro* results might in part explain how neutrophil migration into the lung tissue can be prevented effectively by nedocromil sodium and sodium cromoglycate (Hutson *et al.*, 1988; 1989).

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Action of atrial natriuretic peptide (ANP) on dog cerebral arteries: evidence that neurogenic relaxation is not mediated by release of ANP

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1 Atrial natriuretic peptide (ANP) (10^{-9} to 10^{-8} M) produced a concentration-related relaxation in helical strips of dog cerebral arteries partially contracted with prostaglandin $F_{2\alpha}$. The relaxation was not affected by treatment with ouabain, quinidine, oxyhaemoglobin, methylene blue, or removal of endothelium.

2 Relaxations induced by nicotine or transmural electrical stimulation were not reduced in arteries in which tachyphylaxis to ANP had developed.

3 In arteries exposed to Ca^{2+}-free media under severe hypoxia, contractions due to prostaglandin $F_{2\alpha}$ and Ca^{2+} were attenuated by treatment with ANP, whereas the reoxygenation-induced contraction was unaffected.

4 The results suggest that ANP does not mediate neurogenic relaxation of dog cerebral arteries. The ANP-induced relaxation is not associated with activation of the sodium pump but is due to an inhibitory action on the release and influx of Ca^{2+}, probably as a result of stimulation of guanylate cyclase.

Introduction

Atrial natriuretic peptide (ANP), secreted from mammalian atria, may play an important role in regulating fluid and electrolyte balance and vascular resistance (DeBold *et al.*, 1981; Kangawa & Matsuo, 1984). The peptide relaxes isolated arteries and veins from a variety of animals (Winquist, 1985). However, the magnitude of relaxation differs in blood vessels from different regions. In the rabbit, the response is greater in renal and iliac arteries than in basilar, saphenous and ear arteries (Faison *et al.*, 1985). Rabbit facial veins respond to the peptide with a marked relaxation, whereas femoral, iliac and saphenous veins do not respond at all (Faison *et al.*, 1985). The ANP-induced relaxation is resistant to blockade by a wide variety of pharmacological antagonists but appears to be associated with an activation of particulate guanylate cyclase (Winquist *et al.*, 1984a).

Transmural electrical stimulation and nicotine produce a cerebroarterial relaxation, possibly by activating of vasodilator nerves innervating the arterial wall (Toda, 1975; Lee *et al.*, 1975). The neurotransmitter mediating this relaxant response has not been identified but the involvement of β-receptors, muscarinic receptors and prostaglandin release have been excluded, as has the activation of purinergic and peptidergic (substance P and vasoactive intestinal peptide) nerves (Toda, 1975; 1982) and activation of the Na^+-pump. However this neurogenic relaxation is not endothelium-dependent (Lee, 1980) and can be inhibited by haemoglobin (Lee *et al.*, 1984), haemolysate and methylene blue (Toda, 1988). ANP is a potent vasodilator which mediates relaxation of cerebral arteries via an endothelium-independent mechanism. Since the presence of ANP has been demonstrated in hypothalamic neurones (Currie *et al.*, 1985; Sudoh *et al.*, 1988), we have investigated the possibility that neurogenically mediated relaxations in dog cerebral arteries are caused by the release of ANP. In addition, we have studied the mechanisms of the relaxant response to ANP.

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Methods

Preparation

Mongrel dogs of either sex, weighing 7 to 15 kg, were anaesthetised with sodium pentobarbitone (30 mg kg^{-1}, i.v.) and killed by being bled from the common carotid arteries. The brain was rapidly

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removed, and basilar and middle cerebral arteries (0.6 to 0.8 mm outside diameter) were isolated. The arteries were cut helically into strips, approximately 20 mm long. The strips were vertically fixed between hooks in a muscle bath containing a modified Ringer-Locke solution, which was maintained at 37 ± 0.3°C and aerated with a mixture of 95% O_2 and 5% CO_2. The hook anchoring the upper end of the strip was connected to the lever of a force-displacement transducer (Nihonkohden-Kogyo Co., Tokyo, Japan). The resting tension was adjusted to 1.5 g, which is optimal for inducing a maximal contraction (Toda *et al.*, 1978). The composition of the nutrient solution was as follows (mM): NaCl 120, KCl 5.4, $NaHCO_3$ 25.0, $CaCl_2$ 2.2, $MgCl_2$ 1.0, and dextrose 5.6. The pH of the solution was 7.36–7.41. Before the start of experiments, preparations were allowed to equilibrate for 60 to 90 min in the bathing media, during which time the solutions were replaced every 10 to 15 min.

Transmural stimulation of innervating nerves

The arterial strips were placed between a pair of stimulating electrodes made of platinum plates, 5 × 15 mm in size and approximately 2 mm apart from each other (Toda, 1971). The gap between the strip and the electrodes was wide enough to allow undisturbed contractions, and yet sufficiently narrow to produce effective stimulation of intramural nerves. The strips were transmurally stimulated by a train of 0.2 ms square wave pulses of supramaximal intensity (approximately 10 V), at frequencies of 2, 5 and 20 Hz for periods of 100, 40 and 10 s, respectively.

Recording

Isometric contractions and relaxations were displayed on an ink-writing oscillograph (Nihonkohden-Kogyo Co.). Two basilar or two middle cerebral arterial strips were obtained from each dog. The contractile response to KCl (30 mM) was initially recorded and the preparations were washed three times with fresh medium. In order to induce tone, the strips were exposed to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 10^{-7} to 10^{-6} M) which caused contraction amounting to 30–45% of the contraction induced by KCl (30 mM). Transmural stimulation was applied repeatedly at 10 min intervals until regular responses were obtained. Responses to atrial natriuretic peptide (ANP; 10^{-9}, 3×10^{-9} and 10^{-8} M), nicotine (10^{-4} M) and KCl (5 mM) in single concentrations were obtained by direct addition to the bathing medium. If necessary, responses to ANP applied cumulatively were obtained. Relaxant responses to transmural stimulation, ANP or other vasodilator agents were measured as a % of the relaxant response to papaverine (10^{-4} M) which was added to the bath at the end of the experiment. In some strips, the intimal surface was gently rubbed with a cotton pellet to remove the endothelium. The removal of endothelium was confirmed histologically and by testing the relaxant response to substance P and Ca ionophore A23187. In experiments in which vasodilator responses were obtained in the presence of pharmacological antagonists, the antagonists were added 20–30 min before transmural stimulation or addition of the vasodilator agents.

In Ca^{2+}-free experiments, contractile responses to $PGF_{2\alpha}$ (10^{-5} M) were obtained twice after the equilibration period. The tonic contraction of the second response was taken as a control. Arterial strips were then exposed to Ca^{2+}-free medium containing 0.1 mM EGTA for 60 min, during which time the media were replaced twice every 20 min. The gas mixture (95% O_2 and 5% CO_2) was replaced with a mixture of 95% N_2 and 5% CO_2 after 20 min exposure to the Ca^{2+}-free medium. The Po_2 of the nutrient solution was rapidly lowered from 570–600 mmHg to approximately 10 mmHg. Twenty min after replacement of the gas mixture, one of the paired strips was treated for 20 min with ANP (10^{-8} M) (after 60 min exposure to Ca^{2+}-free medium and 40 min under hypoxic conditions), then, $PGF_{2\alpha}$ (10^{-5} M) was added. The other strip, to which no peptide was added, served as control. When the response had levelled off, Ca^{2+} (2.2 mM) was added. After the response to Ca^{2+} had stabilized, the gas was returned to a mixture of 95% O_2 and 5% CO_2. In some experiments, paired strips were treated identically until the responses to Ca^{2+} (2.2 mM) had stabilized under hypoxic conditions, then, ANP (10^{-8} M) was added to one of the paired strips before the gas was returned to 95% O_2 and 5% CO_2, the other strip served as control.

Statistics and drugs

Results shown in the text, table and figures are expressed as mean values ± s.e.mean. Statistical analysis for two groups was made by use of Student's paired or unpaired *t* tests. Tukey's method after one-way analysis of variance was used for multiple comparisons. A probability of 0.05 or less was considered significant.

Drugs used were nicotine, methylene blue, quinidine sulphate, hexamethonium bromide (Nakarai Chemicals Ltd, Kyoto, Japan), calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP), α-human atrial natriuretic peptide (ANP), substance P (Peptide Institute, Inc, Minoh, Japan: impurities detectable by ordinary gradient h.p.l.c. systems are less than 1%), EGTA (ethylene glycol-*bis*-(β-aminoethyl-ether)-N,N'-tetraacetic acid; Wako

Pure Chemical Ind, Osaka), prostaglandin $F_{2\alpha}$ (Ono Pharmaceutical Co, Osaka), ouabain octahydrate (E. Merck, Darmstadt, West Germany), tetrodotoxin (Sankyo Co, Ltd, Tokyo), A23187 (Boehlinger Mannheim, West Germany), papaverine hydrochloride (Dainippon Pharmaceutical Co., Osaka) and sodium pentobarbitone (Abbott Lab, North Chicago, IL, U.S.A.). Oxyhaemoglobin was prepared by adding a 10 fold molar excess of the reducing agent, sodium dithionite ($Na_2S_2O_4$), to a 1 mM solution of commercial haemoglobin (Canine; Sigma, St. Louis, MO, U.S.A.) in distilled water. Sodium dithionite was then removed by dialysis twice against 250 volumes of distilled water for 4 h at 4°C. The solutions were frozen in aliquots at −20°C and stored for up to 14 days.

Results

Relaxations induced by ANP

In cerebral arteries partially contracted with $PGF_{2\alpha}$, ANP (10^{-9} to 10^{-8} M) produced a concentration-dependent relaxation; mean values at 10^{-9}, 3×10^{-9} and 10^{-8} M were $4.3 \pm 0.8\%$ ($n = 9$), $19.9 \pm 2.8\%$ ($n = 13$) and $48.2 \pm 2.7\%$ ($n = 29$), respectively. These responses were obtained by adding single concentrations to each strip, because of the rapid development of tachyphylaxis to ANP upon cumulative application.

Treatment with ouabain (2×10^{-7} M) did not significantly alter the response to 10^{-8} M ANP (Table 1), whereas the relaxant response to 5 mM K^+ ($44.9 \pm 6.6\%$ relative to the maximum relaxant response induced by 10^{-4} M papaverine) was reversed to a contraction ($4.7 \pm 1.0\%$ of the contractile response produced by 30 mM K^+, $n = 10$). Typical recordings of the responses to K^+ and ANP are shown in Figure 1. Treatment with 10^{-5} M quinidine did not reduce the relaxation to ANP (Table 1) but significantly attenuated the nicotine (10^{-4} M)-induced relaxation (from $40.5 \pm 6.3\%$ to $9.4 \pm 1.9\%$, $n = 8$). Methylene blue (10^{-5} M) and oxyhaemoglobin (1.6×10^{-5} M) failed to inhibit the ANP-induced relaxation (Table 1).

Table 1 Relaxant responses of cerebral arteries to 10^{-8} M atrial natriuretic peptide (ANP) in the absence and presence of antagonists and endothelium

Intervention	n	* *Relaxation* (%) *induced by ANP* *Control*	*Experimental*
Ouabain 2×10^{-7} M	13	41.7 ± 4.4	38.8 ± 3.3
Quinidine 10^{-5} M	10	64.1 ± 4.9	67.1 ± 4.9
Methylene blue 10^{-5} M	3	56.2 ± 8.8	58.5 ± 7.2
Oxyhaemoglobin 1.6×10^{-5} M	9	62.2 ± 5.3	69.0 ± 3.4
Endothelium denudation	9	41.3 ± 7.8	44.7 ± 7.1

* Relative to the relaxation caused by 10^{-4} M papaverine. Data shown are means ± s.e.mean. n = number of preparations used.

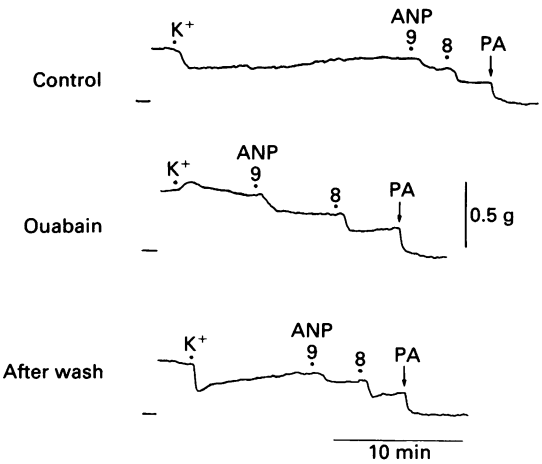

Figure 1 Modification by ouabain (2×10^{-7} M) of the relaxant responses to K^+ (5 mM) and atrial natriuretic peptide (ANP, 10^{-9} and 10^{-8} M) in dog middle cerebral artery strip partially contracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). PA = 10^{-4} M papaverine. Horizontal lines just left of each tracing represent the level before the addition of $PGF_{2\alpha}$.

Removal of endothelium from the cerebral arteries, which prevented the relaxant response to substance P (10^{-7} M) and abolished the response to the Ca ionophore A23187 (10^{-7} M), did not alter the relaxant response to ANP (Table 1). Endothelium-independent relaxations produced by VIP (10^{-8} M) and CGRP (10^{-8} M) were abolished by repeated applications (data not shown). ANP (10^{-8} M)-induced relaxations were unaffected in the arteries, in which tachyphylaxis to VIP and CGRP had developed; average relaxant responses to ANP before and after treatment with VIP were $56.8 \pm 7.1\%$ and $60.4 \pm 6.7\%$ ($n = 5$), respectively and those before and after CGRP were $55.5 \pm 6.5\%$ and $54.5 \pm 6.7\%$ ($n = 4$), respectively.

ANP and the response to vasodilator nerve stimulation

Nicotine (10^{-4} M) produced a transient relaxation of cerebro-arterial strips partially contracted with

$PGF_{2\alpha}$, which was abolished by treatment with 10^{-5} M hexamethonium ($n = 4$). In the arteries in which the relaxant response to ANP (10^{-8} M) was abolished by prior treatment with the same concentration of ANP, the nicotine-induced relaxation was not altered (Figure 2). Mean values of the relaxation before and after development of tachyphylaxis to ANP were $37.9 \pm 4.8\%$ and $37.8 \pm 5.4\%$ ($n = 8$), respectively. Transmural electrical stimulation (2, 5 and 20 Hz) produced a tetrodotoxin-sensitive relaxation, which was not attenuated in the arteries in which tachyphylaxis to ANP had developed; mean values at 5 Hz before and after treatment with ANP were $27.3 \pm 4.8\%$ and $24.6 \pm 4.6\%$ ($n = 3$), respectively.

ANP and contractions induced by $PGF_{2\alpha}$ and Ca^{2+}

The contractile response to 10^{-5} M $PGF_{2\alpha}$ was suppressed by $83.6 \pm 2.0\%$ ($n = 14$) following exposure of the cerebral arterial strips to Ca^{2+}-free, EGTA (0.1 mM)-containing medium and severe hypoxia (Figure 3), as was the response under normoxia (Bian & Toda, 1988). The addition of 2.2 mM Ca^{2+} produced a phasic contraction (A in Figure 3) followed by a relaxation and a tonic contraction (B). Reoxygenation elicited an additional contraction (C). The contraction was preceded by a transient relaxation in 4 out of 8 strips, which was not significantly affected by treatment with ANP; mean values of the relaxant responses in the absence and presence of ANP were 26.3 ± 9.2 and $24.0 \pm 7.5\%$ ($n = 4$), respectively, relative to contractions caused by $PGF_{2\alpha}$ plus Ca^{2+}. Typical responses are demonstrated in the upper trace of Figure 4. Treatment for 20 min with 10^{-8} M ANP attenuated the contraction due to $PGF_{2\alpha}$ in the Ca^{2+}-free medium under hypoxia and also the phasic contraction due to Ca^{2+} (Figures 3 and 4). However, the tonic contractions induced by Ca^{2+} and reoxygenation were not affected. In order to exclude a possible involvement

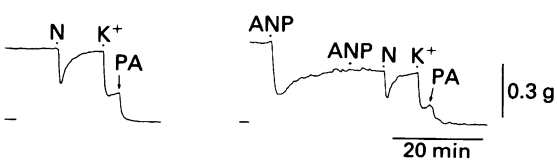

Figure 2 Relaxant responses to nicotine (N, 10^{-4} M) and K^+ (5 mM) of a dog middle cerebral artery strip before and after treatment with 10^{-8} M atrial natriuretic peptide (ANP). ANP (2nd application) did not produce a relaxation, after the relaxant response to first addition of ANP had stabilized. The strip was partially contracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$); the level before the addition of $PGF_{2\alpha}$ is shown as horizontal lines just left of each tracing. PA = 10^{-4} M papaverine.

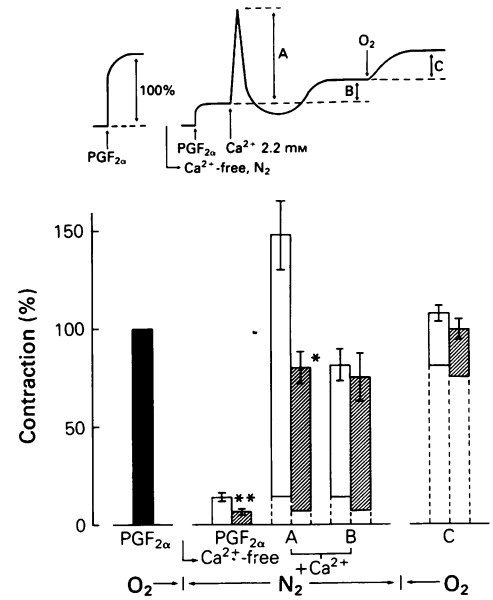

Figure 3 Modification by atrial natriuretic peptide (ANP, 10^{-8} M) of the contractile responses to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 10^{-5} M), Ca^{2+} (2.2 mM; A and B) and reoxygenation (C) in dog cerebral artery strips exposed to Ca^{2+}-free medium under severe hypoxia. Contractions induced by 10^{-5} M $PGF_{2\alpha}$ in control medium (oxygenated) were taken as 100% (solid column); mean absolute values in control (open columns) and experimental series (with ANP; hatched columns) were 1387 ± 223 mg ($n = 6$) and 1327 ± 280 mg ($n = 6$), respectively. For explanations of A, B and C, see the top tracing. Significantly different from control, * $P < 0.02$; ** $P < 0.05$. Vertical bars represent s.e.mean.

of ANP degradation during the incubation period of approximately 60 min (Figure 4), additional experiments were carried out. Two strips obtained from the same dog were exposed to the Ca^{2+}-free medium, and $PGF_{2\alpha}$ and Ca^{2+} were applied. The data are presented as open and hatched columns in Figure 5. After the Ca^{2+}-induced contractions (B in Figure 5) had stabilized, 10^{-8} M ANP was applied to one of the strips, which responded with a significant relaxation. After the relaxation had stabilized, the N_2 gas was returned to the O_2 gas. The reoxygenation-induced contraction in the control and ANP-treated arteries did not differ significantly (Figure 5).

Discussion

ANP produced a concentration-related relaxation in dog cerebral arterial strips partially contracted with $PGF_{2\alpha}$, which is appreciably greater than that in

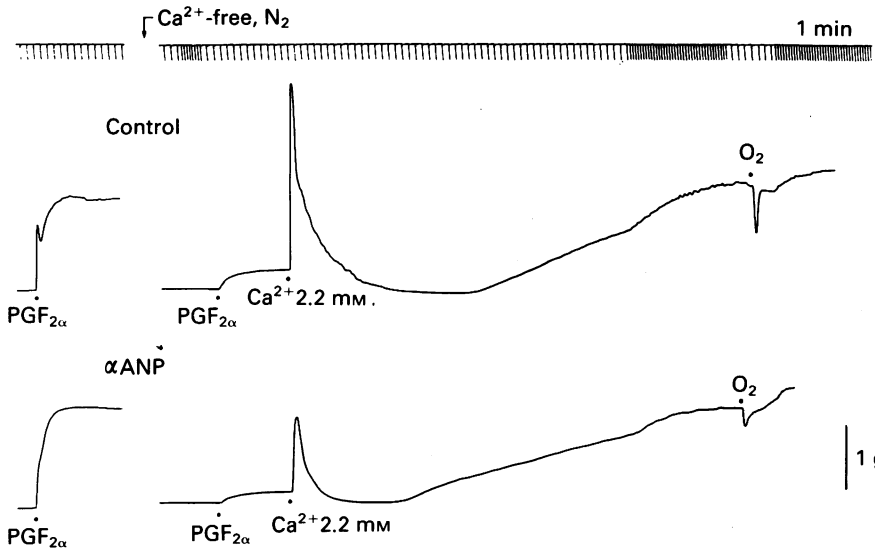

Figure 4 Typical responses to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 10^{-5} M), Ca^{2+} (2.2 mM) and reoxygenation of basilar artery strips exposed to Ca^{2+}-free media under severe hypoxia in control (upper trace) and experimental (10^{-8} M atrial natriuretic peptide (ANP)-treated) series (lower trace). The strips were obtained from the same dog.

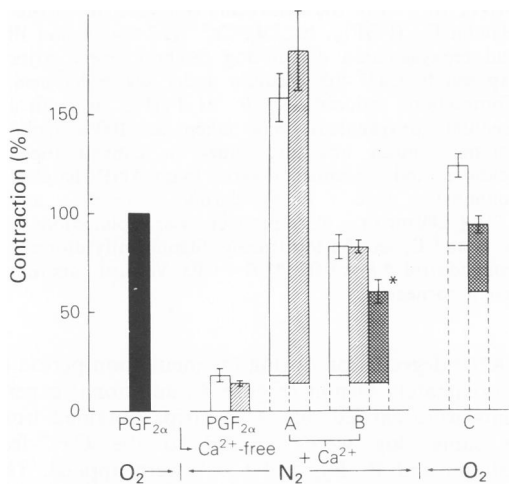

Figure 5 Responses to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 10^{-5} M), Ca^{2+} (2.2 mM; A and B) and reoxygenation (C) of the cerebral artery strips exposed to Ca^{2+}-free medium under severe hypoxia. Atrial natriuretic peptide (ANP, 10^{-8} M) was applied to the strip (cross-hatched columns), after the Ca^{2+}-induced contraction (B) had stabilized (hatched column). Contractions induced by 10^{-5} M $PGF_{2\alpha}$ in control medium (oxygenated) were taken as 100% (solid column); mean absolute values in control (open columns) and experimental series (hatched and cross-hatched columns) were 1263 ± 127 mg ($n = 8$) and 1353 ± 158 mg ($n = 8$), respectively. Two strips were obtained from the same dog. Significantly different from control, $^{*}P < 0.02$. Vertical bars represent s.e.mean.

rabbit basilar arteries but less than the relaxations in rabbit renal and mesenteric arteries (Faison *et al.*, 1985). A component of the ANP-induced relaxation of the rabbit aorta appears to be mediated via activation of Na^+/K^+ ATPase, since the response is attenuated by ouabain and K^+-free medium (Sybertz & Desiderio, 1985; Auguet *et al.*, 1985). However, such a mechanism cannot explain the relaxant response to ANP in the present study in dog cerebral arteries, since ouabain in a concentration sufficient to abolish the relaxant response to KCl (5 mM), which is mediated through the activation of the electrogenic Na^+ pump (Toda, 1985), had no effect. Similar findings have been obtained in rat thoracic aorta (O'Donnell *et al.*, 1987).

Removal of the endothelium did not reduce the relaxation induced by ANP, as seen in cat coronary arteries (Yanagisawa *et al.*, 1987), although a receptor binding study has suggested the presence of ANP receptors in bovine aortic endothelial cells (Schenk *et al.*, 1985). In dog cerebral arteries, substance P- and vasopressin-induced relaxations are dependent on endothelium (Onoue *et al.*, 1988), whereas relaxations elicited by VIP and CGRP are endothelium-independent. However, from our studies ANP would not appear to stimulate the same receptor sites as VIP and CGRP, since ANP-induced relaxation were not attenuated in arteries in which the relaxant response to VIP and CGRP had been abolished.

Vasodilator responses of cerebral arteries from dogs and monkeys to electrical and chemical (by nicotine) stimulation of nerves innervating the

arterial wall are attenuated by quinidine and abolished by treatment with haemolysate and methylene blue (Toda, 1982; 1988). These responses are not dependent on endothelium (Lee, 1980) and are possibly mediated by polypeptides (Toda & Okamura, unpublished data) other than substance P, VIP, vasopressin and CGRP. Although the present study has clearly shown that ANP is a potent relaxant of cerebral vascular smooth muscle via an endothelial-independent mechanism, it cannot be responsible for the neurogenically mediated relaxant responses, since ANP-induced relaxations were not affected by quinidine, methylene blue and oxyhaemoglobin. Furthermore, relaxations induced by transmural nerve stimulation and nicotine were not attenuated in arteries in which tachyphylaxis to ANP had developed (Figure 2).

Treatment of cerebral arteries with ANP attenuated the contraction induced by $PGF_{2\alpha}$ and Ca^{2+} in Ca^{2+}-free medium, suggesting an interference with Ca^{2+} release from intracellular storage sites and the influx of Ca^{2+} through receptor-operated channel. Inhibition by ANP of intracellular Ca^{2+} release has been demonstrated in rabbit isolated aorta (Meisheri *et al.*, 1986). $PGF_{2\alpha}$ is thought to contract the cerebral arteries mainly by activation of a receptor-mediated process rather than activation of a voltage-dependent one, since $PGF_{2\alpha}$-induced contractions are not suppressed in arteries depolarized by excess K^+ (Toda & Onoue, 1988), and $PGF_{2\alpha}$ up to 2.8×10^{-6} M does not depolarize the membrane in guinea-pig basilar arterial smooth muscle (Fujiwara & Kuriyama, 1984). It has been demonstrated that increased influx of $^{45}Ca^{2+}$ by activation of α-adrenoceptors and angiotensin II receptors is decreased by ANP, whereas the influx under membrane depolarization is unaffected (Chiu *et al.*, 1986). In contrast to a significant attenuation of contractions due to $PGF_{2\alpha}$ and Ca^{2+}, the reoxygenation-induced contraction was not inhibited by ANP, which is expected to result mainly from increased Ca^{2+} influxes (Bian & Toda, 1989). The reason for this ineffectiveness could not be determined in the present study. Nitroglycerin, like ANP, inhibits the contractions due to $PGF_{2\alpha}$ and Ca^{2+} but does not alter the reoxygenation-induced contraction (Bian & Toda, 1989). Both ANP and nitroglycerin increase cellular guanosine 3':5'-cyclic monophosphate (cyclic GMP). However, ANP stimulates particulate guanylate cyclase (Winquist *et al.*, 1984a; Waldman *et al.*, 1984), whereas nitrovasodilators stimulate soluble guanylate cyclase (Mittal & Murad, 1977); thus, the intracellular location of cyclic GMP increments may differ (Mittal, 1985). On the basis of the inhibitory action on the contractile responses to different stimuli ($PGF_{2\alpha}$, Ca^{2+} and reoxygenation) in the dog cerebral arteries, ANP is identical to nitroglycerin but quite different from Ca^{2+} entry blockers and prostacyclin or its analogue, that causes vasodilatation possibly by an increase in cellular cyclic AMP (Kukovetz *et al.*, 1979). Similarities between the action of ANP and nitrovasodilators have been presented (Winquist *et al.*, 1984b).

ANP receptors and immunoreactive ANP exist in the brain (Ueda *et al.*, 1987) in addition to the kidney, lung and atrium. ANP has an interesting mode of action in cerebral artery smooth muscle, and may be a physiologically important regulator of brain circulation. However, evidence for a putative role of ANP as a transmitter in vasodilator nerves in cerebral arteries was not obtained.

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Activation of the human neutrophil 5-lipoxygenase by exogenous arachidonic acid: involvement of pertussis toxin-sensitive guanine nucleotide-binding proteins

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1 The mechanism by which incubation of human peripheral blood neutrophils with exogenous arachidonic acid leads to 5-lipoxygenase product synthesis was investigated.

2 Incubation of neutrophils with arachidonic acid caused a concentration- and time-dependent synthesis of leukotriene B_4, its Ω-oxidation products, and 5-hydroxyeicosatetraenoic acid.

3 The threshold concentration of arachidonic acid required for this effect was equal to, or greater than 3.3 μM and the synthesis increased with up to 33 μM arachidonic acid, the highest concentration used. Synthesis induced by arachidonic acid increased with time for up to 15 min and the major products detected were the Ω-oxidation products of leukotriene B_4.

4 Pre-incubation of neutrophils with pertussis toxin inhibited the synthesis of 5-lipoxygenase products induced by arachidonic acid by 75% or more, but had no effect on either arachidonic acid-induced synthesis of the 15-lipoxygenase product, 15-hydroxyeicosatetraenoic acid, or activation of the 5-lipoxygenase induced by the calcium ionophore A23187.

5 Pre-incubation of neutrophils with granulocyte-macrophage colony-stimulating factor lead to enhanced leukotriene synthesis in response to arachidonic acid.

6 These results imply that exogenous arachidonic acid is not only used as a substrate, but also activates the 5-lipoxygenase. Possible mechanisms of action are discussed.

Introduction

By virtue of their potent effects on cells of the immune system, the 5-lipoxygenase metabolites of arachidonic acid are believed to play important roles in inflammation and allergy (Sirois & Borgeat, 1984; Lewis & Austen, 1984). In particular, leukotriene B_4 is a stereospecific activator of neutrophil chemotaxis (Ford-Hutchinson *et al.*, 1980; Malmsten *et al.*, 1980), calcium mobilization (Naccache *et al.*, 1981; White *et al.*, 1983), aggregation (Ford-Hutchinson *et al.*, 1980) and degranulation (Hafstrom *et al.*, 1981). The actions of leukotriene B_4 are receptor-dependent (Goldman & Goetzl, 1984) and are blocked by the guanine nucleotide-binding protein (G-protein) inhibitor, pertussis toxin (Becker *et al.*, 1985).

For 5-lipoxygenase product generation to occur, both release of endogenous arachidonic acid and activation of the 5-lipoxygenase are required. These two events are calcium-dependent (Feinstein & Sha'afi, 1983; Borgeat *et al.*, 1983; Samuelsson *et al.*, 1987). Furthermore, it has recently been demonstrated that activation of the 5-lipoxygenase may require its calcium-dependent translocation to the cell membrane (Rouzer & Samuelsson, 1987; Rouzer & Kargman, 1988). Therefore, although the requirement of calcium for release of endogenous substrate may be overcome by supplying exogenous substrate, it is likely that intracellular calcium mobilization is still necessary for significant activation of the 5-lipoxygenase.

Recently, there has been considerable focus on the action of arachidonic acid on neutrophils. At high concentrations (50–100 μM), arachidonic acid stimulates translocation of protein kinase C (McPhail *et al.*, 1984; Cox *et al.*, 1987). At lower concentrations (0.1–50 μM), incubation of neutrophils with arachidonic acid results in mobilization of intracellular

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calcium (Smith et al., 1987; Sha'afi et al., 1980), aggregation (O'Flaherty et al., 1979), production of superoxide (Maridonneau-Parini & Tauber, 1986; Badwey et al., 1987; Clark et al., 1987), and degranulation (Smith et al., 1987; O'Flaherty et al., 1979). Although the exogenous concentrations of arachidonic acid required to activate these cells are considerably higher than those for other compounds such as Paf, fMet-Leu-Phe and leukotriene B_4, they may be achieved in inflamed tissue (Hammarstrom et al., 1975; Greaves, 1986).

In light of these previous reports which have characterized arachidonic acid as a neutrophil stimulus, we investigated and characterized the effect of exogenous arachidonic acid on the 5-lipoxygenase. The results indicate that the interaction of exogenous arachidonic acid with the human neutrophil 5-lipoxygenase system involves not only the presentation of substrate, but also activation of the 5-lipoxygenase enzyme that is mediated, at least in part, via pertussis toxin-sensitive G-proteins.

Methods

Cell separation

Whole blood was obtained by venepuncture, and following dextran sedimentation of erythrocytes, neutrophils were purified by centrifugation on Ficoll-Paque cushions (Boyum, 1968). The erythrocytes remaining in the final pellet were removed by hypotonic lysis with water (20 s), and the cells were resuspended at a final concentration of 10^7 cells ml^{-1}. The entire cell separation was carried out at room temperature. The percentage of neutrophils in the cell suspensions used in this study exceeded 97%, and cell viability as determined by trypan blue exclusion was greater than 98%.

Cell incubations

The cell suspensions were warmed to 37°C for 5 min before incubation with arachidonic acid. Arachidonic acid, A23187 and 15-HPETE were dissolved in dimethylsulphoxide (DMSO) at a 1000 fold higher concentration than was finally required. The final concentration of DMSO present (maximum of 0.2%) did not stimulate detectable leukotriene synthesis. After the desired incubation time with the stimuli, the cells were inactivated by the addition of 500 μl of an ice-cold mixture of methanol/acetonitrile (50/50, v/v), containing internal standards (25 ng ml^{-1} of prostaglandin B_2 and 19-OH prostaglandin B_2). The samples were then diluted by the addition of 500 μl of distilled water, and stored at −20°C until analysed by r.p.-h.p.l.c.

In the experiments involving pertussis toxin, the cell suspensions were pre-incubated with the toxin at a final concentration of 0.25 μg ml^{-1} for 2 h at 37°C. During this period of time, the cells were gently swirled in a rotary water bath (New Brunswick Scientific, Edison, New Jersey, U.S.A.). After 2 h, the cells were incubated under the desired conditions, inactivated and stored as described above prior to r.p.-h.p.l.c. analysis.

To examine the effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) on 5-lipoxygenase product synthesis in response to arachidonic acid, neutrophils were pre-incubated with 200 pM GM-CSF at room temperature for 1 h. We have previously shown that these incubation conditions are optimal for maximal enhancement of leukotriene synthesis in response to Paf, fMet-Leu-Phe and A23187 (DiPersio et al., 1988). The cells were then warmed to 37°C for 5 min before stimulation with arachidonic acid.

Reverse phase-h.p.l.c. analysis of lipoxygenase products

Analysis of lipoxygenase products was carried out by r.p.-h.p.l.c. as described previously, using an on-line extraction procedure (Borgeat & Picard, 1988). Briefly, the denatured cell suspensions were centrifuged at 600 g for 10 min to remove precipitated material, and the supernatants were injected directly onto a Nova-Pak C_{18} (5 × 100 mm, 4 μm particles, Waters Millipore) protected with a Guard-Pak (μBondaPak C_{18}) cartridge (Waters Millipore). The various lipoxygenase products were eluted at 1.5 ml min^{-1} with gradients of organic solvents. Elution was monitored with fixed wavelength u.v. photometers at 229 and 280 nm. The compounds were identified by comparison of their retention times with those of authentic standards and by the ratio of their u.v. absorbances at 229 and 280 nm. 5,15 diHETE was measured at 229 nm under the same assay conditions as described above. Quantitation was performed by comparing peak heights of calibrated standards and corresponding compounds in samples after correction for recovery using the internal standard PGB_2. The lower level of detection was 0.5–1 ng.

Chemicals

Calcium ionophore A23187 and 5, 8, 11, 14 (all-*cis*) eicosatetraenoic acid (arachidonic acid) were obtained from the Sigma Chemical Company (St. Louis, Missouri, U.S.A.). Hank's Balanced Salt Solu-

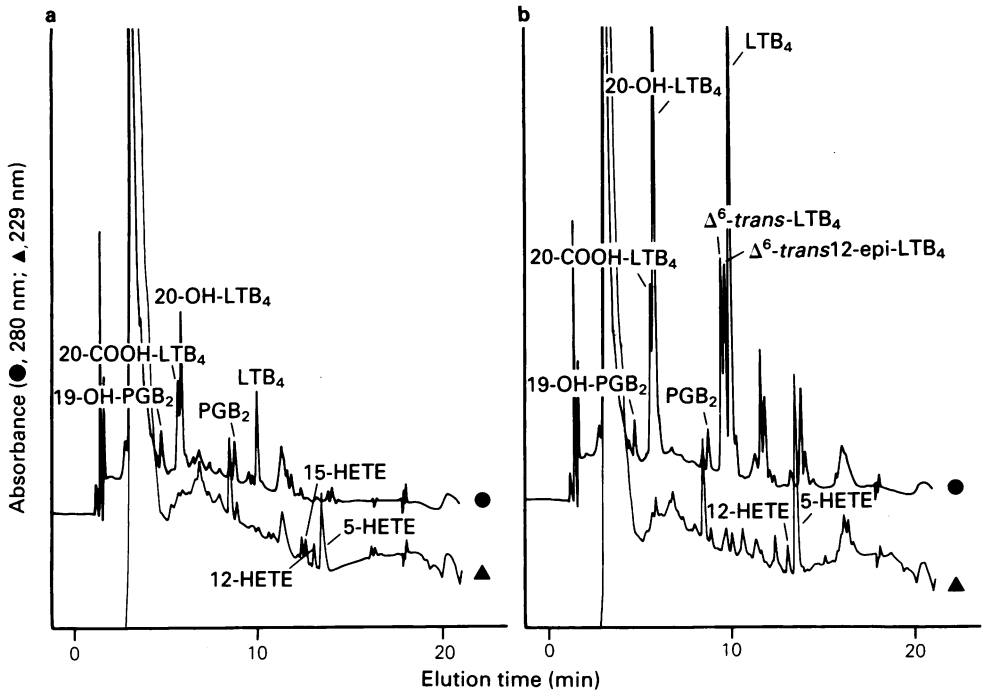

Figure 1 Reverse-phase h.p.l.c. chromatograms of lipoxygenase products from human neutrophils. Neutrophils from healthy human donors were prepared as described (see Methods), resuspended at a final concentration of 10^7 cells ml^{-1}, and incubated at 37°C for 15 min under the following conditions: (a) addition of 33 μM arachidonic acid, or (b) addition of 1.0 μM calcium ionophore A23187. After 15 min, the reactions were stopped by the addition of 500 μl of an ice-cold mixture of methanol/acetonitrile containing 12.5 ng of the internal standards (PGB_2 and 19-OH-PGB_2), and diluted to approximately 2 ml with 500 μl of distilled water. After centrifugation, the entire supernatant was injected. The amounts of PGB_2 and 19-OH-PGB_2 present in each chromatogram are therefore 12.5 ng. Elution, detection and quantitation were achieved as described (see Methods). Attenuation settings were 0.02 and 0.05 a.u.f.s. for 280 nm and 229 nm respectively. These results are from one experiment that is representative of at least ten others.

tion (HBSS) was from GIBCO (Burlington, Ontario, Canada), Ficoll-Paque was purchased from Pharmacia (Dorval, Québec, Canada) and all solvents were h.p.l.c. grade from Anachemia (Montréal, Canada). Granulocyte-macrophage colony-stimulating factor (GM-CSF) was a generous gift from the Genetics Institute (Cambridge, Massachussetts, U.S.A.), fura-2 was purchased from Molecular Probes Inc. (Eugene, Oregon, U.S.A.). 15S-hydroperoxy-eicosatetraenoic acid (15-HPETE) was synthesized from soy-bean lipoxygenase as previously described (Gardner, 1975), using arachidonic acid as substrate. The fatty acids (arachidonic acid and 15-HPETE) were purified either by silicic acid chromatography or reverse-phase high performance liquid chromatography (r.p.-h.p.l.c.) prior to use. Pertussis toxin was purchased from List Biological Labs Incorporated (Campbell, California, U.S.A.).

Results

Reverse phase-h.p.l.c. lipoxygenase products from arachidonic acid-stimulated human neutrophils

When human peripheral blood neutrophils were exposed to arachidonic acid they produced leukotriene B_4, its Ω-oxidation products (20-OH and 20-COOH-leukotriene B_4) and 5-HETE. Occasionally, we also observed synthesis of the non-enzymatically derived all-*trans* isomers of leukotriene B_4. However, this was not a consistent response during the course of this study. Figure 1a shows the characteristic arachidonic acid metabolite profile when the cell suspensions were exposed to 33 μM arachidonic acid for 15 min. Under these conditions, the major product was leukotriene B_4 (taking into account the combined synthesis of leukotriene B_4 and its Ω-

oxidation products). Production of the 15-lipoxygenase metabolite, 15-HETE was also observed under these conditions as was 12-HETE, which was derived from contaminating platelets. The 5-lipoxygenase product synthesis induced by arachidonic acid was not as powerful as that observed with the calcium ionophore A23187 (Figure 1b), where larger quantities of leukotriene B_4 and its metabolites as well as 5-HETE were detected (no 15-HETE was observed when A23187 was used as a stimulus). In addition to these compounds, A23187 also induced the production of the non-enzymatically-derived diHETEs.

Concentration-dependence of the effect of arachidonic acid on 5-lipoxygenase product synthesis

To characterize further the effect of exogenous arachidonic acid on 5-lipoxygenase product synthesis, neutrophils were incubated with increasing concentrations of the fatty acid for 15 min (Figure 2). A concentration-dependence was observed, with maximal formation of 5-lipoxygenase products at the highest dose of arachidonic acid used (33 μM). The threshold in all experiments was between 3.3 μM and 10 μM, depending on the donor. The major products were 20-OH- and 20-COOH-leukotriene B_4. The synthesis of the 15-lipoxygenase product, 15-HETE, also increased with increasing concentrations of arachidonic acid. Maximal stimulation of leukotriene synthesis by arachidonic acid was observed after an incubation time of 15 min. At this time point, the product profile was characterized by a marked increase in the levels of 20-OH and 20-COOH-leukotriene B_4 while levels of 5-HETE, 15-HETE and leukotriene B_4 had plateaued (data not shown).

Effect of pre-incubation of neutrophils with pertussis toxin on the 5-lipoxygenase product synthesis induced by arachidonic acid

To examine the hypothesis that arachidonic acid not only acts as a substrate but also stimulates 5-lipoxygenase product synthesis via a pertussis toxin-sensitive G-protein, neutrophils were preincubated with 0.25 $\mu g\,ml^{-1}$ of pertussis toxin for 2 h at 37°C before stimulation. These incubation conditions with pertussis toxin have been previously shown to cause significant inhibition of G-protein function in other systems (Becker *et al.*, 1985; Bradford & Rubin, 1985). These experimental conditions were chosen in order to maximize cell viability and experimental reproducibility. Pre-incubation of neutrophils with pertussis toxin prior to stimulation with arachidonic acid significantly inhibited 5-lipoxygenase product synthesis (Figure 3). The inhibitory effect of the toxin was observed even at the highest dose of arachidonic

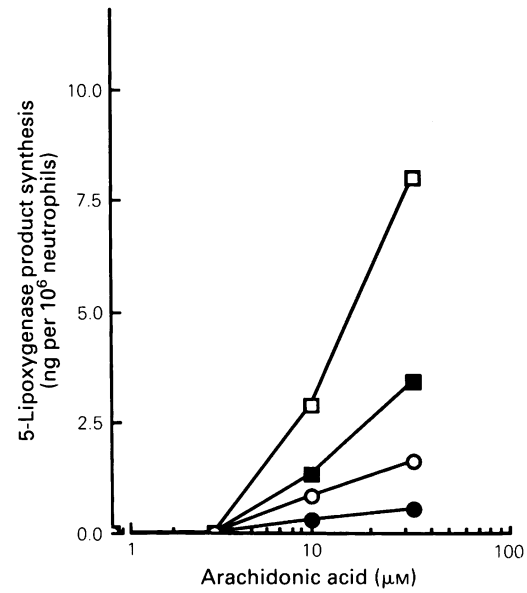

Figure 2 Concentration-dependence of the transformation of arachidonic acid by human neutrophils into 5-lipoxygenase products. Neutrophils (10^7 cells per ml) were stimulated at 37°C for 15 min with increasing concentrations of arachidonic acid. The reactions were then stopped and the resultant 5-lipoxygenase product synthesis was determined by r.p.-h.p.l.c. as described (see Methods): (●) leukotriene B_4; (■) 5-HETE; (○) 15-HETE; (□) Σ(20-OH- and 20-COOH-leukotriene B_4). The values are expressed as the mean of triplicate determinations from a single experiment which is representative of five others. Standard deviations of the mean were less than 5% of the mean.

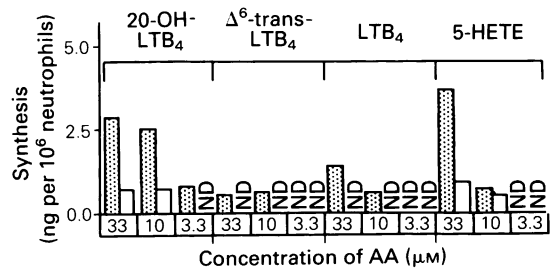

Figure 3 Effect of pre-treatment of human neutrophils with pertussis toxin (PT) on the 5-lipoxygenase product synthesis induced by arachidonic acid (AA). Neutrophils were pre-incubated for 2 h at 37°C with either 0.25 $\mu g\,ml^{-1}$ PT or an equivalent volume of diluent control, prior to stimulation for 15 min with one of 3 different concentrations of arachidonic acid. The results are expressed as described in the legend for Figure 2. ND = not detectable. Open column with PT; stippled column without PT.

acid used (33 μM), where production of leukotriene B_4 and its all-*trans* isomers was not detected in the cells pre-treated with pertussis toxin. Furthermore, levels of Ω-oxidation products of leukotriene B_4 and 5-HETE were reduced to less than 30% of control. The same trend was observed when 10 μM arachidonic acid was used as a stimulus, and pre-treatment of the cells with pertussis toxin completely inhibited the response to 3.3 μM arachidonic acid.

In support of a specific effect of pertussis toxin on 5-lipoxygenase product synthesis, pre-incubation of neutrophils with pertussis toxin had no effect on the arachidonic acid-induced synthesis of the 15-lipoxygenase product 15-HETE while the synthesis of 5-lipoxygenase products was inhibited by more than 60% under the same conditions (data not shown).

Effect of pre-incubation of human neutrophils with pertussis toxin on the transformation of 15-HPETE into 5,15 diHETE induced by A23187 or arachidonic acid

In order to determine whether the toxin was blocking the activation pathway of the 5-lipoxygenase or directly inhibiting the enzyme, we studied its effect on the activation of the 5-lipoxygenase induced by the calcium ionophore A23187, a non receptor-dependent agonist. In these experiments, 5-lipoxygenase activity was assessed by the measurement of the formation of 5,15 diHETE from exogenous 15-HPETE, a compound previously shown to be an excellent substrate for the 5-lipoxygenase in A23187-stimulated neutrophils (Borgeat *et al.*, 1983). By using an exogenous substrate, the possibility that pertussis toxin reduced 5-lipoxygenase product synthesis via an effect at the level of substrate release was eliminated. Neutrophils were pre-incubated with or without pertussis toxin under the conditions described above, and then incubated with both 10 nM A23187 and 3.3 μM 15-HPETE for 15 min at 37°C (Figure 4). Similar experiments were carried out on neutrophils incubated with both 3.3 μM arachidonic acid and 3.3 μM 15-HPETE. At the concentrations used, neither A23187 nor arachidonic acid stimulated detectable synthesis of 5,15 diHETE when added alone (data not shown). Incubation of neutrophils with 15-HPETE alone resulted in the formation of small amounts of 5,15 diHETE (Figure 4), a response that was unaffected by pre-treatment with pertussis toxin. In combination with 15-HPETE, both arachidonic acid and A23187 induced a significant increase in the conversion of 15-HPETE to 5,15 diHETE, indicating that both compounds stimulate 5-lipoxygenase mediated metabolism of 15-HPETE. Pre-incubation of neutrophils with pertussis toxin had no significant

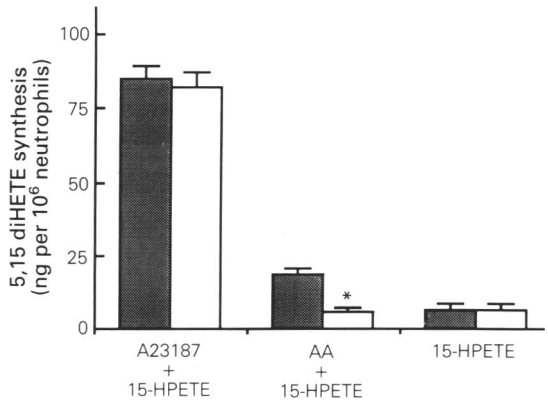

Figure 4 Effect of pre-treatment of human neutrophils with pertussis toxin (PT) on the activation of the 5-lipoxygenase by the calcium ionophore A23187 or arachidonic acid. Neutrophils were pre-incubated for 2 h at 37°C with 0.25 μg ml^{-1} PT (open columns) or the equivalent volume of diluent control (solid columns). The cells were then incubated under the following conditions: 3.3 μM 15-HPETE and 10 nM A23187, 3.3 μM 15-HPETE and 3.3 μM arachidonic acid (AA), or 3.3 μM 15-HPETE alone. After 15 min, the reactions were stopped and the resulting synthesis of 5,15 diHETE measured by r.p.-h.p.l.c. as described (see Methods). Synthesis of 5,15 diHETE under stimulation by either 10 nM A23187 or 3.3 μM arachidonic acid alone was not detectable. The results are expressed as mean of triplicate determinations of a single experiment that is representative of three; vertical bars show s.d. Significantly different from control values at $^*P < 0.01$ (Student's t test, $n = 3$).

effect on the synthesis of 5,15 diHETE induced by A23187 while under the same conditions, the synthesis of 5,15 diHETE induced by arachidonic acid was completely inhibited.

Effect of pre-incubation of neutrophils with GM-CSF on leukotriene synthesis in response to arachidonic acid

Neutrophils (5×10^6 ml^{-1}) were pre-incubated with GM-CSF (200 pM) for 1 h at room temperature before stimulation with increasing concentrations of arachidonic acid for either 5 or 15 min. The resulting synthesis of leukotriene B_4 and its Ω-oxidation products are shown in Figures 5a and b respectively. As shown in the previous results, increased leukotriene synthesis was observed with increasing concentrations of arachidonic acid. In these experiments, the lowest concentration of arachidonic acid with which we could detect leukotriene synthesis was 10 μM. However, when the cells were pre-incubated with GM-CSF, leukotriene synthesis was consistently detected when the cells were incubated with 3.3 μM

and sometimes as little as 1.0 μM arachidonic acid (data not shown). Furthermore, pre-treatment of the cells with GM-CSF lead to enhancement (between two and ten fold depending on the concentration of exogenous arachidonic acid) of leukotriene synthesis in response to concentrations of arachidonic acid which induced leukotriene synthesis in the cells untreated with GM-CSF.

Discussion

It has previously been shown that incubation of several different cell types with exogenous arachidonic acid results in the formation of 12- and 15-lipoxygenase products (Jorg *et al.*, 1982; Goldyne *et al.*, 1984; Lagarde *et al.*, 1984). In the case of 5-lipoxygenase products, however, the situation is different, and it is generally accepted that leukotriene synthesis in neutrophils (the system most extensively studied) requires activation of the enzyme (Borgeat *et al.*, 1983). The calcium ionophore A23187 has been shown to be a strong stimulus of the neutrophil 5-lipoxygenase (Borgeat & Samuelsson, 1979a), whereas inflammatory stimuli such as Paf and fMet-Leu-Phe activate the enzyme to a lesser degree (Salari *et al.*, 1985; Borgeat *et al.*, 1988). It is also recognized that exogenous arachidonic acid induces a small but consistent synthesis of 5-lipoxygenase products in neutrophils (Borgeat & Samuelsson, 1979b). In the present work, through the use of pertussis toxin, we have clearly demonstrated that in addition to being used as substrate, exogenous arachidonic acid also activates the human neutrophil 5-lipoxygenase, thereby reinforcing the concept that 5-lipoxygenase product synthesis requires activation of the 5-lipoxygenase.

Our studies with the G-protein inhibitor, pertussis toxin, provide strong evidence for receptor-dependent activation of the 5-lipoxygenase by exogenous arachidonic acid. G-proteins have been identified as early elements in the excitation response sequence of many cell types, and have been invariably associated with the transduction of receptor occupancy into cellular responses (Gilman, 1984). To date, the G-proteins represent the earliest known molecular components interacting with cell surface receptors (Gilman, 1984). The signalling transduction pathway in human neutrophils contains a G-protein which is linked to the cell surface receptors for many neutrophil agonists including leukotriene B_4 (Becker *et al.*, 1985), Paf (Naccache *et al.*, 1986) and fMet-Leu-Phe (Lad *et al.*, 1985), and the association between this G-protein and these receptors is specifically inhibited by pertussis toxin (Becker *et al.*, 1985; Lad *et al.*, 1985; Naccache *et al.*, 1986).

In the present work, the inhibitory effects of the toxin were specifically directed towards the stimulation of 5-lipoxygenase product synthesis by arachidonic acid, since we did not observe inhibition of the synthesis of the 15-lipoxygenase product 15-HETE. In addition, using the calcium ionophore A23187 as a 5-lipoxygenase stimulus, we were able to determine that pertussis toxin did not directly inhibit the 5-lipoxygenase enzyme. In this assay, we used the hydroperoxy fatty acid 15-HPETE as a substrate and measured its conversion into 5,15 diHETE as an indication of 5-lipoxygenase activation (Borgeat *et al.*, 1983). 15-HPETE was metabolized by neutrophils to 5,15 diHETE only to a minor extent unless A23187 or arachidonic acid were also added (Figure 5). Pertussis toxin had no effect on the ability of A23187 to activate the 5-lipoxygenase and stimulate

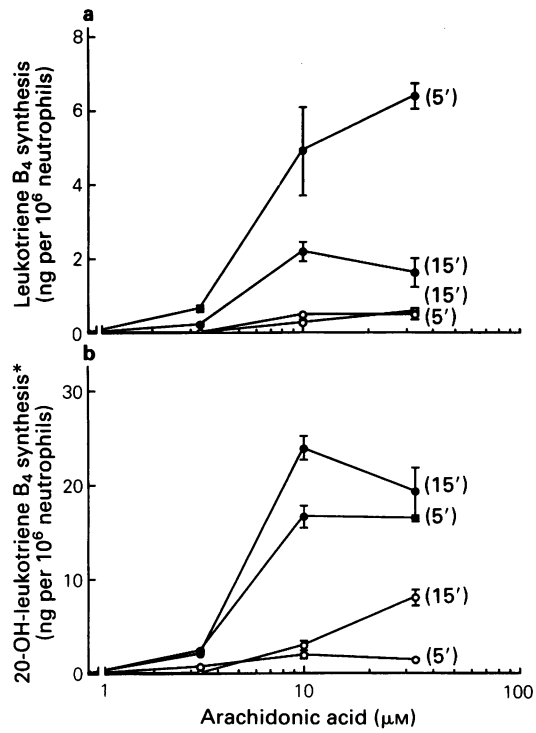

Figure 5 The effect of pre-incubation of neutrophils with GM-CSF on the synthesis of 5-lipoxygenase products induced by exogenous arachidonic acid. Neutrophils were pre-treated with 200 pM GM-CSF for 1 h at room temperature prior to stimulation with increasing concentrations of arachidonic acid for either 5 or 15 min: (●) plus GM-CSF; (○) without GM-CSF. The resultant synthesis of leukotriene B_4 and 20-OH-leukotriene B_4^* (i.e. Σ20-OH- and 20-COOH-leukotriene B_4) are shown in (a) and (b) respectively. The results are expressed as mean of triplicate determinations from one experiment that is representative of four; vertical bars show s.d.

the conversion of 15-HPETE into 5,15 diHETE, indicating that it does not directly inhibit the 5-lipoxygenase or interfere with activation of the 5-lipoxygenase by A23187. It is also important to note that arachidonic acid stimulated the transformation of 15-HPETE into 5,15 diHETE, and furthermore, that this transformation was inhibited by pertussis toxin. These results confirm our previous observation that pertussis toxin inhibits arachidonic acid-induced leukotriene synthesis and further supports our hypothesis that arachidonic acid activates the 5-lipoxygenase via a G-protein-dependent mechanism. Our results demonstrating that pre-incubation of neutrophils with GM-CSF enhanced the synthesis of leukotrienes in response to arachidonic acid also provide support to our hypothesis that arachidonic acid stimulates the 5-lipoxygenase, since we and others have recently shown that pre-incubation of neutrophils with GM-CSF enhances the ability of several other neutrophil agonists such as fMet-Leu-Phe (DiPersio *et al.*, 1988), C5a (Dahinden *et al.*, 1988) and Paf to stimulate leukotriene synthesis.

In the present study, it has proved difficult to establish the mechanism(s) involved in the activation of the 5-lipoxygenase and the stimulation of leukotriene synthesis by arachidonic acid. However, considering the established role of calcium in activation of the 5-lipoxygenase, our recent investigations on the effects of arachidonic acid on the mobilization of intracellular calcium, and the observation that leukotriene B_4 can also activate the 5-lipoxygenase, it is possible that activation of the 5-lipoxygenase by arachidonic acid is related to its ability to promote calcium mobilization, and involves activation of the leukotriene B_4 receptor, probably by a positive feedback loop due to *de novo* synthesis of leukotriene B_4 (Figure 6). The latter mechanism could be due to transformation of exogenous arachidonic acid into leukotriene B_4 by 5-lipoxygenase which is already active, or by a direct effect of arachidonic acid on intracellular calcium stores (Beaumier *et al.*, 1987), resulting in the activation of the 5-lipoxygenase, leukotriene B_4 synthesis from exogenous arachidonic acid, and subsequent activation of the leukotriene B_4 receptor. In both cases, stimulation of the leukotriene B_4 receptor would enhance the activation of the 5-lipoxygenase and consequently, leukotriene synthesis from exogenous arachidonic acid. In a separate study, we have obtained evidence that both of these mechanisms exist and account for the effect of arachidonic acid on calcium mobilization. Additional support for this hypothesis arises from the fact that the characteristics of arachidonic acid and leukotriene B_4 as neutrophil agonists are strikingly similar. Indeed, stimulation of human neutrophils by leukotriene B_4 elicits a similar range of functions as those previously described for arachidonic acid

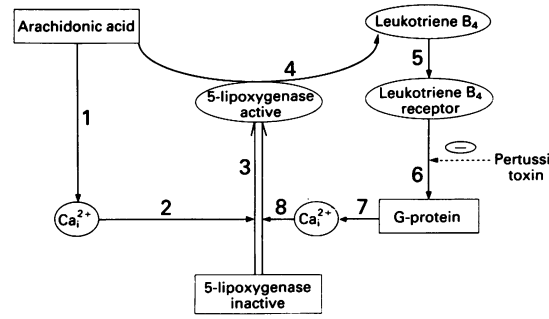

Figure 6 Possible mechanism for the stimulation of the 5-lipoxygenase by exogenous arachidonic acid. Numbers represent the postulated sequence of events. This scheme implies a critical role for calcium in the activation of the 5-lipoxygenase, and also that leukotriene B_4 activates this enzyme. In this scheme, the triggering event for the induction of 5-lipoxygenase product synthesis by arachidonic acid would be the direct effect of arachidonic acid on intracellular levels of calcium, which in turn would lead to the activation of the 5-lipoxygenase (events 1 to 3). Formation of leukotriene B_4 would occur, leading to a positive feedback effect of leukotriene B_4 on the activation of the 5-lipoxygenase (events 4 to 9). The positive feedback could eventually be switched off due to desensitization of the leukotriene B_4 receptor by leukotriene B_4.

(O'Flaherty *et al.*, 1979; Hafstrom *et al.*, 1981; White *et al.*, 1983; Smith *et al.*, 1987). Furthermore, and most importantly, cellular responses to both leukotriene B_4 (Becker *et al.*, 1985) and arachidonic acid are sensitive to pertussis toxin. The positive feedback loop could finally be switched off as a consequence of desensitization of the leukotriene B_4 receptor by leukotriene B_4, as has previously been reported (Sha'afi *et al.*, 1981).

The ability of arachidonic acid to activate the human neutrophil 5-lipoxygenase may be of considerable patho-physiological importance *in vivo*. The fact that micromolar concentrations of arachidonic acid are required to observe these effects *in vitro* does not diminish the importance of this finding since large quantities of arachidonic acid have been described in inflammatory lesions (Hammarstrom *et al.*, 1975; Greaves, 1986). Furthermore, in this work, we have demonstrated that exposure of neutrophils to GM-CSF *in vitro* increases the sensitivity of these cells to arachidonic acid (Figure 5). If GM-CSF has the same effect *in vivo*, it may have a dramatic influence on inflammation by increasing the activation of the 5-lipoxygenase not only in response to agents such as Paf and C5a (Dahinden *et al.*, 1988), but also arachidonic acid.

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Apparent affinity of 1,3-dipropyl-8-cyclopentylxanthine for adenosine A_1 and A_2 receptors in isolated tissues from guinea-pigs

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1 The classification of adenosine receptor subtypes (A_1 and A_2) in intact tissues has been based on the order of agonist potency. In this study the apparent affinity of 1,3-dipropyl-8-cyclopentylxanthine (CPX), an antagonist which has been reported to be A_1 selective, and the non-selective antagonist 1,3-dimethyl-8-phenylxanthine (8PT) has been evaluated on isolated tissues from the guinea-pig.

2 The isolated tissues used were atria (bradycardic response, proposed A_1 sub-type), aorta and trachea (relaxant response, proposed A_2 sub-type).

3 Both the xanthines antagonized responses to adenosine in the three tissues but had little or no effect on responses to carbachol (atria), sodium nitrite (aorta) or isoprenaline (trachea).

4 pA_2 values for 8PT were similar on the three tissues (6.3–6.7), however, the pA_2 value for CPX on the atria (7.9–8.4) was greater than that on the aorta (6.6) or trachea (6.6).

5 These results support the suggestion that the adenosine receptors which mediate bradycardia in the atrium are of the A_1 sub-type and that those which mediate relaxation in the aorta and trachea are of the A_2 type.

Introduction

It has been proposed that adenosine receptors can be subdivided into A_1 and A_2 sub-types (Van Calker *et al.*, 1979). This subdivision is based on the relative potency or binding affinity of a series of adenosine analogues in a variety of biochemical and isolated tissue preparations (Collis, 1985). However, there have been few reports of antagonists which show selective affinity for the proposed subtypes of adenosine receptors, particularly in intact tissues (Collis *et al.*, 1987). Recently, 1,3-dipropyl-8-cyclopentylxanthine (CPX) has been reported to exhibit selective A_1 receptor affinity in rat brain membranes (Bruns *et al.*, 1987) and in membranes from adipocytes and platelets (Martinson *et al.*, 1987). It has also been reported to antagonize the bradycardic effect of an adenosine analogue in the rat isolated heart without effects on coronary flow (Haleen *et al.*, 1987). In the present study we have evaluated the apparent affinity of this xanthine in guinea-pig tissues which are thought to possess either A_1 (atria, Collis, 1983; Collis & Saville, 1984) or A_2 receptors (aorta and trachea, Brown & Collis, 1982; Collis & Brown, 1983) based on the relative potency of agonists. For comparison, 1,3-dimethyl-8-phenyl xanthine (8PT) was also evaluated since it is thought to be non-selective for adenosine receptor sub-types (Collis *et al.*, 1985).

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Methods

Rings of isolated thoracic aorta and trachea or atrial pairs from guinea-pigs were mounted in organ baths containing modified Krebs solution at 37°C (composition mM: NaCl 118.4, $NaHCO_3$ 25.0, glucose 5.5, KCl 4.7, $CaCl_2$ 2.5, $KHPO_4$ 1.2, $MgSO_4$ 1.2, $CaNa_2$ EDTA 0.03) gassed with 95% O_2:5% CO_2. Isometric tension development was recorded with UF1 strain gauges and 'Lectromed' polygraphs.

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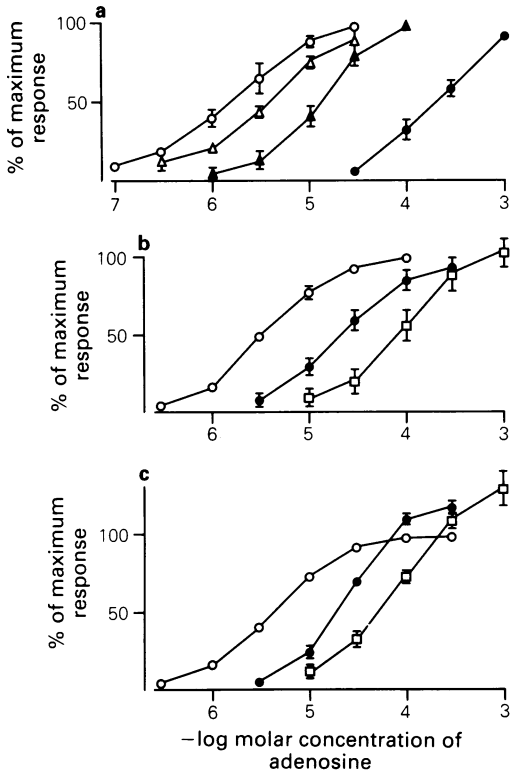

Figure 1 The effect of 1,3-dipropyl-8-cyclopentyl-xanthine (CPX) on responses of guinea-pig isolated tissues to adenosine. (a) Negative chronotropic responses of isolated atria: (○) control ($n = 12$); (△) 10 nM CPX ($n = 3$); (▲) 100 nM CPX ($n = 4$) and (●) 1 μM CPX ($n = 5$). (b) Relaxant responses of isolated aorta: (○) control ($n = 10$); (●) 1 μM CPX ($n = 6$) and (□) 5 μM CPX ($n = 4$). (c) Relaxant responses of isolated trachea: (○) control ($n = 11$); (●) 1 μM CPX ($n = 4$); (□) 5 μM CPX ($n = 7$). Responses are expressed as a % of the maximum obtained on the first concentration response curve.

The nucleoside transport inhibitor dipyridamole (10 μM) and the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl) adenine (10 μM) were routinely present in the Krebs solution when adenosine was used as the agonist. When 2-chloroadenosine was used as the agonist, dipyridamole (10 μM) was present in the Krebs solution. Indomethacin (5 μM) was added to the Krebs solution when tracheal preparations were used to reduce spontaneous activity and prevent contractile effects of adenosine (Advenier *et al.*, 1982; Farmer *et al.*, 1987).

The tissues were placed under a resting tension of 1 g and allowed to equilibrate for 1 h. Sub-maximal contractions (close to 50% maximal) of aortic and tracheal rings were evoked by 3 μM phenylephrine or 0.1 μM carbachol, respectively. Adenosine was then added cumulatively to the bath contents and the evoked relaxation measured. The atrial pairs were allowed to beat spontaneously and the decrease in rate evoked by adenosine or 2-chloroadenosine was measured. Concentration-response curves were also generated to carbachol (atria) to sodium nitrite (aorta) and to isoprenaline (trachea, in presence of 10 μM phentolamine) to check for effects of the xanthines which were not related to adenosine receptors. After generation of the first agonist concentration-response curve, the tissues were incubated with one of the xanthines and the agonist concentration-response curve was repeated. Preliminary experiments established the incubation period needed for equilibrium to be reached with the xanthines (30 min). The effect of the vehicle for CPX (dimethylsulphoxide (DMSO), final concentration 0.005%) on concentration-response curves to adenosine and 2-chloroadenosine was also determined. The magnitude of the xanthine-induced rightward shift of the agonist concentration-response curve was assessed at the 50% maximal level, (in trachea where CPX caused an increase in maximal response to adenosine the concentration-ratio was measured at an equal level of relaxation which was 50% of the maximal response on the control concentration-response curve).

Concentration-ratios over a range of xanthine concentrations were analysed as described by Arunlakshana & Schild (1959) using regression analysis of the pooled data to generate pA_2 values. At least three replicates were performed for each concentration of antagonist. The maximum concentration of each antagonist used was dictated by its aqueous solubility (5 μM for CPX, 10 μM for 8PT).

Data are expressed as mean ± s.e.mean or mean with 95% confidence limits in the case of pA_2 values. Significant differences ($P < 0.05$) were calculated by Student's t test.

Compounds used

Adenosine (Sigma), adenosine deaminase (EC 3.5.4.4., Sigma), carbachol (Sigma), 2-chloroadenosine (Sigma), 1,3 dipropyl-8-cyclopentyl xanthine (CPX, synthesized by Dr R. James, ICI), dipyridamole (Persantin, Boehringer Ingelheim), erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA, Burroughs Wellcome), phentolamine mesylate (Ciba), (−)-phenylephrine hydrochloride (Sigma), 8-phenyltheophylline (Sigma), (±)-isoprenaline hydrochloride (Sigma), indomethacin (Sigma), sodium nitrite (Analar). CPX was dissolved in 1% aqueous

DMSO at 1 mM, 8PT in 80% v/v methanol containing 0.2 M NaOH at 10 mM.

Results

Both xanthines antagonized responses to adenosine, causing dextral displacements of the agonist concentration-response curves. The effect of a range of concentrations of CPX on responses in the atria, aorta and trachea are shown in Figure 1. CPX was a more potent antagonist in the atria (e.g. concentration-ratio at 1 μM = 116) than in the aorta and trachea (e.g. concentration-ratios at 1 μM = 6 and 4 respectively). In the trachea, but not in the aorta, CPX (1 and 5 μM) enhanced the maximum relaxation evoked by adenosine. 8PT appeared to be an equi-effective antagonist in the three tissues (e.g. concentration-ratios at 10 μM = 19, 27, 28 in atria, aorta and trachea respectively).

The Schild plot slopes obtained with CPX and 8PT in the three tissues did not differ significantly from unity over the antagonist concentration-ranges that could be used. The pA_2 values for 8PT were similar in all three tissues (Table 1, Figure 2), however CPX exhibited a higher pA_2 on atria compared with aorta and trachea (Table 1, Figure 2).

At the highest concentrations used the xanthines did not alter responses of atria to carbachol or of aortae to sodium nitrite ($n > 4$), however CPX 5 μM caused a small but significant potentiation of responses to isoprenaline on the trachea (control − log $EC_{50} = 8.04 \pm 0.05$, in presence of CPX = 8.27 ± 0.03, $P < 0.05$). The vehicle for CPX had no significant effect on concentration-response curves to adenosine or 2-chloroadenosine ($n > 4$).

In the absence of exogenous adenosine, neither CPX nor 8PT altered the amplitude of the contractile responses evoked by phenylephrine or carbachol in the aorta and trachea respectively. Both antagonists increased the rate of beating of isolated atria, e.g. CPX increased rate from 151.8 ± 6.8 beats min^{-1} to 208.9 ± 4.9 beats min^{-1} ($n = 15$, $P < 0.001$). Since this increase in rate could be due to antagonism of the negative chronotropic action of endogenous adenosine, further experiments were performed to investigate the effects of adenosine deaminase (1 U ml^{-1}) (EHNA was omitted). In these experiments 2-chloroadenosine was used as the agonist, as it is resistant to deamination.

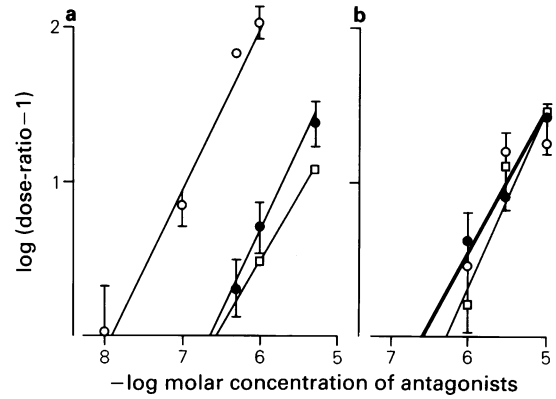

Figure 2 Schild plots of the interaction of (a) 1,3-dipropyl-8-cyclopentylxanthine and of (b) 8-phenyltheophylline with adenosine evoked responses in guinea-pig isolated atria (○), aorta (●), and trachea (□); $n = 3$ or more for each concentration of antagonist.

In the absence of adenosine deaminase, CPX had a pA_2 of 8.14 (7.7–8.7) against 2-chloroadenosine (Schild plot slope = 0.91, $n = 15$). In the presence of adenosine deaminase, the initial beating rate of the atria was 203.4 ± 8.1 beats min^{-1}, and this was not significantly enhanced by CPX (209.8 ± 7.3 beats min^{-1}, $n = 15$). In the presence of adenosine deaminase, the pA_2 value of CPX was increased to 8.41 (7.7–9.6, Schild plot slope = 0.95, $n = 15$); however, this increase was not statistically significant.

Table 1 Antagonist data for 1,3-dimethyl-8-phenylxanthine (8-PT) and 1,3-dipropyl-8-cyclopentylxanthine (CPX) in guinea-pig atria, aorta and trachea (adenosine as agonist)

Xanthine	*Tissue*	*Concentration range* (μM)	*Schild plot slope*	pA_2*	n
8PT	Atria	1–10	0.84 ± 0.22	6.68(5.8–8.6)	14
	Aorta	1–10	0.82 ± 0.19	6.69(6.0–7.9)	11
	Trachea	1–10	1.20 ± 0.20	6.3 (5.6–7.2)	15
CPX	Atria	0.01–1	1.08 ± 0.12	7.91(7.2–8.8)	15
	Aorta	0.5–5	1.05 ± 0.24	6.63(6.4–7.16)	14
	Trachea	1–5	0.87 ± 0.08	6.55(6.3–6.9)	11

* 95% confidence intervals.

Discussion

The results of this study confirm those obtained with membrane preparations which have indicated that CPX is an A_1 selective adenosine receptor ligand. 8-PT, in contrast, did not show selectivity between the receptors in the three tissues, confirming previous suggestions that this compound is non-selective between adenosine receptor sub-types. The pA_2 values for 8PT reported in this paper are similar to those previously published (Collis *et al.*, 1985). The pA_2 values obtained for CPX in the aorta and trachea (6.55–6.6) are close to the $-\log K_i$ values reported in binding or adenylate cyclase based assays at A_2 receptors (6.47–7.16, Martinson *et al.*, 1987; Bruns *et al.*, 1987; Lohse *et al.*, 1987). It is difficult to reach a conclusion as to the nature of the antagonism evoked by CPX or 8PT in the aorta and trachea, because of the limited range of concentrations employed. However, higher concentrations could not be used because of the poor aqueous solubility of these xanthines. The enhancement of maximal responses to adenosine on the trachea may have been a consequence of a non-specific enhancement of relaxant responses in this tissue as evidenced by the effect of CPX on isoprenaline-induced relaxation.

Antagonism of adenosine-evoked responses by CPX in the guinea-pig atrium appears to be consistent with a competitive interaction over the concentration-range used. The pA_2 value for CPX at receptors in the atrium (7.9) is markedly less than the $-\log K_i$ values reported in cyclase based or binding studies with rat adipocytes or CNS membranes (8.9–9.3, Martinson *et al.*, 1987; Bruns *et al.*, 1987; Lohse *et al.*, 1987; Ukena *et al.*, 1986), and slightly less than that in binding data from guinea-pig tissues (8.4, Ukena *et al.*, 1986). One possible reason for the lower pA_2 values found in the guinea-pig atria could be that it is due to the increase in basal beating rate which the antagonist evokes. Kenakin & Beek (1987) have shown that an increase in basal activity of atria caused by an adenosine antagonist can lead to an underestimation of antagonist affinity. Kenakin & Beek (1987) used pharmacological resultant analysis to compensate for the stimulatory effects of the antagonists that they used. We have used a different method, that of removing the effects of endogenous adenosine with a concentration of adenosine deaminase known to abolish responses to large doses of the purine in guinea-pig atria (Collis & Pettinger, 1982). When this procedure was used the pA_2 for CPX in the guinea-pig atria became similar to the $-\log K_i$ reported for guinea-pig CNS tissue (Ukena *et al.*, 1986).

The reason why the affinity of CPX for A_1 receptors is apparently less in guinea-pig tissue and membranes than in rat cell membrane preparations is unclear (present study and Ukena *et al.*, 1986; Martinson *et al.*, 1987; Bruns *et al.*, 1987; Lohse *et al.*, 1987). It has been shown that there are species differences in antagonist binding affinity at A_1 receptors in the mammalian brain and that the affinities are higher in membranes from the rat than from guinea-pig tissue (Ukena *et al.*, 1986; Ferkany *et al.*, 1986). A systematic study of antagonist affinity in isolated tissues from the two species is required to establish whether there is a consistent species-related difference. Preliminary studies have indicated, however, only small differences between the apparent affinities of some xanthines in rat and guinea-pig atria (Collis *et al.*, 1988).

In conclusion, the results of this study demonstrate that CPX is an adenosine receptor antagonist which exhibits a higher affinity for receptors in guinea-pig atria than those in the aorta and trachea of this species. The selectivity of this antagonist supports the hypothesis that there are two subtypes of adenosine receptors and that the A_1 type mediates the negative chronotropic effect of adenosine in the atrium whereas the A_2 subtype mediates the relaxant effect of the purine in the trachea and the aorta.

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Effects of caffeine and ryanodine on depression of post-rest tension development produced by Bay K 8644 in canine ventricular muscle

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1 Post-rest inotropy in canine ventricular myocardium has proved a useful indicator of sarcoplasmic reticular calcium release. This phenomenon is converted to rest depression by the calcium channel activator (agonist), Bay K 8644 as well as other chemically diverse agents such as caffeine and ryanodine.

2 Rapid cooling contractures and post-rest contraction amplitude were used as independent measures of sarcoplasmic reticular calcium content and release. Simultaneous recordings of transmembrane action potentials and their accompanying contractions were obtained to determine the association between electrophysiological and mechanical events. The present study was designed to elucidate the mechanism by which Bay K 8644, caffeine and ryanodine alter force production after variable periods of rest.

3 Bay K 8644 (1 μM) increased steady state contraction in response to a constant train of stimulation, caused rest-depression after 2 and 8 min rest, prolonged action potential duration and increased action potential plateau amplitude. Augmented steady state tension was not accompanied by any change in time to peak tension or rapid cooling contracture amplitude. However, the post-rest rapid cooling contracture was greatly diminished compared to that observed prior to Bay K 8644 treatment.

4 Caffeine (3 and 5 mM) caused rest-depression with an increase in steady state contraction amplitude. Along with this there was a slight decrease in action potential duration and plateau amplitude and an increase in time to peak tension. The rapid cooling contractures were virtually abolished at all conditioning intervals. The effect of caffeine on twitch tension and cooling contracture is consistent with the ability of this compound to inhibit calcium sequestration by the sarcoplasmic reticulum.

5 A combination of Bay K 8644 and caffeine caused significantly less rest-depression than that seen with Bay K 8644 alone. The augmented twitch tension was accompanied by a long time to peak tension and action potential duration. However, there was no increase in the amplitude of the rapid cooling contracture, either after a regular train of stimulation or after rest, compared to that seen after Bay K 8644.

6 Ryanodine (10 nM), produced rest-depression, reduced steady state twitch tension and augmented the rest-depression produced by Bay K 8644. The steady state rapid cooling contracture remained unchanged when both agents were present simultaneously, while the post-rest rapid cooling contracture was significantly depressed compared to that observed with Bay K 8644 alone.

7 Bay K 8644 and ryanodine appear to have similar actions with respect to promoting diastolic loss of calcium from the sarcoplasmic reticulum. Although caffeine also decreases post-rest potentiation, it antagonizes rest-depression caused by Bay K 8644. The data from these experiments

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suggest that this reversal is a result of depressed intracellular calcium buffering and enhanced myofilament sensitivity produced by caffeine in the presence of increased transmembrane calcium influx promoted by Bay K 8644.

Introduction

Previous studies of the effect of the racemic dihydropyridine Bay K 8644 on microscopic currents in cardiac tissue have shown that it facilitates the inwardly directed calcium current (Thomas *et al.*, 1985a) by increasing the open time of membrane-bound voltage-gated channels (Hess *et al.*, 1984). In mechanical studies on preparations of isolated ventricular muscle, the addition of Bay K 8644 increases peak isometric tension development in response to rhythmic electrical stimulation (Thomas *et al.*, 1985a; Hryshko & Bose, 1985; Bose *et al.*, 1987). However, in addition to increasing twitch tension in response to steady state stimulation, this inotropic agent also significantly impaired the ability of the muscle to develop tension after a period of rest (Hryshko, 1987; Bouchard *et al.*, 1988; Bose *et al.*, 1988a,b; Hryshko *et al.*, 1989).

Contraction amplitude in ventricular muscle from several mammalian species is transiently increased after brief periods of rest. This potentiation is biphasic in that a maximum is observed following some characteristic period of rest (Koch-Weser & Blinks, 1961; 1963; Schouten *et al.*, 1987). The sarcoplasmic reticulum has been implicated in rest-potentiation (Bers, 1985; Bose *et al.*, 1988a; Walsh & Tormay, 1989). In contrast to the phenomenon of post-rest inotropy which is observed with short duration of rest, extended periods of rest lead to depressed post-rest contractions in most mammalian species (Koch-Weser & Blinks, 1961; 1963), similar to the effect observed with Bay K 8644. This depression is due to a progressive loss of calcium from the sarcoplasmic reticulum (King & Bose, 1983; Bers, 1985; Hilgemann, 1986a; Bridge, 1986). In atrial preparations (Thomas *et al.*, 1985a) even after prolonged rest there is a substantial residual 'rested state' contraction, believed to be due to release of calcium from the sarcoplasmic reticulum. This is in contrast to the 'rested state' contraction in ventricular muscle from some mammalian species which is predominantly due to trans-sarcolemmal calcium influx and is characterized by a slower onset of a very small contraction (Reiter *et al.*, 1978). The inhibition of post-rest force production of the type seen in the presence of Bay K 8644 is similar to that observed during long rest intervals in control muscles, suggesting that this agent might enhance diastolic calcium loss from the sarcoplasmic reticulum leading to a reduction in the availability of activator calcium from the release site in this organelle.

Caffeine and ryanodine are commonly used to study excitation-contraction coupling in mammalian cardiac muscle as they inhibit calcium release from the sarcoplasmic reticulum or its uptake into this organelle (Weber & Herz, 1968; Blinks *et al.*, 1972; Fabiato, 1981; Bers *et al.*, 1987). The objective of the present study was to design experiments which would make use of the known properties of these compounds to reveal the mechanism by which Bay K 8644 affects post-rest force production. To achieve this, Bay K 8644 was tested alone and in combination with either caffeine or ryanodine to determine if they had a similar mechanism of action on post-rest tension development. It was reasoned that this should make the combined effect of Bay K 8644 and any of the other agents on rest potentiation additive. Rapid cooling contractures were also used to assess indirectly sarcoplasmic reticular calcium content. Simultaneous recordings of transmembrane action potentials and twitch contractions were obtained to determine the relationship between electrical and mechanical events within a given contraction. Preliminary data have been presented to the Federation of American Societies for Experimental Biology (Bouchard *et al.*, 1988).

Methods

Experimental preparation

Mongrel dogs of either sex weighing 8–12 kg were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.v.). Hearts were rapidly excised through a lateral thoracotomy and the coronary vasculature flushed with 50 ml cold (4°C) Krebs-Henseleit (KH) solution. KH solution had the following composition (mM): NaCl 118, KCl 4.7, CaCl 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.4, $NaHCO_3$ 25 and glucose 11. Thin, free running right ventricular trabeculae (0.2–0.5 mm diameter) were tied with 8-0 silk (Matuda) and dissected free from the wall of the heart.

Tension measurements

Isolated muscles were obtained as mentioned previously and transferred to a 2 ml horizontal recircu-

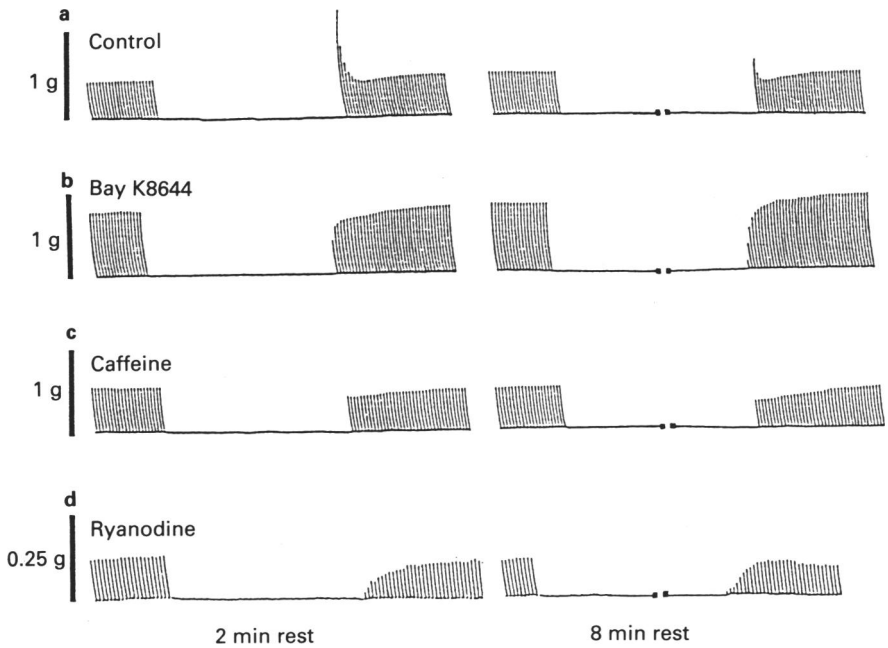

Figure 1 Typical response of canine ventricular trabeculae to varying periods of rest in an untreated muscle (a) and after treatment with 1 μM Bay K 8644 (b), 5 mM caffeine (c) and 10 nM ryanodine (d). Rest durations of 2 (left) and 8 (right) min were imposed after a continuous train of stimulation at a frequency of 0.5 Hz.

lating bath containing oxygenated (95% O_2/5% CO_2) KH solution maintained at a pH of 7.4 and a temperature of 37°C. Muscles were field stimulated by pulses delivered by a computer-driven stimulus sequencer (Boyechko & Bose, 1982) at a frequency of 0.5 Hz and an amplitude 75–100% above threshold voltage and equilibrated for 45–60 min after which they were stretched to an optimal length for maximal isometric force production. Isometric contractions were recorded with a force transducer (Grass FT03C). Periods of rest ranging from 30–240 s were randomly imposed on the otherwise constant (steady state) stimulus pattern.

Rapid cooling contracture

The muscle chamber formed part of a recirculation apparatus with a single bath and two reservoirs connected in parallel with the bath. This system allowed virtually instantaneous (1 s) switching of bathing solutions. Rapid cooling contractures were elicited by cooling the preparation from 37° to 0–2°C by changing the solution entering the muscle chamber through a parallel by-pass system. Identical rest periods to those used in the twitch contraction experiments were examined, where the rapid cooling contractures were measured in the place of a regularly driven beat (steady state rapid cooling contracture) or after variable periods of inactivation (post-rest rapid cooling contracture).

Electrophysiology

For simultaneous recordings of transmembrane action potentials and contractions, one end of the trabecula was fixed with stainless steel insect pins to a silicone rubber base in the bath and the other end was connected to an isometric force transducer. Glass micropipettes were pulled with a Brown & Flaming micropipette puller (Model P-77; Sutter Instruments Co.) and filled with 3M KCl (tip impedance 8–25 MΩ) to record transmembrane action potentials from the surface cells of the trabeculae. Electrodes were connected to a high input impedance amplifier (Neuroprobe 1600; Transidyne General Corp.). A 50 mV calibration signal and 0 mV absolute voltage level were recorded prior to each action potential. These signals, together with simultaneous recordings of action potentials and contractions were recorded on a thermal chart recorder (Model 8500; Astro Med Inc.) and a Zenith VHS recorder (Model VP-2220; Zenith Corp.) coupled to

an analog to digital converter and PCM adapter (Model VR-10; Instrutec Corp.). Signals were monitored during experiments with a 4 channel Hewlett-Packard storage oscilloscope.

Drugs

Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5 - carboxylate) was a generous gift from Dr A. Scriabine (Miles Institute of Preclinical Research) and was dissolved in 100% ethanol. The final concentration of ethanol in the solution did not exceed 0.1% and was usually 0.01%. In the presence of 0.01% there was no decrease in steady state contractions while with 0.1% there was a 25% decrease in contractile force. However there was no decrease in rest potentiation with this entire range of concentrations. Caffeine and ryanodine were obtained from Sigma Chemicals and were dissolved in twice distilled deionized water. All chemicals for physiological solutions were reagent grade. Bay K 8644 was used at a concentration of 1 μM. Although rest depression could be demonstrated with much lower concentrations (i.e., 0.01 μM or more), the onset and stabilization of the effect were much slower. The same was true for ryanodine which was effective in concentrations as low as 1 nM. The concentration range of caffeine chosen (3–5 mM) was similar to the one shown by us previously to impair sarcoplasmic reticulum-mediated contractions (King & Bose, 1983; Hryshko *et al.*, 1989). Further increase in the concentrations of caffeine produced greater effects but the tissue response deteriorated.

Statistics

Statistical significance was determined with analysis of variance followed by Duncan's new multiple range test for multiple comparisons and Student's paired t test for self-controlled experiments (Steel & Torrie, 1960). The level of significance chosen for all experiments was $P < 0.05$. Data have been expressed as mean $\pm$ s.e.mean.

Results

Rest potentiation

Figure 1 depicts the contractile response of canine isolated right ventricular trabeculae to 2 and 8 min rest periods. The control muscle (Figure 1a) shows post-rest potentiation after both rest intervals. Addition of Bay K 8644 (1 μM, 1b), caffeine (3 mM, 1c) and ryanodine (10 nM, 1d) resulted in rest-depression. The amplitudes of post-rest contractions were consistently found to be smaller after 8 min rest compared to those observed after rest for 2 min in all preparations studied.

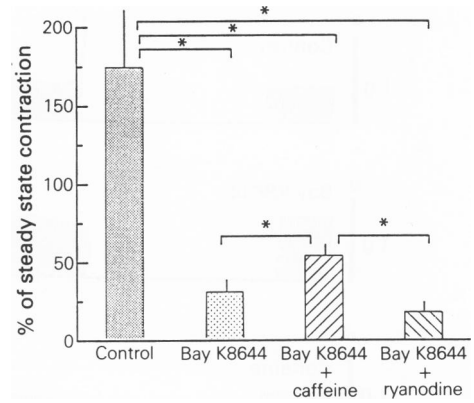

Figure 2 Pooled data from 6 experiments showing the effect of combining caffeine (5 mM) or ryanodine (10 nM) with Bay K 8644 (1 μM) on tension development of the first post-rest beat after 8 min rest. Results are expressed as a percentage of steady state tension of the contraction preceding the test interval. Statistical significance of observed differences were analyzed with an analysis of variance followed by Duncan's new multiple range test for multiple comparisons. * $P < 0.05$.

Figure 2 illustrates the effect of combining Bay K 8644 with caffeine or ryanodine on tension developed after 8 min of rest. As may have been expected if these compounds acted by a common functional mechanism, further addition of ryanodine resulted in an increase in the depression of post-rest twitch tension compared to that observed with Bay K 8644 alone. In contrast, the addition of caffeine to the Bay K 8644-treated preparation reduced the rest-depression. Both combinations of treatments produced a slight depression of tension development in response to a rhythmic train of stimulation, but this was statistically not significant. The most interesting difference between the contractile response in the presence of Bay K 8644 alone or in combination with one of the two other inotropes, was seen in contractions following a period of rest. Qualitatively similar changes were seen after rest for 2 min (this study; Hryshko, 1987; Bouchard *et al.*, 1988; Hryshko *et al.*, 1989).

Rapid cooling contractures

Rapid cooling contractures were elicited to estimate the functional capacity of the sarcoplasmic reticulum

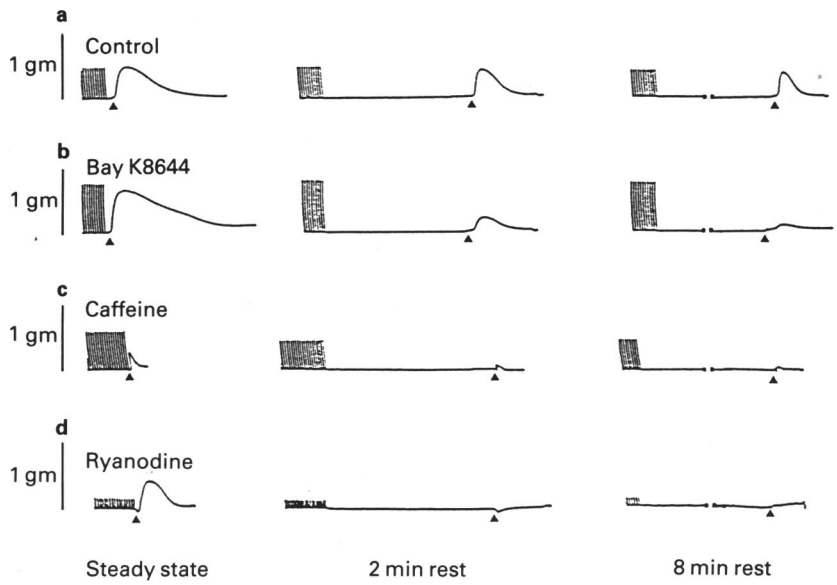

Figure 3 Typical responses of canine ventricular trabeculae to a rapid change in temperature from 37° to 0°C in less than 1 s. The rapid cooling contractures were obtained in the place of a regularly driven beat (left), or after 2 (centre) and 8 (right) min rest. The moment of solution change is indicated by the arrow under the individual recordings: (a) represents the control trace and (b)–(d) after administration of Bay K 8644 (1 μM), caffeine (5 mM), and ryanodine (10 nM). The stimulus frequency was 0.5 Hz for all rapid cooling contracture experiments.

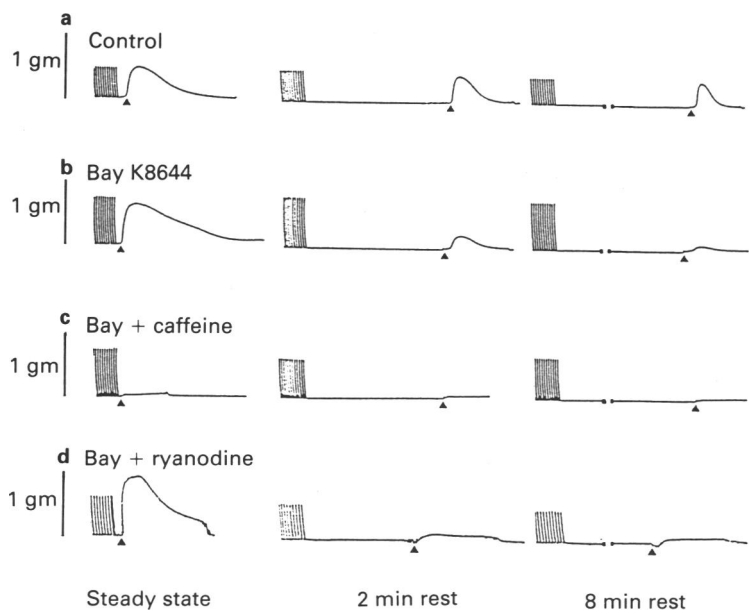

Figure 4 Effect of combining Bay K 8644 (1 μM) with caffeine (5 mM) or ryanodine (10 nM) on rapid cooling contracture amplitude. The protocol was similar to that described in Figure 3.

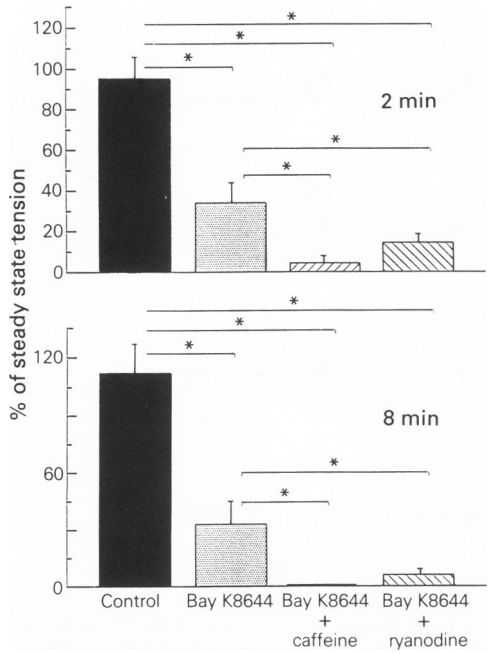

Figure 5 Pooled data from 6 rapid cooling contracture experiments showing the effect of combining Bay K 8644 (1 mM) with caffeine (5 mM) or ryanodine (10 nM) on rapid cooling contractures obtained after 2 and 8 min of rest. Data are expressed as a percentage of contraction prior to initiating the rest interval. Significant differences between treated and untreated groups were determined by analysis of variance followed by Duncan's new multiple range test for multiple comparisons. * $P < 0.05$.

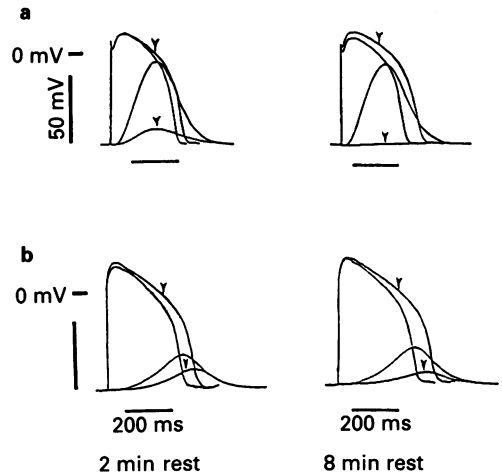

Figure 6 Simultaneous recording of action potentials and accompanying contractions from isolated trabeculae after treatment with 1 μM Bay K 8644 (top panel; a) or Bay K 8644 and 3 mM caffeine (bottom panel; b) after 2 (left) and 8 (right) min rest. In each case, the post-rest action potential and contraction (indicated by arrow) have been superimposed on the steady state beat preceding the period of rest. The stimulation frequency during all electrophysiological studies was 0.5 Hz.

to store and release activator calcium (Kurihara & Sakai, 1985; Bridge, 1986).

Figure 3 illustrates the typical response of the isolated trabeculae to rapid cooling. Figure 3a shows a progressive decrease in the amplitude of the cooling contracture with progressive increase in the rest period prior to cooling. In the presence of Bay K 8644 (1 μM; Figure 3b) there was a greater decrease in rapid cooling contracture at all the rest durations studied. On the other hand Bay K 8644 slightly increased the amplitude of rapid cooling contracture immediately following a train of regular stimuli. This may be a reflection of an inability of the sarcoplasmic reticulum to retain calcium during rest without an impairment of the calcium uptake process. Caffeine (5 mM; Figure 3c) also decreased the amplitude of the rapid cooling contracture after the various durations of rest. Unlike Bay K 8644, caffeine markedly reduced the amplitude of the rapid cooling contracture immediately following a train of regular stimuli. Ryanodine (10 nM; Figure 3d) resembled Bay K 8644 in inhibiting post-rest rapid cooling contractures without inhibiting cooling contractures immediately after steady stimulation. Unlike Bay K 8644, ryanodine had a negative inotropic effect on the trabeculae.

The effect of combining Bay K 8644 with either caffeine (Figure 4) or ryanodine is shown in Figure 4. Responses of control muscles and those after treatment with Bay K 8644 are shown for reference (Figure 4a and b). On further addition of caffeine (5 mM) to the Bay K 8644-treated muscle, rapid cooling contractures were virtually abolished immediately following a train of stimuli or after rest (Figure 4c). Despite this, tension development in response to regular stimulation was maintained. The maintenance of tension development in response to rhythmic stimulation, in spite of the absence of rapid cooling contracture in the place of a regularly driven beat, may reflect the dependence of steady state force generation on trans-sarcolemmal calcium influx in the presence of caffeine. Ryanodine (10 mM) further decreased the post-rest (2 and 8 min) rapid cooling contracture already reduced by Bay K 8644. It should be noted that unlike caffeine, ryanodine did

Table 1 Effect of Bay K 8644, caffeine and ryanodine on twitch parameters

		% change			
Treatment	n	*TPT*ss	*RT*ss	*TPT*r	*RT*r
Bay K 8644 (1 μM)	5	107.0 ± 6.7	116.6 ± 8.5*	112.9 ± 10.4	129.8 ± 11.5*
Caffeine (5 mM)	6	124.5 ± 1*	135.3 ± 7.1*	113.0 ± 3.5*	134.0 ± 10.6*
Ryanodine (10 nM)	6	162.5 ± 7.9*	277.5 ± 11.3*	125.0 ± 7.5*	290.5 ± 11*

Values are mean ± s.e.mean.
TPT = Time to peak tension.
RT = Relaxation time.
ss and r denote steady-state and post-rest contractions respectively.
* denotes $P < 0.05$ compared to control.

not decrease the cooling contracture immediately following a train of stimuli. The results of the rapid cooling experiment indicates that the spectrum of action of ryanodine resembles that of Bay K 8644. The pooled data from 6 experiments are shown in Figure 5.

Electrophysiological studies

Transmembrane action potentials and accompanying twitch contractions were obtained to determine if the apparent trans-sarcolemmal calcium entry associated with membrane depolarization due to caffeine was antagonizing the effect of Bay K 8644 in decreasing post-rest twitch tension. Time to peak tension can be influenced by both sarcoplasmic reticular calcium uptake and release. Thus, an increase in this measure of contraction may be a manifestation of either enhanced and/or prolonged trans-sarcolemmal calcium entry or a reduction of calcium uptake by the sarcoplasmic reticulum.

The effects of Bay K 8644 (1 μM) alone and in combination with caffeine (3 mM) or ryanodine (10 nM) on the transmembrane action potential and accompanying contraction are illustrated in Figures 6 and 7. The recordings in each figure were made from a single trabecula. Data from 7 such experiments are summarized in Figures 8 and 9. Figure 8 represents the pooled data showing the effect of Bay K 8644, caffeine and ryanodine on the action potential duration at 50% repolarization during contractions obtained in response to regular stimulation or after 2 and 8 min rest.

As demonstrated in earlier experiments (Saha *et al.*, 1989, Hryshko *et al.*, 1989) administration of Bay K 8644 was found to prolong significantly the action potential duration at all intervals studied (see Figures 5 and 7). Similar effects on the time course of membrane depolarization were observed when caffeine was combined with Bay K 8644 (Figures 6 and 8). The addition of ryanodine to the Bay K 8644-treated muscle produced a dramatic increase in action potential duration during all test beats (Figures 7 and 8). Thus, the combination of either caffeine or ryanodine with Bay K 8644 was seen to have similar, albeit quantitatively different effects on transmembrane action potential duration. Presumably, these interventions may have similar effects on

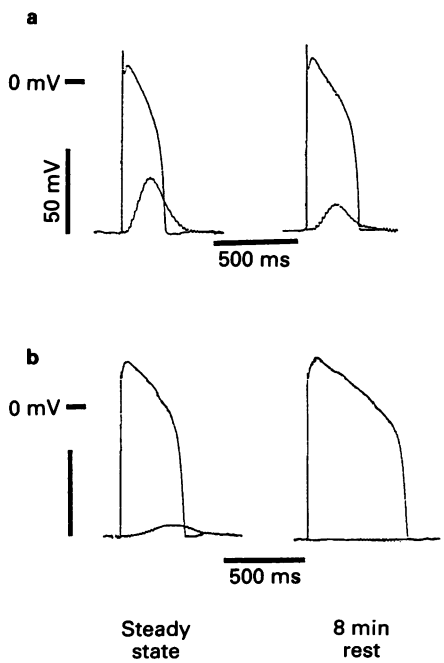

Figure 7 Simultaneous recording of action potentials and accompanying contractions from isolated trabeculae after treatment with 1 μM Bay K 8644 (a) or Bay K 8644 and 10 nM ryanodine (b) in response to rhythmic stimulation (left) or after 8 min rest (right).

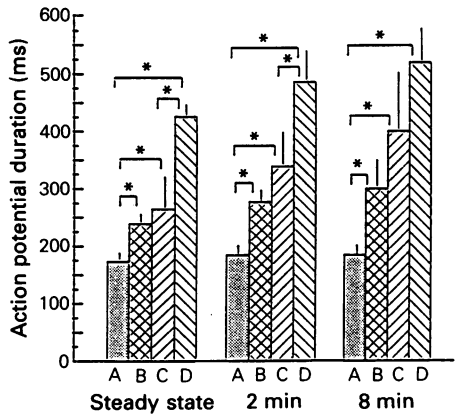

Figure 8 Pooled data from 7 experiments showing control action potential duration for 50% repolarization (A), the effect of Bay K 8644 (1 μM; B) and a combination of Bay K 8644 with caffeine (3 mM; C) or ryanodine (10 nM; D). Measurements were made during steady state contractions and after 2 and 8 min rest. Significance of differences between treated and untreated groups were determined by an analysis of variance followed by Duncan's new multiple range test for multiple comparisons. * $P < 0.05$.

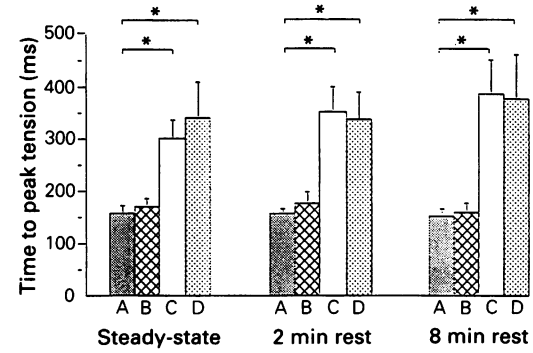

Figure 9 Pooled data from 7 experiments illustrating control time to peak tension (A), the effect of Bay K 8644 (1 μM; B) and the combination of Bay K 8644 with caffeine (5 mM; C) or ryanodine (10 nM; D). Measurements were made during steady state contractions and after 2 and 8 min rest. Statistical analysis of this data was similar to the tests performed in earlier figures. * $P < 0.05$.

trans-sarcolemmal calcium influx associated with the prolonged action potentials, which would help to explain the partial protection of Bay K 8644-induced rest-depression by caffeine but not the contractile response of the trabeculae to the combination of Bay K 8644 and ryanodine.

Table 1 illustrates the effect of the three test agents on time to peak tension and relaxation time for contractions elicited in response to a regular train of stimuli and after 2 min rest. Addition of Bay K 8644 had no effect on time to peak tension. However, a small but statistically significant prolongation of the terminal portion of relaxation occurred (i.e., the final 10%). In comparison, both caffeine and ryanodine significantly prolonged the time to peak tension as well as relaxation time, ryanodine having a much greater effect than caffeine.

Pooled data from 7 experiments showing the effect of combining Bay K 8644 with caffeine or ryanodine on time to peak tension measurements obtained during steady state contractions and those elicited after 2 and 8 min rest are illustrated in Figure 9. A single dose of Bay K 8644 had a significant effect on the time to peak tension at any conditioning interval. In contrast, the combination of either caffeine or ryanodine with Bay K 8644 significantly enhanced the time course of contraction during continuous stimulation and after rest and thus was greater than the change produced by either agent alone. In this regard, it is important to stress that the combination of either caffeine or ryanodine with Bay K 8644 produced similar directional changes in action potential duration and time to peak tension but only caffeine appeared to protect partially against the Bay K 8644-induced rest-depression. Furthermore, these data suggest that trans-sarcolemmal calcium influx alone may not be the primary mechanism responsible for the ability of caffeine to antagonize Bay K 8644-induced rest depression.

Discussion

The new observation of this study is that although Bay K 8644, caffeine and ryanodine convert rest-potentiation in canine ventricular trabeculae to rest-depression, there are several fundamental differences in the spectrum of actions of these three agents. The conversion of rest-potentiation to rest-depression by Bay K 8644, first described by us (Bose *et al.*, 1987; Hryshko, 1987; Bouchard *et al.*, 1988; Hryshko *et al.*, 1989), is indicative of depletion of sarcoplasmic reticular calcium stores during diastole.

Considerable experimental evidence suggests that redistribution of calcium within the sarcoplasmic reticulum during diastole may be responsible for the potentiation of contraction amplitude (rest-potentiation) immediately following a period of rest (Koch-Weser & Blinks, 1961; 1963; King & Bose, 1983; Bers, 1985; Bers *et al.*, 1987; Hilgemann,

1986a,b; Bridge, 1986; Schouten *et al.*, 1987). From similar experiments it has also been suggested that there may be a continuous but slow loss of calcium from the sarcoplasmic reticulum during diastole. The latter, if allowed to continue for an extended period of time results ultimately in a decrease in contractility (rest-depression; Koch-Weser & Blinks, 1961; 1963; Bose *et al.*, 1988a). In the present study, the addition of Bay K 8644 to the bathing solution caused rest-depression which is similar to that seen normally with prolonged rest. This was in addition to its contrasting action of enhancing the amplitude of the steady state contraction. Based on a consideration of several models of excitation-contraction coupling in mammalian cardiac muscle proposed by various investigators (Koch-Weser & Blinks, 1963; Wood *et al.*, 1969; Morad & Goldman, 1973; Wohlfart & Noble, 1982; Schouten *et al.*, 1987; Bose *et al.*, 1988a), rest-depression due to Bay K 8644 might reflect one of two processes: (i) an acceleration of ongoing diastolic calcium loss from the sarcoplasmic reticulum passing through the sarcoplasm so as to influence the contractile apparatus or (ii) a pathway by which calcium lost from this organelle bypasses the contractile system. Experiments involving rapid cooling of the cardiac muscle to measure sarcoplasmic reticular calcium content as well as others using caffeine or ryanodine as pharmacological tools were done to obtain some insight into the ability of Bay K 8644 to perturb intracellular calcium handling by the sarcoplasmic reticulum.

Bay K 8644

We have shown previously that a racemic mixture of the dihydropyridine chemical Bay K 8644 prolongs action potential duration both during steady stimulation as well as after rest (Bose *et al.*, 1987; Hryshko *et al.*, 1989; Saha *et al.*, 1989). This is consistent with the ability of Bay K 8644 to increase transsarcolemmal calcium entry. The increase in amplitude of the steady state contraction can be explained on this basis. Of particular interest is the unusual effect of this compound not only to decrease rest-potentiation but to convert it to rest-depression. This is indicative of a decrease in sarcoplasmic reticular calcium release. Such an effect can be due either to increased refractoriness of the calcium release mechanism or to a depletion of the release pool. Despite this, the ability of the sarcoplasmic reticulum to take up calcium from the myoplasm is not affected, as shown by the lack of change of the time to peak tension seen in this study and others (Thomas *et al.*, 1985a). Further evidence in support of the maintained ability of the sarcoplasmic reticulum to pump calcium in the presence of Bay K 8644 is the increase in amplitude of rapid cooling contracture following a regular train of stimuli, suggesting an increase in sarcoplasmic reticular calcium content. However, the rapid decrease in the amplitude of the rapid cooling contractures after initiation of rest indicates that the loss of calcium from the sarcoplasmic reticulum is increased by Bay K 8644. Previous investigations have failed to demonstrate any effect of this agent on sarcoplasmic reticular function in skinned cardiac muscle fibres or on calcium transport in sarcoplasmic reticular vesicles from skeletal muscle (Thomas *et al.*, 1985b; Zorzato *et al.*, 1985). One factor which may contribute to this unusual effect of Bay K 8644 on post-rest contraction, as opposed to contractions resulting from continuous stimulation, may be the functional junction between the t-tubule and sarcoplasmic reticulum which is retained only in the intact ventricular preparation. The inhibitory effect of Bay K 8644 on post-rest contraction is not seen in atrial preparations (D. Bose, unpublished observations) which are poor in t-tubules (Sommer & Johnson, 1979).

Post-rest force production has previously been shown to be strongly influenced by compounds that affect calcium handling by the sarcoplasmic reticulum (Hilgemann 1986a,b; Bers, 1987; Bers *et al.*, 1987). In such experiments, a gradual diastolic increase in the content of calcium in the extracellular space of cardiac muscle was found to be facilitated by the administration of ryanodine and was accompanied by a decrease in post-rest tension development. Thus, it appears that post-rest contraction in most mammalian species is governed to a greater extent by intracellular calcium release. It should be noted that while post-rest rapid cooling contracture amplitude decreases continuously with increasing rest durations, post-rest contractions evoked by electrical stimulation are biphasic in amplitude. Such a finding is consistent with the notion that rapid cooling contracture better represents total sarcoplasmic reticular calcium content while electrically evoked post-rest contractions better represent the calcium pool in the release site of the sarcoplasmic reticulum. This explanation, however, is incomplete as it raises the question of the route which this calcium presumably takes in moving from the sarcoplasmic reticulum, eventually to the extracellular space.

We have suggested that the the apparent 'leak' of calcium from the sarcoplasmic reticulum takes place close to the t-tubule-lateral cisternal junctions. This is based on experiments performed on skinned fibres and sarcoplasmic reticular vesicles mentioned previously (Thomas *et al.*, 1985b; Zorzato *et al.*, 1985), and those measuring asynchronous diastolic sarcomere motion mediated by sarcoplasmic reticular calcium release and measurements of time to peak tension of contractions (Bose *et al.*, 1987; present

study). Scattered light intensity fluctuation of coherent light due to wavelike myofilament motion is believed to be caused by spontaneous oscillatory calcium release from the sarcoplasmic reticulum of ventricular muscle (Lappe & Lakatta, 1980; Lakatta & Lappe, 1981). Since these oscillations are measured during diastole, their depression after the addition of Bay K 8644 (Bose *et al.*, 1987; 1988a,b) suggests that calcium present in the myoplasm during diastole is decreased by Bay K 8644. This could result from decreased release from the lateral cisternae, probably due to decreased content. Ryanodine, which is known to increase calcium 'leak' from the sarcoplasmic reticulum (Bers *et al.*, 1987) also reduces scattered light intensity fluctuation (Sutko *et al.*, 1986b; Kobayashi & Bose, unpublished observations). The time to peak tension associated with contractions produced by continuous electrical stimulation or after variable periods of rest has been consistently reported to be unaffected by treatment with Bay K 8644 (Thomas *et al.*, 1985a; Hryshko, 1987; Bouchard *et al.*, 1988). This suggests that prolongation of the duration of active state (due to alterations of sarcoplasmic reticular calcium uptake and release) do not contribute to the positive inotropy produced by this agent. Failure to slow relaxation rate throughout the twitch, as opposed to slowing of the final 10% of the relaxation phase, suggests that sarcoplasmic reticular calcium pump activity is also not markedly impaired by Bay K 8644. Diastolic 'leak' of calcium from the sarcoplasmic reticulum should be expected to result in a uniform slowing of relaxation, as occurs with caffeine or ryanodine alone. As this did not occur in the presence of Bay K 8644, it may be concluded that the pathway taken by calcium after it leaves the sarcoplasmic reticulum during diastole does not include primarily the myofilaments and this release may indeed be from regions of the sarcoplasmic reticulum closer to the sarcolemma. In this regard it should be noted that low external sodium partially reverses depression of post-rest force production by agents or procedures which cause rest-depression, including Bay K 8644 (Sutko *et al.*, 1986a; Bose *et al.*, 1987). This suggests that the Na-Ca exchange process may be mediating the 'apparent' diastolic loss of calcium in muscles, including those treated with Bay K 8644.

Bay K 8644 plus caffeine

In most species the effect of administration of caffeine on excitation-contraction coupling in heart muscle appears to be biphasic. This reflects the diversity of actions of this compound. The initial response to caffeine is an enhancement of calcium-induced release of calcium from the sarcoplasmic reticulum as measured by increased force production, increased scattered light intensity fluctuation and ^{45}Ca flux (Fabiato, 1981; Stern *et al.*, 1985; Sutko *et al.*, 1986; Rasmussen *et al.*, 1987). The second action of caffeine is gradually to deplete the intracellular calcium pool(s), and in turn, calcium release from the sarcoplasmic reticulum. The small positive inotropy observed with this agent is probably a manifestation of a number of intracellular processes which favour myofilament activation, including inhibition of sarcoplasmic reticular calcium ATPase (Weber & Herz, 1968), enhanced myofilament sensitivity (McClellan & Winegrad, 1978) and inhibition of phosphodiesterase (Butcher & Sutherland, 1962).

Caffeine has been previously shown to inhibit post-rest tension development (Bers, 1983; Hryshko, 1987). The interaction between caffeine and Bay K 8644 was tested to gain insight into the depressant action of Bay K 8644 on the post-rest beat. It was hypothesized, that if these agents utilized a similar mechanism to produce rest-depression, then the effect of combining them should be additive. This effect was not observed. Instead, caffeine was found to reverse partially the depressant effect of Bay K 8644 on post-rest twitch tension. Moreover, rapid cooling contractures observed under the influence of both drugs retained the characteristics of that observed with caffeine alone, which suggests that sarcoplasmic reticular calcium stores were severely depleted during continuous stimulation under the influence of both inotropes, even though the post-rest tension was less depressed. Thus, it seems unlikely that the caffeine-induced protection against post-rest twitch tension depression was due to enhanced calcium release from the sarcoplasmic reticulum. This explanation is supported by the absence of significant cooling contractures when evoked immediately after a train of regular stimuli or after periods of rest or in studies where other indirect measures of intracellular calcium movements were found to be depressed by caffeine administration (Sutko *et al.*, 1986b; Rasmussen *et al.*, 1987; Bers, 1987).

In addition to the possibility of enhanced transsarcolemmal calcium entry associated with the effect of caffeine to prolong action potential duration, increased myofilament activation during the post-rest beat may be a likely explanation for the caffeine-induced antagonism of the effect of Bay K 8644 on post-rest tension development. It could be argued that enhanced activation of contraction may be related either to the prolongation of action potential duration and a possible increase in the calcium current, or time to peak tension under these conditions. However, as discussed below, similar electrophysiological changes were found with Bay K 8644 alone and in combination with ryanodine, both of which resulted in greater rest-depression. Further-

more both caffeine and ryanodine prolong time to peak tension when present alone or in combination with Bay K 8644 to a similar extent. Despite the similarity in their effect on the twitch profile, antagonism of Bay K 8644-induced rest depression was observed only with the combination of Bay K 8644 and caffeine. These data do not support the hypothesis that enhanced trans-sarcolemmal calcium influx alone is responsible for the reduction of Bay K 8644-induced rest-depression by caffeine. It appears that despite the characteristic rest-depression produced by either caffeine or Bay K 8644 alone, the decreased depression when the two are combined may result from the augmented calcium current due to Bay K 8644 as well as sensitization of the contractile apparatus and reduced sarcoplasmic reticular calcium sequestration due to caffeine.

Bay K 8644 plus ryanodine

Ryanodine is a negative inotrope, which reacts specifically with the calcium release channels localized on the terminal cisternae of the sarcoplasmic reticulum (Inui *et al.*, 1987). Its binding is modulated by caffeine (Pessah *et al.*, 1986), although in this study the two were never present simultaneously. This alkaloid decreases ventricular contractility during regular stimulation and after rest by decreasing the content of calcium in the sarcoplasmic reticular pool, measured by rapid cooling (Bers *et al.*, 1987; Bouchard *et al.*, 1988), increasing the passage of calcium from the intracellular stores to the extracellular space (Hilgemann, 1986a,b; Bers *et al.*, 1987), decreasing diastolic calcium-mediated myofilament motions (Sutko *et al.*, 1986b), while only slightly reducing ^{45}Ca uptake (Rasmussen *et al.*, 1987).

Addition of ryanodine in the presence of Bay K 8644 further decreased post-rest twitch tension and cooling contractures. The data are in agreement with the prediction that the two drugs share a similar mechanism of action. This is supported by the lack of change in rapid cooling contracture amplitude immediately following a train of regular beats and the further increase in action potential duration observed under the influence of both agents. Thus, although the release of calcium from the release site of the sarcoplasmic reticulum is impaired by ryanodine (Hilgemann, 1986a,b; Bers *et al.*, 1987; Rousseau *et al.*, 1987), the ability of this organelle to sequester myoplasmic free calcium appears to be unaffected. One effect of ryanodine which does not parallel that of Bay K 8644, however, is the increase in time to peak tension (162.5%) and marked increase in relaxation time (277.5%). Other effects such as those observed on the action potential duration under the influence of both drugs differ significantly from the response seen with Bay K 8644. This suggests that even in the face of a possible large increase in the duration of trans-sarcolemmal calcium entry the sarcoplasmic reticular storage pool for calcium is unable to retain its content of calcium over the course of time, similar to that observed with Bay K 8644 alone. If one assumes that calcium pumping by the sarcoplasmic reticulum is not impaired by the combination of Bay K 8644 and ryanodine (on the basis of rapid cooling contractures not being impaired during steady stimulation), then the significant increase in the time taken for both peak tension development and relaxation with this treatment indicates that calcium may be passing through the myofilaments on its way out from the sarcoplasmic reticulum during the process of 'leak'. Caffeine which causes an intermediate amount of prolongation in time to peak contraction and relaxation time compared with ryanodine may be acting by a different mechanism, as suggested earlier. However it would be impossible to rule out increased 'leak' of calcium from the sarcoplasmic reticulum under its influence. Studies on calcium release channels in the lateral cisternae indicate that they are converted to a high conductance state by caffeine (Rousseau & Meissner, 1989). Ryanodine in contrast keeps the channels open in a low conductance state (Rousseau *et al.*, 1987) which may explain a longer duration of calcium 'leak' leading to a greater prolongation of tension development and recovery. The fact that a similar extent of change is not seen with only Bay K 8644 may be because in this case the 'leak' may be occurring from a very limited site on the sarcoplasmic reticulum.

In summary, the results of the experiments where Bay K 8644 was coadministered with either caffeine or ryanodine suggest that the latter two drugs do not share a similar mechanism of action in depressing post-rest tension development in canine ventricular muscle. Of the two agents tested in combination with Bay K 8644, ryanodine produced contractile (post-rest) and electrophysiological changes which were somewhat similar to those observed with Bay K 8644 alone. It is probable that the depression of post-rest contraction amplitude by Bay K 8644 is indicative of acceleration of ongoing diastolic calcium loss from the sarcoplasmic reticulum into the extracellular space, without influencing the myofilaments significantly during transit. This leaves less activator calcium available for release upon resumption of stimulation.

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3-Aminopropylphosphinic acid—a potent, selective $GABA_B$ receptor agonist in the guinea-pig ileum and rat anococcygeus muscle

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1 3-Aminopropylphosphinic acid, a γ-aminobutyric acid (GABA) analogue, was tested for activity on guinea-pig isolated ileum and rat isolated anococcygeus muscle preparations. The effects of 3-aminopropylphosphinic acid were compared with those of GABA and baclofen.

2 In the electrically stimulated ileum, 3-aminopropylphosphinic acid, like GABA and baclofen, caused a concentration-dependent inhibition of the cholinergic twitch contraction, the IC_{50} value being $1.84 \pm 0.23\,\mu M$ ($n = 12$). Unlike GABA, but like baclofen, 3-aminopropylphosphinic acid did not produce an initial contraction.

3 The inhibitory effects of 3-aminopropylphosphinic acid and baclofen in the guinea-pig ileum were not significantly antagonized by bicuculline ($10\,\mu M$), phentolamine plus propranolol (both $1\,\mu M$), yohimbine ($1\,\mu M$), naloxone ($1\,\mu M$), impromidine ($1\,\mu M$) or 8-phenyltheophylline ($10\,\mu M$). The inhibitory effects of 3-aminopropylphosphinic acid, but not of baclofen, were however antagonized by phaclofen ($500\,\mu M$). In addition the effects of 3-aminopropylphosphinic acid were abolished by baclofen desensitization in the guinea-pig ileum.

4 3-Aminopropylphosphinic acid, GABA and baclofen reduced the twitch contraction evoked by electrical field stimulation in the rat anococcygeus muscle. The IC_{50} for 3-aminopropylphosphinic acid inhibition of the anococcygeus contraction was $0.89 \pm 0.15\,\mu M$ ($n = 8$).

5 It is concluded that 3-aminopropylphosphinic acid is a potent, selective $GABA_B$ agonist, being seven times more potent than baclofen in the guinea-pig ileum and five times more potent than baclofen in the rat anococcygues muscle preparations.

Introduction

In the guinea-pig ileum, γ-aminobutyric acid (GABA) causes both contraction and inhibition of the electrically-induced cholinergic contractions (Krantis *et al.*, 1980). In the presence of bicuculline, a $GABA_A$ receptor antagonist, GABA causes inhibition of the ileal twitch contraction by an action on presynaptic $GABA_B$ receptors (Bowery *et al.*, 1981; Kaplita *et al.*, 1982; Ong & Kerr, 1983). The β-*p*-chlorophenyl analogue of GABA, baclofen, a selective $GABA_B$ agonist, mimics this effect but is no more potent than GABA itself (Bowery *et al.*, 1981; Giotti *et al.*, 1983). Several compounds, have been reported to show $GABA_B$ receptor agonist activity (Bowery, 1982), but to date, none have shown an increase in potency over baclofen.

In binding studies on rat brain membranes, a new compound, 3-aminopropylphosphinic acid (Figure 1) has been claimed to have an affinity for the $GABA_B$ receptor some 20 times greater than the affinity of baclofen (Figure 1) (Dingwall *et al.*, 1987a, b). We have investigated the pharmacological activity of 3-aminopropylphosphinic acid in the guinea-pig ileum and rat anococcygeus muscle *in vitro*.

Methods

Guinea-pig ileum

Male guinea-pigs (300–450 g) were killed by a blow to the head and bled out. Segments of ileum of approximately 3 cm in length were removed from an

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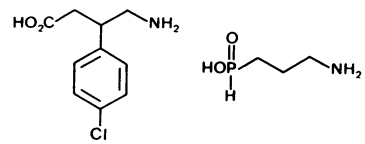

Figure 1 Chemical structures of (left) baclofen (±)-4-amino-3-(4-chlorophenyl) butanoic acid and (right) 3-aminopropylphosphinic acid. In this study racemic baclofen was used.

area 10–15 cm proximal to the ileo-caecal junction. Preparations were immediately placed in a modified Krebs solution (Bülbring, 1953) which was continually bubbled with 95% O_2 and 5% CO_2. Segments were freed of their mesenteric attachment and suspended in 10 ml organ baths containing Krebs solution under an isometric tension of 1 g. Isometric contractions to transmural stimulation (Paton, 1954) were recorded with strain gauge transducers (Biomed Dynamometer UF1) and displayed on an Ormed Multitrace pen recorder. Electrical stimulation of preparations was achieved by passing rectangular pulses (duration 0.5 ms; frequency 0.1 Hz; supramaximal voltage (25–35 V)), from a Grass SD11 stimulator via platinum electrodes. Preparations were allowed to equilibrate for 1 h prior to addition of compounds to the organ bath.

Rat anococcygeus

Male Wistar rats (200–300 g) were killed by a blow to the head and bled out and their anococcygeus muscles removed as previously described (Gillespie, 1972). The muscles were mounted in organ baths (10 ml) containing modified Krebs solution which was continually gassed with 95% O_2 and 5% CO_2. A resting tension of 0.5 g was applied and the preparations were field stimulated from Grass SD11 stimulators via platinum ring electrodes with the following stimulus parameters: pulse duration 1 ms; frequency 10 Hz; for 1 s. Isometric muscle responses were measured with a strain gauge transducer (Biomed UF1 dynamometer) and displayed on an Ormed Multitrace pen recorder.

Typical tension responses generated in either preparation as a result of electrical stimulation were between 2 and 4 g force. Preparations generating less tension were rejected.

In both preparations, sequential agonist concentration-response curves were constructed, allowing 30 min between additions of agonist to minimize tachyphylaxis. When antagonists were used, concentration-response curves to 3-aminopropylphosphinic acid in the presence of the antagonist were constructed after an initial equilibration period of 10 min. $GABA_B$ receptor desensitization was achieved by 3 additions of baclofen (100 μM) without washout from the tissue.

Drugs used

The following drugs were used: γ-amino-n-butyric acid (Sigma), (±)-baclofen (Research Biochemicals Inc.), (±)-propranolol (ICI), phentolamine mesylate (Ciba-Geigy), yohimbine hydrochloride (Sigma), naloxone hydrochloride (Sigma), 8-phenyltheophylline (Aldrich Chem. Co), impromidine oxylate (SK&F), bicuculline methiodide (Sigma), phaclofen (Tocris Neuramin) and 3-aminopropylphosphinic acid (prepared by the method of Dingwall *et al.*, 1987a, b). With the exception of 8-phenyltheophylline, all compounds were dissolved in distilled water, subsequent dilutions being made in distilled water and compounds were added to the organ bath in volumes no greater than 1% total volume. 8-Phenyltheophylline was made up in 80% methanol/2 M NaOH, subsequent dilutions being made in distilled water and all vehicle controls were negative. Propranolol and phentolamine were added directly to the Krebs solution.

Statistical analysis

EC_{50} values for concentration-response curves were derived by use of a pharmacological data handling package (PDH, SK&F software) on a VAX computer. Statistically significant differences between concentration-response curves were estimated using "Allfit' (De Lean *et al.*, 1977) and P values of less than 0.05 were taken to be significant. Where n numbers are given in the text, they refer to number of animals.

Results

Guinea-pig ileum

GABA and baclofen produced a concentration-related inhibition of the cholinergic twitch response in the guinea-pig ileum (Figures 2, 3). Concentrations inducing 50% inhibition (IC_{50}) for the inhibition of contraction were 13 ± 1.63 μM ($n = 8$) and 12.6 ± 1.8 μM ($n = 8$) for GABA and baclofen respectively. 3-Aminopropylphosphinic acid produced an inhibition of the electrically stimulated guinea-pig ileal twitch response which had the same characteristics as the GABA and baclofen responses (Figure 2). However, like baclofen but unlike GABA, the inhibitory response to 3-aminopropylphosphinic acid (up to 100 μM) was not preceded by a contraction. The IC_{50} for 3-aminopropylphosphinic acid

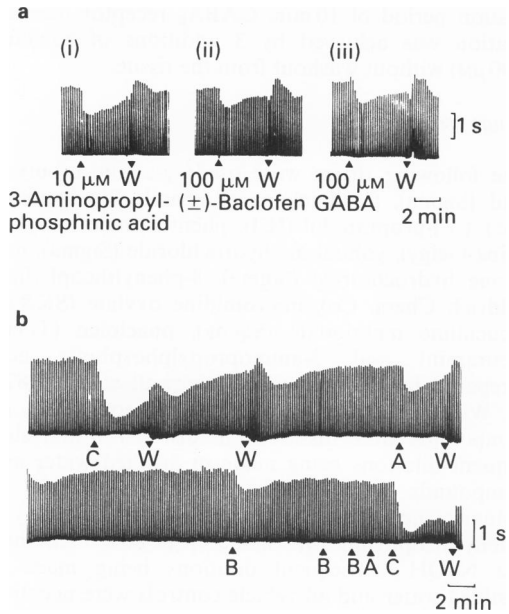

Figure 2 (a) Effect of 3-aminopropylphosphinic acid, (10 μM) (i), baclofen (100 μM) (ii), and GABA (100 μM) (iii), on the contractions of the guinea-pig ileum induced by field stimulation. (b) The effect of $GABA_B$ receptor desensitization on the responses to 3-aminopropylphosphinic acid (A) (10 μM) and clonidine (C) (0.1 μM). Control 3-aminopropylphosphinic acid (10 μM) and clonidine (0.1 μM) responses are shown prior to baclofen (B) (100 μM). Repeated applications of baclofen (100 μM) were without effect, thus indicating desensitization of $GABA_B$ receptors. Although under these circumstances 3-aminopropylphosphinic acid (10 μM) was without effect, clonidine, which reduces the twitch through an α_2-receptor mechanism, was as effective as it was prior to $GABA_B$ receptor densitization. The trace is continuous. Stimulus parameters were 0.5 ms duration, 0.1 Hz at supramaximal voltage.

inhibition of twitch contractions was 1.84 ± 0.23 μM ($n = 12$). As with the GABA and baclofen responses, the inhibition reached a maximum within approximately 1 min, and began to return to control levels within approximately 2 min even though the compound was still in contact with the tissue. The total duration of inhibition of twitch contractions in the presence of 3-aminopropylphosphinic acid without washout was 4–5 min.

When the guinea-pig ileum was desensitized to baclofen by three additions of baclofen (100 μM) at 2–6 min intervals, 3-aminopropylphosphinic acid (10 μM), was completely without effect (Figure 2b), whereas relaxations to clonidine (0.1 μM) persisted ($n = 8$). Similarly, the guinea-pig ileum was desensitized to 3-aminopropylphosphinic acid (10 μM) by three repeated additions of the compound and found to be unresponsive to baclofen 100 μM ($n = 4$).

The selectivity of the 3-aminopropylphosphinic acid inhibition was evaluated by use of a number of pharmacological antagonists at concentrations previously shown to cause significant antagonism of the relevant agonist responses. The control concentration-response curves to either 3-aminopropylphosphinic acid or baclofen were not significantly altered by incubation of tissue with either bicuculline (10 μM), propranolol plus phentolamine (both 1 μM), yohimbine (1 μM), 8-phenyltheophylline (10 μM), impromidine (1 μM), or naloxone (1 μM).

This suggests that 3-aminopropylphosphinic acid is not inhibiting the cholinergic twitch contraction in the guinea-pig ileum by interacting with $GABA_A$, β- or α-adrenoceptors, α_2-adrenoceptors, adenosine, histamine H_3 or opioid receptors, respectively.

Phaclofen, a new compound reported to have $GABA_B$ antagonist activity (Kerr *et al.*, 1987), significantly antagonized the 3-aminopropylphosphinic acid concentration-response curve in the guinea-pig ileum at a concentration of 500 μM ($P = 0.03$) ($n = 4$) but not at a concentration of 200 μM ($n = 8$). However, no significant antagonism of the baclofen concentration-response curve was obtained at concentrations of phaclofen up to 500 μM ($n = 7$).

Rat anococcygues muscle

As previously reported, GABA and baclofen inhibited the nerve-mediated contractions of the rat anococcygeus muscle produced by electrical field stimulation. The IC_{50} values obtained in the present experiments were 3.08 ± 0.45 μM ($n = 4$) for GABA and 5.47 ± 0.68 μM ($n = 4$) for baclofen. The inhibition produced by GABA and baclofen was much more long-lasting than that seen in the guinea-pig ileum. The agonists could be left in contact with the tissue for 15 min with no diminution in the response.

3-Aminopropylphosphinic acid inhibited the contractions of the rat anococcygeus in a concentration-dependent manner ($IC_{50} = 0.89 \pm 0.15$ μM ($n = 8$)) (Figure 3.) As with GABA and baclofen, the inhibition was more long-lasting than the inhibition seen in the ileum with 3-aminopropylphosphinic acid, and could be sustained for periods in excess of 15 min.

When baclofen (100 μM) was repeatedly added to the rat anococcygeus until no further inhibitory response could be obtained, 3-aminopropylphosphinic acid had no further inhibitory effect. However, since the effects of baclofen are long lasting in this preparation, the contractions did not return to normal during repeated application, and therefore no firm conclusions could be drawn from these data.

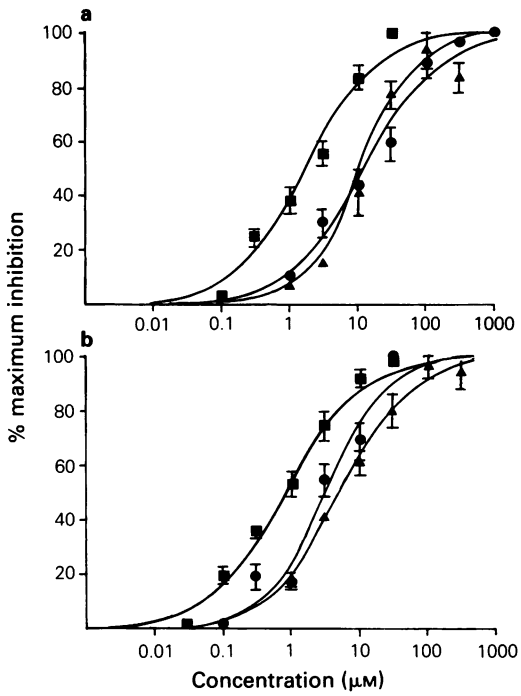

Figure 3 Concentration-response curves to agonists in the guinea-pig ileum (a) and rat anococcygeus muscle (b). (a) Concentration-response curves for the % maximum inhibition of the field stimulated guinea-pig ileum to 3-aminopropylphosphinic acid (■), baclofen (▲) and GABA (●). Stimulus parameters were 0.5 ms duration, 0.1 Hz at supramaximal voltage. The mean IC_{50} value for 3-aminopropylphosphinic acid was significantly less than the mean IC_{50} values for either baclofen or GABA ($P < 0.05$). There was no statistically significant difference between the mean IC_{50} values for GABA and baclofen. (b) Concentration-response curves for the % maximum inhibition of the field stimulated rat anococcygeus to 3-aminopropylphosphinic acid (■), baclofen (▲) and GABA (●). Stimulus parameters were 1 ms duration, 10 Hz for 1 s every 10 s, at supramaximal voltage. The mean IC_{50} value for 3-aminopropylphosphinic acid was significantly less than mean IC_{50} value for either baclofen or GABA, ($P < 0.05$). In addition, the mean IC_{50} value for GABA was significantly different from the mean IC_{50} value for baclofen ($P < 0.05$). The points represent mean for at least four preparations from separate animals; s.e. mean shown by vertical bars.

Discussion

There have been several studies on the structural requirements for $GABA_B$ receptor activation from which it would appear that even minor manipulations of the baclofen molecule result in total loss of activity (Olpe *et al.*, 1980; Krogsgard-Larsen, 1988). To date, no $GABA_B$ agonist has been shown to be more potent than baclofen on the *in vitro* systems described here. 3-Aminopropylphosphinic acid was found to be seven times more potent than racemic baclofen in the guinea-pig ileum and five times more potent than racemic baclofen in the rat anococcygeus muscle. In studies reported by Dingwall *et al.* (1987a, b), 3-aminopropylphosphinic acid has been reported to have an affinity for the $GABA_B$ receptor that is twenty times more than the affinity of baclofen.

It is interesting to compare the structures and activities of the phosphinic analogue of GABA, described here as a potent agonist, to the phosphonic analogue which has been described as a weak partial agonist/antagonist (Luzzi *et al.*, 1986), although the only difference in structure between the two is the acidic moiety. 3-Aminopropylphosphinic acid has a distorted tetrahedral arrangement of atoms around the phosphorus, has only one acidic proton and has the negative charge distributed over two oxygen atoms, thus it is similar in many respects to GABA. While 3-aminopropylphosphoninic acid has a near tetrahedral arrangement of atoms around the phosphorus, has two acidic protons (depending on pH) and has the negative charge distributed over three oxygen atoms.

In the guinea-pig ileum, it would appear that 3-aminopropylphosphinic acid is interacting with $GABA_B$ receptors located prejunctionally on cholinergic terminals. Since 3-aminopropylphosphinic acid did not cause an initial contraction, it is likely to be devoid of $GABA_A$ agonist activity. Although final validation of this assumption awaits a more specific and potent $GABA_B$ antagonist, the results with a number of known specific receptor antagonists suggest that 3-aminopropylphosphinic acid is not interacting with any other class of receptor. Further, tissues desensitised to baclofen, no longer responded to 3-aminopropylphosphinic acid. Clonidine, which was employed to test the specificity of the $GABA_B$ receptor desensitization, was equieffective both before and during $GABA_B$ receptor desensitization. Phaclofen has been claimed to be a weak but selective $GABA_B$ antagonist (Kerr *et al.*, 1987; Dutar & Nicholl, 1988). We were able to demonstrate inhibition of the 3-aminopropylphosphinic acid responses in the guinea-pig ileum but not those of baclofen. It is unclear why this should be so, although since phaclofen has more recently been claimed to show $GABA_B$ antagonist activity in a variety of test systems (Karlsson *et al.*, 1988; Soltesz *et al.*, 1988), it does support our hypothesis that 3-aminopropylphosphinic acid, is interacting with $GABA_B$ receptors.

The time course of the 3-aminopropylphosphinic acid response in both the guinea-pig ileum and the rat anococcygeus muscle was similar to that of GABA and baclofen. The response to $GABA_B$ agonists in the guinea-pig ileum is commonly observed to be transient in nature while in the rat anococcygeus muscle it is more long-lasting (Bowery *et al.*, 1981; Muhyaddin *et al.*, 1982).

Our pharmacological investigations support the claims of Dingwall *et al.* (1987a, b) that 3-aminopropylphosphinic acid is a highly potent, selective $GABA_B$ agonist. Further experiments with this compound may shed light on the physiological role of $GABA_B$ receptors in the mammalian intestine.

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The pharmacological properties of the peptide, endothelin

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1 The effect of endothelin (ET-1) has been studied on isolated vascular and non-vascular preparations, using both functional and competition radioligand binding techniques. The effects of endothelin on blood pressure were studied in both anaesthetised, chemically denervated normotensive and spontaneously hypertensive rats (SHR).

2 Endothelin elicited contractile responses in the rat thoracic aorta, perfused mesenteric bed, rabbit mesenteric artery and portal vein. The maximal responses in the rat aorta were enhanced by removal of the endothelium, and were reduced in the presence of either a cyclo-oxygenase inhibitor (indomethacin) or a thromboxane receptor antagonist (SQ 29,548). In terms of potency, the most sensitive preparation was the rat endothelium-denuded aorta and rat perfused mesenteric bed ($-\log EC_{50}$ values = 8.2 ± 0.07 and 8.2 ± 0.12, mean ± s.e.mean, $n = 4$, respectively). In the perfused mesenteric bed of the rat the maximum response to endothelin (219 ± 12 mmHg, $n = 4$) was greater than that to either phenylephrine (maximal response = 67 ± 9 mmHg; $n = 4$) or KCl (maximal response = 110 ± 6 mmHg, $n = 4$).

3 Endothelin elicited contractile responses of the guinea-pig isolated ileum, oesophageal muscularis mucosae and uterus. Responses were also observed in the rat fundic strip and paced left atria. The guinea-pig urinary bladder, trachea, rat vas deferens and anococcygeus exhibited little or no response to endothelin at the concentrations studied (1×10^{-12}–3.2×10^{-8} M). Of the above preparations, the ileum and oesophageal muscularis mucosae were the most sensitive to endothelin ($-\log EC_{50} = 8.5 \pm 0.11$ and 8.4 ± 0.06, $n = 6$, respectively), exhibiting potencies similar to those observed in the endothelium-denuded aorta of the rat.

4 In competition-radioligand binding studies, endothelin did not displace either [^{3}H]-PN 210-100 or [^{125}I]-(−)-ω-conotoxin GVIA from binding sites in membranes from rat cerebral cortex and, skeletal muscle or from guinea-pig cerebral cortex and hippocampus, respectively. This indicates a lack of direct interaction of endothelin at the dihydropyridine binding site and the N-type calcium channel, respectively. However, in functional studies, contractile responses to endothelin (1×10^{-8} M) in the endothelium-denuded aorta of the rat were potently reversed by nifedipine, verapamil, and prenylamine ($-\log IC_{50}$ values = 8.0 ± 0.13, 7.2 ± 0.09 and 6.6 ± 0.08, $n = 4$–8, respectively). In addition, the responses to endothelin were virtually abolished in the presence of Krebs physiological salt solution containing no calcium but with 1×10^{-5} M EDTA added. Pre-equilibration with either (−)-ω-conotoxin (1×10^{-6} M) or tetrodotoxin (1×10^{-6} M) did not affect responses to endothelin.

5 In chemically denervated rats, endothelin (1 pmol kg^{-1}–10 nmol kg^{-1}, i.v.) exhibited pressor responses, which were unaffected by a 3 h pretreatment with indomethacin. In the SHR, the effects on blood pressure were not significantly different from those observed in normotensive animals at any of the doses studied. A transient (duration < 30 s) depressor response was also observed in all groups studied at a dose of 0.1–1 nmol kg^{-1} i.v.

6 In conclusion, endothelin is a potent contractile agonist in both vascular and non-vascular muscle. It appears to elicit responses partly via the entry of extracellular calcium (by a mechanism distinct from that of other calcium facilitators) and partly by release of endoperoxides.

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Introduction

Endothelin is a 21 amino acid residue polypeptide released from the vascular endothelium which exhibits potent vasoconstrictor responses both *in vitro* and *in vivo* (Yanagisawa *et al.*, 1988; Spokes *et al.*, 1988). Endothelin, in studies using porcine tissue, is synthesized from a precursor molecule, pre-proendothelin, by proteolytic cleavage (Itoh *et al.*, 1988) and exhibits effects both on intact tissue (Yanagisawa *et al.*, 1988) and a culture derived from vascular smooth muscle cells (Hirata *et al.*, 1988; Miasiro *et al.*, 1988). These actions are mediated through binding sites which are specific for endothelin and distinct from catecholamine receptors or sites sensitive to dihydropyridines (Yanagisawa *et al.*, 1988; Hirata *et al.*, 1988; Kanse *et al.*, 1988).

The mechanisms by which the effects of endothelin are elicited are not precisely known. Vasoconstrictor responses to endothelin are highly sensitive to a reduction in extracellular calcium concentration, and are antagonised by calcium entry blockers such as nicardipine (Yanagisawa *et al.*, 1988) or nifedipine (Hughes *et al.*, 1988). However, Auguet *et al.* (1988) have found that, in contrast, responses of the rat isolated aorta are insensitive to nifedipine, D600 or diltiazem. Increases in intracellular calcium, determined by an increase in fura-2 fluorescence, occurred in vascular smooth muscle cultures in response to endothelin (Hirata *et al.*, 1988). These data are consistent with the postulate of Yanagisawa *et al.* (1988) that endothelin may be an agonist for the dihydropyridine-sensitive calcium channel. Miasiro *et al.* (1988) have shown that endothelin increases $^{45}Ca^{2+}$ efflux even in calcium-free solution, and propose, conversely, that the peptide causes the release of calcium from intracellular stores.

The action of endothelin may differ from that of synthetic calcium facilitators such as Bay K 8644, since the binding of [^{125}I]-endothelin to sites present on vascular smooth muscle culture was unaffected by dihydropyridine calcium entry blockers and (−)-ω-conotoxin (Hirata *et al.*, 1988). In the present study, the effect of endothelin at dihydropyridine and at the (−)-ω-conotoxin GVIA binding sites has been investigated directly, using competition radioligand binding studies.

We have also compared the action of endothelin on a number of isolated pharmacological preparations, including vascular and non-vascular smooth muscle. D'Orléans-Juste *et al.* (1988) have demonstrated that endothelin contracts the rat superfused stomach strip in addition to vascular tissue, whilst Uchida *et al.* (1988) have shown that endothelin contracts the guinea-pig isolated trachea. Ishikawa *et al.* (1988) have shown that the peptide elicits positive chronotropic and inotropic effects, which may be mediated by a release of atrial natriuetic peptide (ANF), since Fukuda *et al.* (1988) have shown that endothelin releases ANF from rat atrial myocytes.

The role of cyclo-oxygenase products in the responses of vascular tissue have been investigated both *in vitro* and *in vivo*, since endothelin has been shown to release prostacyclin (PGI_2) and thromboxane A_2 (TxA_2) from guinea-pig perfused lung (Antunes *et al.*, 1988). Finally, we have compared the pressor responses to endothelin in both normotensive and spontaneously hypertensive rats (SHRs). Tomobe *et al.* (1988) have shown that endothelin is more potent at eliciting contractions of isolated renal arteries from the SHR in comparison to renal arteries from normotensive animals.

A preliminary account of some of this work was presented at the joint meeing of the American Society for Cell Biology and the American Society for Biochemistry and Molecular Biology, San Francisco, 1989 (Eglen *et al.*, 1989).

Methods

Isolated tissues

Functional studies Rings (2 mm) of thoracic aorta were prepared from male Sprague-Dawley rats (250 g) and rings of mesenteric artery and portal vein from male, New Zealand rabbits (3 kg). These preparations were placed in Krebs physiological salt solution (see below for composition) and maintained at pH 7.4, 37°C. The tissues were then placed in 10 ml organ baths (previously treated with SIGMACOAT) containing Krebs solution (pH 7.4, 37°C) and suspended, under 1.0 g tension, between stainless steel hooks. The perfused mesenteric bed was prepared after the method of McGregor (1965), using tissue isolated from male Sprague-Dawley rats (250 g), anaesthetised by ether inhalation and pretreated with 10,000 u kg^{-1}, i.p. heparin. The isolated mesenteric bed was removed, and the upper major branches of the mesenteric artery were cauterised. The tissues were perfused with prewarmed, oxygenated (37°C, pH 7.4) Krebs solution at a rate of 3 ml min^{-1}.

Non-vascular tissues were prepared as follows: guinea-pig (Dunkin-Hartley, 300 g, male) urinary bladder (Eglen & Whiting, 1987), proximal ileum (Clague *et al.*, 1985), oesophageal muscularis mucosae (Kamikawa *et al.*, 1985), zig-zag tracheal strips (Emmerson & McKay, 1979), uterus (Edinburgh Staff, 1968), rat (Sprague-Dawley, 250 g, male) paced (2 Hz, threshold voltage +10%, 1 ms duration) left atria (Blinks, 1966), vas deferens (Edinburgh Staff, 1968), anococcygeus muscle (Gillespie, 1972) and fundic strip (Coleman, 1987).

All preparations were removed from animals previously killed by CO_2 asphyxiation. The uteri were removed from animals pretreated 18 h previously with 0.1 mg kg^{-1} (i.p.) stilboestrol. The preparations were placed in either Krebs physiological salt solution (left atria, vas deferens, trachea, fundic strip and anococcygeus tissue), Sund's physiological salt solution (see below for composition: uterus) or Tyrode solution (see below for composition; remaining tissues). Experiments were conducted at pH 7.4, 37°C, with the exception of the studies using the uterus which were conducted at 30°C in order to inhibit spontaneous activity. Indomethacin (1×10^{-6} M) was routinely added to inhibit cyclooxygenase activity, except where indicated.

The preparations were allowed to equilibrate for 60 min during which time the bathing solution was replaced every 15 min. Tissues were then exposed to 5×10^{-2} M KCl for 5 min, in order to estimate the maximum contractile activity and viability of the tissues; paced left atrial preparations were exposed to 1×10^{-7} M isoprenaline. All the tissues were then washed three times at 10 min intervals over the next 30 min, during which time the tissues relaxed, and the baseline tension was restored.

Cumulative concentration-response curves were then constructed to agonists, using concentrations spaced at 0.5 log intervals. Incremental concentrations of agonists were added, once a sustained contraction was attained to the previous agonist concentration. The time between additions varied according to the preparation, agonist, and the concentration employed. Treatment with endothelin required 10–15 min to attain sustained responses, although the vascular preparations required longer (15–30 min) at the highest concentrations. In some experiments, the endothelium of the rat aortic preparations was removed by gently rolling the tissue between moistened fingers 10 times. The effectiveness of this procedure was assessed by abolition of relaxant responses to acetylcholine (1×10^{-6} M).

Endothelin has been shown (Warner *et al.*, 1988) to relax the perfused mesenteric bed preparation of the rat, when precontracted with methoxamine. This was investigated using a method similar to that of Warner *et al.* (1988). Endothelin was administered as a continuous infusion in these studies, at a rate of 3 ml min^{-1}. Preliminary experiments showed that relaxations were observed to acetylcholine ($-\log EC_{50} = 6.8 \pm 0.08$, mean ± s.e.mean, $n = 4$). The ability of endothelin to relax the rat isolated aorta precontracted with phenylephrine (0.1 μM), in which the endothelium was intact was also studied. This preparation also showed relaxations to acetylcholine ($-\log EC_{50} = 6.5 \pm 0.08$, $n = 6$).

The effects of changing the ionic composition of the Krebs solution on responses of the rat aortic rings (with endothelium removed as described above) was studied. In separate experiments, responses to endothelin were determined both in Krebs solution from which $CaCl_2 \cdot 6H_2O$ was omitted, resulting in a free calcium concentration of less than 1×10^{-8} M and in Krebs solution containing no $CaCl_2 \cdot 6H_2O$ and 1×10^{-5} M EDTA, in order to reduce the free calcium ion concentration further. Secondly, a series of experiments was conducted in which the concentration of KCl was increased from 4.6×10^{-3} M to 1.5×10^{-2} M, whilst NaCl was correspondingly decreased from 0.154 M to 0.139 M to retain equivalent osmotic tension. This method has been shown to partially depolarise the vascular smooth muscle and be a prerequisite for eliciting contractions to the dihydropyridine calcium facilitator, Bay K 8644 (Schramm *et al.*, 1983; Auguet *et al.*, 1988).

The effects of cumulative addition of the calcium entry blockers (nifedipine, verapamil and prenylamine) were studied using rat rubbed aortic rings precontracted with endothelin (1×10^{-8} M) for 10 min. In separate experiments, rat aortic rings were also exposed to the above antagonists (1×10^{-7} M) for a period of 60 min before construction of a concentration-response curve to endothelin. Similar experiments were conducted in which tissues were equilibrated with either the N-calcium channel blocker, (−)-ω-conotoxin (1×10^{-6} M), or the sodium channel blocker, tetrodotoxin (1×10^{-6} M).

The involvement of the products of cyclooxygenase activity were studied using the endothelium-denuded aorta of the rat in which concentration-response curves were constructed to endothelin in the presence of either indomethacin (1×10^{-6} M) or the thromboxane receptor antagonist, SQ 29,548 (1×10^{-6} M), following an initial equilibrium period of the tissues with these agents for 60 min.

In the functional studies, responses were measured using either Hugo Sachs K30 or grass FT03 force transducer, connected to a Watanabe-Graphtec 34303 linearecorder or a Beckman Dynograph R611 recorder respectively. In experiments on the rat atria and fundic strip preparations, responses were also analysed using a COMPAQ 386 computer.

Radioligand binding studies

[^{3}H]-PN 200-110 experiments Membrane fractions were prepared from rat cerebral cortex and skeletal muscle according to the method of Gould *et al.* (1984). Dihydropyridine binding sites in these preparations were labelled with [^{3}H]-PN 200-110 (3×10^{-11}–1×10^{-10} M) for 2 h at 25°C in a final volume of 1.25 ml of 5×10^{-2} M Tris HCl buffer (pH 7.4) and were terminated by vacuum filtration over polyethylenemine (0.1%) pretreated glass fibre filters.

Non-specific binding was defined using 1 μM nitrendipine. In studies using endothelin (Penninsula Labs and Peptides International) bovine serum albumin (BSA) was present at 0.1–0.2%.

(−)-ω-Conotoxin experiments Guinea-pig cerebral cortex and hippocampus and other tissues of interest were homogenised in 5×10^{-2} M Tris HCl (pH 7.4), centrifuged at 30,000 *g* for 12 min at 4°C and the pellets resuspended in fresh buffer containing 0.1% BSA, 0.1 μM captopril, 5×10^{-4} M phenanthroline, 5×10^{-4} M dithiothreitol and 70 μg ml^{-1} bacitracin at 0.25–0.5 mg tissue ml^{-1}. Tissue homogenates (0.4 ml) were incubated with unlabelled ω-conotoxin or endothelin (50 μl aliquots with 30 min pre-equilibration) and [^{125}I]-(−)-ω-conotoxin GVIA (50 μl; 10 pM final concentration) for 60 min at 22°C. Assays were terminated by vacuum filtration through 0.1% polyethylenemine-soaked glass fibre filters and the bound radioactivity determined on a Packard gamma counter. The non-specific binding was defined by using 1×10^{-7} M unlabelled conotoxin and it accounted for 10–15% of the total binding (Sharif *et al.*, 1989).

In vivo *studies*

The effects of endothelin on heart rate and blood pressure of normotensive and spontaneously hypertensive (Okamoto) rats (SHRs) were studied using the method described by Yanagisawa *et al.* (1988). The animals were anaesthetised with urethane (1.25 g kg^{-1}, i.p.) and the femoral artery and vein were cannulated for blood pressure recording and compound administration respectively. All animals were pretreated, 15 min before administration of endothelin or vehicle (saline), with atropine (0.25 mg kg^{-1}, i.v.), propranolol (1 mg kg^{-1}, i.v.) and prazosin (1 mg kg^{-1}, i.v.). The baseline mean arterial blood pressure (MAP) of the normotensive animals and SHRs after administration of these drugs, but prior to endothelin administration, were 84 ± 4 and 79 ± 4 mmHg (n = 16–20), respectively.

Endothelin (0.001–10 nmol kg^{-1}) was administered intravenously, and the blood pressure and heart rate were monitored for both magnitude and duration of effect. Blood pressure responses were recorded using a Gould-Stratham P23Gb transducer connected to a Beckman Dynograph R611 recorder. Heart changes were displayed using a Beckman cardiotachometer, triggered by the blood pressure recording.

In a separate series of experiments, the effect of cyclo-oxygenase inhibition was studied in normotensive rats pretreated with indomethacin (10 mg kg^{-1}, i.p.) or saline 3 h before the injection of endothelin. The remainder of the experiment was conducted as described above.

Analysis of results

In vitro *studies* All responses were measured as changes in isometric tension (mg), except in experiments on the rat perfused mesenteric bed, in which changes in perfusion pressure (mmHg) were determined. These changes were expressed as a percentage of response observed to either 5×10^{-2} M KCl or 1×10^{-7} M isoprenaline in the case of the atrial studies. The potency of an agonist was assessed by determination of the EC_{50}, by means of a non-linear iterative curve fitting procedure (Parker & Waud, 1971) using RS1 software (BBN Corporation). Saturation binding data were analysed by use of LIGAND (Munson & Rodbard, 1980), while competition binding data were analysed by an iterative curve fitting program (Michel & Whiting, 1984) based upon the method of Parker & Waud (1971).

In vivo *studies* The effects of endothelin on blood pressure were measured by recording changes in MAP (mmHg). In all studies, the mean (±s.e.mean) changes in the experimental parameters were calculated. Significant differences were determined by use of a one tail Student's t test, with $P < 0.05$ being considered significant.

Drugs used

Acetylcholine (Sigma), atropine (Sigma Chemical Co. Ltd), Bay K 8644 ((methyl-2,6-dimethyl-5-dinitro-4-(2-trifluoromethylphenyl)-1,4-dihydro-3-pyridine carboxylate) Bayer), (−)-ω-conotoxin GVIA (Peninsula Labs), [^{125}I]-ω-conotoxin GVIA (2200 Ci mmol^{-1}; NEN), endothelin (ET-1 Peninsula Labs and Peptides International), heparin (Eli Lilly), indomethacin (Sigma), isoprenaline (Sigma), nifedipine (Bayer), prazosin (ACIC, Inc.), prenylamine (Hoechst-Roussel), propranolol (Sigma), phenylephrine (Sigma), stilboestrol (Sigma), tetrodotoxin (Calbiochem), urethane (Sigma), [^{3}H]-PN 200-110 (isopropyl-4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-methoxy-carbonyl-pyridine; 85 Ci mmol^{-1}; Amersham), verapamil (Knoll AG), SQ 29,548 (15-[1α,2β(5Z),3β,4α]-7-[3-[2-(phenylamino)-carbonyl]hydrazino]methyl]-7-oxobicyclo-[2.2.1]hept-2-yl]-5-heptenoic acid).

Composition of the physiological salt solutions (mM)

Krebs solution: NaCl 118.4, KCl 4.6, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2, glucose 10.0, $NaHCO_3$ 25.0 and $CaCl_2 \cdot 6H_2O$ 2.5. Tris-Krebs solution: NaCl 118.4, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2, glucose 10.0, $NaHCO_3$ 25.0, $CaCl_2 \cdot 6H_2O$ 2.5 and Tris-HCl 10.0. Tyrode solution: NaCl 136.9, KCl 2.7,

Table 1 Responses of isolated vascular preparations to phenylephrine and endothelin

Species	Preparation	*Phenylephrine* Potency ($-\log EC_{50}$)	*Phenylephrine* Maximal response (g)	*Endothelin* Potency ($-\log EC_{50}$)	*Endothelin* Maximal response (g)
Rat	Aorta (−endothelium)	8.4 ± 0.06	1.3 ± 0.10	8.2 ± 0.07	1.6 ± 0.04
	Aorta (+endothelium)	8.2 ± 0.10	1.4 ± 0.03	8.2 ± 0.11	1.3 ± 0.19
	Perfused Mesenteric Bed	5.2 ± 0.12	67 ± 9[a]	8.2 ± 0.12	219 ± 12[a]
Rabbit	Mesenteric artery (−endothelium)	6.5 ± 0.07	5.4 ± 1.5	7.6 ± 0.09	2.5 ± 0.7
	Portal vein (−endothelium)	6.8 ± 0.05	1.5 ± 0.12	8.2 ± 0.13	1.3 ± 0.16

Values are mean ± s.e.mean, $n = 4$–10.
[a] Responses were recorded as changes in perfusion pressure (mmHg).

$MgCl_2 \cdot 6H_2O$ 1.1, NaH_2PO_4 0.4, glucose 5.6, $NaHCO_3$ 11.8 and $CaCl_2 \cdot 6H_2O$ 1.8. Sund's solution: NaCl 154.0, KCl 5.6, $CaCl_2 \cdot 6H_2O$ 0.54, $MgCl_2 \cdot 7H_2O$ 0.95, $NaHCO_3$ 5.9, glucose 2.7. All solutions were gassed with 95% O_2 plus 5% CO_2.

Results

In vitro *studies*

Functional experiments All vascular preparations responded to phenylephrine, with the rat tissue being generally more sensitive than the rabbit (see Table 1). Endothelin also elicited contractile responses in all these preparations, acting as a full agonist (with respect to phenylephrine) in the rat aorta and rabbit portal vein. In the rabbit mesenteric artery, endothelin elicited a maximal response which was approximately half that observed to phenylephrine. The potency and maximal response of endothelin in the rubbed aorta of the rat was significantly ($P < 0.05$) enhanced in comparison to unrubbed preparations. Endothelin caused concentration-dependent increases in the perfusion pressure of the rat mesenteric bed and the $-\log EC_{50}$ value was similar to that observed in the rubbed aorta of the rat. However, the maximal response to endothelin (219 ± 12 mmHg, $n = 4$) was over 2 fold that observed to maximal concentrations of either phenylephrine (67 ± 9 mmHg, $n = 4$) or KCl (110 ± 6 mmHg, $n = 4$). These data are shown in Figure 1.

Non-vascular smooth muscle preparations

Table 2 Responses of isolated smooth muscle and atria to carbachol, isoprenaline and endothelin

Species	Preparation	*Carbachol* % *max*	*Carbachol* Potency ($-\log EC_{50}$)	*Endothelin* % *max*	*Endothelin* Potency ($-\log EC_{50}$)
Guinea-pig	Urinary bladder	100	5.9 ± 0.08	29	<7.5
	Ileum	100	7.2 ± 0.12	31	8.5 ± 0.11
	OMM	100	7.0 ± 0.07	84	8.4 ± 0.06
	Uterus	100	7.0 ± 0.05	74	7.8 ± 0.12
	Trachea	100	6.8 ± 0.08	37	<7.5
Rat	Vas deferens	100	6.8 ± 0.10	NR	NR
	Fundic strip	100	6.5 ± 0.12	100	7.7 ± 0.03
	Anococcygeus	100	7.1 ± 0.08	11	<7.5
	Left atria	100	8.5 ± 0.03[a]	53	<7.5

Values are mean ± s.e.mean, $n = 4$–8. NR – no response up to and including 3.2×10^{-8} M endothelin. Endothelin was studied in all tissues between 1×10^{-11}–3.2×10^{-8} M. OMM – oesophageal muscularis mucosae. [a] Responses to isoprenaline.

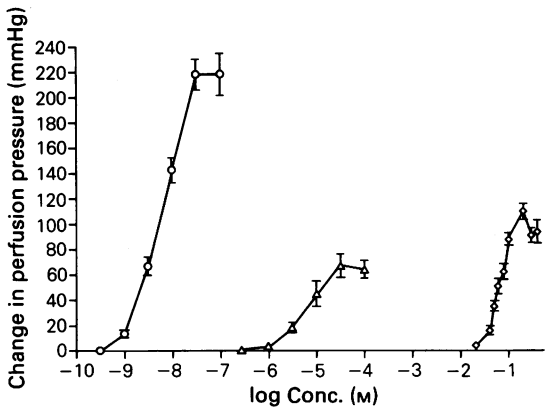

Figure 1 Effect of endothelin (○), phenylephrine (△) and KCl (◇) on the perfusion pressure of the isolated perfused rat mesenteric bed. Values are mean and vertical lines show s.e.mean ($n = 4$).

responded to carbachol with similar potencies, with the exception of the urinary bladder which was about 10 fold less sensitive than the other tissues (Table 2). Endothelin was a full agonist (with respect to carbachol) in the fundic strip preparations and a partial agonist in the oesophageal muscularis mucosa, ileum and uterus preparations. Contractions were also observed in the ileum, in which endothelin was a potent agonist ($-\log EC_{50} = 8.5 \pm 0.08$), but attained a maximum response (31%) much less than that observed to carbachol (100%) or KCl (100%; Table 2). The potencies of endothelin in the ileum and oesophageal muscularis mucosae were not significantly different ($P > 0.05$) in comparison to that observed in the endothelium-denuded aorta of the rat. Endothelin was less potent in the uterus, urinary bladder and trachea in comparison to the above tissues. In the studies with the trachea a maximum response was not attained to the highest concentration studied (3.2×10^{-8} M). No response was observed to endothelin in the vas deferens, and only a small, but significant ($P < 0.05$) response was observed in the anococcygeus muscle. A positive inotropic response was observed to endothelin in the paced left atria, which at the highest concentration used (3.2×10^{-8} M) attained 55% of the response to isoprenaline (1×10^{-7} M).

In view of the potency of endothelin in the rat aortic preparation, this tissue was used to study further the mechanism of action of endothelin. In the rat aorta precontracted with phenylephrine and in which the endothelium was intact (as adjudged by relaxation to acetylcholine), no relaxation was observed to endothelin at concentrations between 1×10^{-11} and 3.2×10^{-8} M. This aspect of the action of endothelin was further studied by using the perfused mesenteric bed of the rat, after the method of Warner *et al.* (1988). The perfusion pressure was elevated from 40 ± 7 to 106 ± 13 mmHg ($n = 4$) by infusion with phenylephrine (3×10^{-5} M). No significant ($P > 0.05$) decrease in perfusion pressure was observed in response to an infusion of endothelin at the concentrations studied (1×10^{-14}–1×10^{-7} M). Further increases in perfusion pressure were observed to an infusion of endothelin at concentrations between 1×10^{-10} to 1×10^{-7} M.

The responses of endothelin were studied in the endothelium denuded aorta of the rat in Krebs solution containing either 4.6×10^{-3} M or 1.5×10^{-2} M KCl. No significant ($P > 0.05$) change in the development of isometric tension in response to endothelin (1×10^{-10}–3.2×10^{-8} M) was observed in Krebs containing either 4.6×10^{-3} M KCl (maximum response = 832 ± 128 mg, $n = 4$) or 1.5×10^{-2} M KCl (maximum response = 786 ± 132 mg, $n = 4$).

The responses of the endothelium-denuded aorta of the rat to endothelin were highly sensitive to reductions in extracellular calcium. Omission of the $CaCl_2 \cdot 6H_2O$ from the Krebs solution resulted in a reduction in the response to 3.2×10^{-8} M endothelin. The change in isometric tension was reduced from 1.40 ± 0.49 to 0.55 ± 0.1 g ($n = 4$). In calcium-free Krebs solution containing 1×10^{-5} M EDTA, the response to 3.2×10^{-8} M endothelin was further reduced to 0.22 ± 0.15 g ($n = 6$).

The effects of various ion channel antagonists were studied. The responses of the endothelium-denuded aorta of the rat to endothelin were unaffected in the presence of either 1×10^{-6} M tetrodotoxin or (−)-ω-conotoxin. However, pre-exposure of the tissues to 1×10^{-7} M nifedipine, verapamil or prenylamine completely abolished any subsequent responses to endothelin. In addition, in tissues precontracted with endothelin (1×10^{-8} M), nifedipine, verapamil and prenylamine inhibited the contraction in a concentration-dependent manner. The concentration of these compounds which inhibited the contracture by 50% ($-\log IC_{50}$) were: nifedipine, 8.0 ± 0.13; verapamil, 7.2 ± 0.09 and prenylamine, 6.6 ± 0.08 ($n = 4$–8).

The effects of cyclo-oxygenase products on the response of the endothelium-denuded aorta of the rat were also studied. Endothelin in the presence of indomethacin (1×10^{-6} M) did not affect the potency of endothelin but the maximal response was significantly ($P < 0.05$) reduced from 1.56 ± 0.04 mg to 1.09 ± 0.16 mg ($n = 4$). A similar finding was observed when indomethacin was omitted but the thromboxane receptor antagonist SQ 29,548 (1×10^{-6} M) was present. The potency of endothelin was again not significantly affected but the maximal response was reduced to 770 ± 0.06 mg ($n = 4$). These data are shown in Figure 2.

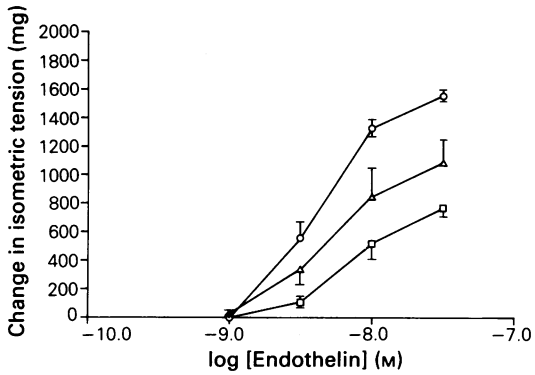

Figure 2 Effect of indomethacin (1×10^{-6} M; △), SQ 29,548 (1×10^{-6} M, □) on responses to endothelin in rat isolated aortic rings. (○) Control responses to endothelin. Values are mean and vertical lines show s.e.mean. ($n = 5$).

Radioligand binding studies In competition studies conducted at 25°C Bay K 8644, nitrendipine, prenylamine and verapamil inhibited [^{3}H]-PN 200-110 binding to the dihydropyridine binding sites in cerebral cortex and skeletal muscle membranes (Table 3). In contrast, endothelin at concentrations ranging from 1×10^{-12}–9×10^{-7} M had no effect on [^{3}H]-PN 200-110 binding in cortical or skeletal muscle membranes. These data are shown in Table 3. Endothelin (1×10^{-12}–9×10^{-7} M) was also without effect on [^{3}H]-PN 200-110 binding to cerebrocortical and skeletal muscle membranes when assays were conducted at 4°C for 2.5 h or at 37°C for 1 h (data not shown). Endothelin at a concentration of 9×10^{-8} M did not affect the ability of Bay K 8644, prenylamine or verapamil to inhibit [^{3}H]-PN 200-110 binding in the two membrane preparations used (Table 3). To investigate if endothelin was being proteolytically degraded in our experiments, additional studies were conducted at 37°C both using a shorter incubation period and including protease inhibitors in the assay. Even when assays were conducted at 37°C for either 5, 15 or 30 min in the presence of iodacetamide (1×10^{-3} M), leupeptin ($1\,\mu g\,ml^{-1}$), phenylmethylsulphonylfluoride (1×10^{-4} M), pepstatin A (1×10^{-6} M), aprotonin ($1\,\mu g\,ml^{-1}$), soybean trypsin inhibitor ($10\,\mu g\,ml^{-1}$) and bacitracin ($0.6\,mg\,ml^{-1}$) endothelin was completely without effect.

[^{125}I]-(ω)-conotoxin binding to N-type Ca^{2+}-channels on guinea-pig hippocampal and cerebrocortical membranes was competitively displaced by unlabelled (ω)-conotoxin. The inhibition constants (IC_{50}) for unlabelled conotoxin were 20.5 ± 1.5 pM ($n = 4$) (Hill coefficients = 1.4 ± 0.1) and 23.5 ± 2.4 pM ($n = 4$; Hill coefficients = 1.5 ± 0.2) for the cortex and hippocampus respectively. Endothelin (10^{-12}–10^{-7} M) did not influence [^{125}I]-conotoxin binding in the cortex or hippocampus ($n = 2$–3) under the present assay conditions.

In vivo *studies*

Endothelin elicited prolonged increases in blood pressure in all animals studied. The threshold for this effect was $0.1\,nmol\,kg^{-1}$ and at the highest doses, i.e. 3 and $10\,nmol\,kg^{-1}$ i.v., lethal effects were observed following large pressure responses. The magnitude and duration of the pressure responses were dose-dependent. The increases in blood pressure at $1\,nmol\,kg^{-1}$ i.v. of endothelin were accompanied by an initial decrease in heart rate (Figure 3). It was also observed that at doses of 0.1–$10\,nmol\,kg^{-1}$ endothelin induced a hypotensive response (duration > 300 s) as shown in Figure 3. This effect, which was significantly ($P > 0.05$) different from responses in animals injected with vehicle, was not observed with the lower doses ($1\,pmol\,kg^{-1}$–$10\,pmol\,kg^{-1}$).

Table 3 The effect of calcium channel ligands and endothelin on binding in rat cortex and skeletal muscle at [^{3}H]-PN 200-110 25°C

	Cortex			*Skeletal muscle*		
Compound	pIC_{50}	*nH*	% *inhibition**	pIC_{50}	*nH*	% *inhibition**
Bay K 8644	8.1	(0.7)	100	6.6	(0.9)	100
Endothelin	NA	NA	+2	NA	NA	−3
Nitrendipine	8.7	(1.1)	100	7.9	(0.7)	100
Prenylamine	6.3	(0.9)	100	7.4	(0.8)	92
Verapamil	8.0	(0.7)	48	7.5	(0.7)	83

pIC_{50} is the negative logarithm of the IC_{50} value. nH is the Hill coefficient of the displacement curve. NA = not applicable. Values are the mean of 3 experiments (s.e.mean < 5%). Identical results were obtained when assays were conducted in the presence of 9×10^{-8} M endothelin.

* % inhibition refers to % inhibition of specific binding achieved at the highest concentration of each drug test.

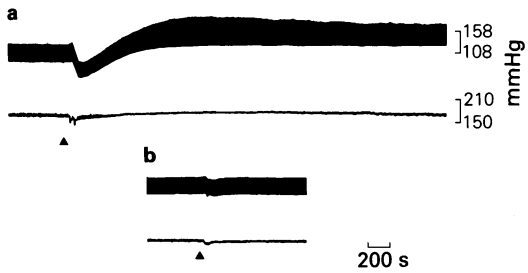

Figure 3 Effects of endothelin (a; 1 nmol kg^{-1} i.v.) or vehicle (b; saline) on blood pressure (mmHg) and heart rate (beats min^{-1}) of the anaesthetised normotensive rat.

The hypertensive and transient hypotensive responses were not significantly different ($P > 0.05$) in animals pretreated with indomethacin at any dose studied. Finally, responses to endothelin at any of the doses used were not significantly different in the anaesthetised, chemically denervated SHR.

Discussion

Endothelin is an endogenous peptide (Yanagisawa *et al.*, 1988) which has been shown to enhance the entry of extracellular calcium in vascular tissue (Hirata *et al.*, 1988). The high potency of the peptide has led workers to speculate that it may play a role in the pathophysiology of hypertensive disease and coronary vasospasm (Yanagisawa *et al.*, 1988). In the present study, we have examined the action of endothelin in a number of tissues, both vascular and non-vascular.

Endothelin contracted the rat aorta and displayed high potency in vessels both with and without an intact endothelium. The absolute change in isometric tension was greater in rat aortae denuded of endothelium. This has been shown (Martin *et al.*, 1986) with other vasoconstrictors, such as α_2-adrenoceptor agonists, and has been ascribed to a basal release of endothelium-derived relaxing factor (EDRF), which functionally antagonises constrictor responses (see Alosachie & Godfraind, 1988 for references). Warner *et al.* (1988) have suggested that endothelin in the perfused rat mesenteric bed may release EDRF which could also account for the enhanced response in tissues denuded of endothelium. Alternatively, the enhancement may be due to removal of EDRF which has been shown (Spedding *et al.*, 1986) to modulate calcium channel activity in the rat aorta. In contrast to the data of Warner *et al.* (1988), no relaxation was observed to endothelin in the present study in either the isolated aorta or in the perfused mesenteric bed (in which the endothelium was intact) of the rat. The reason for this disparity is unclear.

The potency of endothelin in the endothelium-denuded aorta of the rat observed in the present study ($-\log EC_{50} = 8.2$) was less than observed in the pig coronary artery ($-\log EC_{50} = 9.40$; Yanagisawa *et al.*, 1988), but similar to values obtained in the rat isolated renal artery ($-\log EC_{50} = 8.76$; Tomobe *et al.*, 1988), aorta ($-\log EC_{50} = 8.39$; Auguet *et al.*, 1988) and human omental artery ($-\log EC_{50} = 8.55$; Hughes *et al.*, 1988).

Endothelin was less potent in the rabbit mesenteric artery, in comparison to the rabbit portal vein. These data are in accordance with the results obtained by D'Orléans-Juste *et al.* (1988), and may be related to the varying sensitivities of vascular tissue to the entry of extracellular calcium. It is clear therefore that endothelin exhibits different potencies depending upon the vessel and species employed as a bioassay.

The responses to endothelin in the perfused mesenteric bed were different from those in other vascular studies, in that the absolute vasoconstriction was much greater than that observed to either phenylephrine or KCl. In the present study, the basal perfusion pressure was 40 mmHg, which was higher than has been employed in previous studies with this preparation (10 mmHg, Randall *et al.*, 1988). This disparity may have been due to the method of preparation, which was modified from that described by McGregor (1965). Specifically, the cauterisation of the upper major branches of the mesenteric artery may have raised the perfusion pressure. The response to endothelin in this preparation suggests that endothelin may act through mechanisms unavailable to either phenylephrine or KCl. Additional experiments are required to elucidate these mechanisms, but it is clear that the perfused mesenteric bed of the rat forms a highly sensitive bioassay for endothelin. This finding may indicate that, *in vivo*, endothelin could exert pressor responses by profound effects on the microvasculature.

The ability of the isolated ileum, oesophageal muscularis mucosae, fundic strip and uterus to contract in response to endothelin provides evidence that contractile effects of endothelin are not restricted to the vasculature. It has been previously shown (D'Orleans-Juste *et al.*, 1988) that the rat stomach strip is sensitive to endothelin and the present study suggests that other types of gastrointestinal smooth muscle are also sensitive. It should be noted that due to a limited supply of the peptide, it was not possible to obtain a definite maximum response in the uterine and fundic strip preparations. Consequently, the $-\log EC_{50}$ values obtain in these tissues may differ from the true $-\log EC_{50}$. The mechanism of action of endothelin on these tissues is unknown, but it

seems reasonable to suggest that it is also due to facilitation of the entry of extracellular calcium. The effect of endothelin was specific, however, in that some types of muscle, such as the anococcygeus and vas deferens, were insensitive.

These differences between tissues in response to endothelin may reflect either differences in the number of endothelin binding sites, or differences in the sensitivity of smooth muscles to the entry of extracellular calcium (Tomita *et al.*, 1985). A further consideration is that there are at least two classes of voltage-sensitive calcium channels (Aaronson *et al.*, 1986). Additional studies are required to elucidate further the exact site at which endothelin acts to increase intracellular calcium. Miasiro *et al.* (1988) have shown a transient increase in intracellular calcium (as measured by an increase in $^{45}Ca^{2+}$ efflux in calcium free medium) in response to endothelin which could occur by an increase in inositol phospholipid-hydrolysis. However, in vascular smooth muscle cultures endothelin does not elicit a stimulation of inositol phospholipid hydrolysis (Hirata *et al.*, 1988), although converse results have been obtained by Sugiura *et al.* (1989) and Marsden *et al.* (1989).

The potency of endothelin in the present study in the guinea-pig isolated trachea was lower ($-\log EC_{50} > 7.5$) than that found by Uchida *et al.* (1988) ($-\log EC_{50} = 9.27$). One explanation for this disparity is that in the present study cyclo-oxygenase activity was inhibited; it was not in the study by Uchida *et al.* (1988). Antunes *et al.* (1988) have shown that endothelin can elicit the release of PGI_2 and TxA_2 from guinea-pig lung tissue, and it has been shown (Jones *et al.*, 1982) that the trachea is highly sensitive to the contractile effects of thromboxanes. The maximal change in isometric tension, but not potency, to endothelin was reduced in the presence of either the cyclo-oxygenase inhibitor, indomethacin, or the thromboxane receptor antagonist, SQ 29,548. This suggests that a component of the response to endothelin *in vitro*, is mediated by TxA_2 release. However, the physiological significance of this finding *in vivo* is unclear, since pretreatment of rats with indomethacin did not significantly affect the hypertensive responses to endothelin.

The pressor responses to endothelin described in the present study are similar to those obtained by Yanagisawa *et al.* (1988) and Spokes *et al.* (1988). The effective concentration range of endothelin is narrow, with threshold effects occurring at $0.01\,nmol\,kg^{-1}$ and limited by lethal effects occurring at 3 and $10\,nmol\,kg^{-1}$. Paradoxically a transient, dose-dependent depressor response was also observed. This may be due to the release of EDRF by endothelin (Warner *et al.*, 1988), although we have been unable to confirm this action in the present *in vitro* studies. An alternative explanation is the transient release of atrial natriuretic peptide (ANF). This peptide is hypotensive and rapidly metabolised (Lang *et al.*, 1987) and endothelin is a potent ($-\log EC_{50} = 9.6$) secretagogue for ANF in rat atrial myocytes (Fukuda *et al.*, 1988). Additional studies are warranted to clarify this action. The ability of endothelin to elicit a positive inotropic response in rat isolated left atria is in agreement with the data of Ishikawa *et al.* (1988). However, a greater maximal response to endothelin was observed in the present study. Facilitation of calcium entry by a direct action of endothelin has been shown to occur in this tissue in response to endothelin (Ishikawa *et al.*, 1988) which is similar to the effect of Bay K 8644 (Schramm *et al.*, 1983).

The mechanism by which endothelin elicits contractile responses in vascular tissue appears to involve the entry of extracellular calcium, although Miasira *et al.* (1988) observed no increase in the rate of $^{45}Ca^{2+}$ influx in the presence of endothelin. The high dependency of the responses to endothelin on extracellular calcium is in agreement with other groups (Yanigisawa *et al.*, 1988; Hirata *et al.*, 1988; Hughes *et al.*, 1988) but in contrast to the findings of Auguet *et al.* (1988). However, it appears to act at a different site to that of Bay K 8644 in this tissue.

In the present study, it has been shown that endothelin does not interact directly at the dihydropyridine binding site, labelled by [^{3}H]-PN 200-40 or at the N-channel labelled with ω-conotoxin, a finding in accordance with the data of Gu *et al.* (1989) and Hirata *et al.* (1988) which show that dihydropyridines and ω-conotoxin do not interact at the endothelin binding site. Furthermore, responses to endothelin were not enhanced by elevating the extracellular K^+ concentration, a procedure shown (Schramm *et al.*, 1988) to be essential in detecting vascular response to Bay K 8644 *in vitro*. Auguet *et al.* (1988) have demonstrated a small but significant potentiation of the responses to endothelin in the rat aorta under similar conditions, but this was not confirmed in the present study. In agreement with other workers (see Introduction), the responses to endothelin were potently antagonised by calcium entry blockers, irrespective of their class, but no direct interaction was observed in radioligand binding studies.

It is possible that endothelin binds to a different state of the calcium channel than that studied in the competition radioligand binding studies. In binding studies, the interaction is essentially at the inactivated state of the channel (Bean, 1984) whereas in functional studies an interaction at the resting or open state of the channel is inferred. The lack of effect of conotoxin in both functional and binding studies suggests that endothelin does not interact at

the N-calcium channel. Finally, the lack of effect of tetrodotoxin suggests that endothelin also does not interact at the sodium channel. This finding is supported by data which show that endothelin (1×10^{-7} M) does not displace the binding of either [^{3}H]-saxitoxin or [^{3}H]-batrachotoxin A to membranes from rat cerebral cortex (A.D. Michel, unpublished observations).

In conclusion, endothelin is a potent vasoconstrictor *in vitro* and *in vivo*, which may act by enhancing extracellular calcium entry. Some of the effects *in vitro* may be attributable to cyclo-oxygenase product release, but this is not a major factor *in vivo*. The effects of endothelin are not restricted to the vasculature, although it cannot be concluded from the present studies whether these effects are mediated directly by an enhancement of calcium entry or indirectly by neurotransmitter release.

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Effects of N-ethylmaleimide on 5-hydroxytryptamine transport and sodium content in rabbit platelets

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1 The present study analysed the mechanism underlying the inhibitory action of N-ethylmaleimide (NEM) on the 5-hydroxytryptamine (5-HT) uptake by blood platelets.

2 Rabbit platelets suspended in protein-free buffer were first preincubated for 45 min in the absence and presence of NEM (20 to 160 μM) or ouabain (0.5–2.0 μM) and then either analysed for their Na^+ and K^+ content or incubated (15 s) with various concentrations of [^{3}H]-5-HT (0.13–4.03 μM) to determine K_m and V_{max} for 5-HT uptake.

3 Both NEM and ouabain produced concentration-dependent decreases in V_{max} with IC_{50} values of 52 and 0.58 μM, respectively. Neither drug changed K_m significantly.

4 Both NEM and ouabain increased the Na^+ and decreased the K^+ content of platelets in a concentration-dependent manner.

5 There was a linear correlation between V_{max} (expressed in % of control) and the reciprocal cellular Na^+ content, with the results for both drugs falling onto one and the same regression line ($r = 0.992$; $n = 8$). This regression showed that an increase in Na^+ content by 69% sufficed to reduce V_{max} by 50%.

6 At concentrations that reduced 5-HT uptake by about 60%, neither NEM nor ouabain altered the potency of imipramine for inhibition of 5-HT uptake.

7 Hence, NEM inhibits 5-HT transport by inhibiting the Na^+/K^+-ATPase and not by a direct interaction with the 5-HT carrier. The consequential increase in the intracellular Na^+ concentration reduces the transmembrane Na^+ gradient and, therefore, hinders 5-HT inward transport. This action of the drug does not affect the ability of the carrier to bind 5-HT or imipramine.

Introduction

Several lines of evidence indicate that [^{3}H]-imipramine specifically binds to a membrane site which is associated with the 5-hydroxytryptamine (5-HT) carrier present in the plasma membrane of blood platelets and 5-hydroxytryptaminergic neurones (Raisman *et al.*, 1979; Briley *et al.*, 1979; Talvenheimo *et al.*, 1979; Rehavi *et al.*, 1980; Langer *et al.*, 1980; 1981). Since the inhibition by imipramine of 5-HT transport is competitive in nature (Born *et al.*, 1972; Tuomisto, 1974; Marcusson *et al.*, 1986; Wölfel *et al.*, 1988) and since 5-HT competitively displaces [^{3}H]-imipramine specifically bound to plasma membranes of platelets (Talvenheimo *et al.*, 1979; Marcusson & Tiger, 1988) and brain tissue (Marcusson *et al.*, 1986), it would seem reasonable to assume that imipramine binds to the substrate recognition site of the 5-HT carrier or else to a carrier site whose occupation mutually excludes 5-HT from entering transport. However, the finding that substrates of the carrier (e.g., 5-HT and tryptamine) slow down the dissociation of bound [^{3}H]-imipramine (Sette *et al.*, 1983; Wennogle & Meyerson, 1983; Segonzac *et al.*, 1985) led to the suggestion that the site involved in [^{3}H]-imipramine binding and the carrier protein are distinct, but allosterically coupled entities. This 'two-site hypothesis' of imipramine action, which was challenged by others (Marcusson *et al.*, 1986; Marcusson & Tiger, 1988; Humphreys *et al.*, 1988), states that the site labelled with [^{3}H]-imipramine represents a receptor which, when occupied, modulates 5-HT transport by an allosteric interaction with the carrier.

Recently, Meyerson *et al.* (1987) reported that N-ethylmaleimide (NEM), an agent known to alkylate

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sulphhydryl groups, inhibits 5-HT uptake by human platelets in a concentration-dependent manner without affecting [^{3}H]-imipramine binding to these cells. These results were interpreted as indicating that the 5-HT carrier is more susceptible to alkylation by NEM than is the [^{3}H]-imipramine binding site and, hence, were taken to support the 'two-site hypothesis' described above. While this is one possible explanation, there is an alternative one. NEM also inactivates the Na^+/K^+-ATPase and, therefore, reduces Na^+ pump activity (Akera & Brody, 1978). Consequently, NEM may well indirectly inhibit 5-HT transport by reducing the transmembrane Na^+ gradient which is the main force driving 5-HT uptake (Sneddon, 1969; Rudnick, 1977). If the Na^+/K^+-ATPase were more susceptible to alkylation by NEM than the 5-HT carrier, the results of Meyerson *et al.* (1987) could be explained without the need to postulate a binding site for imipramine other than the 5-HT carrier.

The experiments described here were carried out to test this possibility. Ouabain was included in the study as a 'positive' control. This drug was previously shown to inhibit 5-HT uptake by reducing the transmembrane Na^+ gradient (Sneddon, 1971). A preliminary account of the present results, which were obtained in rabbit platelets, was presented to the German Pharmacological Society (Wölfel, 1988).

Methods

Rabbits of either sex (2.5–3.5 kg) were anaesthetized by 0.25 ml kg^{-1} i.m. Hypnorm (Janssen). Blood (20–40 ml) was collected from an ear artery in plastic syringes containing trisodium citrate (final concentration 10.6 mM).

Preparation of washed platelets

Platelet-rich plasma (PRP) was obtained by centrifugation of blood at room temperature (175 *g*, 15 min). After the addition of prostaglandin E_1-α-cyclodextrine (PGE_1; 30 nM), the PRP was centrifuged (1000 *g*, 8 min, 25°C). The resulting supernatant was discarded and the cells supended in buffer of the following composition (in mM): NaCl 140.0, KCl 5.0, $MgSO_4$ 1.2, glucose 10.1, Tris 10.0, PGE_1 0.00003, pargyline 0.01 (to inhibit monoamine oxidase); the buffer was saturated with O_2 and its pH adjusted to 7.4 with HCl. The platelets were kept in buffer for 30 min at 25°C, then centrifuged again (1000 *g*, 8 min, 25°C) and finally resuspended in buffer. The platelet count in the final platelet-in-buffer suspension (PRB) was determined by use of the Sysmex Platelet Counter PL 100 (Colora, Lorch, F.R.G.); it amounted to 0.6–1.3 × 10^6 platelets μl^{-1}. All experiments were carried out with freshly prepared PRB and, unless stated otherwise, at 37°C.

Uptake of tritiated 5-hydroxytryptamine ([^{3}H]-5-HT)

Samples of PRB were first preincubated for 45 min in the absence and presence of either NEM (20–80 μM) or ouabain (0.5–2.0 μM). Thereafter, the incubation with [^{3}H]-5-HT was started by diluting 100 μl PRB with 900 μl of buffer containing 33 nM [^{3}H]-5-HT (specific activity 20.0 Ci mmol^{-1}) and unlabelled 5-HT to make up final 5-HT concentrations ranging from 0.13 to 4.03 μM. Uptake of 5-HT was determined as described previously (Wölfel *et al.*, 1988). Briefly, after 15 s of incubation with [^{3}H]-5-HT, uptake was terminated by rapid dilution (with ice-cold buffer) followed by filtration. The radioactivity remaining on the filter was taken to reflect 5-HT uptake. The carrier-mediated component of uptake was determined from the difference between simultaneous measurements of uptake in the absence and presence of 10 μM imipramine, respectively (Wölfel *et al.*, 1988). To determine K_m and V_{max} for the inward transport of 5-HT, eight substrate concentrations ranging from 0.13 to 4.03 μM were tested in each experiment.

The inhibition of 5-HT uptake by imipramine (5–40 nM) was determined at a [^{3}H]-5-HT concentration of 30 nM. Platelets were then exposed to imipramine not only during the 15 s of incubation with [^{3}H]-5-HT, but also during the last 15 min of preincubation.

Determination of Na^+ and K^+ content of platelets

Samples of 750 μl PRB were preincubated for 45 min in the absence and presence of various concentrations of NEM (20–160 μM) or ouabain (0.5–2.0 μM). At 30 min during preincubation, the PRB was thoroughly mixed with 150 μl of a solution containing 250 mM sucrose, 1 mM sorbitol and trace amounts of [^{14}C]-sorbitol. After preincubation, the tubes containing PRB were chilled in a water bath maintained at 2–4°C. All the following steps were done at this temperature. The platelet suspension (750 μl) was transferred into conical 1.5 ml tubes by carefully layering it on top of 200 μl of 10% dextran 40 containing 75 mM sucrose. After centrifugation (7500 *g*, 3 min), a 100 μl portion of the supernatant buffer layer was analysed for its ^{14}C content, with the rest of the buffer as well as dextran 40 layer being discarded. The platelet pellet was washed with 1 ml of 250 mM sucrose, spun down again (7500 *g*, 2 min) and finally lysed in 1 ml of deionized water by ultrasonification. The resulting supernatant was then used to determine ^{14}C radioactivity by liquid scintil-

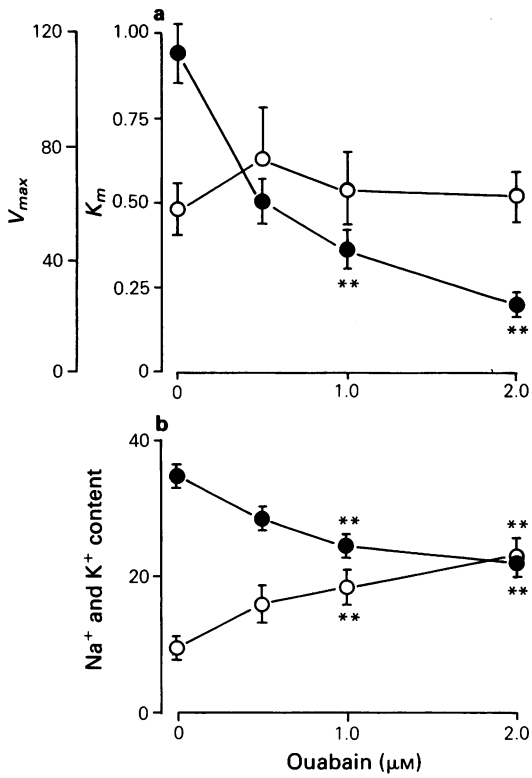

Figure 1 Effects of ouabain on the kinetic parameters for 5-hydroxytryptamine (5-HT) uptake (a) and the Na^+ and K^+ content (b) in rabbit platelets. Ordinates: (a) V_{max} in pmol per 10^8 platelets min^{-1} (●) and K_m in nM (○) for 5-HT uptake; (b) cellular Na^+ (○) and K^+ (●) content in nmol per 10^8 platelets. Abscissae: concentration of ouabain in μM during 45 min of preincubation. Shown are geometric means of 14 (a) and 12 (b) observations each; vertical bars show s.e.mean. Asterisks indicate significant differences from control values (H-test according to Kruskal and Wallis followed by Nemenyi's procedure): $^{**}P < 0.01$.

lation counting (LKB Rackbeta, Freiburg, F.R.G.) and to measure the Na^+ and K^+ content with an Eppendorf flame photometer (Hamburg, F.R.G.). After correction for cation contamination due to trapping of extracellular Na^+ and K^+ (estimated from the [^{14}C]-sorbitol space), the Na^+ and K^+ content of the platelets was expressed in nmol per 10^8 platelets.

Calculations

To determine values of K_m and V_{max} for 5-HT uptake, a least squares non-linear regression analysis was used to fit a logistic function equivalent to Hill's equation to the saturation curves relating initial rates of uptake to the concentration of 5-HT (Wölfel *et al.*, 1988). The saturation curves obtained under the present experimental conditions exhibited mean apparent Hill coefficients that did not differ from unity. IC_{50} values for inhibition of 5-HT uptake by imipramine were obtained by fitting Hill's equation for multisite inhibition to inhibition curves (i.e., uptake in % of control vs. imipramine concentration) as described previously (Schömig & Bönisch, 1986). Regression lines ($y = a + bx$) were calculated according to conventional procedures (Sachs, 1984).

Statistics

Results are given as geometric means ± s.e.mean or, when stated in the text, together with their 95% confidence limits. The significance of differences between means was assessed by non-parametric procedures: Wilcoxon's signed rank test for paired observations; Friedman's test followed by the procedure of Wilcoxon and Wilcox for multiple comparisons of dependent observations; H-test according to Kruskal and Wallis followed by Nemenyi's procedure for multiple comparisons of independent observations (Sachs, 1984). Differences were taken to be statistically significant when $P < 0.05$.

Materials

The following were used: 5-hydroxytryptamine creatinine sulphate, pargyline hydrochloride, N-ethylmaleimide and ouabain octahydrate (Sigma, München, F.R.G.); imipramine hydrochloride (Ciba-Geigy, Basel, Switzerland); 5-[1,2-^{3}H(N)]-hydroxytryptamine creatinine sulphate (NET 498; NEN, Dreieich, F.R.G.); dextran 40 (Rheomacrodex 10%; Schiwa, Glandorf, F.R.G.); Hypnorm (Janssen Pharmaceutica, Beerse, Belgium), prostaglandin E_1-α-cyclodextrine (Schwarz Pharma, Monheim, F.R.G.).

Results

Effects of NEM and ouabain on K_m and V_{max} for 5-HT uptake and the cellular Na^+ and K^+ content

Preincubation of the platelets with ouabain (0.5–2.0 μM) caused a concentration-dependent decrease in the V_{max} of 5-HT uptake ($IC_{50} = 0.58$ μM), whilst leaving the value of K_m for 5-HT unchanged (Figure 1a). Qualitatively similar results were obtained after

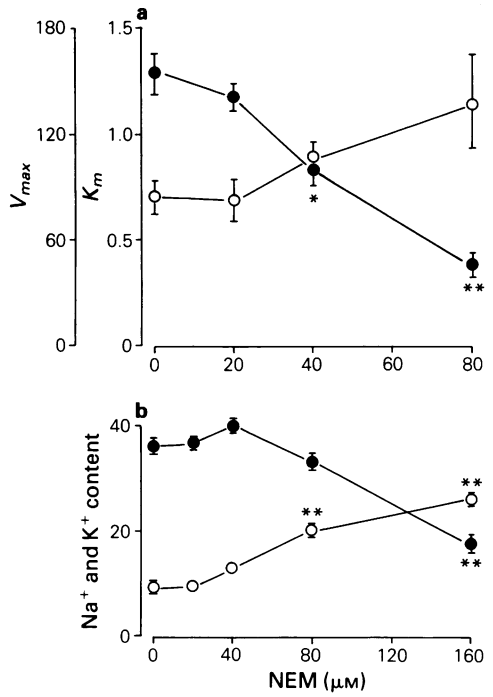

Figure 2 Effects of N-ethylmaleimide (NEM) on the kinetic parameters for 5-hydroxytryptamine (5-HT) uptake (a) and the Na^+ and K^+ content (b) in rabbit platelets. Ordinates: (a) V_{max} in pmol per 10^8 platelets min^{-1} (●) and K_m in nM (○) for 5-HT uptake; (b) cellular Na^+ (○) and K^+ (●) content in nmol per 10^8 platelets. Abscissae: concentration of NEM in μM during 45 min of preincubation (note the difference in scale between a and b). Shown are geometric means of at least 14 (a) and 17 (b; except at 160 μM NEM, where $n = 7$) experiments each; vertical bars show s.e.mean. Asterisks indicate significant differences from control values (H-test according to Kruskal and Wallis followed by Nemenyi's procedure): $*P < 0.05$; $**P < 0.01$.

preincubation with NEM (20–80 μM; IC_{50} for inhibition of $V_{max} = 52$ μM), even though the K_m value tended to rise with increasing NEM concentration (Figure 2b). At a NEM concentration of 160 μM, initial rates of uptake were too low to get reliable estimates of K_m and V_{max}.

In parallel experiments, the Na^+ and K^+ content was determined in platelets pre-exposed to various concentrations of ouabain (0.5–2.0 μM) or NEM (20–160 μM). As shown in Figures 1b (ouabain) and 2b (NEM), both drugs caused the Na^+ content of the cells to increase and the K^+ content to decrease. However, while ouabain-induced increases in Na^+ content went always hand in hand with decreases in K^+ content (Figure 1b), preincubation with up to 80 μM NEM induced a rise in Na^+ content without a statistically significant change in K^+ content (Figure 2b).

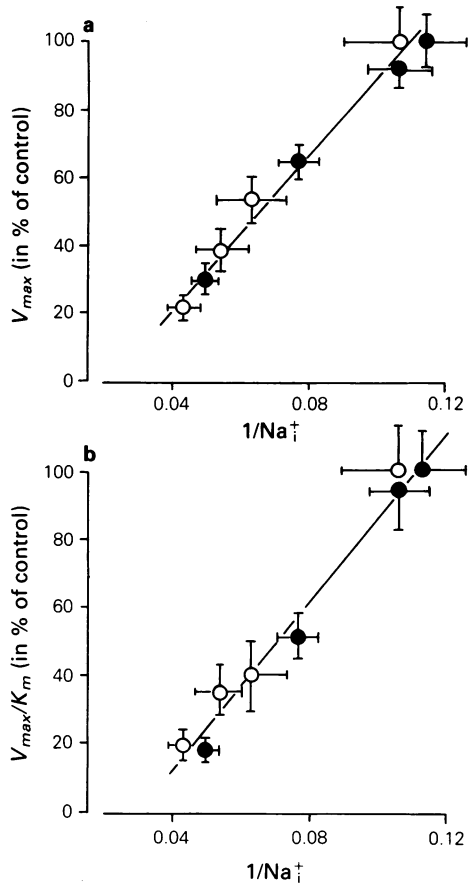

Figure 3 Linear correlation between V_{max} (a) or V_{max}/K_m (b) for 5-hydroxytryptamine (5-HT) uptake and the reciprocal cellular Na^+ content. Ordinates: V_{max} and V_{max}/K_m in % of control. Abscissae: reciprocal of the cellular Na^+ content in nmol per 10^8 platelets. Shown are geometric means of at least 12 observations each; vertical and horizontal bars show s.e.mean. Results obtained with ouabain and N-ethylmaleimide (NEM) are shown as (○) and (●), respectively. Control V_{max} was 113 (95% confidence limits: 81 to 156; $n = 16$) and 154 (131 to 181; $n = 22$) pmol per 10^8 platelets min^{-1} in experiments with ouabain and NEM, respectively; the corresponding values of V_{max}/K_m were 0.235 (0.176 to 0.313) and 0.220 (0.172 to 0.283) pmol per 10^8 platelets min^{-1} nM^{-1}, respectively. The regression lines ($y = a + bx$) obtained from the mean group results ($n = 8$ in both panels) were drawn to fit the following parameters: $a \pm S_a = -24 \pm 4.8$ (a) and -36 ± 4.3 (b); $b \pm S_b = 1118 \pm 59$ (a) and 1237 ± 71 (b). The correlation coefficient was 0.992 in a and 0.990 in b.

When the V_{max} and the ratio of V_{max}/K_m (both being expressed in % of control) were each plotted

against the reciprocal cellular Na^+ content (which, under our experimental conditions, is a measure of the transmembrane Na^+ gradient), linear relationships with similar slopes were obtained (Figure 3). Moreover, irrespective of whether V_{max} (Figure 3a) or the ratio of V_{max}/K_m (Figure 3b) was related to the reciprocal of the Na^+ content, the results obtained for both drugs were virtually indistinguishable and fell on one and the same regression line. This finding indicates that NEM, like ouabain, inhibits 5-HT uptake at non-saturating (V_{max}/K_m) as well as highly saturating (V_{max}) substrate concentrations by reducing the transmembrane Na^+ gradient. According to the regression lines of Figure 3, V_{max} and V_{max}/K_m were halved when the Na^+ content of the cells had risen by 69% (95% confidence limits: 60 to 79%) and 57% (49 to 66%), respectively. Under control conditions, the cellular Na^+ content was 9.0 (7.4 to 11.0) nmol per 10^8 cells ($n = 34$).

Effect of ouabain and NEM on the potency of imipramine

After pre-exposure of platelets to 0.5 μM ouabain or 40 μM NEM for 45 min, initial rates of uptake were determined at a [^{3}H]-5-HT concentration of 30 nM, both in the absence and presence of various concentrations of imipramine (5–40 nM). The concentration-effect curves for the imipramine-induced inhibition of 5-HT uptake obtained under these and under control conditions are shown in Figure 4a. Although the inhibitory effect of the highest imipramine concentration tested here (40 nM) was somewhat less pronounced after pre-exposure to NEM than in controls (Figure 4a), the IC_{50} for imipramine was affected neither by NEM nor by ouabain (Figure 4b); it amounted to 19 (16 to 23) nM in the control group of experiments ($n = 10$).

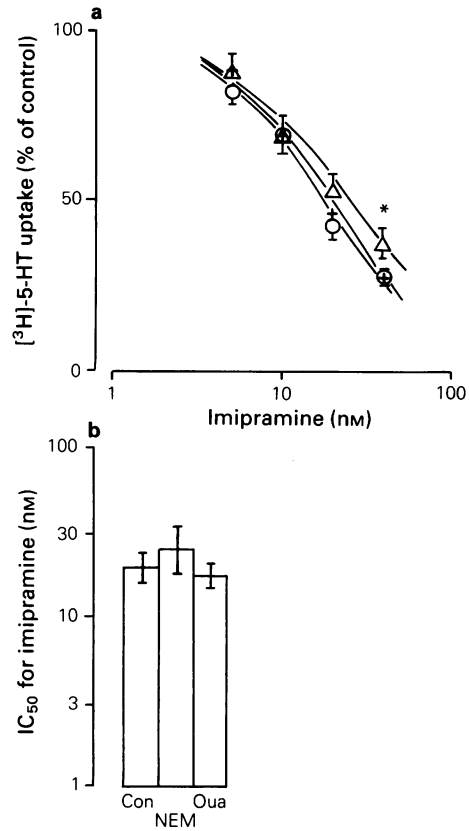

Figure 4 Failure of ouabain and N-ethylmaleimide (NEM) to affect the potency of imipramine in inhibiting 5-hydroxytryptamine (5-HT) uptake. Ordinate scale in (a): uptake of 5-HT in % of control. Abscissa scale in (a) (log scale): concentration of imipramine in nM. Height of columns in (b) (log scale): IC_{50} for uptake inhibition by imipramine in nM. Uptake of 5-HT (30 nM) was determined in the absence and presence of various concentrations of imipramine (5, 10, 20 and 40 nM) both, under control conditions (Con, +) and after pre-exposure (45 min) to either 0.5 μM ouabain (Oua, ○) or 40 μM NEM (△). Shown are geometric means with s.e.mean shown by vertical bars (a) or ±95% confidence limits (b) of 10 dependent observations. Control uptake was 9.5 (Con), 3.7 (NEM) and 3.5 (ouabain) pmol per 10^8 platelets min^{-1}. *$P < 0.01$ for the difference between NEM and control group with respect to the inhibitory effect of 40 nM imipramine (Friedman's test followed by the procedure of Wilcoxon and Wilcox).

Discussion

Pre-exposure of platelets to 40 μM NEM for 45 min reduced 5-HT uptake by about 50%, but did not alter the potency of imipramine for inhibition of 5-HT uptake. Since the potency value for imipramine is the functional counterpart of the dissociation constant for imipramine binding, the present results corroborate those of Meyerson *et al.* (1987). However, when the ouabain-like action of NEM is taken into consideration, our findings are clearly against the view of Meyerson *et al.* (1987) who suggested that the 5-HT carrier and the imipramine binding site differ in their susceptibility to alkylation by NEM. In other words, the ability of NEM to impair 5-HT uptake without affecting imipramine binding was taken to support the hypothesis that the 5-HT carrier and the imipramine binding site represent distinct plasma membrane proteins. The results of Figure 3 demonstrate that NEM resembles ouabain in reducing the transmembrane Na^+ gradient and that this action fully accounts for the drug-induced inhibition of 5-HT uptake. Hence, it is not

the carrier protein but rather the Na^+/K^+-ATPase which is highly susceptible to SH-group alkylation by NEM. Therefore, a binding site for imipramine other than the 5-HT carrier need not be postulated to explain the results of Meyerson *et al.* (1987).

Although, within the range of NEM concentrations tested here (20–80 μM), the inhibitory action of the drug on 5-HT uptake was predominantly indirect in nature (i.e., consequent to the impairment of Na^+ pump activity), NEM concentrations higher than 80 μM may well interfere with 5-HT transport by direct inactivation (i.e., alkylation) of the carrier protein. In support of this view it has been reported that NEM concentrations substantially higher than those used here inhibit imipramine binding (Davis, 1984; Meyerson *et al.*, 1987). This may be the explanation for the present finding that 80 μM NEM tended to increase the K_m for 5-HT uptake. Another Na^+-dependent amine carrier system in which NEM exerts a dual inhibitory action is the noradrenaline carrier associated with the plasma membrane of noradrenergic neurones or PC-12 cells. Schömig *et al.* (1988) found NEM to be 75 times more potent in inhibiting the noradrenaline uptake by intact PC-12 cells than in inhibiting the noradrenaline uptake by plasma membrane vesicles derived from these cells. Since the Na^+ gradient required for noradrenaline uptake to take place is artificially generated in membrane vesicles but brought about through the action of the Na^+ pump in intact cells, these results provide direct evidence for the neuronal noradrenaline carrier being much less susceptible to inactivation by NEM than the Na^+/K^+-ATPase. Hence, the noradrenaline and the 5-HT carrier system resemble each other with respect to their liability to be affected by NEM.

Ouabain- and NEM-induced increases in the Na^+ content of platelets reduced the V_{max} of 5-HT uptake but did not change the K_m of the carrier system for 5-HT or the IC_{50} for uptake inhibition by imipramine. As the extracellular Na^+ concentration was held constant in the present experiments, our results can be taken to mean that the Na^+ gradient across the plasma membrane determines the velocity of 5-HT transport at non-saturating (i.e. V_{max}/K_m = rate constant characterizing the unsaturated carrier) and highly saturating (V_{max}) substrate concentrations, while having no influence on the K_m value and the affinity of imipramine binding. The extracellular Na^+ concentration, on the other hand, appears to be the main determinant of the values of K_m for 5-HT and IC_{50} for imipramine. Therefore, the observation of Talvenheimo *et al.* (1983) that the V_{max} of 5-HT uptake increases with increasing external Na^+ concentration was probably due to changes in the transmembrane Na^+ gradient and not the result of changes of the external Na^+ concentration *per se*.

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Antagonism of responses to excitatory amino acids on rat cortical neurones by the spider toxin, argiotoxin$_{636}$

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1 Several low molecular weight spider toxins have recently been shown to block potently glutamatergic neuromuscular transmission at the invertebrate neuromuscular junction. The aim of the present investigation was to evaluate the effects of one such toxin, argiotoxin$_{636}$, on excitatory amino acid receptor-mediated responses in mammalian neurones.

2 Membrane currents were recorded from rat cortical neurones after 2–6 weeks in cell culture, by the whole-cell variant of the patch-clamp technique. N-methyl-D-aspartate (NMDA) and kainate were used as selective agonists for their respective receptor subtypes. α-Amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) was used as a selective agonist for the quisqualate receptor subtype.

3 Responses to these agonists were characterised with respect to their concentration and voltage-dependence. Argiotoxin$_{636}$ (3–30 μM) was found to attenuate markedly responses to NMDA in an agonist- and voltage-dependent manner. Thus, argiotoxin$_{636}$ progressively reduced successive responses to NMDA when membrane potentials were voltage clamped between −40 mV to −100 mV. The more negative the membrane potential the more rapid the development of the block of inward current.

4 The antagonism of NMDA-induced currents by argiotoxin$_{636}$ could be reversed by clamping the membrane at positive potentials (+20 to +60 mV) and reapplying NMDA.

5 Responses to AMPA and kainate were less affected by argiotoxin$_{636}$, with an antagonist action only becoming evident at a concentration of 100 μM.

6 These results suggest that argiotoxin$_{636}$ is an open-channel blocker of the NMDA activated ion-channel in mammalian neurones. Furthermore, our results indicate at least a 30 fold selectivity for NMDA over the quisqualate- and kainate-activated ion-channels.

Introduction

Low molecular weight toxins isolated from the venom of various spiders have recently been shown to block potently glutamatergic synaptic transmission at invertebrate neuromuscular junctions (see review by Jackson & Usherwood, 1988). It is thought that these spider toxins exert a selective effect on the postsynaptic cationic channel which is gated by glutamate and its structural analogue, quisqualate. (Kawai *et al.*, 1982a; Abe *et al.*, 1983; Kerry *et al.*, 1988).

Glutamate is also a major excitatory transmitter in the mammalian central nervous system (CNS), where it acts upon at least three subtypes of excitatory amino acid receptors. These have been classified as N-methyl-D-aspartate (NMDA), quisqualate and kainate, according to their preferred agonists (Watkins & Evans, 1981; Foster & Fagg, 1984). In agreement with the invertebrate data it has been found that spider toxins also block glutamatergic transmission in the mammalian CNS, again by an action at quisqualate and/or kainate gated ion-channels (Kawai *et al.*, 1982b; Akaike *et al.*, 1987).

At the invertebrate neuromuscular junction, spider toxins appear to act as open-channel blockers of the glutamate receptor-gated cationic channel (Usherwood & Duce, 1985; Grishin *et al.*, 1986; Kerry *et al.*, 1988). This channel has a very large unitary conductance of 120–150 pS (Anderson *et al.*, 1978; Patlak *et al.*, 1979; Cull-Candy *et al.*, 1981), which is much larger than any of the channel conductances activated by glutamate on mammalian neurones. In the latter case the largest conductance

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is around 50 pS (Nowak *et al.*, 1984; Cull-Candy & Ogden, 1985; Ascher *et al.*, 1988).

This channel conductance is preferentially associated with NMDA receptors and is blocked in a voltage-sensitive manner by Mg^{2+} ions (Nowak *et al.*, 1984; Mayer *et al.*, 1984; Ascher & Nowak, 1988a). Mammalian non-NMDA receptors appear to activate preferentially smaller conductance channels (Cull-Candy & Usowicz, 1987; Jahr & Stevens, 1987; Ascher & Nowak, 1988b). It is unclear, therefore, why toxins which block the large conductance invertebrate channel should remain specific for quisqualate-activated receptors on mammalian neurones, when they activate channels with a much smaller conductance(s) (Ascher & Nowak, 1986; Cull-Candy & Usowicz, 1987; Ascher & Nowak, 1988b). Indeed, evidence to the contrary has been shown for the dissociative anaesthetic, phencyclidine (PCP), which is an open channel blocker of the invertebrate glutamate receptor ion channel (Idriss & Albuquerque, 1985) but blocks NMDA, and not non-NMDA, receptor-mediated responses on mammalian neurones (Anis *et al.*, 1983). This antagonism of NMDA receptors by PCP also results from an open channel blockade of the receptor-activated ion-channel. (Honey *et al.*, 1985; MacDonald *et al.*, 1987).

Recently, the active toxins of some of these spider venoms have been identified (Jackson & Usherwood, 1988). We have used synthetic argiotoxin$_{636}$, a toxin originally isolated from the spider *Argiope lobata* and named argiopine (Grishin *et al.*, 1986), to investigate the specificity of these toxins for mammalian glutamate receptor ion-channels. By use of the whole-cell patch clamp technique, the effect of argiotoxin$_{636}$ on NMDA and non-NMDA receptor-mediated responses has been evaluated on rat cultured cortical neurones. A preliminary account of this work has appeared in abstract form (Kemp *et al.*, 1988).

Methods

Preparation and maintenance of primary-dissociated rat cortical cells

Dissociated cortical cells were prepared from the cerebral hemispheres of neonatal (1–2 days old) Sprague-Dawley rats according to the procedure described by Kaplan *et al.* (1986), with minor modifications. Briefly, dissected cortices were minced with fine scissors, in cold Hank's balanced salt solution (HBSS, Gibco) containing 100 units ml^{-1} streptomycin and 100 mg ml^{-1} penicillin (Sigma) under aseptic conditions. The tissue was transferred to a conical tube (maximum of 1 cortex per tube) and the supernatant aspirated and replaced with 2 ml of HBSS containing 0.25% trypsin (Sigma) and 0.05% deoxyribonuclease-1 (DNAase, Type II, Sigma). The tissue was then agitated at room temperature for 12 min in a shaking water bath. An equal volume of 0.25% soybean trypsin inhibitor (Type 1-S, Sigma) in HBSS was added to each tube to inhibit further proteolysis. The tissue was then centrifuged at 2000 r.p.m. for 5 min, the supernatant removed and replaced with 2 ml of HBSS containing 0.025% DNAase and the tissue completely dissociated by repeated trituration using a fire-polished Pasteur pipette. The cell suspension was then centrifuged at 2000 r.p.m. for 10 min and washed once with 2 ml HBSS. Following a further centrifugation the individual tissue pellets from each tube were combined and resuspended in 1 ml of growth medium of the following composition: Dulbecco's Modified Eagles Medium (DMEM, Gibco) and Ham's F12 nutrient mixture (F12, Gibco) in a 2:1 ratio with the following supplements: donor horse serum (Gibco), 10%; L-glutamine 2 mM (Flow). The number of viable cells was estimated by use of a haemocytometer and the Trypan Blue exclusion procedure (Patterson, 1979). Cell density was adjusted to give a final value of 5×10^5 cells ml^{-1} and 0.2 ml of this suspension was plated onto glass coverslips previously coated with poly-D-lysine (Sigma) according to the method of Pettman *et al.* (1979). The cells were placed in an incubator and maintained at 37°C and saturated humidity with 5% ambient CO_2 for a period of 1 h following which a further 2 ml of growth medium was added. The medium was changed after a further 15 h and 40 h. Cytosine arabinofuranoside (10^{-5} M, Sigma) was added to each dish to inhibit non-neuronal cell proliferation after attaining confluency (usually 4–5 days after plating).

Whole cell patch clamp recordings

For electrophysiological recordings coverslips were transferred to a glass bottomed, Perspex recording chamber mounted on the stage of a Nikon Diaphot inverted microscope. Cultures were observed using phase-contrast optics, and were continuously perfused ($\simeq$1 ml min^{-1}) with an equilibrated (5% CO_2, 95% O_2) artificial cerebrospinal fluid (aCSF) of the following composition (in mM): NaCl 124, KCl 2, KH_2PO_4 1.25, $MgCl_2$ 2, $CaCl_2$ 2, $NaHCO_3$ 25, D-glucose 11.

Patch pipettes with an approximate tip diameter of 2 μm and resistance of 4 MΩ were pulled by means of a Mechanex BBCH puller using borosilicate glass (Clark Electromedical). No additional fire polishing was performed and pipettes were not treated to reduce capacitance. The patch pipette solution comprised (in mM) CsF 120, CsCl 10, HEPES 10, EGTA

10, NaCl 4, $CaCl_2$ 0.5, pH adjusted to 7.25 with CsOH.

Whole-cell currents were recorded from cultured cells 2 to 6 weeks after plating, using a LIST EPC-7 amplifier. After forming a high resistance seal with the cell under investigation capacitance transients were minimised by using the C-FAST facility of the EPC-7. No further capacitance compensation was applied and no compensation was made for series resistance which, after the membrane had been ruptured, measured $7.16 \pm 0.45\,M\Omega$ (mean ± s.e.mean, $n = 6$). The mean seal resistance of 15 cells measured immediately before the membrane patch was ruptured was $44 \pm 5\,G\Omega$.

Drug solutions were applied to individual cells by a double-barrelled pipette assembly, as described by Johnson & Ascher (1987). The arrangement enabled rapid equilibration in either control or test solutions. For experiments involving kainic acid (Sigma) and α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA, CRB) the solution in the perfusion barrels was the same as the external perfusate with the addition of tetrodotoxin (TTX, 1 μM, Sigma) to block voltage-activated Na^+ channels. AMPA was used in preference to quisqualate because of its greater selectivity for the quisqualate receptor subtype. For experiments involving N-methyl-D-aspartic acid (NMDA, Tocris) the solution in the perfusion pipettes was modified by omitting $MgCl_2$ and including glycine (1 μM, Sigma) and TTX (1 μM). Samples of synthetic argiotoxin$_{636}$ were prepared by Dr T.L. Shih (MSDRL, Rahway).

Results

Characteristics of the current responses to excitatory amino acid agonists

NMDA, AMPA and kainate all evoked inward currents at negative membrane potentials. When glycine was omitted from the NMDA perfusate the current response was markedly attenuated, in agreement with the findings of Johnson & Ascher (1987). NMDA characteristically evoked current responses which were associated with a large increase in the current noise level (Figure 1a). The degree of desensitisation of the response depended on the concentration of NMDA applied, with little fade occurring at concentrations below 30 μM and more prominent desensitisation occurring at high concentrations (> 100 μM). Responses to AMPA characteristically showed a rapidly declining initial component, followed by a steady state level which was not associated with as prominent a noise increase as that produced by NMDA (Figure 1b). Kainate responses were usually well maintained and only occasionally

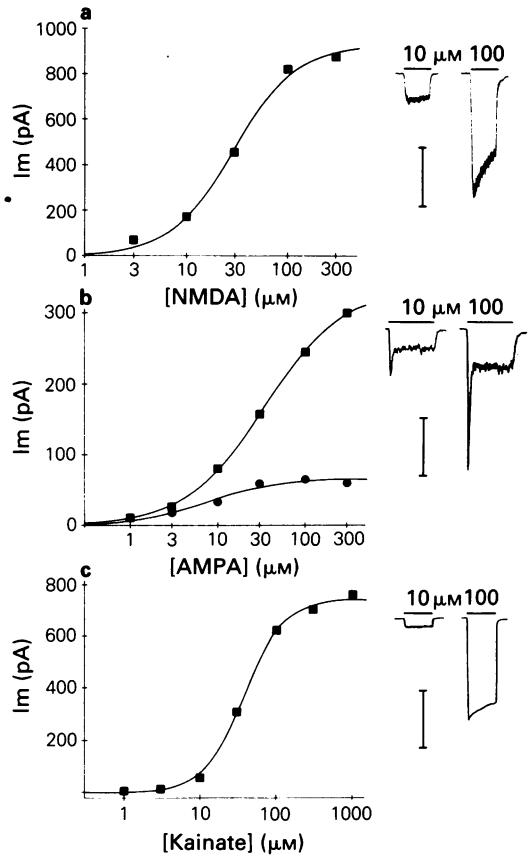

Figure 1 Typical concentration-response curves for excitatory amino acid-evoked inward currents. Different cortical cells were used in the construction of each graph and in all cases the membrane potential was clamped at −60 mV. Lines represent least squares best fits to the equation $I = Imax/(1 + (EC_{50}/\text{agonist concentration})^{nH})$ determined using an iterative procedure on a VAX computer with Research System 1 (RS1) software (BBN Software products corporation). (a) Concentration-response curve for N-methyl-D-aspartate (NMDA). Inset: responses of the same cell to 5 s applications of NMDA at 10 and 100 μM. Note the fade during the response to 100 μM NMDA, peak currents were used in the construction of the graph. (b) Concentration-response curves for the peak (■) and sustained (●) components of the response to α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA). Inset: responses to 10 and 100 μM AMPA on the same cell. The trace speed was increased to illustrate better the nature of the initial transient response. (c) Concentration-response curve to kainate. Inset: responses to 10 and 100 μM on the same cell, kainate responses showed less fade and were not associated with a prominent increase in noise level. Vertical bars in (a) and (c) = 400 pA, (b) = 100 pA. Duration of drug applications were 5 s in each case as denoted by horizontal bars.

showed a tendency to fade. They were not associated with a prominent increase in the current noise level (Figure 1c). Membrane current responses to all three agonists were clearly voltage-dependent with similar reversal potentials: NMDA = 8.89 ± 1.96 mV ($n = 9$ cells); AMPA = 5.17 ± 2.22 mV ($n = 6$ cells); kainate = 5.50 ± 1.43 mV ($n = 6$ cells). These positive values presumably reflect the slightly higher external cation concentration used in these experiments. The slopes of the current-voltage (I/V) curves were approximately linear over the voltage range −60 to +60 mV. However, for all three agonists a region of reduced slope conductance usually occurred at cell potentials more negative than −60 mV. In some instances this was particularly prominent with NMDA, the I/V curve often showed a region of negative slope conductance over the potential range −60 to −100 mV (Figure 2a). This may have been due to residual quantities of magnesium in our nominally magnesium-free medium.

Agonist potency was compared by constructing concentration-response curves at a holding potential of −60 mV using at least 5 agonist concentrations. Representative curves are shown in Figure 1. In all cases lines were fitted through the data points by using a computer generated least squares iterative procedure which also calculated EC_{50} and Hill coefficient values. The corresponding values for NMDA and kainate are given in Table 1. Analysis of AMPA responses was made difficult because of their biphasic nature. Consequently, both initial and late phases of the AMPA response were plotted as a function of concentration (Figure 1b). The initial transient component appeared to increase almost linearly with increasing agonist concentrations. The possibility that this represented a proton activated current was considered. However, identical responses were obtained when all drug solutions were buffered with HEPES (25 mM) and adjusted to pH 7.4. Furthermore, it would appear that the initial desensitising component is not simply a feature of AMPA itself since similar responses were obtained with quisqualate (data not shown). The late sustained component of the AMPA response reached a maximum value at relatively low concentrations (ca 30 μM) and concentration-response curves were predictably shallow. The total amount of current generated during the sustained AMPA response was small compared to that seen with the other agonists or of the peak AMPA response. Detailed quantitative analyses of AMPA concentration-response curves ($n = 3$) were not undertaken because of their complex nature.

Antagonism of NMDA responses: agonist and voltage-dependency

Argiotoxin$_{636}$, 3–30 μM, produced a markedly voltage-dependent block of NMDA-induced currents on a total of six cells studied. This was demonstrated by constructing a control NMDA I/V curve by applying NMDA (30 μM) at different membrane holding potentials (Figure 2a), usually −100 to +60 mV. The membrane potential was then clamped at +60 mV and the cell continuously perfused with argiotoxin$_{636}$ (3 μM). NMDA was reapplied three times (5 s duration) at each membrane potential descending from +60 mV in 20 mV steps. The block of NMDA-induced currents was only apparent at potentials below −20 to −40 mV using this procedure. On returning the membrane potential to more positive values, however, even these responses were now reduced (3 cells), i.e. the I/V plot (in the presence of argiotoxin$_{636}$) showed a marked hysteresis. Higher concentrations of argiotoxin$_{636}$ (30 μM) showed a similar profile in that NMDA responses evoked at positive holding potentials were unaffected, but at this concentration the effects of argiotoxin$_{636}$ were apparent at all membrane potentials negative to and including 0 mV (Figure 2b). The effects of argiotoxin$_{636}$ were difficult to reverse if the membrane was held at negative potentials, even with repeated NMDA applications. However, recovery could be achieved by holding the membrane at positive potentials and repeatedly applying NMDA (3 cells).

The NMDA-antagonist actions of argiotoxin$_{636}$ were dependent not only upon holding potential but also on addition of the agonist. This phenomenon was demonstrated at three different holding potentials on the same cell. The cell was initially held at −80 mV and three consecutive control responses obtained to NMDA (30 μM). Argiotoxin$_{636}$ (3 μM)

Table 1 Quantitative analyses of agonist concentration-response curve parameters

Agonist	*pEC_{50} value*	*Hill coefficient*	n
NMDA	4.60 ± 0.06	1.31 ± 0.07**	16
Kainate	3.84 ± 0.22	1.56 ± 0.17*	4

Values are shown as mean ± s.e.mean. n = number of cells.
Slopes significantly different from 1, * $P < 0.05$, ** $P < 0.01$; t test.

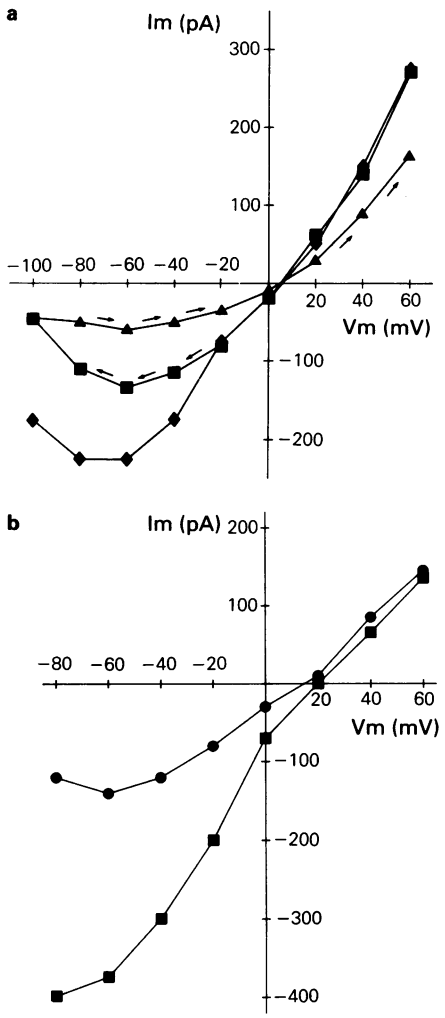

Figure 2 Current-voltage curves for N-methyl-D-aspartate (NMDA) showing the effects of argiotoxin$_{636}$ (3 and 30 μM). Data were obtained from two different cells. (a) Peak currents to control applications of NMDA (30 μM, ◆) were measured at the membrane potentials indicated on the abscissa scale. The cell was then voltage clamped at +60 mV and argiotoxin$_{636}$ (3 μM) continuously applied. Three NMDA applications (5 s duration) were then made at each membrane potential, working from +60 mV to −100 mV and the peak of the third response plotted (■, left arrows). The procedure was then repeated in the reverse direction, i.e. commencing from −100 mV (▲, right arrows). Note that initially, argiotoxin$_{636}$ blocked inward currents to NMDA at membrane potentials more negative than −20 mV. Once established, however, the argiotoxin$_{636}$ block was increased at all negative holding potentials and persisted even at positive potentials. Partial recovery from the effects of argiotoxin$_{636}$ was obtained, the curve has been omitted from the figure for clarity. (b) A similar experiment showing the effects of 30 μM argiotoxin$_{636}$. Control responses to NMDA 30 μM (■) and in the presence of argiotoxin$_{636}$ (●). At this higher concentration the effects of the toxin were apparent at membrane potentials more negative than 0 mV. Outward current responses at membrane potentials positive to +20 mV remained unaffected.

was then continuously perfused for a period of 2 min before reapplying NMDA (Figure 3). The initial response to NMDA + argiotoxin$_{636}$, after 2 min exposure to argiotoxin$_{636}$ alone, showed no attenuation compared to control responses. However, subsequent agonist applications were progressively reduced in amplitude. The membrane was then held at +60 mV to facilitate full recovery from argiotoxin$_{636}$ and the procedure repeated at −100 mV. At this membrane potential the onset of the antagonism was more pronounced with each subsequent agonist application, but the response to the initial application after argiotoxin$_{636}$ still showed no decrement. Finally, the procedure was repeated at −60 mV, after argiotoxin$_{636}$ had been washed out. In this case very little antagonism was evident despite repeated agonist exposures.

Antagonism of responses to AMPA and kainate

High concentrations (100 μM) of argiotoxin$_{636}$ reduced the inward current responses evoked by the other excitatory amino acids, AMPA (2 cells) and kainate (5 cells), at holding potentials more negative than −40 mV (Figures 4 and 5). The blocking effect of argiotoxin$_{636}$ was once again, voltage- and use-dependent for both AMPA and kainate. Concentrations of argiotoxin$_{636}$ of 30 μM and below did not overtly antagonise responses to either of these agonists (n = 13).

Discussion

The present results demonstrate antagonism of NMDA receptor-mediated responses on mammalian neurones by the synthetic spider toxin, argiotoxin$_{636}$. The use of selective excitatory amino acid receptor agonists and voltage-clamp techniques on dissociated cortical neurones in cell culture has enabled a detailed assessment of the pharmacological characteristics of the action of argiotoxin$_{636}$.

NMDA, AMPA and kainate evoked responses which were clearly different in character. Analysis of the NMDA and kainate concentration-response curves revealed Hill coefficients which were significantly greater than unity. The precise significance of slopes of 1.31 and 1.56 for NMDA and kainate,

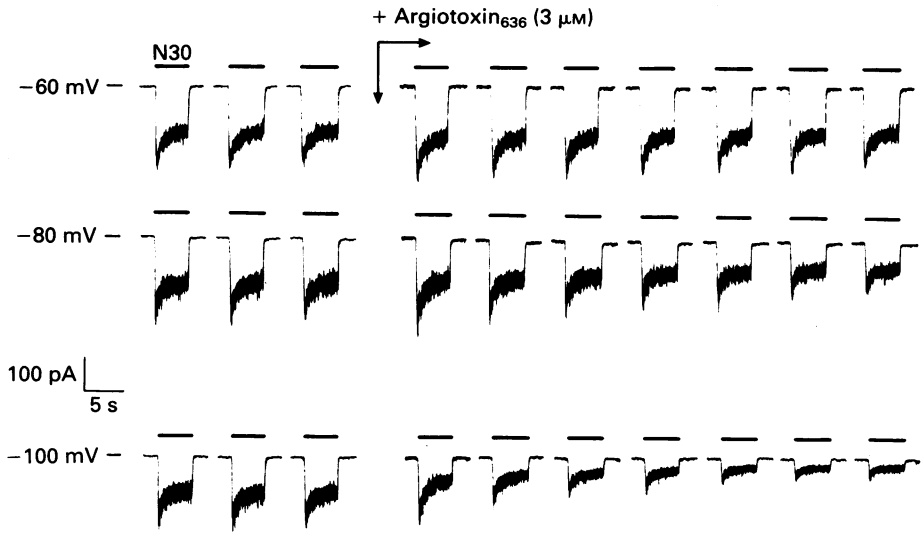

Figure 3 The block of N-methyl-D-aspartate (NMDA) responses by argiotoxin$_{636}$ was agonist-dependent. The figure shows the results obtained from a single cell, the continuous chart record has been broken for clarity. The membrane potential was initially clamped at −80 mV and three control responses obtained to NMDA (30 μM) applications (duration indicated by the horizontal bars). Argiotoxin$_{636}$ (3 μM) was then continuously perfused and following a 2 min period NMDA (30 μM) was reapplied at 6 s intervals. The initial response to NMDA showed no attenuation, however, subsequent inward current responses to NMDA were progressively reduced. After 7 such applications in the presence of the toxin the cell was reperfused with control aCSF. The effects of argiotoxin$_{636}$ were reversed by clamping the membrane potential at +60 mV and repeatedly applying NMDA. When fully recovered the cell was clamped at either −60 mV or −100 mV and the procedure repeated. Note that the rate of onset of the block by argiotoxin$_{636}$ was influenced by membrane potential such that no antagonism was apparent at −60 mV whereas a rapid block developed at −100 mV. The increase in the responses to the initial applications of NMDA after the argiotoxin$_{636}$ perfusion period, seen at −60 and −80 mV holding potentials, was not a consistent observation and was not, therefore, deemed to be significant.

respectively, is unclear, although these values are strikingly similar to those recently obtained for NMDA and kainate responses in *Xenopus* oocytes injected with rat brain mRNA (Verdoorn & Dingledine, 1988). Desensitisation at high agonist concentrations is likely to lead to an underestimation of the true Hill coefficient. Overall, these results suggest that more than one agonist molecule may be required to activate NMDA and kainate receptors.

Responses to AMPA deserve further consideration. AMPA concentrations above 10 μM showed a prominent desensitising component which appeared to increase almost linearly up to 1000 μM. This was not simply a property of AMPA itself since quisqualate evoked similar biphasic responses. The possibility that the desensitising component represented a proton activated current (Krishtal & Pidoplichko, 1981; Konnerth *et al.*, 1987) was considered. However, biphasic responses to AMPA persisted in HEPES (25 mM) buffered solution adjusted to pH 7.4. It is possible that this response profile is due to a solution change which is not much faster than the rate of desensitisation. Thus, the true peak response which would be achieved if the change were instantaneous may, in practice, be reduced by desensitisation before the solution change is complete.

Argiotoxin$_{636}$ produced a voltage- and agonist-dependent block of NMDA responses at concentrations which did not affect responses to either AMPA or kainate. These characteristics suggest that its antagonist effects result from an action as an open ion-channel blocker of the NMDA receptor complex, in common with its action at the invertebrate muscle glutamate receptor. Use- or agonist-dependency implies that the ion-channel must first be opened before the antagonist may bind and any block develop. The voltage-dependency also suggests that the binding site is within the ion channel and therefore influenced by the transmembrane field potential. Using a Boltzman distribution to describe the voltage-dependency of ketamine, MacDonald *et al.* (1987) have calculated that it binds to a site within the NMDA ion-channel, half-way across the membrane field. It has not been possible to obtain similar estimations for argiotoxin$_{636}$, since under our experimental condi-

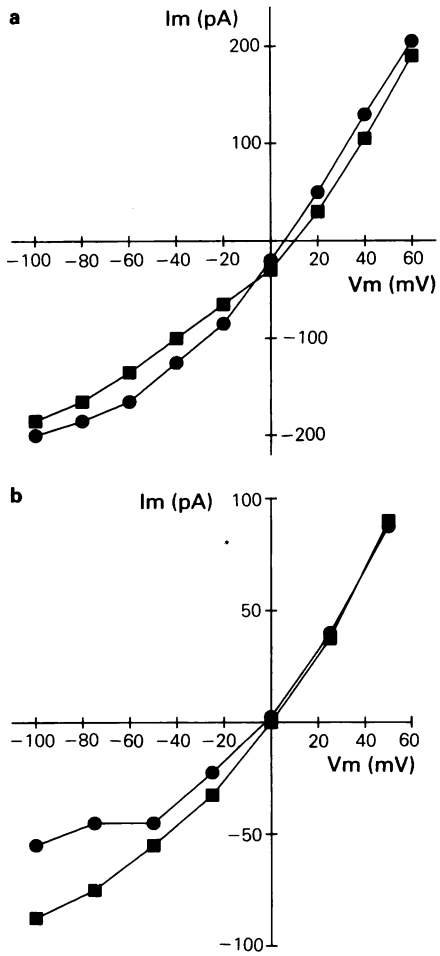

Figure 4 Effects of argiotoxin$_{636}$ on membrane currents evoked by α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA). Current-voltage plots were constructed for peak AMPA responses using the protocol described in the legend to Figure 2. (a) Control responses to AMPA (30 μM, ■) were not affected by 30 μM argiotoxin$_{636}$ (●). (b) Inward, but not outward, current responses to AMPA (30 μM, ■) were attenuated in the presence of 100 μM argiotoxin$_{636}$ (●).

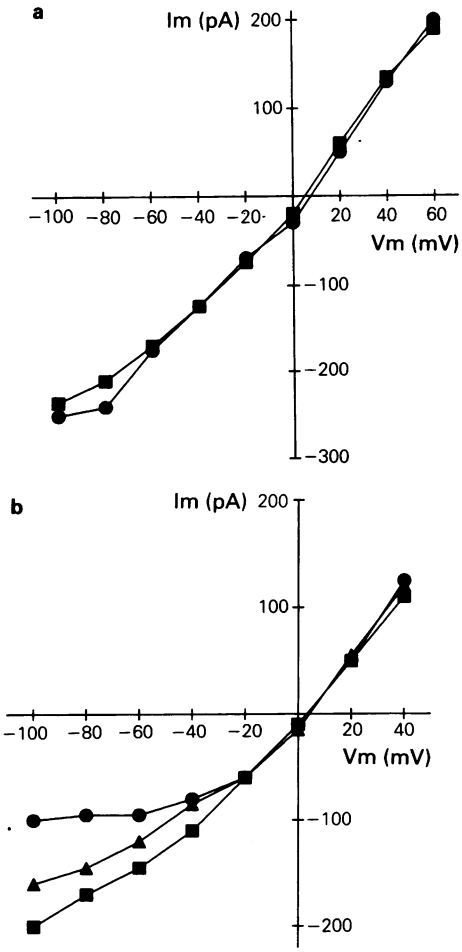

Figure 5 Effects of argiotoxin$_{636}$ on membrane currents evoked by kainate. Current-voltage plots were constructed for peak kainate responses using the protocol described in the legend to Figure 2. (a) Control responses to kainate (30 μM, ■) were not affected by argiotoxin$_{636}$ (30 μM, ●). (b) Inward current responses to kainate (30 μM, ■) at membrane potentials more negative to −20 mV were attenuated by argiotoxin$_{636}$ (100 μM, ●). Partial recovery (▲) was obtained in control aCSF after clamping the cell membrane at +60 mV and repeatedly applying kainate.

tions equilibrium block was not obtained at each membrane potential, as evidenced by the hysteresis in Figure 2a.

The selectivity of argiotoxin$_{636}$ for NMDA responses is perhaps not as surprising as it may at first seem, given that other open-channel blockers of the invertebrate muscle glutamate receptor (e.g. PCP, ketamine) also selectively block the NMDA receptor ion-channel on mammalian neurones (Anis *et al.*, 1983; MacDonald *et al.*, 1987). The selectivity of compounds acting uncompetitively as excitatory amino acid receptor antagonists is clearly going to be determined by the structural similarities of the gated ion-channel rather than the ligand specificity of the agonist recognition site. However, the Joro spider toxin (JSTX), which is structurally very

similar to argiotoxin$_{636}$, has been shown to block potently responses to both quisqualate and kainate in rat isolated hippocampal pyramidal cells (Akaike *et al.*, 1987), although these effects were not use-dependent. Unfortunately, these authors were unable to determine any potential NMDA-blocking effects since their preparations failed to respond to this agonist.

Purified JSTX has been found to block excitatory synaptic transmission to CA1 pyramidal cells in guinea-pig hippocampal slices and to block the effects of iontophoretically applied glutamate but not aspartate (Saito *et al.*, 1985). Again, an action at a quisqualate-type receptor was inferred. In *Xenopus* oocytes injected with rat brain mRNA, JSTX has also been shown to block kainate-activated currents (Sugiyama *et al.*, 1987). However, a possible action of NMDA currents was not investigated.

Although these results are difficult to reconcile with our present data it may be that, whilst closely related structurally, the different toxins have different profiles as excitatory amino acid receptor antagonists on mammalian neurones. However, it has been reported, that argiotoxin$_{636}$ (7.5×10^{-8}–2.3×10^{-7} M) itself blocks dorsal root evoked ventral root potentials and the action of glutamate in the frog isolated spinal cord (Antonov *et al.*, 1987). Both of these effects are presumed to be mediated largely by non-NMDA receptors. Further experiments are required, therefore, in order to clarify this situation.

Our results suggest that argiotoxin$_{636}$ is an open-channel blocker of the NMDA-activated ion-channel in mammalian neurones, with at least a 30 fold selectivity over channels activated by quisqualate and kainate receptors. This finding is in agreement with previous studies with PCP and ketamine which showed that these substances block both the invertebrate glutamate receptor ion-channel and the NMDA receptor ion-channel but not quisqualate- or kainate-receptor activated ion-channels. Our results suggest that, far from being a selective antagonist of quisqualate and kainate responses, argiotoxin$_{636}$ displays a preference for NMDA-gated ion channels in rat cultured cortical neurones under the voltage-clamp conditions used here.

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Comparison of the prejunctional β-adrenoceptor stimulating actions of adrenaline and isoprenaline in the dog mesenteric vein

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1 The prejunctional β-adrenoceptor stimulating actions of adrenaline and isoprenaline were compared by recording junction potentials from smooth muscle cells of the dog mesenteric vein.

2 The potency of adrenaline and isoprenaline on β-adrenoceptors in the postjunctional membrane was estimated from hyperpolarization of the membrane in smooth muscle cells of the guinea-pig facial vein. In the presence of yohimbine, both agents hyperpolarized the membrane to a similar extent.

3 In the dog mesenteric vein, amplitude of the excitatory junction potential (e.j.p.) and slow depolarization was inhibited by adrenaline and potentiated by isoprenaline; the former but not the latter was accompanied by depolarization of the smooth muscle membrane.

4 In the presence of yohimbine, adrenaline inhibited the e.j.p. without depolarization of the smooth muscle membrane; the action was weaker than in the absence of yohimbine. The isoprenaline-induced potentiation of the e.j.p. was further enhanced by yohimbine.

5 It is concluded that adrenaline and isoprenaline have similar stimulating actions on postjunctional β-adrenoceptors in the guinea-pig facial vein, but have different actions on prejunctional β-adrenoceptors in the dog mesenteric vein; isoprenaline but not adrenaline stimulates this β-adrenoceptor to facilitate the release of transmitter substances from perivascular noradrenergic nerves.

Introduction

In noradrenergic nerve endings, many types of receptor system may be involved in the regulation of the release of transmitter substances. In particular, the prejunctional α-adrenoceptor is thought to be involved in the regulation of transmitter release by endogenous noradrenaline (NA) (i.e., the α-autoregulation mechanism, Starke, 1987). β-Adrenoceptors are also found in the prejunctional membrane and they are involved in the facilitation of the release of NA (Langer, 1977; Starke, 1977; Vanhoutte *et al.*, 1981). However, this system does not work in every tissue, and in the rabbit pulmonary artery, stimulation of prejunctional β-adrenoceptors by isoprenaline does not increase the evoked release of NA (Nedergaard, 1987).

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Adrenaline is a stimulant of both α- and β-adrenoceptors, and low concentrations (about 10^{-8} M) facilitate the evoked release of NA through activation of prejunctional β-adrenoceptors in human omental (Stjärne & Brundin, 1975), pulmonary (Göthert & Hentrich, 1985) and saphenous vessels (Molderings *et al.*, 1988) or the rat portal vein (Dahlöf *et al.*, 1978; Westfall *et al.*, 1979). However, such an increase in the release of NA is observed only in the presence of α-adrenoceptor antagonists (Molderings *et al.*, 1988).

Neuromuscular transmission in vascular tissues can be studied by measuring the membrane responses of the postjunctional muscle membrane evoked by electrical stimulation of perivascular nerves. In the majority of vascular tissues, stimulation of perivascular nerves evokes an excitatory

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junction potential (e.j.p.) or slow depolarization, or both (Kuriyama *et al.*, 1982; Bolton & Large, 1986). The slow depolarization may be generated by NA, because this potential could be blocked by α-adrenoceptor antagonists, while the e.j.p. may be generated either by NA acting through receptors other than α-adrenoceptors (i.e. the γ-receptor, Hirst & Neild, 1981), or by adenosine triphosphate (ATP) released together with NA (Sneddon & Burnstock, 1985; Burnstock & Kennedy, 1986). However, the linear relationship between the amplitude of the e.j.p. and the slow depolarization allows the estimation of the amount of NA released indirectly, from the e.j.p. (Suzuki, 1984; Suzuki *et al.*, 1984).

We investigated the effects of adrenaline and isoprenaline on the isolated mesenteric vein of the dog, the objective being to compare the β-adrenoceptor stimulating actions of these agonists mainly on the prejunctional membrane. The effects of these agonists on postjunctional β-adrenoceptors were estimated in the guinea-pig facial vein, by recording the membrane hyperpolarization which was produced by activation of β-adrenoceptors (Seki *et al.*, 1988b). The effects of stimulation of prejunctional β-adrenoceptors on noradrenergic transmission were estimated from the e.j.p. and slow depolarization recorded from smooth muscle cells of the dog mesenteric vein. A brief report of the preliminary experiments was presented at the 61st Japanese Pharmacological Congress in Fukuoka (Seki *et al.*, 1988a).

Methods

Male mongrel dogs, weighing 10–15 kg, were anaesthetized with Na pentobarbitone (40 mg kg^{-1}, i.v.), and bled from the saphenous artery. The mesenteric vascular beds distributing to the ileal region were dissected, and the third branch of the mesenteric vein (external diameter, 0.3–0.5 mm) was excised together with the artery, lymphatic vessels, sympathetic nerves and mesenteric membrane. The isolated tissue was mounted in an organ bath made from Lucite plate, with a capacity of about 2 ml. A silicon rubber plate (Shin-etsu Kagaku, KE-66) was fixed at the bottom of the chamber, on which the vessel was immobilized by using tiny pins (the membranous tissues surrounding the vessel were adopted for supporting the vessel on the plate). The organ bath was perfused with warmed (35°C) Krebs solution at a rate of 2–3 ml min^{-1}.

Electrical responses of smooth muscle cells were recorded with a glass capillary microelectrode filled with 3 M KCl (resistance, 40–80 MΩ). The electrode was inserted into the smooth muscle cells through the membranous tissues and adventitia of the vessel, and this allowed resting membrane potentials greater than −70 mV to be recorded (in one case when the membranous tissues were removed, the membrane potential ranged −60 to −65 mV, possibly due to damage of smooth muscle cells locating on the surface of the vessel). Perivascular nerves were stimulated by the point stimulation method, and electrical responses were displayed on a pen-writing recorder (Recticorder, Nihon Kohden RJG-4124).

The Krebs solution had following ionic compositions (mM); Na^+ 137.4, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 134, HCO_3^- 15.5, $H_2PO_4^-$ 1.2 and glucose 11.5. The solution was aerated with O_2 containing 3% CO_2, and the pH of the solution was maintained at 7.2–7.3.

Drugs used were adrenaline HCl (Daiich), atropine sulphate (Merk), guanethidine sulphate (Tokyo Kasei), (−)-isoprenaline HCl (Sigma), phentolamine mesylate (Ciba Geigy), propranolol HCl (Sumitomo Kagaku), tetrodotoxin (Sankyo) and yohimbine HCl (Tokyo Kasei).

Experimental values were expressed as the mean ± s.d., and statistical significance was determined by Student's *t* test. Probabilities of less than 5% ($P < 0.05$) were considered significant.

Results

Effects of adrenaline and isoprenaline on postjunctional β-adrenoceptors

Experiments were first carried out to determine the potency of adrenaline and isoprenaline on postjunctional β-adrenoceptors, by using the guinea-pig facial vein which possesses predominantly β-adrenoceptors, stimulation of which hyperpolarizes the smooth muscle membrane (Seki *et al.*, 1988b).

The resting membrane potential of smooth muscle cells of the guinea-pig facial vein ranged between −60 and −70 mV (mean value, −65.0 ± 5.0 mV, $n = 33$ from 5 tissues), and the potential was stable with occasional small fluctuations, as observed in the rabbit facial vein (Komori *et al.*, 1989) or the guinea-pig mesenteric vein (Suzuki, 1981). The fluctuations in resting membrane potential were resistant to tetrodotoxin (3×10^{-7} M), guanethidine (5×10^{-6} M), phentolamine (10^{-6} M) and atropine (10^{-6} M), suggesting that it was myogenic in origin (Seki *et al.*, 1988b). Smooth muscle of the facial vein possessed α- and β-adrenoceptors through which the membrane can be depolarized or hyperpolarized, respectively

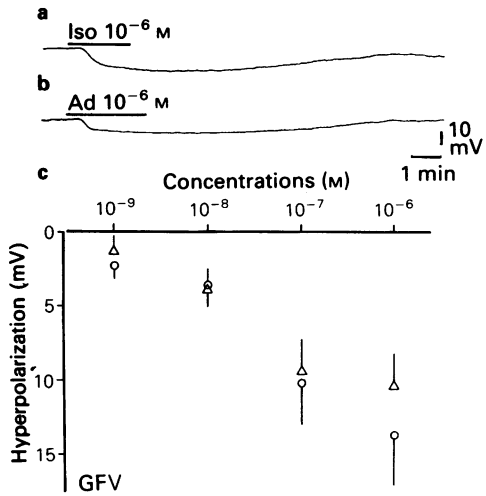

Figure 1 Hyperpolarization by isoprenaline (Iso) (a) and adrenaline (Ad) (b) of smooth muscle membrane of the guinea-pig facial vein, in the presence of yohimbine (3×10^{-7} M). (c) Concentration-response relationship of the adrenaline (△)- and isoprenaline (○)-induced hyperpolarization in the guinea-pig facial vein. Yohimbine (3×10^{-7} M) was present throughout. Mean with s.d. shown by vertical lines. ($n = 5$–8).

(Prehn & Bevan, 1983; Komori *et al.*, 1989). Experiments were therefore carried out in the presence of yohimbine which inhibits the α-adrenoceptor-mediated depolarizations produced by endogenous and exogenous noradrenaline (Komori *et al.*, 1989). In the presence of 10^{-7} M yohimbine, adrenaline and isoprenaline hyperpolarized the membrane, and the potency of these agonists was much the same at any given concentration (10^{-9}–10^{-6} M) (Figure 1). In the presence of a combination of yohimbine and propranolol (10^{-7} M), neither adrenaline nor isoprenaline, in concentrations up to 10^{-5} M (data not shown) produced hyperpolarization.

Thus, adrenaline and isoprenaline have a similar potency in stimulating the β-adrenoceptors in smooth muscle cells of the guinea-pig facial vein.

Effects of adrenaline and isoprenaline on smooth muscle cells of the dog mesenteric vein

The resting membrane potential of smooth muscle cells in the dog mesenteric vein ranged between −65 and −75 mV (mean value, -70.5 ± 3.5 mV, $n = 55$), and the potential was quiescent, as reported previously (Suzuki, 1984; Kou *et al.*, 1984). Application of adrenaline (10^{-7} M) depolarized the smooth muscle membrane and, at higher concentrations ($>10^{-6}$ M), generated slow oscillatory potentials (Figure 2). In the presence of yohimbine (3×10^{-7} M), adrenaline did not alter the membrane potential (Figure 2d). Application of isoprenaline (up to 10^{-6} M) did not produce any detectable change in the membrane potential, either in the presence or absence of yohimbine.

Figure 3 summarizes the effects of adrenaline and isoprenaline on membrane potential of smooth muscle cells in the dog mesenteric vein. The adrenaline-induced depolarization was antagonized by yohimbine (3×10^{-7} M).

Thus in smooth muscle cells of the dog mesenteric vein, adrenaline depolarizes the membrane by stimulating mainly α_2-adrenoceptors. After blockade of α_2-adrenoceptors by yohimbine, stimulation of β-adrenoceptors by adrenaline or isoprenaline does not alter the membrane potential.

Effects of adrenaline and isoprenaline on adrenergic transmission

The effects of adrenaline and isoprenaline on sympathetic transmission in the isolated mesenteric vein of the dog were determined by recording excitatory junction potentials (e.j.p.) evoked following perivascular nerve stimulation. In this vein, electrical stimulation of the perivascular nerves evokes an e.j.p. and a following slow depolarization, and application of repetitive stimuli at 0.1–1 Hz produce e.j.ps of successively increasing amplitude (facilitation) superimposed on a slow depolarization (Suzuki, 1984).

Figure 4 shows the effects of adrenaline and yohimbine on the e.j.p. and the slow depolarization. Stimulation of perivascular nerves with a train of 10 pulses at 0.33 Hz evoked e.j.ps with increasing amplitude in the initial 4–5 shocks. The slow depolarization, developed over 20–25 s to reach a steady depolarization of about 5 mV. Application of 10^{-7} M adrenaline depolarized the smooth muscle membrane by about 8 mV (Figure 3), and reduced the amplitudes of e.j.ps and the slow depolarization to about 40% of the control (Figure 4b). Yohimbine (3×10^{-7} M) reduced the e.j.p. amplitude to about 70% of the control value and blocked the generation of the slow depolarization. In the presence of yohimbine, application of adrenaline inhibited e.j.p. amplitude by about 80–90%, with no significant alteration of the faciltation of the e.j.ps (Figure 4d).

The effects of adrenaline and isoprenaline on the e.j.p. and slow depolarization are summarized in Figures 5 and 6, respectively. In these figures, the amplitudes of the e.j.p. and slow depolarization are expressed relative to that before the application of adrenaline or isoprenaline. As shown in Figure 5,

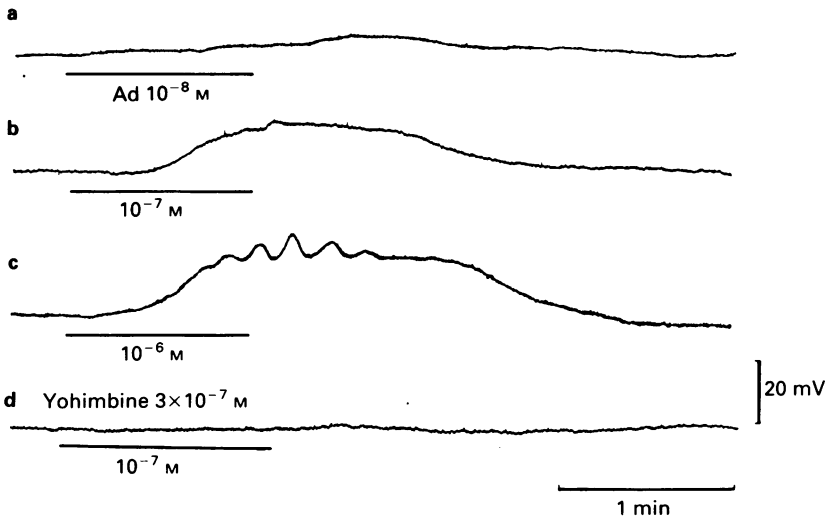

Figure 2 Electrical responses produced by adrenaline (Ad) in smooth muscle cells of the dog mesenteric vein, before (a–c) and after (d) application of yohimbine (3×10^{-7} M). Adrenaline (a, 10^{-8} M; b and d, 10^{-7} M; c, 10^{-6} M) was applied at the bar under each record. All the responses from the same tissue. The resting membrane potential; −70 mV.

adrenaline or isoprenaline consistently inhibited or enhanced the amplitude of the e.j.p., respectively, either in the presence or absence of yohimbine. The adrenaline-induced inhibition of the e.j.p. was antagonized by yohimbine, while the isoprenaline-induced increase in amplitude of the e.j.p. was slightly enhanced by yohimbine.

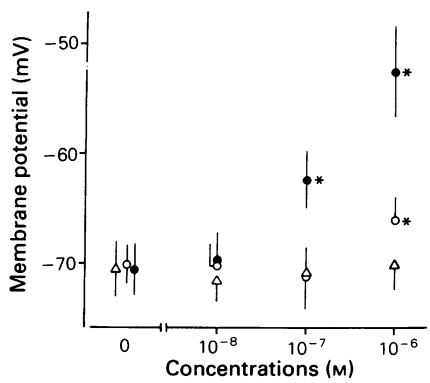

Figure 3 Membrane potentials of smooth muscle cells of the dog mesenteric vein, in the presence of isoprenaline (△), adrenaline (●) and adrenaline with yohimbine 3×10^{-7} M (○) are shown as mean values with s.d. indicated by vertical lines. (n = 8–15).

The amplitude of the slow depolarization was inhibited by adrenaline and slightly increased by isoprenaline. The former but not the latter effect was accompanied by a depolarization of the smooth muscle membrane (Figure 3).

Discussion

The present experiments demonstrated that in the dog mesenteric vein, isoprenaline and adrenaline have different actions on sympathetic transmission, as the e.j.p. amplitude was increased by isoprenaline and decreased by adrenaline. The α-adrenoceptors involved in the pre- and post-junctional membrane of this vein are mainly of the α_2-subtype which is antagonized by yohimbine (Suzuki, 1984; Kou *et al.*, 1984). The present experiments were carried out in the presence of yohimbine, and the membrane potential of smooth muscle cells remained unaltered by the addition of adrenaline or isoprenaline. Therefore, the increase in amplitude of the e.j.p. induced by isoprenaline can mainly be attributed to a prejunctional effect. The reduction in amplitude of the e.j.p. by adrenaline may be mainly due to the α_2-adrenoceptor-mediated depolarization of the postjunctional membrane, and the adrenaline-induced inhibition of the e.j.p. was antagonized after blockade of the depolarization by yohimbine. Comparison of the β-adrenoceptor stimulating actions between adrenaline and isoprenaline revealed that

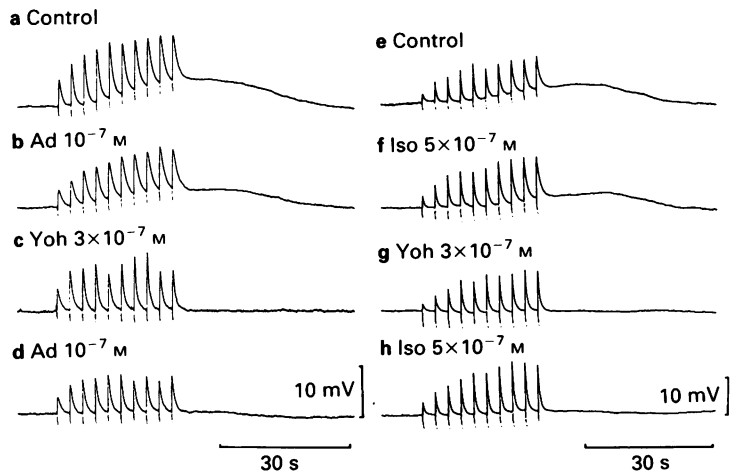

Figure 4 Effects of adrenaline (Ad) and isoprenaline (Iso) on the e.j.p. and slow depolarization in the dog mesenteric vein, before (a, b, e and f) and after (c, d, g and h) application of yohimbine (Yoh, 3×10^{-7} M). Nerve stimulation; (a–d) 0.05 ms duration, 25 V intensity, 10 stimuli at 0.3 Hz frequency; (e–h) 0.03 ms duration, 30 V intensity, 10 stimuli at 0.3 Hz frequency. (a–d) and (e–f) were recordings from different tissues. Membrane potential; (a–d), −71 mV; (e–h), −70 mV.

both of these agonists had similar potencies on the postjunctional β-adrenoceptors, as estimated by changes in the amplitude of membrane hyperpolarizations in the guinea-pig facial vein. Thus, adrenaline and isoprenaline seem to have differential actions on prejunctional β-adrenoceptors.

Alternatively, the prejunctional α-adrenoceptor is less sensitive to yohimbine, and the concentration of yohimbine used in the present experiments (3×10^{-7} M) is not sufficient to block the effects of adrenaline on the prejunctional α-adrenoceptors which are involved in α-autoregulation of transmitter release (Starke, 1987). However, in the dog mesenteric vein, yohimbine antagonizes the noradrenaline-induced inhibition of the e.j.p. by blocking prejunctional α_2-adrenoceptors (Suzuki, 1984). Therefore, it is unlikely that absence of a potentiating action of adrenaline on the e.j.p. in the presence of yohimbine is due to prejunctional α-adrenoceptor stimulation; rather it is probably due

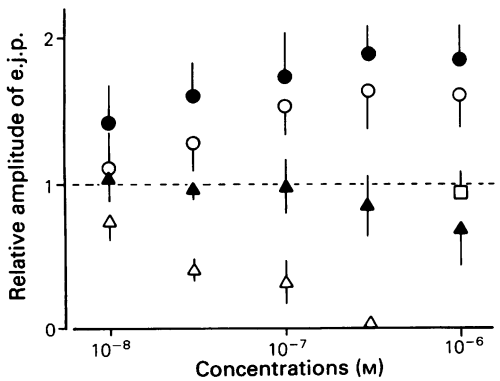

Figure 5 Concentration-response relationship of the effects of adrenaline and isoprenaline on the e.j.p. in the dog mesenteric vein. The e.j.p. was evoked by single stimuli (0.03–0.05 ms duration, 25–40 V intensity), and the amplitude relative to the control is shown as the value with s.d. indicated by vertical lines (n = 11–35): (△) adrenaline; (▲) adrenaline plus yohimbine 3×10^{-7} M; (○) isoprenaline; (●) isoprenaline plus yohimbine 3×10^{-7} M; (□) isoprenaline with propranolol 10^{-7} M.

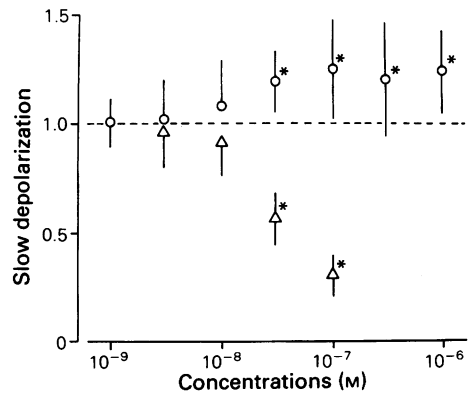

Figure 6 Concentration-response relationship of the effects of adrenaline and isoprenaline on the slow depolarization produced by perivascular nerve stimulation (10 stimuli at 0.3 Hz frequency) in the dog mesenteric vein: (△) adrenaline; (○) isoprenaline. Mean values are shown with s.d. indicated by vertical lines. (n = 5–8).

to the weak β-adrenoceptor stimulating actions of adrenaline in the prejunctional membrane of the dog mesenteric vein.

The role of prejunctional β-adrenoceptors in sympathetic transmission is equivocal, and stimulation of this receptor by isoprenaline increases (Adler-Graschinsky & Langer, 1975; Guimarês *et al.*, 1978; Westfall *et al.*, 1979; Misu *et al.*, 1984; Molderings *et al.*, 1988) or does not change (Endo *et al.*, 1977; Nedergaard, 1987) the evoked release of noradrenaline from perivascular sympathetic nerves. It has also been reported that isoprenaline enhances the release of noradrenaline only in the presence of α-adrenoceptor antagonists such as yohimbine or phentolamine (Dahlöf *et al.*, 1978; Majewski *et al.*, 1981). Electrophysiological measurments of e.j.ps demonstrate that prejunctional β-adrenoceptor-mediated facilitation of transmitter release by isoprenaline can be seen in the mesenteric artery of the guinea-pig but not in the rabbit (Kuriyama & Makita, 1984). Thus, variations between tissues have also to be considered.

Stimulation of postjunctional β-adrenoceptors by isoprenaline or adrenaline in the presence of yohimbine, hyperpolarized the smooth muscle membrane in the guinea-pig facial vein but not in the dog mesenteric vein. Although the present experiments did not investigate further the ionic mechanisms involved in the hyperpolarization, potassium ions may be the main contributor, as in the rabbit facial vein stimulation of postjunctional β-adrenoceptors by endogenous or exogenous noradrenaline increases the membrane permeability to potassium ions (Komori *et al.*, 1989). Further experiments are required to elucidate the mechanisms behind the difference in potency of the β-adrenoceptors involved in the generation of the membrane hyperpolarization between facial and mesenteric veins. There is also indirect evidence which demonstrates that stimulation of β-adrenoceptors does not change the membrane potential of vascular smooth muscle cells, i.e., in the guinea-pig mesenteric vein, application of noradrenaline in the presence of α-adrenoceptor antagonists does not change the membrane potential (Suzuki, 1981). Thus, differences may arise perhaps from regional variations, in the distribution of β-adrenoceptor populations.

It is concluded that in the dog mesenteric vein, adrenaline and isoprenaline have different stimulating actions at prejunctional β-adrenoceptors and adrenaline but not isoprenaline facilitates the evoked release of transmitter substances from perivascular nerves.

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Microelectrode study on the ionic mechanisms which contribute to the noradrenaline-induced depolarization in isolated cells of the rabbit portal vein

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1 Experiments were carried out to determine the identity of the ionic mechanisms which contribute to the noradrenaline-evoked depolarization recorded with microelectrodes in freshly dispersed rabbit portal vein cells.

2 In normal physiological salt solution with microelectrodes containing 1 M NaCl the reversal potential (E_r) of the noradrenaline-induced response was -7.6 ± 2.9 mV. When the external NaCl was replaced by equipmolar concentrations of NaI, NaBr and $NaNO_3$, E_r was -33 ± 3.5 mV, -29.1 ± 5.2 mV and -18.4 ± 1.1 mV, respectively.

3 In physiological salt solution E_r of noradrenaline-evoked responses recorded with electrodes filled with 1 M NaI or 1 M $NaNO_3$ was $+16.3 \pm 3.9$ mV and $+10.0 \pm 7.6$ mV, respectively. These results suggest that an increase in anion conductance contributes to the depolarization to noradrenaline.

4 Data from experiments with organic anions indicated that glutamate behaves as a less permeant anion but that benzenesulphonate blocks the anion conductance to unmask another conductance mechanism activated by noradrenaline.

5 When external NaCl was substituted by choline Cl and Tris Cl E_r was -21.3 ± 3.7 mV and -20.5 ± 2.8 mV, respectively. These results suggest that noradrenaline also activates a cation conductance mechanism in freshly dispersed rabbit portal vein cells. It is concluded that the depolarization to noradrenaline recorded with a microelectrode is produced by the simultaneous activation of an anion channel and a separate cation channel.

Introduction

In a previous study with patch pipette techniques we provided evidence in isolated cells of the rabbit portal vein that noradrenaline produces an increase in membrane conductance to chloride ions and to cations (Byrne & Large, 1988b). There appeared to be a curious interaction between these two separate conductance mechanisms. When sodium chloride was the major salt in the patch pipette (experiments were carried out in potassium-free conditions to remove a prominent calcium-activated potassium conductance increase) the response to noradrenaline could be described mainly by an increase in chloride conductance. However, when sodium glutamate was the major constituent of the patch pipette the inward current evoked by noradrenaline appeared to be carried by cations. Moreover the reversal potential of the noradrenaline-induced response was about 70 mV more positive than the chloride equilibrium potential. Thus in these conditions it appeared that noradrenaline produced little or no increase in chloride conductance. It was observed that the amplitude of the responses was similar whether the current was carried by either chloride or cations. It would seem that the contents of the patch pipettes and subsequent dialysis of the cell interior have a profound influence on the qualitative nature of the conductance mechanisms evoked by noradrenaline in freshly dispersed rabbit portal vein cells. These results raise doubts concerning the physiological implications of the ionic mechanisms evoked by noradrenaline which are identified with patch pipette techniques. It is possible that membrane mechanisms may be obscured or exaggerated in patch pipette studies. For example the most prominent response to noradrenaline recorded with patch pipettes in freshly dispersed rabbit ear artery cells is hyperpolarization

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(or outward current) produced by an increase in potassium conductance (Benham, Bolton, Byrne and Large, unpublished). In whole tissue experiments α-adrenoceptor activation by exogenous noradrenaline and nerve stimulation evokes only depolarization (Suzuki & Kou, 1983). In the present study we have carried out experiments to ascertain whether the depolarization recorded with microelectrodes is produced by an increase in chloride and/or cation conductance in cells isolated from the rabbit portal vein. It is evident that in isolated smooth muscle cells microelectrodes produce a much smaller disturbance of the intracellular milieu than is found with patch pipettes. For example, with microelectrodes it is possible to obtain many reproducible responses to noradrenaline in freshly dispersed cells. This is not the case with patch pipettes and in many cells only a single response to noradrenaline can be obtained (see Byrne & Large, 1987a; 1988b). In this paper we provide evidence which suggests that the noradrenaline-induced depolarization recorded with a microelectrode is mediated by an increase in both chloride and cation membrane conductance and therefore supports a physiological role for both ionic mechanisms.

Methods

Rabbits (2–2.5 kg) of either sex were killed by overdose of i.v. sodium pentobarbitone. Isolated cells were obtained from rabbit portal vein by digestion with papain according to a method described in detail previously (Byrne & Large, 1988a,b). Cells were stored on cover slips at 4°C and were used on the same day as dispersion.

A single microelectrode was used for recording membrane potential and passing current with an Axoclamp-2A amplifier. Depolarizations or hyperpolarizations to noradrenaline were recorded under current clamp with the Bridge circuit of the Axoclamp-2A amplifier. Under voltage clamp, membrane currents were measured in the discontinuous single-electrode voltage clamp mode. In this mode, the function of the microelectrode alternates between current passing and voltage recording at a high frequency. The voltage is measured only when the discontinuous current is zero, allowing a precise measurement of membrane potential. The duty cycle used was current passing for 30%, and voltage recording for 70% of each cycle. The procedure was to select a sampling frequency at which the headstage voltage after current passing returned to true baseline. The sampling frequency was between 1.0 and 2.0 kHz. Microelectrodes had resistance of 100–200 MΩ when filled with either 1 M NaCl or 1 M KCl.

The normal bathing solution contained (mM): NaCl 126, KCl 6, $MgCl_2$ 1.2, $CaCl_2$ 1.5, HEPES 10 and glucose 11, buffered to pH 7.2 with NaOH and experiments were carried out at room temperature (20–24°C). In external substitution experiments 126 mM NaCl was replaced with equimolar concentration of choline Cl, Tris Cl, NaI, NaBr, $NaNO_3$, Na glutamate or Na benzenesulphonate. The external solution always contained 10^{-6} M propranolol to block any β-adrenoceptor-mediated hyperpolarization (Byrne & Large, 1988a).

Junction potentials produced by anion substitution in the external solution were minimized by placing an agar bridge between the recording bath and the chamber with the indifferent electrode. With this configuration junction potentials were small (< 5 mV) and corrections were made as described previously (Byrne & Large, 1987a).

In a few experiments 1 M NaCl in the microelectrode was replaced by either 1 M NaI or $NaNO_3$. Noradrenaline was applied by ionophoresis.

The values given in the text are the mean ± s.e.mean. Statistical significance of changes in the reversal potential from the normal value (1 M NaCl electrode and 126 mM NaCl external solution) was estimated with Student's t test.

Drugs used were: atropine sulphate, bovine albumin (fatty acid-free), dithiothreitol, noradrenaline bitartrate, papain type IV (all Sigma), and (±)-propranolol hydrochloride (ICI).

Results

General observations

In the present experiments microelectrodes filled with NaCl were used in order to remove the calcium-activated potassium current evoked by noradrenaline in these cells which would allow more accurate measurement of the reversal potential of the depolarization to noradrenaline. There is evidence to indicate that with NaCl-filled microelectrodes, substantial amounts of intracellular K^+ had been replaced with Na^+. Firstly, an outward current to depolarizing voltage steps could not be recorded if NaCl was used as the electrolyte in the microelectrodes. Figure 1 illustrates currents evoked by depolarizing steps to −30 mV and +30 mV from a holding potential of −50 mV when either a KCl (Figure 1a) or a NaCl (Figure 1b) electrode was used. The currents at −30 mV are similar with both KCl and NaCl electrodes (upper records Figure 1a and b) and essentially reflect the input resistance of the cell. A hyperpolarizing 20 mV step produced a current response of similar amplitude. However a command step to +30 mV evokes a large outward current with

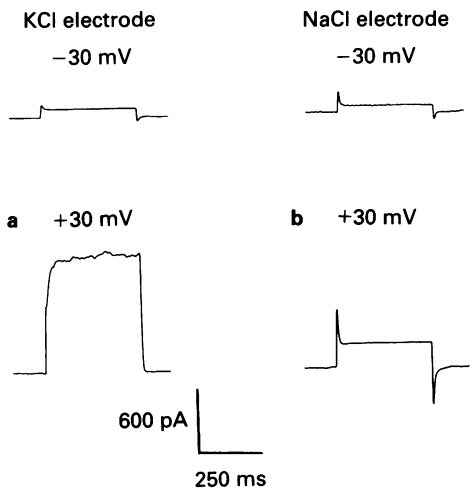

Figure 1 Evidence for the lack of a voltage-dependent outward current with the use of a NaCl-filled microelectrode. The records are current responses to depolarizing voltage commands to −30 mV (upper traces) and +30 mV (lower traces) with the use of a microelectrode filled with either 1 M KCl or 1 M NaCl. Holding potential: −50 mV. Comparison of the lower traces illustrate that the step to +30 mV evokes a large outward (potassium) current with a KCl microelectrode (a) but there is no apparent voltage-dependent current with a NaCl microelectrode (b) and the amplitude of the current response in (b) reflects the input resistance of the cell.

a KCl electrode (lower record Figure 1a). In contrast, with a NaCl electrode there was no evidence of a voltage-dependent response at +30 mV and the amplitude of the current at the end of the command pulse (lower record of Figure 1b) is about 4 times larger than the corresponding value at −30 mV (i.e. an indication of the input resistance). These data suggest that with NaCl-filled microelectrodes there is little (or no) voltage-dependent potassium current.

A second line of evidence which suggests that potassium had been largely replaced by sodium is that there was no evidence of a calcium-activated increase in potassium conductance to noradrenaline which has been demonstrated if the intracellular electrode is filled with KCl (Byrne & Large, 1988a,b). Although these results indicate that use of microelectrodes filled with NaCl eliminate potassium currents we have no data on the intracellular concentration of sodium achieved in our experiments. It is possible that there may be substantial amounts of potassium remaining in the cells and that the intracellular concentration of sodium was sufficiently high to block the potassium currents. For example, it has been shown that internal sodium ions block large unitary calcium-dependent potassium currents in bovine adrenal chromaffin cells (Marty, 1983). An estimate of the internal sodium activity could be obtained in experiments with a Na-sensitive microelectrode which would also yield interesting information regarding the change in intracellular sodium concentration with time. However, technical difficulties of impaling an isolated smooth muscle cell with both a recording and a Na-sensitive microelectrode preclude such observations at the present time.

It is possible that the increased intracellular concentration of Na^+ would stimulate the electrogenic sodium/potassium ATPase which might in turn influence the observed membrane potential changes to noradrenaline. However it seems unlikely that this occurred in the present experiments as the resting membrane potential was about 0 mV when recorded with NaCl-filled electrodes. If the electrogenic sodium/potassium ATPase was active it might be expected that the resting membrane potential would be at more hyperpolarized values. In addition the amplitude and time course of the noradrenaline-evoked depolarizations recorded in the present experiments were similar to the characteristics of the responses recorded with microelectrodes filled with 1 M KCl (Byrne & Large, 1988a).

On most occasions the ionophoretic application of noradrenaline produced a monophasic depolarization when recorded with a NaCl-filled microelectrode (see Figure 2a). Sometimes biphasic depolarizations were recorded but these responses were not reproducible and overall there was no convincing evidence that the response could be differentiated temporally into two components representing two conductance mechanisms. We investigated whether a chloride and/or a cation conductance increase was involved in the noradrenaline-evoked depolarization by altering independently the cation and anion gradients. A change in the reversal potential (E_r) by these experimental manipulations would implicate the appropriate conductance mechanism in the response to noradrenaline.

Responses to noradrenaline in different external anion solutions

Figure 2a illustrates the measurement of the reversal potential (E_r) of the noradrenaline-induced response in a cell with a NaCl-filled microelectrode in normal bathing solution (126 mM NaCl). In these conditions the resting membrane potential is close to 0 mV and small amounts of inward and outward current were passed through the recording electrode to obtain the holding membrane potentials indicated. The ionophoretic application of noradrenaline-evoked depolarization at a membrane potential of −52 mV and the amplitude of the response declined as the membrane

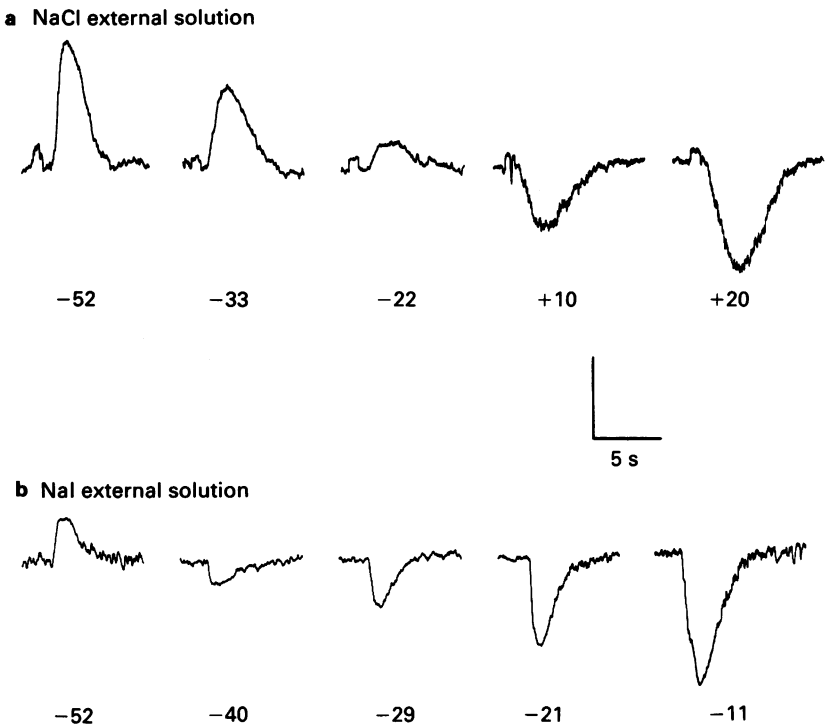

Figure 2 Responses to noradrenaline in either external 126 mM NaCl (a) or 126 mM NaI (b) recorded with a NaCl-filled electrode. Membrane potentials are indicated under each trace. Noradrenaline was applied by iontophoresis: 10 nA for 500 ms in (a) or 200 ms in (b). A small iontophoretic artefact (small upward deflection) can be seen just prior to the responses in (a). Vertical calibration bar: 20 m V in (a) and 10 m V in (b).

potential was reduced and reversed to hyperpolarization at between −22 and +10 mV.

The amplitude of the responses are plotted as a function of holding potential in Figure 3 (circles) and E_r was −9 mV. In eight experiments with a NaCl-filled electrode with 126 mM NaCl bathing solution the mean reversal potential was −7.6 ± 2.9 mV (Table 1). In the conditions used, this value of the reversal potential may be close to both the chloride and sodium equilibrium potentials although we do not have independent estimates of E_{Cl} and E_{Na}.

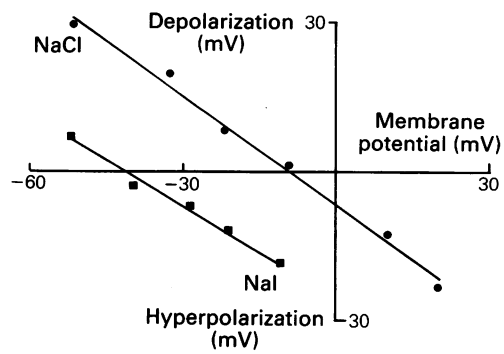

Figure 3 Relationship between the amplitude of the noradrenaline-evoked response and membrane potential in either external 126 mM NaCl (●) or 126 mM NaI (■). Data taken from responses shown in Figure 2.

Figure 2b shows responses to noradrenaline when 126 mM NaCl in the bathing solution was replaced by an equimolar concentration of NaI. It is evident that the reversal potential was between −52 mV and −40 mV. The results are plotted in Figure 3 (squares) and the interpolated equilibrium potential is −42 mV. The mean E_r in external NaI solution was −33.0 ± 3.5 mV (Table 1) and is statistically significant from the value found in external NaCl solution ($P < 0.001$). Similar experiments were carried out with 126 mM NaBr and $NaNO_3$ and the E_rs were respectively −29.1 ± 5.2 mV and −18.4 ± 1.1 mV (Table 1). The change in the reversal potential of the noradrenaline-evoked responses produced by substitution of external Cl^- with I^-, Br^- and NO_3^- suggests that an anion conductance increase at least partially underlies the depolarization to noradrenaline.

Table 1 Reversal potential of the noradrenaline-induced response in various conditions

External solution†	*Electrode solution*††	*Reversal potential* (mV)	(n)
NaCl	NaCl	-7.6 ± 2.9	(8)
NaI	NaCl	-33.0 ± 3.5*	(5)
NaBr	NaCl	-29.1 ± 5.2*	(8)
$NaNO_3$	NaCl	-18.4 ± 1.1**	(8)
Na glutamate	NaCl	$+19.3 \pm 5.7$*	(6)
Na benzenesulphonate	NaCl	-8.7 ± 4.5	(7)
NaCl	NaI	$+16.3 \pm 3.9$**	(3)
NaCl	$NaNO_3$	$+10.0 \pm 7.6$***	(3)
Choline Cl	NaCl	-21.3 ± 3.7**	(6)
Tris Cl	NaCl	-20.5 ± 2.8**	(4)

Statistically different from control value with external NaCl and NaCl electrode: $*P < 0.001$, $**P < 0.005$ and $***P < 0.05$.

† All salts at a concentration of 126 mM and †† electrode solutions at 1 M.

Other experiments were carried out with relatively large organic anions which normally do not permeate chloride channels very well. Figure 4 illustrates the results of two experiments in which external NaCl was replaced either by Na glutamate (squares) or Na benzenesulphonate (circles) and the respective E_r values were +25 mV and −10 mV. The overall mean values were $+19.3 \pm 5.7$ mV and -8.7 ± 4.5 mV (Table 1) respectively for Na glutamate and Na benzenesulphonate. The statistically significant shift of the reversal potential of the noradrenaline-induced response to a more positive potential than the control value (−7.6 mV) is consistent with the idea that glutamate acts simply as a less permeant anion. This is patently not so for benzenesulphonate as E_r with this anion was similar to the control E_r. It seems extremely unlikely that benzenesulphonate is as permeable as chloride and we feel that benzenesulphonate does not simply act as an impermeant anion but has some other action. We would like to suggest that benzenesulphonate somehow blocks the anion response which unmasks the cation conductance mechanism (see Discussion for details).

Replacement of Cl^- in the microelectrode with other anions

In a few experiments the recording electrodes were filled with 1 M NaI and $NaNO_3$ (rather than NaCl) with 126 mM external NaCl. With NaI and $NaNO_3$ electrodes the E_r of the noradrenaline-induced response was respectively $+16.3 \pm 3.9$ mV (Table 1) and $+10.0 \pm 7.6$ mV (Table 1). The shift of E_r to positive potentials with NaI and $NaNO_3$ electrodes support the above data that an anion current contributes to the noradrenaline-evoked depolarization and that I^- and NO_3^- permeate the anion channel more readily than Cl^- ions.

Substitution of sodium with other cations in the external solution

Experiments were carried out in which most of the external sodium was replaced by cations that normally permeate cation channels rather poorly. In these conditions the E_r of a cation conductance would move to more negative potentials. Figure 5

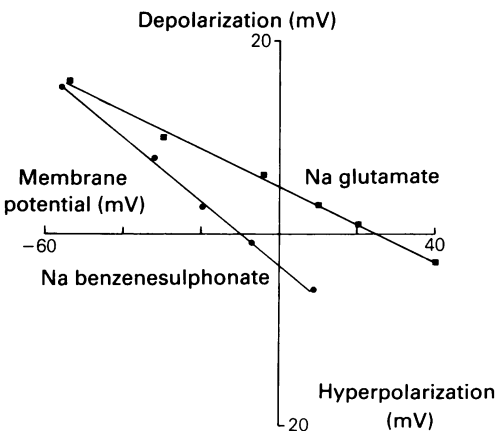

Figure 4 Relationships between noradrenaline-evoked responses and membrane potential when the major external anion was either benzenesulphonate (●) or glutamate (■). See text for further details.

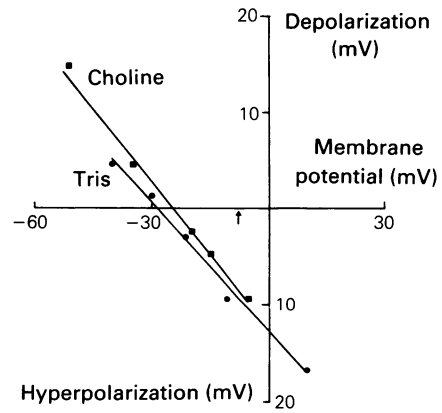

Figure 5 Effect of membrane potential on the responses to noradrenaline when either 126 mM Tris Cl (●) or choline Cl (■) was present in the bathing solution. The arrow indicates the mean E_r with NaCl in the external solution.

shows the results from two experiments in which external 126 mM NaCl was replaced by equimolar concentrations of either choline chloride (squares) or Tris chloride (circles). It can be seen that in these experiments E_r of the noradrenaline-induced depolarization was −25 mV (choline) and −28 mV (Tris). The mean E_r values for external choline and Tris were -21.3 ± 3.7 mV and -20.5 ± 2.8 mV (Table 1) respectively. The shift of the reversal potential to more negative potentials in the presence of external choline and Tris indicates that a cation conductance mechanism also contributes to the noradrenaline-evoked depolarization in freshly dispersed rabbit portal vein cells.

Discussion

The present experiments were undertaken to see if anionic and cationic mechanisms identified with patch pipette techniques contributed to the noradrenaline-induced depolarization observed with microelectrodes. In experiments where external Cl^- was largely replaced by more permeant anions (I^-, Br^- and NO_3^-) the reversal potential of the noradrenaline-induced responses was shifted to more negative potentials as predicted if an anion conductance increase contributed to the noradrenaline-induced depolarization. This postulate was supported by the results from studies where NaI and $NaNO_3$ were used as the microelectrode filling solution in which the reversal potential was shifted to positive membrane potentials. Thus the overall evidence supports strongly the idea that an increase in chloride conductance subscribes to the noradrenaline-evoked depolarization. Experiments with less permeant anions produced apparently conflicting data. Whereas glutamate appeared to behave simply as a less permeant substitute the results with benzenesulphonate were not as straightforward. When external NaCl was replaced by Na benzenesulphonate there was no change in E_r of the response to noradrenaline. This suggests that either benzenesulphonate and chloride are similarly permeable (extremely unlikely) or that the presence of benzenesulphonate reveals another conductance mechanism. We feel that the latter proposal is more likely to be valid since previously it has been suggested that benzenesulphonate blocks a chloride channel in the rat anococcygeus muscle (Large, 1984). Van Helden (1988) has claimed that another organic anion, isethionate, may also block a chloride conductance mechanism in the guinea-pig mesenteric vein. Thus in our experiments it is possible that the depolarization to noradrenaline recorded in Na benzenesulphonate is brought about by an increase in membrane cation conductance. Experiments in which external Na was replaced by choline and Tris moved the reversal potential of the noradrenaline-induced response to more negative membrane potentials. The only reasonable explanation for these results is that an increase in cation conductance contributes to the depolarization to noradrenaline. Thus, in conclusion, it would appear that the depolarization to noradrenaline is mediated by an efflux of chloride through an anion channel and an influx of cations through a separate cation-selective channel. The fact that both cationic and anionic conductance mechanisms to noradrenaline can be observed with microelectrode recording is evidence that both membrane mechanisms may be activated by α-adrenoceptor stimulation in physiological conditions.

If it is assumed that the relative contribution to the cation conductance mechanisms does not alter in the different anion solutions, then the values of the reversal potentials to noradrenaline suggest the following permeability sequence through the anion channel: $I^- > Br^- > NO_3^- > Cl^-$ > glutamate. This interpretation would be incorrect if variable amounts of the anions used entered the cells through resting leak anion channels. If this occurred the value of the equilibrium potential of the anions would vary from anion to anion depending on the concentration gradient established across the cell membrane. However, the shift of the reversal potential of the noradrenaline-induced responses to positive potentials when NaI and $NaNO_3$ were used in the microelectrodes indicates that our conclusions are likely to be valid. The order of halide permeability ($I^- > Br^- > Cl^-$) has also been found in rat lacrimal glands cells (Evans & Marty, 1986), cultured Schwann cells (Gray *et al.*, 1984) and in *Xenopus laevis* oocyte membranes reconstituted into planar bilayers (Young *et al.*, 1984). Moreover the same sequence is found for the channel opened by glycine and γ-aminobutyric acid (GABA) in mouse cultured spinal neurones (Bormann *et al.*, 1987). In a previous publication we noted the similarity between the calcium-activated chloride conductance increase in rat lacrimal glands (Marty *et al.*, 1984) and the rat anococcygeus muscle (Byrne & Large, 1987b). Interestingly in rat lacrimal gland cells the chloride channel appears to be more permeable to NO_3^- than to Br^- (Evans & Marty, 1986) whereas the reverse order was found in our experiments. There is no obvious explanation for this discrepancy unless the structures of the channels are different. Further experiments with patch pipettes are needed to substantiate this point.

Finally, it is becoming increasingly evident that chloride currents may have an important physiological role in smooth muscle. Evidence for a chloride current has been presented in cells from rat common

carotid artery (Shoemaker *et al.*, 1985), in the ctenophore *Mnemiopsis* (Stein *et al.*, 1985), rat anococcygeus muscle (Byrne & Large, 1987a), guinea-pig uterus (Coleman & Parkington, 1987), rabbit portal vein (Byrne & Large, 1988b) and the guinea-pig mesenteric vein (van Helden, 1988). In the guinea-pig vas deferens it has been demonstrated that the chloride equilibrium potential is about −25 mV (Aickin & Brading, 1982). Therefore any transmitter or local mediator which increases chloride conductance will evoke depolarization and consequent contraction if the voltage threshold for opening of voltage-dependent calcium channels is reached.

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Perinatal lead exposure impairs opioid but not non-opioid stress-induced antinociception in developing rats

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1 The development of opioid systems has been shown to be sensitive to perinatal exposure to lead. We have studied the effects of such exposure on opioid and non-opioid mediated stress-induced antinociception in developing rats.

2 Lead was administered in the maternal drinking water from conception to postnatal day 14 at 300 and 1000 p.p.m. Twenty and 25 day old rats were subjected to swimming stress and antinociception measured using the tail immersion test.

3 A 3 min swim-stress induced an opioid-mediated antinociceptive response in 20 day old rats which was attenuated by 300 p.p.m. lead and by 1000 p.p.m. lead treatment in a dose-related manner. A 10 min swim-stress induced a non-opioid mediated antinociceptive response in 25 day old rats which was not antagonised by 300 or 1000 p.p.m. lead.

4 Naloxone antagonised the residual antinociception observed in 20 day old animals treated with 300 p.p.m. lead and had no effect on antinociception in control or lead-treated 25 day old rats.

5 Using a lead exposure model considered to represent subclinical lead toxicity in man, it was shown that perinatal lead exposure disrupts opioid but not non-opioid mediated stress antinociception.

Introduction

There are clear indications that the developing child is particularly susceptible to neurotoxic effects of lead and indeed that environmental exposure to this metal might be responsible for certain behavioural abnormalities (Royal Commission Report, 1983). Experimental animal studies also indicate susceptibility to lead toxicity during the perinatal period and some neurotransmitter systems have been identified as being particularly sensitive to exposure to this metal. In this respect we have observed marked toxic effects of low level perinatal lead exposure on the development of opioid peptides (Bailey & Kitchen, 1985), opioid receptors (McDowell & Kitchen, 1988) and opioid-induced antinociception (Kitchen *et al.*, 1984; Kitchen & McDowell, 1985) in the rat, using a model of lead exposure which gives blood levels representative of subclinical lead toxicity.

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We have extended our investigations on opioid-induced antinociception to examine the effect of perinatal lead exposure on the phenomenon of stress-induced antinociception early in development. Recently we demonstrated that short swims produce opioid-mediated stress-induced antinociception in 20 day old rats, whereas longer swims at later ages (25 days) induce antinociception via non-opioid mechanisms (Jackson & Kitchen, 1989). Accordingly, the effects of lead exposure on opioid and non-opioid stress-induced antinociception have been studied at day 20 and day 25 respectively.

Methods

Animals and lead dosing

Wistar albino rats (University of Surrey strain) of either sex were used in all experiments and maintained at a constant temperature (21 ± 1°C) and

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Table 1 Effect of lead on nociceptive responses in unstressed 20 and 25 day old rats using the tail immersion test

Dose of lead (p.p.m.)	*Response latency* (s) *20 days*	*25 days*
0	2.0 ± 0.1	2.7 ± 0.2
300	1.6 ± 0.1	2.0 ± 0.1*
1000	1.5 ± 0.1*	2.0 ± 0.1*

Each value is the mean of at least 16 observations. The data represent pretest values for animals subsequently used as unstressed control groups or swim stressed groups. Significant differences from 0 p.p.m. groups are denoted by $*P < 0.05$.

light/dark cycle (lights on at 07h 00min). Litters were culled to 8 pups per dam at birth. Lead was administered as originally described by Carmichael *et al.* (1981) with the modifications of McDowell & Kitchen (1988), In brief, lead was administered in the maternal drinking water (as the acetate dissolved in distilled water acidified with glacial acetic acid) from conception (−21 days) to postnatal day 14. Two doses of lead were used in this study (300 p.p.m. and 1000 p.p.m.) and mothers of control litters were treated with a corresponding solution of acetic acid (28 mM). Maternal weight and fluid intake were monitored throughout and litter size and neonate weight gain measured in the postnatal period. Neonates were weaned at day 21.

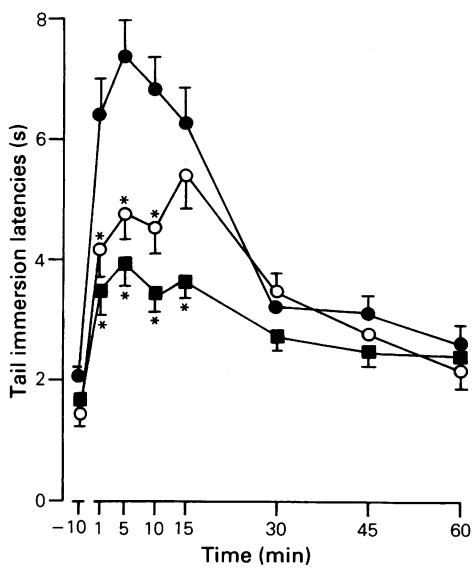

Figure 1 Effect of perinatal lead exposure on antinociception induced by a 3 min swim in 20 day old rats. Values represent means for groups of at least 8 animals (vertical lines show s.e.mean). (●) 0 p.p.m. Pb; (○) 300 p.p.m. Pb; (■) 1000 p.p.m. Pb. Significant differences from the 0 p.p.m. Pb control group are denoted by $*P < 0.05$.

Stress-induced antinociception

Stress-induced antinociception was examined in 20 and 25 day old pups treated with 0 p.p.m., 300 p.p.m. or 1000 p.p.m. lead. Warm water swimming (20°C) for 3 min at day 20 or 10 min at day 25 was used as the stressor. All animals used in this study were handled regularly and injected on the day of test to control for any stressful effects of these procedures and to enable meaningful comparison between all experimental groups. Procedures were carried out in a quiet, windowless, air conditioned laboratory and animals equilibrated to these conditions for 2 h before use. Rat pups remained in the home cage except during drug administration, swimming and antinociceptive testing. Each series of determinations represents measurements made on at least four separate litters in each lead treatment group, on at least two separate days. Each animal was tested only once and all experiments were carried out between 13 h 00 min and 17 h 00 min.

Each litter was divided into four groups. One group received 0.9% saline i.p. and remained in the home cage as an unstressed control group. The other 3 groups were injected i.p. with either saline or with 1 or 10 mg kg^{-1} doses of the opioid antagonist naloxone (using a blind procedure and a dose volume of 0.1 ml 20 g^{-1}). Ten minutes later animals were placed individually in a swimming area 14 cm in diameter and 12 or 20 cm deep for 20 and 25 day old rat pups, respectively. At the end of the swimming period pups were gently dried. Antinociception was assessed using the tail immersion test. Rats were hand-held over a hot water bath with the terminal 1–2 cm portion of their tails in water at a temperature of 50°C. This noxious stimulus produced a distinct tail flick response and tail immersion latencies (i.e. the time taken for rats to remove their tails from the water) were measured to the nearest 0.1 s using a stop-watch. Nociceptive responses were determined 10 min before swimming stress and 1, 5, 10, 15, 30, 45 and 60 min after swimming. Treatment groups were statistically compared by use of analysis of variance and Dunnett's test.

Results

Baseline nociceptive latencies in unstressed lead-treated rats were lower than in the corresponding unstressed control (0 p.p.m.) group, an effect

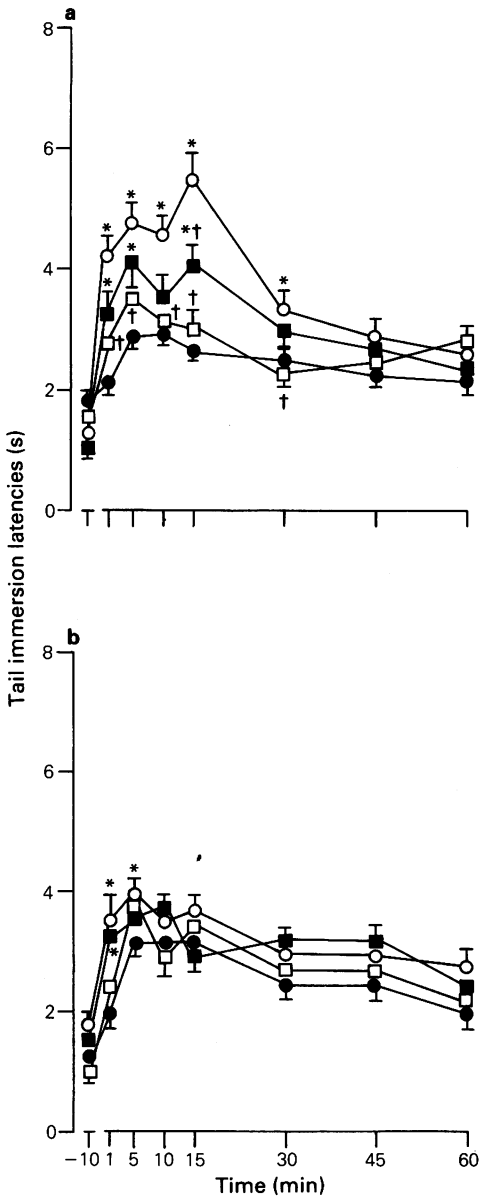

Figure 2 Effect of naloxone on antinociception induced by a 3 min swim in 20 day old rats exposed to (a) 300 p.p.m. and (b) 1000 p.p.m. lead. Values represent means for groups of at least 8 animals (vertical lines show s.e.mean). (●) Saline injected, unstressed; (○) saline injected, swim-stressed; (■) naloxone ($1\,mg\,kg^{-1}$), swim-stressed; (□) naloxone ($10\,mg\,kg^{-1}$), swim-stressed. Significant differences from unstressed controls are indicated by $*P < 0.05$ and from the swim-stressed controls by $\dagger P < 0.05$.

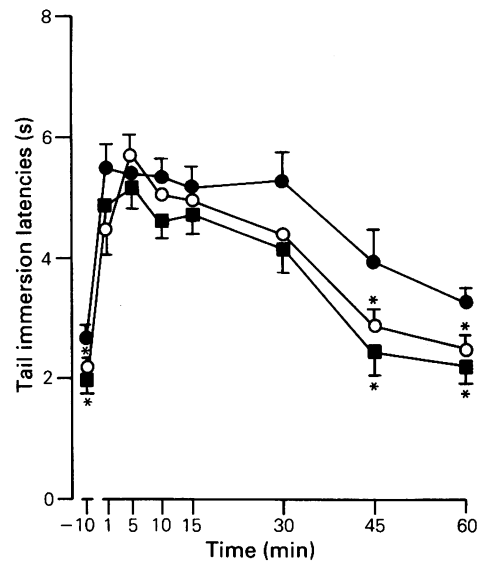

Figure 3 Effect of perinatal lead exposure on antinociception induced by a 10 min swim in 25 day old rats. Values represent means for groups of at least 8 animals (vertical lines show s.e.mean). (●) 0 p.p.m. Pb; (○) 300 p.p.m. Pb; (■) 1000 p.p.m. Pb. Significant differences from the 0 p.p.m. Pb control group are shown by $*P < 0.05$.

observed in both 20 and 25 day old animals (Table 1). A 3 min swim stress in 20 day old rats produced a 3–4 fold increase in nociceptive latency 5 min after the stress in the control (0 p.p.m.) group. This stress-induced antinociception persisted for 15 min (Figure 1). A 3 min swim produced significantly less antinociception in 20 day old rats treated with either 300 p.p.m. lead or 1000 p.p.m. lead than in the 0 p.p.m. control group. The nociceptive latencies of 300 p.p.m.-treated rats subjected to a 3 min swim stress were, however, still greater than those of the unstressed 300 p.p.m.-treated group, as shown in Figure 2a. The antinociception induced by swimming in these 20 day old animals was partially attenuated and completely antagonised by 1 and $10\,mg\,kg^{-1}$ naloxone respectively, an effect also observed in the control 0 p.p.m. group (data not shown). The much smaller increases in tail immersion latencies induced by a 3 min swim in 1000 p.p.m.-treated pups were only significantly elevated over those of unstressed controls during the first 5 min post-stress (Figure 2b). These responses were not significantly reversed by naloxone, although nociceptive latencies of stressed animals treated with the highest dose of naloxone were similar to those of controls at 1 and 5 min after swimming, suggesting that naloxone was having

some effect on the residual stress-induced antinociception observed in these animals. The doses of naloxone used in the present study had no effects on baseline tail immersion latencies *per se* (data not shown).

In 25 day old rats, a 10 min swim produced a 2–3 fold increase in nociceptive latency 5 min after stress and this antinociception was observed in control and both lead-treated groups. However, nociceptive latencies in lead-treated animals returned to baseline values more rapidly and were significantly lower than those of the 0 p.p.m. group 45 and 60 min after stress (Figure 3). Swim stress-induced antinociception in 25 day old control groups was unaffected by 1 and 10 mg kg^{-1} naloxone (data not shown) and this lack of effect of the opioid antagonist was also observed in 300 (Figure 4a) and 1000 p.p.m. lead-treated animals (Figure 4b). Figure 4a and b also show the tail immersion latencies of unstressed 300 and 1000 p.p.m.-treated pups. These animals showed small increases in response latencies during the first 15 min of the test. This effect was consistently seen throughout the study (see also Figure 2a and b) and is likely to reflect the stress induced by an injection of saline.

There were no significant effects of lead on maternal or neonate weight gain or on maternal fluid intake.

Discussion

Several studies have shown changes in nociception threshold after inorganic lead treatment and the majority point to a reduced sensitivity to pain (Baraldi *et al.*, 1985; Bondy *et al.*, 1985; Vickers & Paterson, 1986). In the present study there were indications of the opposite effect and previous experiments in our laboratory have suggested that lead-induced changes in response to noxious stimuli are age- and test-dependent (Kitchen *et al.*, 1984; Kitchen & McDowell, 1985). Antinociception induced by a short period of swimming stress is reversed by naloxone and is clearly opioid in nature (Terman *et al.*, 1986; Jackson & Kitchen, 1989). Stress-induced antinociception has been demonstrated to involve the release of endogenous opioid peptides (for review see Millan, 1981) and lead may produce antagonism of stress-induced antinociception by disruption of synthesis, storage or release of these peptides. In support of this, perinatal lead exposure has been shown to disrupt markedly opioid peptides in the postnatal period (Bailey & Kitchen, 1985) and disruption of dopamine/opioid interneurones by lead has also been proposed (Govoni *et al.*, 1980; Memo *et al.*, 1981). Alternatively, attenuation of this effect by lead might indicate

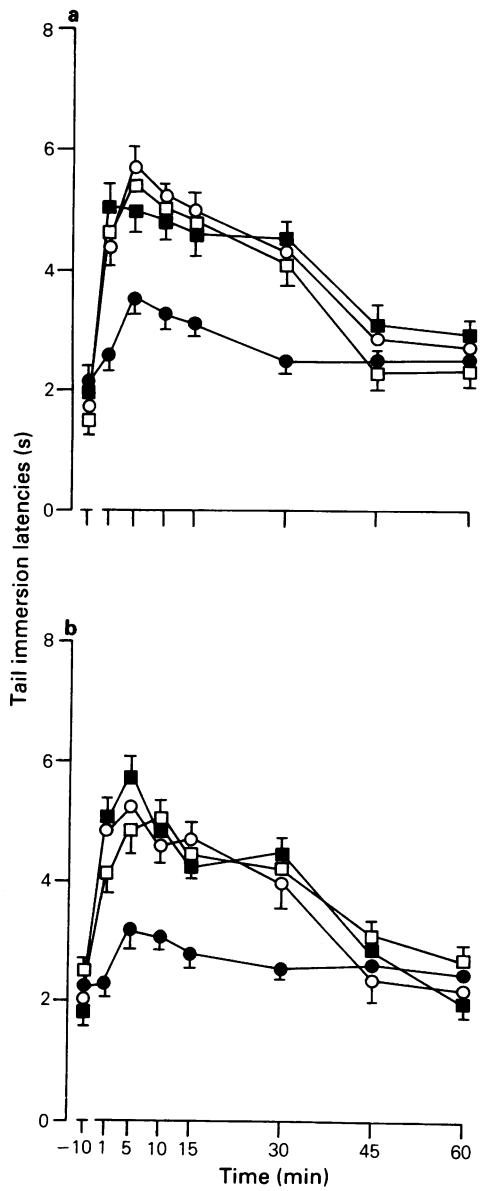

Figure 4 Lack of effect of naloxone on antinociception induced by a 10 min swim in 25 day old rats exposed to (a) 300 p.p.m. and (b) 1000 p.p.m. lead. Values represent means for groups of at least 8 animals (vertical lines show s.e.mean). (●) Saline injected, unstressed; (○) saline injected, swim-stressed; (■) naloxone (1 mg kg^{-1}), swim-stressed; (□) naloxone (10 mg kg^{-1}), swim-stressed. There were no significant differences between swim-stressed controls and naloxone-treated groups. All 3 groups showed significant ($P < 0.05$) antinociception during the first 30 min post-stress.

an opioid receptor blocking activity of this metal. Indeed, reduced antinociceptive effects of morphine and ketocyclazocine have been observed after similar perinatal lead exposure (Kitchen *et al.*, 1984; Kitchen & McDowell, 1985). However, we have been unable to demonstrate antagonism by lead (up to 10^{-6} M) of μ-opioid responses in opioid-sensitive isolated tissues (Kitchen & Kelly, unpublished) and the effects of lead on binding do not indicate major disruption of μ-receptor sites (McDowell & Kitchen, 1988). Therefore lead may also be acting at the post receptor level.

The stress-induced antinociception produced by a 10 min swim in 25 day old rats is insensitive to antagonism by naloxone and hence is mediated via non-opioid mechanisms (Jackson & Kitchen, 1989). The lack of effect of lead on this type of stress-induced antinociception clearly points to toxic effects of lead on opioid function and not to generalised toxicity upon perception of noxious stimuli. Indeed, there were no indications of overt toxicity using the lead dosing protocol and previous studies have demonstrated that blood lead levels at weaning are below $15\,\mu g\,100\,ml^{-1}$ at the highest lead dose (McDowell & Kitchen, 1988).

There is also evidence from biochemical studies that the development of opioid systems are more sensitive to the effects of lead than other neurochemical systems (Bailey & Kitchen, 1986). Whether this developmental disruption of opioid systems produces long-lasting toxic effects into adulthood is unclear, but the question of opioid functioning in man should be addressed where there is evidence of behavioural abnormalities associated with lead exposure.

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Presence of T-kininogen and kinins in sponge-induced exudates in rats

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1 The content of kinins and T-kininogen (the third kininogen) in exudates induced by the subcutaneous implantation of saline-soaked sponges have been measured by radioimmunoassay in normal Wistar rats and in Brown Norway rats from a strain which is deficient in high and low molecular weight kininogens.

2 In both strains, sponge implantation induced a rise of T-kininogen in plasma with subsequent accumulation in the sponge exudate. This accumulation correlated with the extravasation of plasma proteins during the first 6 h. Bioassays showed that the T-kinin moiety was retained in T-kininogen.

3 In Wistar rats, a large release of immunoreactive kinins up to a mean value of 6.4 ng ml^{-1} was observed during the first 6 h and on the second day after the implantation. In Brown Norway rats, the kinin level in the exudates did not exceed 0.53 ng ml^{-1}.

4 Of the kinins present during the first 6 h in the exudates withdrawn from Wistar rats, 60% were identified by high performance liquid chromatography as bradykinin.

5 The volume of the exudate induced by the implantation of dry sponges was smaller in Brown Norway rats than in Wistar rats.

6 We conclude that the role of T-kininogen in this kind of exudate was mainly the inhibition of thiol proteinases and not the release of T-kinin. In Wistar rats, bradykinin acts as a pro-inflammatory factor during the first hours and may play a role during the healing process.

Introduction

Kinins are pro-inflammatory peptides released from plasma kininogens. In most mammalian species, two kinds of kininogens, low and high molecular weight kininogens, have been identified. Tissue kallikreins release bradykinin from both rat kininogens (Alhenc-Gelas *et al.*, 1981), while plasma kallikrein releases bradykinin only from the high molecular weight kininogen. The participation of kinins in some inflammatory reactions has been studied for several years (Review in Lewis, 1970; Garcia Leme, 1978). However, in rats, the interest in this topic was renewed by two recent findings. First, we discovered that the plasma of rats from a particular Brown Norway strain is devoid of both kininogens and poor in plasma prekallikrein (Damas & Adam, 1980; 1985). These animals have been used as a negative index of the participation of kinins in some inflammatory reactions. Oedemas and pleurisies induced by carrageenan, kaolin or urate crystals were greatly reduced in these rats indicating the role of the kinins in these models (Damas *et al.*, 1984; 1986; Oh-ishi *et al.*, 1987). Secondly, Okamoto & Greenbaum (1983a, b) discovered a second low molecular weight kininogen in rat plasma. This kininogen is resistant to the kininogenase activity of kallikreins but releases Ile-Ser-bradykinin or T-kinin after hydrolysis by high concentrations of trypsin. It was thus named T-kininogen. T-kininogen is similar if not identical to the alpha$_1$-cysteine proteinase inhibitor, the major acute phase plasma protein of the rat (Enjyoji *et al.*, 1988). Inflammation induced by adjuvant or turpentine increases the plasma level of T-kininogen from 0.25 mg to 3 to 8 mg ml^{-1} (Barlas *et al.*, 1985a; Bouhnik *et al.*, 1987). During inflammatory processes, T-kininogen could thus represent a large plasma pool of a kinin-releasing substrate (Greenbaum, 1984). Indeed, Barlas *et al.* (1985b)

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have observed the accumulation of large amounts of T-kininogen and the release of T-kinin in carrageenan-induced oedema in an air-pouch model. Brown Norway rat plasma devoid of high and low molecular weight kininogens has been proved to contain T-kininogen (Hayashi *et al.*, 1984; Damas & Adam, 1985). As paw oedema and pleurisy induced by carrageenan in these rats were small, the role of T-kininogen as a kinin-forming substrate was considered as insignificant (Damas *et al.*, 1986; Damas, 1987; Oh-ishi *et al.*, 1987).

However, in the rat, the above reactions are short-lasting as compared to the granulomatous reaction studied by Barlas *et al.* (1985b). Moreover, in the granulomatous tissues, Sakomoto *et al.* (1987) reported the presence of an acid proteinase able to release T-kinin from T-kininogen. A release of T-kinin could thus take place during long-lasting reactions. As we have developed specific radioimmunoassays to quantify T-kininogen and bradykinin (Adam *et al.*, 1989), we measured, during the first three days, the accumulation of T-kininogen and the release of kinins in inflammatory exudates induced by sponge implantation in normal rats and in kininogen-deficient Brown Norway rats. Our observations could shed some light on the role of T-kininogen in long-lasting inflammatory processes as either a kinin-releasing substrate or a cysteine proteinase inhibitor.

Methods

We used male Wistar rats as normal control rats. The deficient Brown Norway rats were bred by the farm of the Catholic University of Leuven (Heverlee, Belgium). The mean weight of the animals was 220 g for Brown Norway rats and 260 g for Wistar rats. Polyvinyl sponges (diameter 15 mm, thickness 4.5 mm) weighing 17–17.5 mg, were first sterilized in ethanol, rinsed in twice distilled water and oven-dried. They were used as such (dry sponges) or soaked in physiological saline. The rats were anaesthetized with ether. After sterilisation of the skin of the back with ethanol, a 15 mm dorsal incision was made and the dermis separated from the muscular layer. One sponge was implanted in each side. Three to seventy-two hours later, the rats were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}); 10 min later, blood was withdrawn by cardiac puncture and mixed with 0.1 vol. 3.8% citrate. Plasma was separated by centrifugation at 2500 *g* for 10 min and frozen at −25°C. The dorsal incision was opened and the sponges removed carefully to avoid local bleeding.

After removal, the sponges were directly immersed in polyethylene tubes containing either 2 ml physiological saline or 0.5 mg ml^{-1} trypsin in 2 ml physiological saline or 80% cold ethanol in water. The tubes containing trypsin were incubated at 37°C for 30 min. Then the sponges were gently squeezed with a syringe plunger and the total cell count was performed by phase contrast microscopy using a Thoma's plate. The other sponges were gently squeezed three times and removed. The exudates diluted in saline were frozen at −25°C until the assay of T-kininogen. The exudates mixed with ethanol were centrifuged at 2500 *g* for 10 min. The supernatant was evaporated and the dried residue was dissolved in phosphate buffer for measurement of the kinin content.

T-kininogen levels in plasma or in exudates were measured by a specific radioimmunoassay previously described (Adam *et al.*, 1989): 100 µl of samples or increasing quantities of unlabelled T-kininogen and antiserum (final dilution 10^{-4}) were incubated with ^{125}I-labelled T-kininogen (20,000 c.p.m.) at 4°C in a total volume of 0.4 ml of incubation buffer $KH_2PO_4/Na_2HPO_4 \cdot 2H_2O$ 200 mM, pH 7.5 containing 5 g bovine albumin, 0.1 g NaN_3 and 0.37 g EDTA per litre. After 4 h, the reaction was stopped by adding 2 ml of isotonic saline containing sheep anti-rabbit gamma globulin antiserum (final dilution 100 fold), polyethylene glycol 6000 (40 g l^{-1}) and microcristalline cellulose (500 mg l^{-1}). After incubation at room temperature for 30 min and centrifugation at 1000 *g* for 15 min, the supernatant was decanted, and the radioactivity of the pellet counted. Our antibodies raised in rabbits against pure rat T-kininogen did not cross-react with T-kinin, bradykinin or rat high molecular weight kininogen.

In order to measure the kinin-releasing activity of T-kininogen in exudates, 0.4 ml of diluted exudate was incubated with 0.1 ml N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (final concentration 1 mg ml^{-1}) in the presence of 0.1 ml EDTA (1 mg ml^{-1} final concentration) and 0.2 ml Tris HCl 0.15 M, pH 7.8, for 30 min at 37°C. The reaction was stopped with 0.2 ml soya bean trypsin inhibitor (SBTI, final concentration: 2.5 mg ml^{-1}) and the released kinins were measured by bioassay using rat stomach strip, rat uterus and rat duodenum superfused with Tyrode solution (2.5 ml min^{-1}) at 37°C and aerated with 95% O_2 and 5% CO_2. The threshold level varied between assays from 0.5 to 2 ng ml^{-1}. The standard bradykinin was prepared in 0.15 M Tris HCl pH 7.8 containing EDTA (1 mg ml^{-1}).

Kinin levels in exudates were measured by a specific radioimmunoassay (Adam *et al.*, 1989). This radioimmunoassay was performed under similar conditions to the one described for T-kininogen, except that albumin was substituted by lysozyme (1 g l^{-1}) in the incubation buffer. The antiserum was

obtained in rabbits against bradykinin coupled to swine thyroglobulin and used at a final dilution 10^{-5}. The radioimmunoassay was standardised using bradykinin but Ile-Ser-bradykinin, kallidin and des-Arg^9-bradykinin showed a complete cross-immunoreactivity on an equimolar basis.

Kinins in exudates were also separated by a reverse phase high performance liquid chromatography (h.p.l.c.); the dried residue of exudates mixed with ethanol was dissolved in 200 μl phosphate buffer; 50 μl were injected and the column (0.46 × 25 cm) of TSK-gel ODS-120 T, 5 μm (LKB) was isocratically eluted with 19% acetonitrile in 0.05 M phosphate buffer pH 2.5 at a flow rate of 1 ml min^{-1}. Fractions of 0.33 ml were analysed by radioimmunoassay for the presence of kinins.

Total proteins in plasma or in exudates were quantified by the Folin-Ciocaltau reagent as described by Lowry *et al.* (1951).

Bradykinin and kallidin were purchased from UCB (Belgium), T-kinin, trypsin, N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and soya bean trypsin inhibitor (SBTI) from Sigma.

Results are given as the mean ± s.e.mean of n determinations. Comparisons between groups were made with two-tailed Student's t test for unpaired data.

Results

Plasma level of T-kininogen

After the implantation of saline-soaked sponges, a progressive and similar increase in the plasma level of T-kininogen in Wistar rats and in Brown Norway rats was observed. This increase was statistically significant from 24 h onwards. The peak increase: 811 μg ml^{-1} was reached, in Wistar rats, at 48 h (Figure 1).

T-kininogen in sponge exudates

Simultaneously, in both strains, an accumulation of T-kininogen occurred in the sponges. The maximum level of T-kininogen, measured by radioimmunoassay, was observed at 72 h (Figure 2). We checked that T-kininogen in the exudates was still able to release a kinin activity. Trypsin (1 mg ml^{-1}) in presence of a kininase inhibitor EDTA (1 mg ml^{-1}) released 12.46 ± 1.26 μg bradykinin equivalent per ml of the exudates withdrawn at 48 h from Wistar rats ($n = 6$). This amount corresponded to about 700 μg ml^{-1} of T-kininogen, a value slightly higher than the level of 553 ± 71 μg ml^{-1} measured by radioimmunoassay. In fact, bioassays measured not only T-kininogen but total kininogens. This could explain the difference between the two results.

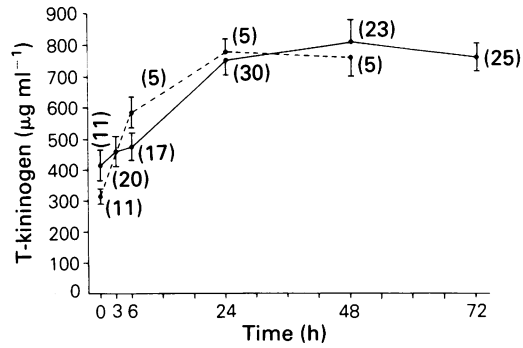

Figure 1 Plasma level (μg ml^{-1}) of T-kininogen after sponge implantation (continuous line) in Wistar rats and (broken line) in Brown Norway rats. Mean (n) determinations; vertical bars show s.e.mean.

The extent of the inflammatory reaction induced by the saline-soaked sponges was determined by the measurement of protein and leucocyte levels in the exudates. The accumulation of proteins in the exudates was rapid in Wistar rats and reached a maximum, about 62 to 76% of the plasma level, from 24 h to 72 h after the implantation (Figure 3). The extravasation of proteins in the exudates was delayed in Brown Norway rats: the protein level was significantly lower at 3 h. Thereafter, it reached 67 to 69% of the plasma level (Figure 3). Simultaneously, leucocytes were found in the inflammatory lesion. Their level, similar in both strains, was maximum at 24 h and then decreased. The diminution was however slower in Brown Norway rats (Figure 3).

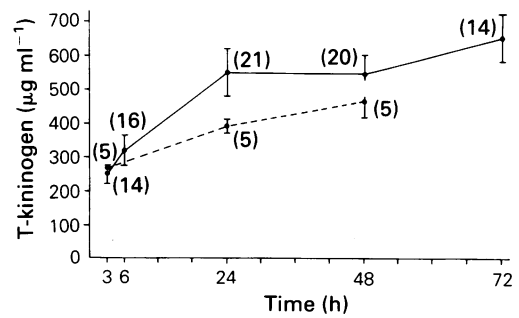

Figure 2 Accumulation of T-kininogen (μg ml^{-1}) in sponge exudates (continuous line) from Wistar rats and (broken line) from Brown Norway rats. Mean (n) determinations; vertical bars show s.e.mean. The levels in Wistar rats were not significantly different from those in Brown Norway rats.

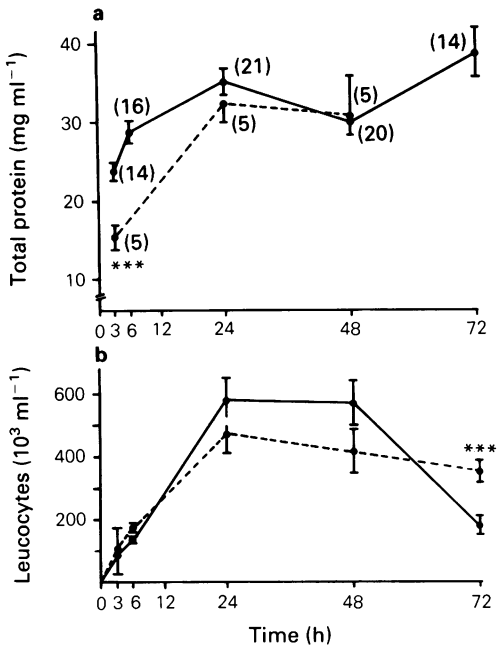

Figure 3 Evolution of the level of total proteins (mg ml^{-1}) (a) and total leucocytes (b) in sponge-induced exudates withdrawn from Wistar rats (continuous line) or from Brown Norway rats (broken line). The figure shows the mean of 10–12 determinations for leucocytes and of (n) determinations for proteins; vertical bars show s.e.mean. ***$P < 0.005$.

In Wistar rats, during the first 6 h, the level of T-kininogen in exudates correlated with the extravasation of proteins in the exudates (Figure 4). Thereafter, the T-kininogen content of the exudates expressed in μg mg^{-1} of proteins did not correlate with the plasma level (Figure 4).

Kinin level in exudates

Figure 5 shows the evolution of kinin levels as measured by radioimmunoassay in sponge-exudates from both rat strains. In Wistar rats, the level of immunoreactive kinins was elevated as soon as the inflammatory reaction started. Thereafter, this level decreased up to 24 h and increased again during the second day up to a maximum level of 6.4 ± 0.93 ng ml^{-1} ($n = 11$). The ranges of the levels observed were from 1.57 to 17.9 ng ml^{-1} at 3 h and from 1.3 to 9.34 ng ml^{-1} at 48 h. We tested for the presence of kinins in the exudates by bioassays. The exudates withdrawn 3 h after the implantation contained: 4.62 ± 2.98 ng ml^{-1} ($n = 12$) and 9.8 ± 1.9 ng ml^{-1} ($n = 5$) after 48 h. On the other hand, in Brown Norway rats, the content of immunoreactive kinins was low, between 0.08 to 0.53 ng ml^{-1}, during the whole inflammatory process (Figure 5).

We tried to determine the nature of the immunoreactive peptides present in the exudates, using separation by a reverse phase h.p.l.c. Successful measurements were only obtained for the exudates withdrawn from Wistar rats, 3 h after the sponge implantation. The kinins present in these exudates were detected partly as bradykinin and partly as unidentified peptides which were eluted between kallidin and bradykinin (Figure 6). Bradykinin accounted for about 60% of the immunoreactive peptides separated by h.p.l.c. ($n = 6$).

Comparison between the inflammatory reaction in Wistar and kininogen-deficient Brown Norway rats

To compare the importance of the inflammatory reactions induced in both rat strains, we implanted dry sponges and weighed them in order to measure the volume of the exudates. The sponges removed from Wistar rats, 3 h after implantation, were heavier than those removed from Brown Norway rats: 201 ± 12 mg ($n = 12$) and 142 ± 14 mg ($n = 12$; $P < 0.005$) respectively. The level of proteins in the exudates was similar: 38.2 ± 3.9 mg ml^{-1} in Wistar rats ($n = 12$) and 33.5 ± 3.3 mg ml^{-1} in Brown Norway rats ($n = 12$).

Discussion

The inflammatory reaction induced by sponge implantation increased the plasma level of T-kininogen. This increase was relatively small in comparison with the levels observed after turpentine (Bouhnik *et al.*, 1987; Adam *et al.*, 1989) or carrageenan injections (Barlas *et al.*, 1985b). This difference would indicate that the inflammatory reaction following the implantation of saline-soaked sponges is relatively weak. The two other kininogens are not acute phase plasma proteins and their level in non-injured rats accounts for 35% of total kininogens (Barlas *et al.*, 1985a; Bouhnik *et al.*, 1987; Adam *et al.*, 1989).

Confirming previous results (Barlas *et al.*, 1985b; Sakamoto & Yoshikawa, 1986), we observed the presence of T-kininogen in the exudate. During the first 6 h of the inflammatory reaction, the accumulation of this substance was proportional to the extravasation of plasma proteins. After 24 h, the concentration of T-kininogen in the exudate exceeded the protein level. This suggests that the kallikrein-resistant kininogen was accumulating in the exudate without releasing kinins during the whole inflammatory process. Indeed, the T-kinin moiety was shown to be present in T-kininogen withdrawn from the exudate, as previously observed by Sakamato &

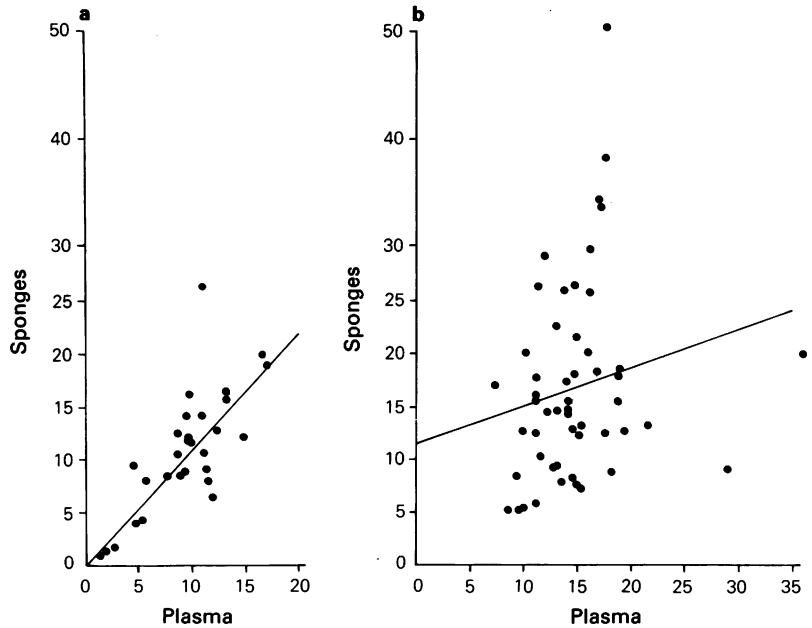

Figure 4 Correlation between the plasma level and the sponge level of T-kininogen in Wistar rats. The concentration of T-kininogen is expressed in $\mu g\,mg^{-1}$ of proteins. In (a) values from sponges at 3 and 6 h; in (b) values from sponges at 24, 48 and 72 h. In (a) $y = 1.09x + 0.07$; $r = 0.75$; $n = 29$; in (b) $y = 0.33x + 11.56$; $r = 0.18$; $n = 52$.

Yoshikawa (1986) for T-kininogen recovered from the exudate filling the granulomatous inflammation induced by carrageenin. Whatever the accumulation of T-kininogen, only a significant release of kinins was observed in Wistar rats. In these rats, the two other kallikrein-sensitive kininogens are present. Furthermore, we did not find T-kinin in the exudates but bradykinin and another immunoreactive peptide

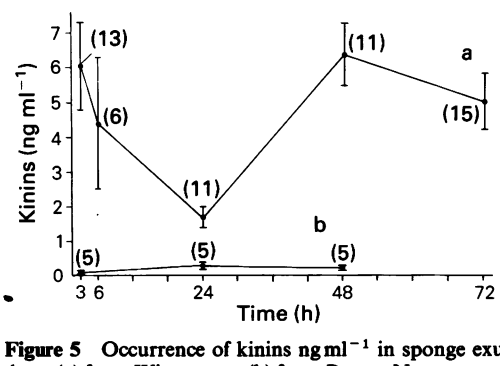

Figure 5 Occurrence of kinins $ng\,ml^{-1}$ in sponge exudates (a) from Wistar rats; (b) from Brown Norway rats. Mean of (*n*) determinations; vertical bars show s.e.mean. The results obtained in Brown Norway rats were significantly different from those recorded in Wistar rats at 3 h and 24 h ($P < 0.02$) and at 48 h ($P < 0.001$).

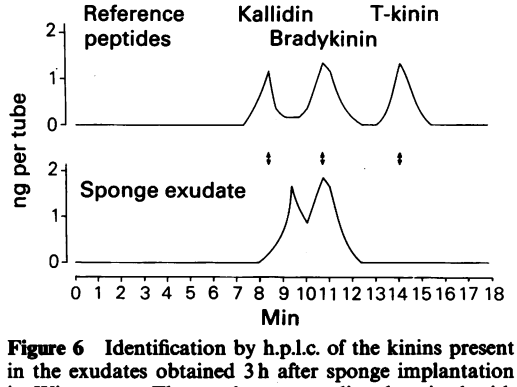

Figure 6 Identification by h.p.l.c. of the kinins present in the exudates obtained 3 h after sponge implantation in Wistar rats. The exudates were directly mixed with ethanol in order to precipitate kininases and kinin-forming enzymes. After centrifugation; the supernatant was lyophilised. Kinins were dissolved in phosphate buffer, separated by reverse phase h.p.l.c. and measured by radioimmunoassay.

which could be a bradykinin metabolite. These results indicate that T-kininogen was not a significant kinin-releasing substrate during the whole inflammatory process induced by sponge implantation.

Its main function might be the inhibition of thiol proteinases like cathepsins released during inflammatory reactions. Indeed, Nakagawa & Hirata (1986) purified two species of cysteine proteinase inhibitors identical with T-kininogen, from the fluid studied by Barlas *et al.* (1985b).

At first sight, our results contrast with the observations of Barlas *et al.* (1985b) who detected the presence of T-kininogen, T-kinin and bradykinin in the pouch fluid withdrawn 24 h after the injection of carrageenin. The level of T-kinin in their study was $0.34\,ng\,ml^{-1}$. This level is close to the values of immunoreactive kinins measured in the exudates of our kininogen-deficient Brown Norway rats. We did not identify the nature of these kinins. But these peptides were released from T-kininogen, the only kinin-forming substrate present in these rats. They could thus be T-kinin. In Wistar rats, plasma contains the three kininogens and the content of the exudates in kinins was higher. According to Kumakura *et al.* (1988), in carrageenin-induced inflammation, a release of bradykinin was detected mainly during the first hour. After 2 h, the level of bradykinin was lower than the assay limits. This could explain the low level of bradykinin found by Barlas *et al.* (1985b) at 24 h. In sponge exudates, the profile of kinin release during the first 24 h was rather similar: the level was high during the first 3 h and decline up to 24 h. Thus at this time, the concentrations of T-kinin in sponge-exudates from Brown Norway rats, and of total kinins measured in Wistar rats were close as in the results of Barlas *et al.* (1988). In both inflammatory reactions, a large release of bradykinin occurred during the first hours, while the level of T-kinin was very small in comparison with the T-kininogen content of the exudates. During the first hours, the difference between the ranges of our values of kinins and the ones reported by Kumakura *et al.* (1988) could be explained in the following way. First, we measured immunoreactive kinins. The volume of the exudate in sponge is very small preventing a dilution of plasma proteins and of kinins. Second, sponge exudate has a lower degree of protein clearance than carrageenin-induced inflammation in air pouches (Hambleton *et al.*, 1988).

On the other hand, our observations suggest that the other kininogens were implicated in the development of the inflammatory reaction induced by sponge implantation. The protein content of the exudate was reduced in Brown Norway rats and the volume of the exudate was diminished in dry sponges withdrawn from these animals, at the beginning of the reaction.

We also observed a significant kinin release in normal rats during the second day after implantation when the healing process is taking place. We have no idea about the mechanism of this release which did not depend on an infectious process as demonstrated by the leucocyte count in the sponges, and did not originate from T-kininogen as shown by the low values recorded in Brown Norway rats. But this release would indicate that the kinins did not only participate in the vascular changes mediating the inflammatory reaction but also could influence the cellular response leading to the formation of the granulomatous tissue. Indeed, some effects of bradykinin on the fibroblasts have been already described (Hong & Levine, 1976; Newcombe *et al.*, 1977; Vincentini & Villereal, 1984).

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MEETINGS CALENDAR

7–12 August 1989

Therapy with Amino Acids and Analogues, Ist International Congress, Vienna, Austria. (G. Lubec, University of Vienna, Dept. of Paediatrics, A-1090 Vienna, Währinger Gürtel 18, Austria).

1–3 September 1989

Second International FIP Symposium on Disposition & Delivery of Peptide Drugs, Leiden, The Netherlands. (Dr. J. Verhoef, Centre for Bio-Pharmaceutical Sciences, PO Box 9502, 2300 RA Leiden, The Netherlands).

10–15 September 1989

Biochemical Society, Harden Conference — Cellular Barriers and Drug Targetting, Ashford, U.K. (Meetings Officer, The Biochemical Society, 7 Warwick Court, London WC1R 5DP).

13–15 September 1989

British Pharmacological Society Autumn Meeting. Manchester, U.K. (Closed meeting for members and guests)

17–20 September 1989

1st International $GABA_B$ Symposium, Cambridge, U.K. (Professor N.G. Bowery, Department of Pharmacology, School of Pharmacy, 29/39 Brunswick Square, London WC1N 1AX.)

19–21 September 1989

Electrochemical Detection, HPLC and *in vivo* monitoring in the Biosciences, Nottingham, U.K. (Dr I.A. MacDonald, Department of Physiology & Pharmacology, Medical School, Queen's Medical Centre, Nottingham, U.K.)

20–21 September 1989

9th Annual Meeting of the Bayliss and Starling Society. Peptides: Physiology, Pharmacology and Therapeutics, Nottingham, U.K. (Professor T. Bennett, Dept. of Physiology & Pharmacology, Medical School, Queen's Medical Centre, Nottingham, U.K.)

28 September 1989

Leukotrienes. London, U.K. (Society for Drug Research, c/o Institute of Biology, 20 Queensbury Place, London SW7 2DZ, U.K.)

22–25 October 1989

Molecular and Cellular Biology of IL-1, TNF and Lipocortins in Inflammation and Differentiation. Siena, Italy. (L. Parente, Sclavo Research Centre, via Florentina 1, 53100 Siena, Italy).

8–11 November 1989

Tenth International Symposium on Drugs affecting Metabolism, Houston, USA. (Meeting Manager, 4550 Post Oak Place, Suite 248, Houston, Texas 77027, U.S.A.)

27–29 November 1989

Biological Actions of Extracellular ATP, Philadelphia, USA. (Conference Dept., New York Academy of Sciences, 2 East 63rd Street, New York, NY 10021, U.S.A.)

4–5 December 1989

Sensory Nerves and Neuropeptides in Gastroenterology, Florence, Italy. (Fondazione Internazionale Menarini, Piazza del Carmino, 4-20121 Milan, Italy).

6–8 December 1989

Presynaptic Receptors — an Examination of Different Views, New York City, USA. (Conference Department, New York Academy of Sciences, 2 East 63rd Street, New York, NY 10021, U.S.A.)

10–14 December 1989

Sino-Brazilian Symposium on Chemistry and Pharmacology of Natural Products, Rio de Janeiro, Brazil. (Secretariat, Fundacao Oswaldo Cruz, Departmento de Farmacodinamica, Aremida, Brasil, 6365, Rio de Janeiro, Brazil).

3–5 January 1990

British Pharmacological Society Winter Meeting. London, U.K. (Closed meeting for members and guests)

7 February 1990

Royal Society of Chemistry, Free Radicals in Biotechnology and Medicine, London, U.K. (Miss P.E. Hutchinson, The Analytical Division, Royal Society of Chemistry, Burlington House, London W1V 0BN).

26–28 March 1990

Brain Research Association National Meeting, Bristol, U.K. (Dr. I.C. Kilpatrick, Dept. of Pharmacology, The Medical School, University Walk, Bristol BS8 1TD).

1–6 April 1990

6th World Congress on Pain, Adelaide, Australia. (Int. Association for the Study of Pain, 909 NE 43rd Street, Suite 306, Seattle, WA 98105, U.S.A.)

2–4 April 1990

Central and peripheral significance of neuropeptide Y and its related peptides, Baltimore, U.S.A. (Conference Department, New York Academy of Sciences, 2 East 63rd Street, New York, NY 10021, U.S.A.).

4–6 April 1990

New Concepts in Anxiety, Castres, France. (Martin Dehaye, Centre de Recherche Pierre Fabre, 17 avenue Jean Moulin, 81100 Castres, France).

18–20 April 1990

British Pharmacological Society Spring Meeting. Sheffield, U.K. (Closed meeting for members and guests)

27–30 May 1990

Heterocycles in Bio-organic Chemistry, Solbacka, Sweden (Secretariat, PO Box 1136, S-11181 Stockholm, Sweden).

29 May–1 June 1990

Fondazione Giovanni Lorenzini Conference on Prostaglandins and Related Compounds, Florence, Italy (Fondazione Giovanni Lorenzini, Via Monte Napoleone, 23, 20121 Milan, Italy).

28–30 June 1990

IUPHAR Satellite — Mediators in Airway Hyperreactivity, The Hague, The Netherlands. (F.P. Nimkamp, Department of Pharmacology, PO Box 80176, 3508 TD Utrecht, Netherlands).

30 June–4 July 1990

6th Southeast Asian/W. Pacific meeting of Pharmacologists, Hong Kong. (Secretary, Department of Pharmacology, Faculty of Medicine, University of Hong Kong, 5 Sassoon Road, Hong Kong).

27–30 June 1990

International Society for Heart Research XI European Section Meeting, Glasgow, UK. (Professor J.R. Parratt, Dept. of Physiology & Pharmacology, Royal College, University of Strathclyde, Glasgow G1 1XW).

1–6 July 1990

IUPHAR 11th International Congress of Pharmacology, Amsterdam, Netherlands (Contact to be advised).

The Indices to Volumes 96, 97 and 98 together with the Cumulative Contents will be published with the December 1989 issue

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