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Br. J. Pharmacol. (1991), **103**, 1011–1012 **SPECIAL REPORT**

5-HT_3 receptor agonism may be responsible for the emetic effects of zacopride in the ferret

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The racemic 5-HT_3 receptor antagonist, zacopride (10–100 $\mu g\,kg^{-1}$, i.m.) evoked an emetic response in ferrets. This property appeared to reside totally in the S-enantiomer which also produced emesis over the same dose range. This emesis could be prevented by pretreatment with ondansetron (1 $mg\,kg^{-1}$, i.m.) or by R-zacopride (100 $\mu g\,kg^{-1}$, i.m.). In urethane-anaesthetized ferrets, S-zacopride (0.3 $\mu g\,kg^{-1}$, i.v.) evoked a profound Bezold-Jarisch reflex which was blocked by both ondansetron (30 $\mu g\,kg^{-1}$, i.v.) and by R-zacopride (100 $\mu g\,kg^{-1}$, i.v.). These results suggest that, in the ferret, S-zacopride possesses 5-HT_3 receptor agonist properties which may be responsible for the emetic effect. In contrast R-zacopride does not appear to possess 5-HT_3 receptor agonist properties in this species.

Keywords: Zacopride; R-zacopride; S-zacopride; emesis; Bezold-Jarisch reflex; 5-HT_3 receptors; GR38032 (ondansetron)

Introduction Zacopride (4-amino-N-[1-azabicyclo(2.2.2)oct-3-yl]-5-chloro-2-methoxybenzamide{E}-2- butenedioate) is a racemic compound described as a potent and selective 5-HT_3 receptor antagonist (Smith *et al.*, 1988a). Racemic zacopride (hereafter referred to as zacopride) has been shown to be an effective anti-emetic agent against the emesis induced by cytotoxins or radiation in dogs, cats, non-human primates and ferrets (Costall *et al.*, 1987; Dubois *et al.*, 1988; King *et al.*, 1988; Smith *et al.*, 1988b; 1989). Recently, zacopride has been shown to be emetogenic following administration of 0.003–0.3 $mg\,kg^{-1}$ p.o. or i.v. to ferrets (King, 1990). We have compared the 5-HT_3 receptor antagonist properties of both stereoisomers of zacopride in ferrets anaesthetized with urethane and have investigated the mechanism of its emetogenic properties in conscious ferrets.

Methods *Zacopride and its enantiomers in anaesthetized ferrets* Female fitch or albino ferrets (0.8–1.3 kg) were anaesthetized with urethane (1.0–1.5 $g\,kg^{-1}$, i.p.). Both jugular veins were cannulated for the administration of drugs and blood pressure was recorded from a carotid artery. Reproducible, submaximal Bezold-Jarisch (B-J) reflex responses to one dose of 5-hydroxytryptamine (5-HT) (10 or 30 $\mu g\,kg^{-1}$, i.v.) were obtained at 10 min intervals and this dose of 5-HT used throughout the experiment. The B-J reflex was measured as the instantaneous increase in heart period (HP, i.e. beat to beat interval averaged over 4 consecutive beats) following the intravenous bolus administration of 5-HT. Control values were obtained by averaging the HP over 10 consecutive beats. Increasing doses of zacopride (0.3–100 $\mu g\,kg^{-1}$, i.v.) were administered and the response to 5-HT retested 10 min after each dose. The next dose of zacopride was administered immediately after the transient response to 5-HT. Additional studies investigated the profile of activity of the stereoisomers of zacopride (see results section). Statistical analysis was by 2-way analysis of variance.

Emesis studies Adult female albino or fitch ferrets (0.8–1.3 kg) were placed singly in an observation cage 30 min before drug administration with free access to food and water. Drugs were dissolved in sterile isotonic saline (standard volume 0.4 ml) and administered intramuscularly, into the biceps femoris of the hind limb. In all experiments in which a premedicant drug was given, a 15 min pretreatment time was allowed and the two injections were made contralaterally. The animals were observed for 60 min during which time the presence or absence of retching and/or vomiting was recorded in 5 min time bins, as were any other overt effects. The number of emetic events (i.e. retches or vomits) was not recorded.

Results *The effect of zacopride and its stereoisomers in the anaesthetised ferret* A small change in HP (approx. 15% of the control HP) generally accompanied the administration of a bolus of cold saline (0.4 ml i.v.), hence a value of 85% inhibition of the B-J reflex evoked by 5-HT was considered to be a complete block of the response.

5-HT (10 $\mu g\,kg^{-1}$) evoked a marked B-J reflex in the anaesthetized ferret with a mean increase in HP of 703 (s.e. 113 ms, $n = 4$) which was blocked by zacopride (3 $\mu g\,kg^{-1}$, i.v., $P < 0.001$, $n = 4$).

Administration of zacopride (0.1–3.0 $\mu g\,kg^{-1}$, i.v.) itself evoked a marked 'B-J-like' effect in these experiments, i.e. a transient but profound bradycardia, hypotension and apnoea, (data not shown) and this phenomenon was subsequently investigated using the two stereoisomers of zacopride.

S-zacopride (0.3 $\mu g\,kg^{-1}$, i.v.) evoked 'B-J-like' response with a mean increase in HP of 7232 (s.e. 3530 ms, $n = 4$). A lower dose of S-zacopride (0.1 $\mu g\,kg^{-1}$, i.v.) did not consistently produce a 'B-J-like' effect. In these experiments the B-J reflex to 5-HT (10 $\mu g\,kg^{-1}$, i.v.) obtained 10 min after administration of S-zacopride (0.3 $\mu g\,kg^{-1}$, i.v.) was not statistically significantly different from the predose response to the same dose of 5-HT (mean change in HP, predose = 465 (s.e. 30 ms) and post S-zacopride = 531 (s.e. 51 ms)). Ten minutes after administration of the selective 5-HT_3 receptor antagonist, ondansetron (30 $\mu g\,kg^{-1}$, i.v. $n = 4$) however, the responses to both 5-HT and S-zacopride were blocked (i.e. > 85% inhibition, $P < 0.001$). Administration of R-zacopride (0.1–1000 $\mu g\,kg^{-1}$, i.v.) did not produce any discernible effects on HP.

In a separate series of experiments, after obtaining reproducible B-J responses to both 5-HT (10 $\mu g\,kg^{-1}$, i.v.) and to S-zacopride (0.3 $\mu g\,kg^{-1}$, i.v.), incremental doses of R-zacopride (1–100 $\mu g\,kg^{-1}$, i.v.) were administered every 10 min. The response to 5-HT was tested 5 min after each dose of R-zacopride and the response to S-zacopride tested 5 min after 5-HT. R-zacopride produced a dose-dependent attenuation of

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both the 5-HT- and S-zacopride-induced B-J responses, the highest dose of **R**-zacopride ($100\,\mu g\,kg^{-1}$, i.v.) producing a 74% and 98% attenuation respectively ($P < 0.05$).

Emesis studies Administration of either zacopride or S-zacopride ($1\,\mu g\,kg^{-1}$, i.m., $n = 2$ and $n = 4$ respectively) produced no adverse effects. Zacopride ($10\,\mu g\,kg^{-1}$, i.m., $n = 7$) was emetogenic in all of the animals studied whereas $100\,\mu g\,kg^{-1}$, i.m. ($n = 3$) produced emesis in only one animal. Similarly, S-zacopride ($10\,\mu g\,kg^{-1}$, i.m.) produced emesis in 4/6 animals and in 3/4 animals following $100\,\mu g\,kg^{-1}$, i.m. Both zacopride and S-zacopride were sedative at $100\,\mu g\,kg^{-1}$, i.m. **R**-zacopride (10 or $100\,\mu g\,kg^{-1}$, i.m., $n = 4$) was not emetogenic although one ferret vomited following **R**-zacopride ($1000\,\mu g\,kg^{-1}$, i.m., $n = 4$).

Additional experiments examined the effects of pretreatment with ondansetron (100 or $1000\,\mu g\,kg^{-1}$, i.m., $n = 4$ per group). Following ondansetron ($100\,\mu g\,kg^{-1}$) no emesis was observed in response to administration of zacopride ($10\,\mu g\,kg^{-1}$, i.m.) and although the mean latency to the first emetic episode following S-zacopride ($10\,\mu g\,kg^{-1}$, i.m.) was increased from 10 min to 21 min, all the animals retched and/or vomited. No emesis was observed following S-zacopride ($10\,\mu g\,kg^{-1}$, i.m.) in animals pretreated with either ondansetron ($1000\,\mu g\,kg^{-1}$) or **R**-zacopride ($100\,\mu g\,kg^{-1}$, i.m.).

Pretreatment with atropine ($100\,\mu g\,kg^{-1}$, i.m.) did not prevent the emesis evoked by either zacopride ($10\,\mu g\,kg^{-1}$, i.m., $n = 3$) or S-zacopride ($10\,\mu g\,kg^{-1}$, i.m., $n = 2$). A higher dose of atropine ($1\,mg\,kg^{-1}$ i.m., $n = 2$), despite producing marked sedation, did not prevent zacopride- ($10\,\mu g\,kg^{-1}$, i.m.) induced emesis.

Discussion We have previously demonstrated that both enantiomers of zacopride behave as 5-HT_3 receptor antagonists in the rat (Middlefell *et al.*, 1990). Experiments in anaesthetized ferrets demonstrated that intravenous administration of S-zacopride evoked a marked B-J reflex which could be blocked by either ondansetron or by **R**-zacopride. Higher intravenous doses of S-zacopride were not tested on the assumption that the profound bradycardia and apnoea which was produced by $0.3\,\mu g\,kg^{-1}$ would prove fatal were the dose to be increased (although this remains to be tested). At no time was a B-J reflex elicited by intravenous administration of **R**-zacopride. We have therefore been unable to demonstrate any 5-HT_3 receptor antagonist effects of S-zacopride in the ferret. In addition it would seem that in this species, the 5-HT_3 antagonist properties of zacopride are conferred by the **R**-enantiomer.

These experiments have demonstrated that emesis is evoked by zacopride following intramuscular administration to ferrets. This emetic action was produced by S-zacopride, but not by **R**-zacopride over the same dose-range. At a substantially higher dose, **R**-zacopride ($1\,mg\,kg^{-1}$, i.m.) evoked an emetic response in one animal, the reason for this is unknown and no attempt was made to block it. Other workers (King, 1990) however, have reported emesis following very high doses of granisetron in the ferret and suggest that this may be a non-5-HT_3-mediated effect. Emesis produced by both zacopride and S-zacopride was prevented by either the selective 5-HT_3 antagonist ondansetron, or by **R**-zacopride. It is noteworthy that the dose of ondansetron required to prevent zacopride-induced emesis only lengthened the latency to vomit following the same dose of S-zacopride. This may be explained by the fact that, assuming a 1:1 mixture of the enantiomers, an equivalent dose of S-zacopride would be half that of the racemate.

These results suggest that zacopride has 5-HT_3 receptor agonist activity in ferrets and that this property resides primarily in the S-enantiomer. Zacopride, like many other benzamides, has been reported to possess 5-HT_4 receptor agonist effects but we do not consider this to be relevant to its emetogenic properties since ondansetron, which is not believed to be an antagonist of 5-HT_4 receptors (Clarke *et al.*, 1989) prevents the emesis.

In conclusion, the S-enantiomer of zacopride appears to possess 5-HT_3 receptor agonist properties in the ferret which may be responsible for its emetogenic effects.

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The effects of probucol on the progression of atherosclerosis in mature Watanabe heritable hyperlipidaemic rabbits

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1 Probucol was administered to mature Watanabe heritable hyperlipidaemic (WHHL) rabbits (≈9 months old). Groups of WHHL rabbits were randomly selected and treated as follows: Group 1 killed at 9 months ($n = 9$); Group II placed on sham-treated diet at 9 months and followed for 6 months ($n = 8$); Group III placed on probucol at 9 months and followed for 6 months ($n = 8$). Probucol was administered by mixing 1% wt/wt drug with standard laboratory diet.

2 Plasma concentrations of probucol increased to $93 \pm 11\,\mu g\,ml^{-1}$ in Group III during the initial 2 weeks and increased further to $149 \pm 24\,\mu g\,ml^{-1}$ at the end of the treatment period.

3 Plasma concentrations of total cholesterol, unesterified cholesterol and phospholipids were significantly reduced overall by probucol, while triglycerides were not affected.

4 No statistically significant differences were observed in the presence of oxidized products in low density lipoproteins (LDL) isolated from plasma of controls compared to probucol-treated rabbits. However, LDL from probucol-treated animals was resistant to oxidation in the presence of Cu^{2+} ($3\,\mu M$).

5 Group I had aortic atherosclerosis covering $70 \pm 5\%$ of intimal area of thoracic aortae, that increased to $91 \pm 3\%$ in Group II. This was associated with cholesterol contents of aortae increasing from $1.4 \pm 0.2\,\mu g\,mg^{-1}$ in Group I to $2.7 \pm 0.3\,\mu g\,mg^{-1}$ in Group II. Probucol administration did not produce a statistically significant reduction of atherosclerotic lesion area ($78 \pm 7\%$). However, probucol treatment reduced cholesterol content to $1.9 \pm 0.3\,\mu g\,mg^{-1}$ ($P < 0.01$). Collagen content of aortae was not affected by probucol treatment.

6 Thus, while probucol did not promote regression, the drug did retard the continued deposition of cholesterol esters into atherosclerotic lesions of mature WHHL rabbits.

Keywords: Probucol; Watanabe heritable hyperlipidaemic rabbits; oxidation; atherosclerosis; cholesterol

Introduction

Probucol (4,4'-(isopropylidenedithio)bis(2,6-di-*t*-butylphenol) is a hypolipidaemic agent that is modestly effective at reducing plasma concentrations of low density lipoprotein (LDL)-cholesterol (Buckley *et al.*, 1989). However, this reduction in LDL-cholesterol concentrations is associated with a consistent reduction in plasma concentrations of high density lipoprotein (HDL)-cholesterol. This effect on HDL-cholesterol has led to speculation that the probucol-induced alteration in lipoprotein profiles may aggravate the atherogenic process.

In addition to its hypolipidaemic properties, probucol is a potent scavenger of hydroxyl radicals. In fact the chemical structure of probucol is related to the commonly used hydroxyl radical scavenger, butylated hydroxytoluene. A current concept of atherogenesis is that lipid peroxidation within vascular tissue contributes to the disease process (Steinberg *et al.*, 1989). Therefore, it has been proposed that probucol may possess specific anti-atherosclerotic properties that are independent of its hypolipidaemic effects (Steinberg, 1986). In support of this hypothesis, Kita *et al.* (1987) and Carew *et al.* (1987) have demonstrated that the oral administration of probucol to Watanabe heritable hyperlidaemic (WHHL) rabbits within the first 2 months of birth has resulted in marked reductions in the extent of aortic atherosclerosis after approximately 7 months of the regimen. The study of Carew *et al.* (1987) discerned that the modest hypolipidaemic effect of probucol was not responsible for the protective action by comparing the probucol-treated animals to a group administered lovastatin. In contrast to probucol, lovastatin afforded no protection against the development of aortic atherosclerosis despite an equivalent hypolipidaemic response.

While there is good evidence that probucol can prevent the initiation of atherosclerosis in WHHL rabbits, the effects of the drug on established disease have not been demonstrated. It has been speculated that probucol may promote regression of atherosclerosis, based on the observations of reductions in xanthoma during treatment of familial hypercholesterolaemic subjects (Yamamoto *et al.*, 1983). Indeed, a preliminary report has provided some evidence of probucol-induced regression of atherosclerotic lesions in rhesus monkeys fed a high-fat, high-cholesterol diet (Wissler & Vesselinovitch, 1983). The purpose of the present study was to determine whether probucol exerted any effects on established atherosclerosis lesions in WHHL rabbits (Havel *et al.*, 1982; Buja *et al.*, 1983).

Methods

Animals

A colony of WHHL rabbits at Washington University School of Medicine was initiated from animals kindly provided by Dr Joseph L. Goldstein (University of Texas). Animals were maintained in isolation from other colonies under aseptic conditions, and given water and a standard laboratory rabbit diet *ad libitum*. A total of 25 rabbits was used. All procedures performed on the animals were approved by the Washington University Animal Studies Committee.

Probucol (a gift from Merrell Dow Research Institute, Cincinnati, OH, U.S.A.) was dissolved in chloroform and sprayed evenly over the diet in the proportion of 1% wt/wt. Diet for the control group was sprayed with chloroform alone. Treated diets were kept under a fume hood until the odour of the solvent had dissipated. Food intake was monitored daily for

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both control and drug-treated groups. Body weights were determined weekly.

Characterization of plasma lipids

Concentrations of triglycerides, cholesterol esters, unesterified cholesterol and phospholipids in plasma and in isolated lipoprotein fractions were determined with commercially available enzyme kits (Wako Chemical Company, Dallas, TX, U.S.A.) Concentrations of phospholipids and triglycerides were calculated based on mean molecular weights of 722 and 866 respectively.

Probucol determinations in plasma

Plasma concentrations of probucol were determined by reverse phase high performance liquid chromatography (h.p.l.c.) using the method of Satonin & Coutant (1986). Concentrations of probucol in plasma were determined relative to an internal standard supplied by the Merrell Dow Research Institute (MDL 27,272).

Oxidation of isolated low density lipoproteins

LDL was isolated by sequential ultracentrifugation in a Beckman L8-55 instrument equipped with a 50.3 Ti rotor between densities of 1.019 to 1.063 g ml^{-1} using sodium bromide (Havel *et al.*, 1955). Isolated lipoprotein fractions were dialyzed against 100 volumes of EDTA (1 mM)/sodium chloride (0.15 M), with three changes of dialysis fluid over 24 h. Volumes of fractions were reduced by placing LDL in dialysis bags that were coated with Aquacide (Calbiochem, La Jolla, CA, U.S.A.). Mass of protein in lipoprotein fractions was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (Pierce Chemical Company, Rockford, IL, U.S.A.) as standard.

Lipid peroxidation was initiated by incubation of LDL fractions with copper sulphate, 3 μM (McLean & Hagaman, 1989). The extent of lipid peroxidation was quantified as thiobarbituric acid-reacting substances (TBARS) as described by Satoh (1978). Standards were prepared by dissolution of malondialydehyde-*bis*-dimethyl-acetal (Aldrich Chemical Company, Milwaukee, WI, U.S.A.) in distilled water immediately before the start of the assay. Samples (100 μg protein in 0.5 ml EDTA/saline) were incubated with sulphuric acid (0.05 M) and thiobarbituric acid (0.2% wt/vol, dissolved in sodium sulphate, 2 M) for 30 min at 100°C. After cooling, n-butyl alcohol (4 ml) was added and mixtures vortexed vigorously. The two phases were separated by centrifugation at 500 *g* for 5 min. The absorbance of the chromogen was determined at 530 nm with a Beckman DU-30 spectrophotometer. Results are expressed as nmol equivalents of malondialdehyde and the assay was linear between 0.5 to 20 nmol.

Characterization of atherosclerosis

Animals were killed by overdosing with pentobarbitone (120 mg kg^{-1}, administered intravenously). Aortae were rapidly dissected free from the ascending arch to the ileal bifurcation. Extraneous tissue was removed and full length incisions exposed intimal areas. Intimal areas were photographed with a Polaroid camera. The areas of grossly discernible normal and atherosclerotic intima were digitized from these photographs by use of a Numonics model 2210 tablet (Numonics Corporation, Lansdale, PA, U.S.A.) and Sigma-Scan (Jandel Scientific, Corte Madera, CA, U.S.A.) run on a DOS-based computer.

Total cholesterol content of vascular tissue was determined by gas liquid chromatography (g.l.c.) as described by Ishikawa *et al.* (1974) with 5-α-cholestane as a standard. For the determination of collagen, weighted segments of tissue were hydrolyzed by incubation in the presence of concentrated hydrochloric acid incubated overnight at 80°C. Hydroxyproline content of the tissue was determined with Erlich's solution as described by Prockop & Undenfreind (1960).

Immediately following the dissection of aortic tissue from the body, segments of tissue were cut in a region that included a branch of an intercostal artery, since this region is a site of predilection for the development of atherosclerosis. Tissue was immersion fixed in formaldehyde (2% wt/vol) dissolved in phosphate-buffered saline at room temperature for 4 h. Further fixation was achieved by overnight incubation in this solution at 4°C. Sections of tissue (5 μm thick) were cut, mounted and stained with haematoxylin and eosin.

Experimental protocol

Twenty five rabbits were randomized into 3 groups when animals were approximately 9 months of age. Group I was killed at this interval and served as the control group for determining the extent of atherosclerosis at the initiation of drug administration. Group II was given a diet that had been soaked in chloroform in a manner identical to that used to coat probucol on the diet, and was followed for 6 months. Group III was fed a diet for 6 months that was coated with probucol.

Statistical analyses

Statistical comparisons between groups were made with Student's *t* test (two-tailed) performed with Stats-+ (Statsoft, Tulsa, OK, U.S.A.). A repeated-measures analysis of variance (ANOVA) was used to assess the statistical significance of overall plasma lipid concentrations between Groups II and III. Duncan's multiple-range test was used to assess the statistical significance between each interval that plasma lipid concentrations were determined for Groups II and III. A probability value of less than 5% was considered statistically significant. Values are presented as means with s.e.means.

Results

General characteristics

The WHHL rabbits used in the present study weighed approximately 3.75 kg at the initiation of the study i.e., when the rabbits were $\approx$ 9 months of age. During the 6 months of observation, Groups II and III did not increase in body weight, and no significant differences in body weight were apparent between them (Figure 1). In accord with this finding, the food consumption was both constant and comparable between the groups. All animals maintained good health during the entire course of this study and all rabbits that entered the study survived to completion of the protocol.

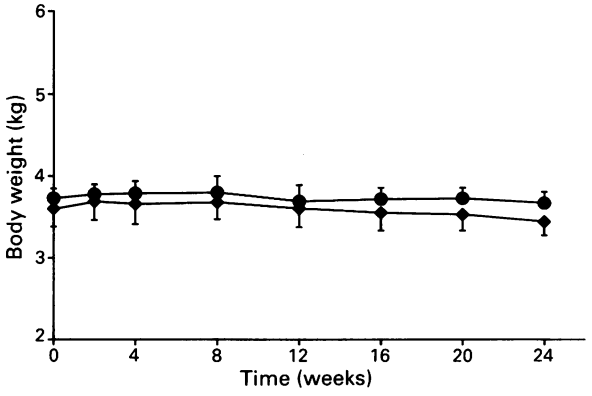

Figure 1 Body weights of control rabbits (Group II, ●) and probucol-treated (Group III), WHHL rabbit (◆). Points represent the means of 8 observations and bars the s.e.mean.

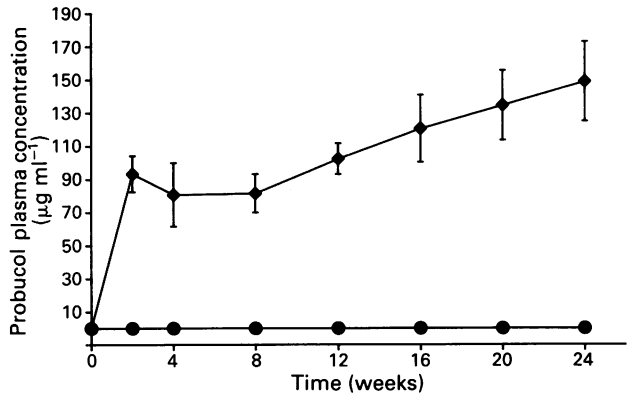

Figure 2 Plasma concentrations of probucol. Plasma concentrations of probucol were determined at selected intervals by h.p.l.c. in control (Group II, ●) and probucol-treated (Group III), WHHL rabbit (◆). Points represent the means of 8 determinations and bars represent the s.e.mean.

Plasma concentrations of probucol were monitored at selected intervals after the initiating treatment as indicated in Figure 2. The mean plasma concentration in the drug treated group was $93 \pm 11\,\mu g\,ml^{-1}$ after 2 weeks and this concentration was maintained to 8 weeks. Thereafter there was a trend toward increasing concentrations, reaching $149 \pm 24\,\mu g\,ml^{-1}$ at the end of the study. As expected, probucol was never detected in the plasma of the control group (Figure 2). A green colouration was present in the plasma of probucol-treated animals that was attributed to the accumulation of oxidized probucol (Barnhart *et al.*, 1990).

Probucol on plasma concentrations of lipoproteins

Plasma concentrations of total cholesterol at the initiation of the study were $674 \pm 80\,mg\,dl^{-1}$ in Group II and $683 \pm 123\,mg\,dl^{-1}$ in Group III (NS). In Group II, plasma concentrations of cholesterol were maintained until the animals were approximately one year old, after which there was a gradual increase. Probucol treatment produced an overall significant decrease in plasma concentrations of total cholesterol ($P < 0.001$) as analyzed by ANOVA. Despite the significance of the overall effect, comparison of each interval by Duncan's multiple-range test showed that only the 8 and 12 week intervals were significantly different between the groups ($P < 0.0375$ and $P < 0.003$, respectively; Figure 3a). Probucol treatment also produced overall reductions in unesterified cholesterol and phospholipids that were significantly different when analyzed by ANOVA ($P < 0.0001$ and $P < 0.0008$, respectively; Figures 3b and 3c). Plasma concentrations of triglycerides were not significantly affected by probucol administration (Figure 3d).

Oxidation of lipoproteins in vitro

LDL was isolated from the plasma of Groups II and III, and the extent of lipid peroxidation was determined by the extent of TBARS present. The concentrations of TBARS in native plasma-derived LDL were low and were not significantly changed compared to LDL isolated from probucol-treated rabbits (Figure 4). LDL from Groups II and III was incubated with copper ($3\,\mu M$) to simulate a microenvironment in which LDL may be subjected to increased oxidative stress. Under these conditions, there was a 110% increase ($P < 0.001$) in the concentrations of TBARS in LDL from untreated animals. In contrast, there was only a modest increase in the concentration of TBARS in LDL derived from probucol-treated animals which was not significantly higher than that in native LDL. Consequently, TBARS were present in significantly ($P < 0.004$) increased concentrations in copper-treated native LDL compared to that isolated from probucol-treated animals.

Extent of atherosclerosis

The extent and nature of atherosclerosis was assessed in all 3 groups of animals. At ≈ 9 months of age (Group I), WHHL rabbits had extensive involvement of the aorta of grossly discernible atherosclerotic lesions of the aorta with 71 ± 5% and

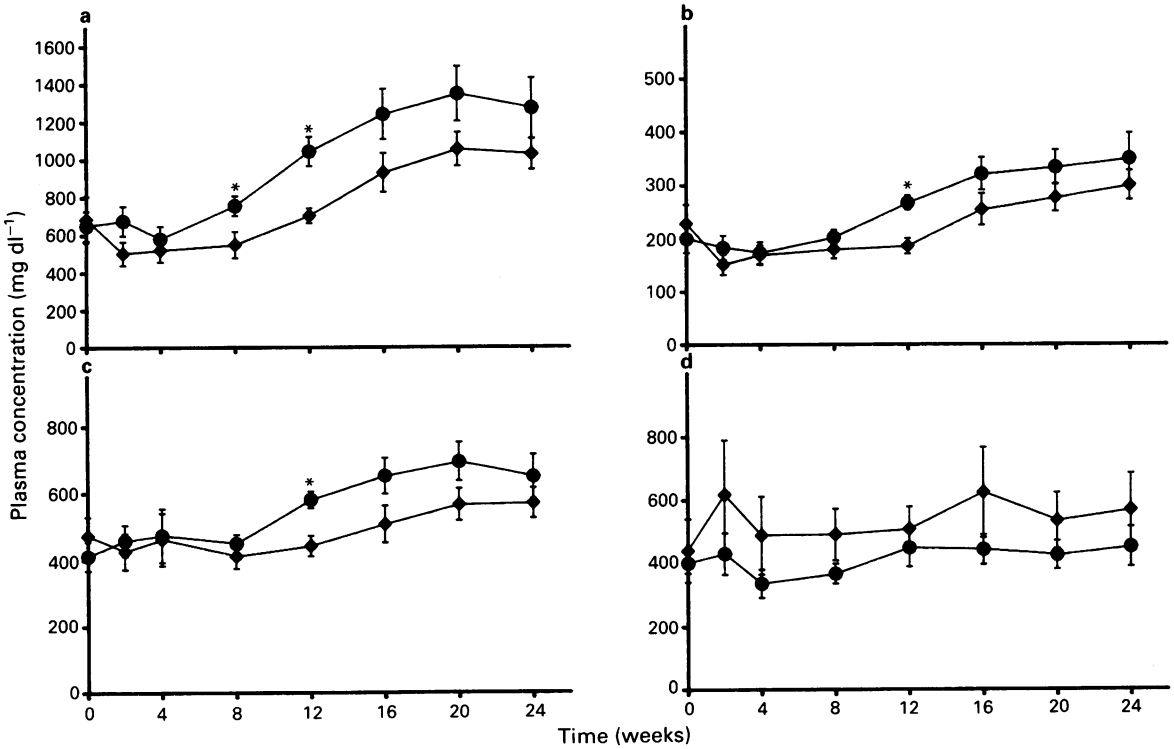

Figure 3 Plasma concentrations ($mg\,dl^{-1}$) of total cholesterol (a), unesterified cholesterol (b), phospholipids (c), and triglycerides (d) of control rabbits (Group II, ●) and probucol-treated (Group III), WHHL rabbit (◆). Points represent the means of 8 determinations and bars represent the s.e.mean.

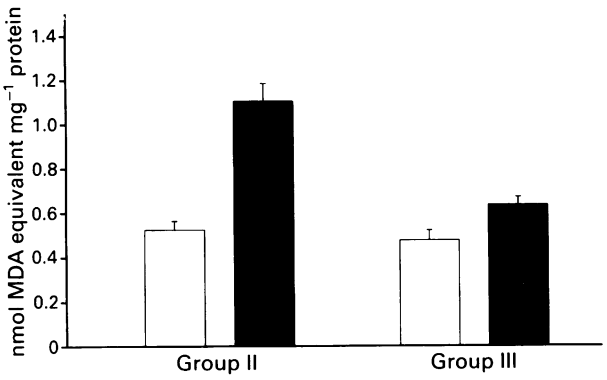

Figure 4 Presence of thiobarbituric acid-reacting substances (TBARS) in low density lipoproteins (LDL) from control (Group II, solid columns) and probucol-treated WHHL rabbits (Group III, open columns). TBARs were determined in the native material and following a 3 h incubation with Cu^{2+} for 18 h. Columns are the means of 4 determinations and bars are s.e.mean.

55 ± 7% of the intimal surface of the thoracic and abdominal segments, respectively, being covered (Figure 5a). The distributions of these atherosclerotic lesions were predominately at the arch and at arterial branch points. After a further 6 months, coverage in Group II had increased to 91 ± 3% and 71 ± 8% for the thoracic and abdominal segments, respectively. The extent of aortic atherosclerosis in Group III was 77 ± 7% and 59 ± 7% for abdominal and thoracic segments respectively. These reductions did not attain statistical significance.

As may be expected, there was a high content of cholesterol in the aortae from WHHL rabbits in Group I (1.4 ± 0.2 μg cholesterol mg^{-1} wet wt; Figure 5b). Both abdominal and thoracic segments in Group II had marked increases in cholesterol content. However, this increased cholesterol content was attenuated in both segments from the probucol-treated Group III ($P < 0.01$).

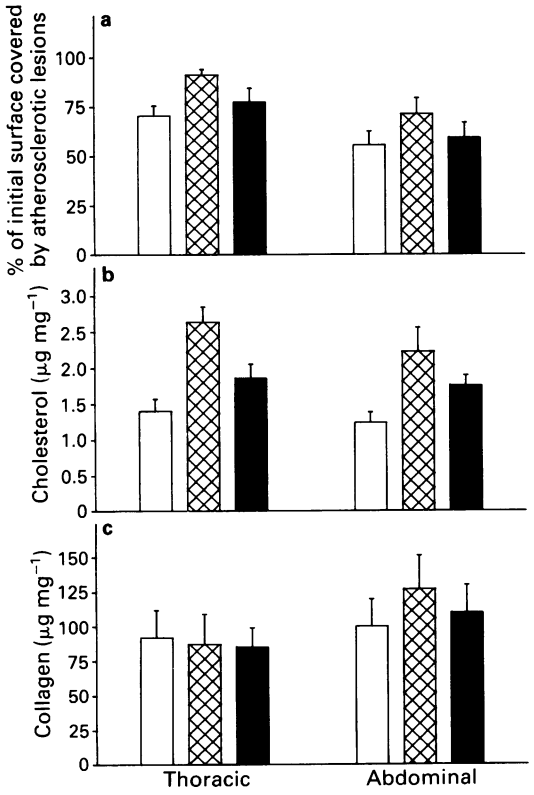

Figure 5 Characterization of aortic atherosclerosis in WHHL rabbits. Parameters determined were the percentage of intimal area that was covered by grossly discernible atherosclerotic lesions (a), total cholesterol content (b), and collagen content (c). Data are represented for Group I (open columns, $n = 9$), Group II (cross-hatched columns, $n = 8$), and Group III (solid columns, $n = 8$). Columns are the means of a minimum of 4 determinations and bars are s.e.mean.

Although there was a decrease in the cholesterol content of the aorta from the probucol-treated animals compared to age-matched controls, no changes in collagen content were detectable as judged by the concentration of hydroxyproline (Figure 5c).

In agreement with these quantitative data, histological sections of aorta demonstrated that there was an increase in the lipid deposition in the core region of lesions comparing Groups I to II. Generally, there was decreased lipid deposition in the core region of Group III. However, there were no major differences in the morphology of the atherosclerotic lesions between the groups studied. Thus, the atherosclerotic lesions differed in extent of lipid deposition rather than in the cellular composition.

Discussion

Probucol has been found to attenuate the initiation and development of atherosclerosis in cholesterol-fed rabbits (Kritchevsky *et al.*, 1971; Tawara *et al.*, 1986; Daugherty *et al.*, 1989) and WHHL rabbits (Kita *et al.*, 1987; Carew *et al.*, 1987). Although several reports have demonstrated anti-atherosclerotic properties of probucol in experimental studies, a negative study has also been published (Stein *et al.*, 1989). Those reports that have described an anti-atherosclerotic effect have generally not ascribed this protection to an alteration in lipid metabolism, but rather to the hydroxyl radical scavenging properties of the drug (Steinberg, 1986). Indeed there is increasing evidence that the changes in metabolic properties that have been described for oxidized LDL *in vitro* (Fogelman *et al.*, 1980; Henriksen *et al.*, 1981; Steinbrecher *et al.*, 1984; Parthasarathy *et al.*, 1986a) may be relevant to the lipoprotein metabolism *in vivo* (Daugherty *et al.*, 1988; Haberland *et al.*, 1988; Boyd *et al.*, 1989; Palinski *et al.*, 1989).

The temporal cellular events that are involved in the progression of atherosclerosis in WHHL rabbits have been described in detail by Rosenfeld *et al.* (1987a,b). Macrophages are deposited in the subintimal space of aortic tissue of WHHL rabbits within the first month following parturition. These initial lesions develop into fatty streaks by the continued deposition of macrophages that are transformed to foam cells, and the persistent deposition of extracellular matrix. Thus, the atherosclerotic lesions investigated in the present study were mature fatty streaks predominantly containing macrophages and numerous smooth muscle cells.

While the determination of a drug to inhibit the initiation of atherosclerosis may have important implications for the prevention of the disease, the ability of a drug to induce regression could have profound implications for therapy of established disease. It has been hypothesized that probucol may induce regression of atherosclerotic lesions since the drug reduces the extent of xanthomas in familial hypercholesterolaemic patients (Yamamoto *et al.*, 1988). Since xanthomas consist of lipid-laden foam cells analogous to atherosclerotic lesions (Fowler *et al.*, 1979; Schaffner *et al.*, 1980), it is thought that these may represent a model of atherosclerotic lesions. The preliminary report of Wissler & Vesselinovitch (1983) demonstrated that there was a modest regression on aortic atherosclerosis as judged by the percentage of intimal area that was covered by grossly discernible lesions. The present study was designed to provide quantitative data to demonstrate whether probucol (A) promoted regression, (B) attenuated the progression, or (C) did not influence the atherogenic process of established atherosclerotic lesions.

Previous reports that have described the temporal cholesterol concentrations in plasma of WHHL rabbits have generally described a gradual reduction with age (Rosenfeld *et al.*, 1987a). In contrast, the present study has described a gradual

increase in plasma concentrations. However, as distinct from other work, the present study was initiated with mature WHHL rabbits that were ≈9 months of age. Furthermore, plasma cholesterol concentrations began to increase only after the rabbits were a year of age. A systematic error in the measurement of plasma cholesterol concentrations is considered unlikely since rabbits were initiated into the study over a 8 month time span. Thus, there was considerable randomization of the plasma cholesterol determinations, which would have negated the effects of a drift in the assay calibration. The mechanism of this increase in plasma cholesterol concentrations in rabbit over one year of age is unknown.

Administration of probucol to WHHL rabbits produced high plasma concentrations of the drug but relatively modest reductions in concentrations of cholesterol for the initial 3 months; although the difference between the groups increased after 1 year of age. Variable responses have been demonstrated for probucol on plasma cholesterol concentrations in WHHL rabbits. Naruszewicz *et al.* (1984) observed a 36% decrease in plasma concentrations of LDL-cholesterol and attributed this reduction to a change in the inherent property of the LDL. However, a later study by the same group failed to demonstrate a hypolipidaemic effect despite comparable plasma concentrations of probucol (Carew *et al.*, 1987).

The moderate reduction in plasma concentrations of cholesterol observed in the present study may have contributed to the observed reduction in cholesterol in the aortae of probucol-treated rabbits. In addition to the hypolipidaemic effect contributing to the anti-atherosclerotic effects, the ability of probucol to scavenge hydroxyl radicals may also have contributed to the reduced cholesterol deposition. Oxidation of LDL in the plasma is likely to be limited because of the combination of the relatively low concentrations of oxidant and the extensive antioxidant defence mechanisms. Despite these considerations, modified LDL has been detected in the plasma of human subjects (Avogaro *et al.*, 1988). However, it is more likely that oxidative modifications of LDL occur in non-intravascular compartments such as the subendothelial space. To simulate this environment of increased oxidative stress, LDL was incubated with low concentrations of copper. Since probucol is highly lipophilic, it is transported by lipoproteins. Indeed, LDL from probucol-treated rabbits was highly resistant to oxidation in the presence of copper. Thus a significant contribution to the decrease of the continued lipid deposition may have been due to prevention of LDL oxidation at the loci of the arterial tissue. The primary protective effects attributable to the antioxidant properties of probucol are likely to be related to an effect on the inhibition of LDL modification rather than influencing the metabolism of the macrophages directly. Hence, although probucol prevents oxidation of LDL and metabolism by macrophages (Parthasarathy *et al.*, 1986b; Yamamoto *et al.*, 1986), the drug has no effect on the metabolism of acetylated LDL by this cell type (Ku *et al.*, 1990). In the present work, it was not feasible to study whether the macrophage metabolism was itself modified after the chronic administration of probucol. Despite this reduction in the extent of lipid deposition, no effect of probucol was demonstrable on the collagen content of aortae following probucol treatment. Thus, the effect on atherosclerotic lesions in established disease appeared to be related only to effects on lipid deposition.

In summary, the present study has demonstrated that the chronic administration of probucol to mature WHHL rabbits with established aortic atherosclerosis does not promote regression but does lead to a reduction in the continued deposition of cholesterol into arterial tissue. This beneficial effect may have been partially due to a slight hypolipidaemic effect that was observed during probucol administration. However, the marked potency in scavenging hydroxyl radicals may have contributed more significantly to the beneficial effect on the atherogenic process.

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Effects of ATP-sensitive K^+ channel blockers on the action potential shortening in hypoxic and ischaemic myocardium

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1 In order to determine whether activation of adenosine triphosphate (ATP)-sensitive K^+ channels exclusively explains the hypoxia- and ischaemia-induced action potential shortening, effects of tolbutamide and glibenclamide on changes in action potential duration (APD) during hypoxia, metabolic blockade or experimental ischaemia were examined in guinea-pig and canine isolated myocardium by standard microelectrode techniques.

2 With use of patch clamp techniques, activity of ATP-sensitive K^+ channels was recorded from open cell-attached patches of guinea-pig isolated ventricular myocytes. The probability of opening of the K^+ channels was decreased by 2 mM tolbutamide and 20 μM glibenclamide to almost the same extent, whereas it was increased by 100 μM pinacidil.

3 In guinea-pig papillary muscles a marked shortening of the action potential produced by 100 μM pinacidil was completely antagonized by 2 mM tolbutamide or 20 μM glibenclamide.

4 In guinea-pig papillary muscles exposed to hypoxic, glucose-free solution or dinitrophenol (10 μM)-containing, glucose-free solution, APD declined gradually and twitch tension decreased. Pretreatment with glibenclamide partially but significantly inhibited the action potential shortening, whereas tolbutamide failed to improve it during hypoxia or metabolic blockade.

5 When in canine isolated myocardium, experimental ischaemia was produced by the cessation of coronary perfusion, APD was gradually shortened. The action potential shortening was partially but not completely inhibited by pretreatment with 20 μM glibenclamide.

6 These results suggest that changes in membrane current(s) other than the outward current through ATP-sensitive K^+ channels also contribute to the action potential shortening in hypoxic or ischaemic myocardium.

Keywords: ATP-sensitive K^+ channel; glibenclamide; tolbutamide; action potential shortening; hypoxia; ischaemia; cardiac muscle

Introduction

It has long been known that hypoxia and metabolic blockade produce a marked shortening of cardiac action potentials (Carmeliet, 1978). Two hypotheses have been proposed to explain the action potential shortening; a decrease in a slow inward current (McDonald & MacLeod, 1973; Schneider & Sperelakis, 1974; Payet *et al.*, 1978) and an increase in a potassium outward current (Trautwein, 1954; MacLeod & Daniel, 1965; Vleugels *et al.*, 1980; Isenberg *et al.*, 1983). Since the discovery of adenosine triphosphate (ATP)-sensitive K^+ channels in heart cells by Noma (1983), it has been assumed that activation of ATP-sensitive K^+ channels plays a major role in the action potential shortening during hypoxia or metabolic inhibition (Trube & Heschler, 1984; Noma & Shibasaki, 1985; Fosset *et al.*, 1988; Sanguinetti *et al.*, 1988; Friedrich *et al.*, 1990) and potentially during myocardial ischaemia. However, any direct evidence showing that ATP-sensitive K^+ channels are activated during myocardial ischaemia or hypoxia has not been provided, probably due to methodological difficulties. Recently it has been shown that sulphonylurea antidiabetic agents can inhibit ATP-sensitive K^+ channels not only in pancreatic β-cells (Sturgess *et al.*, 1985; Trube *et al.*, 1986; Zünkler *et al.*, 1988) but also in cardiac cells (Belles *et al.*, 1987; Fosset *et al.*, 1988). Therefore, in the present study we examined effects of sulphonylureas on the action potential shortening during hypoxia or metabolic blockade in guinea-pig isolated papillary muscles and during experimental myocardial ischaemia in canine coronary-perfused isolated myocardium. By doing so, we expected to assess to what extent the activation of ATP-sensitive K^+ channels contributes to the action potential shortening during myocardial ischaemia or hypoxia. Preliminary accounts of this work have appeared in abstract form (Nakaya *et al.*, 1989; Kanno & Nakaya, 1990).

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Methods

Single channel recordings in guinea-pig isolated ventricular myocytes

Single myocytes were isolated from guinea-pigs by enzymatic dissociation, as described previously (Tohse *et al.*, 1990). Briefly, the heart was removed from the open chest guinea-pig anaesthetized with pentobarbitone sodium. By use of a Langendorff apparatus, the excised heart was perfused with 0.008% collagenase (Yakult, Tokyo, Japan) dissolved in a nominally Ca^{2+}-free Tyrode solution, and then with 0.01% protease (Sigma type XIV, Sigma Chemical Co. Ltd., St. Louis, U.S.A.) in a kraftbrühe (KB) solution (Isenberg & Klockner, 1982). After digestion, the heart was rinsed with the KB solution and cut into small pieces. The cell suspension in the KB solution was stored in a refrigerator for later use.

The composition of Tyrode solution was (in mM): NaCl 143, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.33, glucose 5.5 and HEPES-NaOH buffer (pH 7.4) 5.0. The nominally Ca^{2+}-free Tyrode solution was prepared by omitting $CaCl_2$ from the normal Tyrode solution. The composition of the KB solution was (in mM): KOH 70, 1-glutamic acid 50, KCl 40, taurine 20, KH_2PO_4 20, $MgCl_2$ 3, glucose 10, EGTA 1.0 and HEPES-KOH buffer (pH 7.4) 10.

Unitary current recordings were performed in the open cell-attached configuration of the patch clamp technique (Hamill *et al.*, 1981; Kakei *et al.*, 1985). Patch pipettes were Si-coated, heat-polished and filled with a solution containing 150 mM

KCl, 2 mM $CaCl_2$ and 5 mM HEPES-KOH (pH 7.4). After the gigaohm seal between the patch electrode and the cell membrane was formed in Tyrode solution, the bath solution was changed to an internal solution containing 150 mM KCl, 0.33 mM NaH_2PO_4, 1 mM EGTA and 5 mM HEPES-KOH buffer (pH 7.4). Then, part of the surface membrane on one end of the rod cell was disrupted by penetrating the membrane with a glass pipette (1–2 μm tip diameter) containing the internal solution. After the disruption of the cell membrane, the activity of the ATP-sensitive K^+ channel appeared within a few minutes when the internal solution contained no ATP. Both the membrane potential and current were recorded with a patch clamp amplifier (EPC-7, List, F.R.G.). These signals were stored on video tape through a pulse code modulator (SONY PCM-501ES, Japan) and analysed later with a personal computer (NEC PC-98XA, Japan) equipped with a 20 MByte hard disc and an AD converter (Canoopus Electronics ADX-98, Japan). All these experiments were performed at room temperature (20–25°C).

Action potential and tension recordings from guinea-pig papillary muscles

Guinea-pigs were stunned with a blow on the head and the hearts were quickly removed. Papillary muscles having a diameter less than 1 mm were dissected from the right ventricle. The preparations were transferred to a tissue bath of 5 ml volume and superfused at a rate of 10 ml min^{-1} with a modified Tyrode solution of the following composition (in mM): NaCl 125, KCl 4, $NaHCO_3$ 25, NaH_2PO_4 1.8, $MgCl_2$ 0.5, $CaCl_2$ 2.7 and glucose 5.5. The solution was gassed with 95% O_2 plus 5% CO_2 and the bath temperature was kept constant at 36.0 ± 1.0°C.

Action potentials and contractions were recorded, as previously described (Nakaya *et al.*, 1987). In brief, one end of the preparation was hooked to an extension of the lever arm of a force transducer (Nihon Kohden TB651T, Tokyo, Japan) and the other end was pinned to the bottom of the tissue bath. The length of the muscle was adjusted until resting tension was 200 mg. Stimulating pulses (1 Hz, 1 ms duration, 2 times threshold intensity) were applied to the preparation through platinum field electrodes. Transmembrane potentials were recorded with 3 M KCl-filled microelectrodes (10–30 MΩ) which were connected to a high impedance capacitance neutralizing amplifier (Nihon Kohden, MEZ 8201). The action potentials and tension were displayed on an oscilloscope (Nihon Kohden VC 10), photographed on 35 mm film and recorded on a chart recorder (Watanabe Sokki WR 3101, Tokyo, Japan).

The following action potential variables were analysed by magnification from photographs: resting membrane potential (RMP), action potential amplitude (APA), the maximum upstroke velocity of action potential ($\dot{V}_{max}$), action potential duration at 0 mV (APD_0) and −60 mV level (APD_{-60}). APDs were measured at these fixed potentials, because these APDs would reflect the magnitude of steady-state outward current at respective membrane potentials, regardless of changes in RMP and APA.

After an equilibration period of 2 h, the preparations were superfused with a hypoxic, glucose-free solution for 60 min or glucose-free solution containing 10 μM 2,4-dinitrophenol (DNP) for 45 min. To obtain the hypoxic, glucose-free solution, glucose was omitted from the modified Tyrode solution and the solution in a reservoir was gassed with 95% N_2 plus 5% CO_2, which resulted in Po_2 values of 27.1 ± 2.4 mmHg. In order to examine the influence of sulphonylureas on action potential changes produced by these solutions, some of the preparations were pretreated with 20 μM glibenclamide or 2 mM tolbutamide for 60 min. Action potential and twitch tension were recorded during hypoxia or metabolic blockade after an interval of 5 min. It was difficult to maintain a single impalement in half of the experiments during the interventions because of the increased resting tension. When impalements were not maintained, new stable impalements were secured in the neighbourhood of the former site.

Action potential recordings from canine coronary-perfused isolated myocardium

Mongrel dogs of either sex, weighing 5–12 kg, were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.v.). The heart was rapidly removed and immersed in an oxygenated Tyrode solution having the same composition as the solution used in the guinea-pig papillary muscle study. A branch of the right coronary artery in the right ventricular free wall was cannulated with a small polyethylene tube. The cannula was secured in place with silk sutures and then perfused with the modified Tyrode solution equilibrated with 95% O_2 and 5% CO_2. The perfused area was distinctly delineated by its pale appearance after injection of the modified Tyrode solution. The tissue beyond the perfused area was excised, and the major branches of arteries transected by the dissection were ligated with silk sutures. The cannulated preparation was placed with epicardial surface uppermost in a superfusion chamber and then was superfused with the oxygenated Tyrode solution. The preparation was simultaneously perfused through the coronary cannula with the modified Tyrode solution gassed with 95% O_2 plus 5% CO_2 at a perfusion rate of approximately 1.0 ml g^{-1} wet weight min^{-1} by means of a peristaltic pump (EYELA MP-3, Tokyo, Japan). The temperature of the perfusate and superfusate both were maintained at 36.0 ± 1.0°C.

The preparation was stimulated at a rate of 1.0 Hz through platinum field electrodes. Stumuli were rectangular pulses of 1 ms duration at twice the diastolic threshold. Transmembrane action potentials were recorded with 3 M KCl-filled microelectrodes from the subepicardial muscle cells.

Experimental myocardial ischaemia of 30 min was produced by discontinuing coronary perfusion and starting superfusion with the hypoxic, glucose-containing Tyrode solution gassed with 95% N_2 plus 5% CO_2. It was confirmed that the transmembrane action potentials were not appreciably altered by superfusion with the hypoxic solution when the preparation was continuously coronary-perfused with the oxygenated Tyrode solution.

After an equilibration period of 1–2 h, control action potentials were recorded and experimental ischaemia was produced. Action potentials were recorded at intervals of 5 min during myocardial ischaemia. Multiple impalements were required to provide continuous electrophysiological data. Some preparations were perfused and superfused with the normoxic solution containing 20 μM glibenclamide for 60 min before the cessation of coronary perfusion. These preparations were superfused with the hypoxic Tyrode solution containing the same concentration of the drug during experimental myocardial ischaemia.

Drugs and chemicals

The following drugs were used: tolbutamide (Wako, Osaka, Japan), glibenclamide (Sigma Chemical, St. Louis, U.S.A.), 2,4-dinitrophenol (Wako), pinacidil (Shionogi, Tokyo, Japan). Tolbutamide was dissolved in 1 N NaOH at a concentration of 0.5 M. Glibenclamide was dissolved in 0.05 N NaOH at a concentration of 2 mM. Pinacidil was dissolved in 0.1 N HCl at a concentration of 0.1 M. Dinitrophenol was dissolved in distilled water. Changes in pH of the aerated Tyrode solution, observed after the application of these drugs in concentrations used in the present study, were less than 0.02.

Statistics

All values are presented in terms of mean ± s.e. Statistical analyses were performed by Student's t test for paired and unpaired observations. P values less than 0.05 were accepted as indicating a significant difference.

Results

Effects of tolbutamide and glibenclamide on ATP-sensitive K^+-channels

After the formation of giga ohm sealing, a part of the membrane of guinea-pig ventricular myocytes was disrupted by the other electrode in the high K^+ internal solution without ATP. Within a few minutes, channel activity appeared in the open cell-attached patch, as shown in Figure 1. In this experiment the linear slope conductance obtained from the current-voltage relationship was 80.3 pS and the channel openings were inhibited by the application of 2 mM ATP to the internal surface of the membrane. These findings led us to conclude that the unitary current flowed through ATP-sensitive K^+ channels.

Effects of sulphonylureas and pinacidil on the activity of ATP-sensitive K^+ channels were examined in open-cell attached patches. As shown in Figure 2a and 2b, tolbutamide (2 mM) and glibenclamide (20 μM) inhibited the channel openings in the absence of ATP. In the open cell-attached patches held at −60 mV, tolbutamide and glibenclamide decreased the probability of opening from 0.734 ± 0.060 to 0.136 ± 0.065 ($n = 5$, $P < 0.01$) and from 0.716 ± 0.104 to 0.124 ± 0.072 ($n = 5$, $P < 0.01$), respectively, without affecting unit amplitude. In contrast, pinacidil at a concentration of 100 μM increased the channel openings in the presence of 0.3 mM ATP (Figure 2c). Addition of 0.3 mM ATP to the internal solution decreased the opening probability of ATP-sensitive K^+ channels to 0.148 ± 0.079. It was significantly increased to 0.647 ± 0.100 by 100 μM pinacidil ($n = 6$). Thus, in open cell-attached patches, 2 mM tolbutamide and 20 μM glibenclamide inhibited the opening of ATP-sensitive K^+ channels to the same extent in the absence of ATP, whereas pinacidil activated the channels in the presence of 0.3 mM ATP.

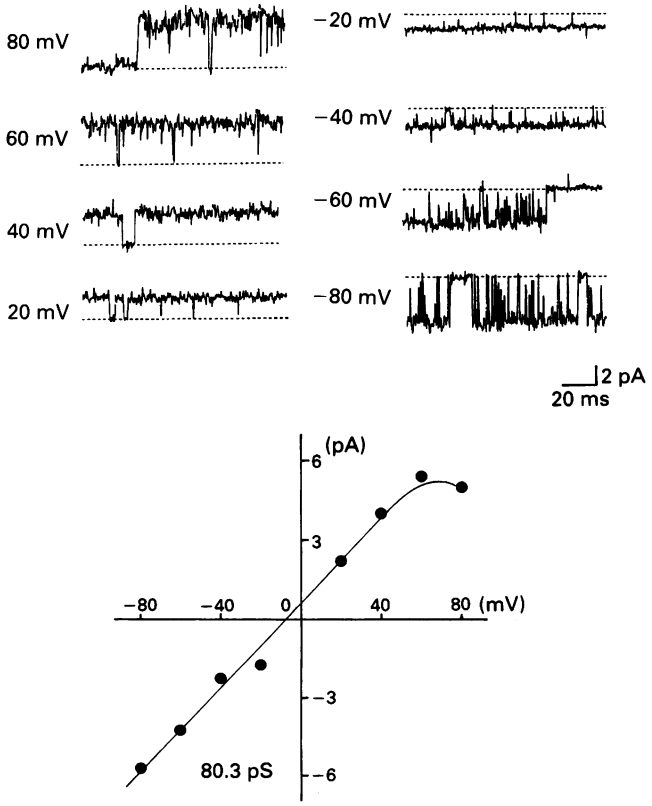

Figure 1 Single channel current recordings in the open cell-attached mode from a guinea-pig ventricular cell. Unitary K^+ currents recorded in a membrane patch held at various voltage levels are shown in the upper panel. The internal solution contained no ATP. Downward deflections are inward directed single channel currents. Current-voltage relationship for the single channel current is shown in the lower panel. Linear slope conductance was 80.3 pS.

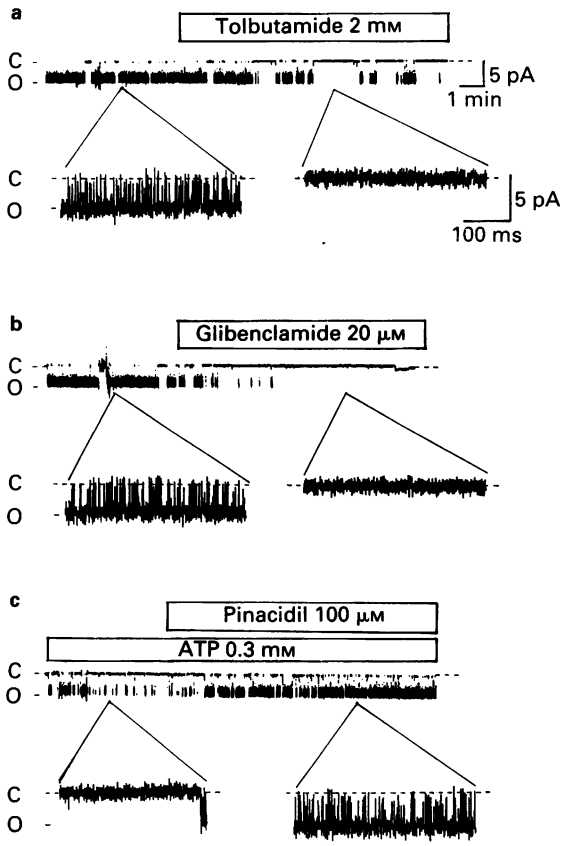

Figure 2 Effects of tolbutamide (2 mM, a), glibenclamide (20 μM, b) and pinacidil (100 μM, c) on ATP-sensitive K^+ channel in an open cell-attached patch held at −60 mV. In each panel expanded records are shown below. Downward deflections are inward single channel currents. C and O indicate the closed and open state of the channels, respectively.

Effects of tolbutamide and glibenclamide on the pinacidil-induced action potential shortening

In the guinea-pig papillary muscles stimulated at 1.0 Hz, pinacidil markedly shortened the action potential and decreased twitch tension (Figure 3). After 30 min exposure to 100 μM pinacidil, action potential duration at the −60 mV level (APD_{-60}) and twitch tension of 6 preparations were significantly decreased by 83.5 ± 1.1% and 94.4 ± 0.9% from the control values of 200.0 ± 6.7 ms and 38.5 ± 19.5 mg, respectively. Addition of 2 mM tolbutamide or 20 μM glibenclamide produced almost complete recovery of APD and less complete recovery of twitch tension, as shown in Figure 3. The antagonistic effect of tolbutamide appeared more rapidly than that of glibenclamide. APD_{-60} at 10 min after 2 mM tolbutamide and 30 min after 20 μM glibenclamide were 100.9 ± 0.9% ($n = 3$) and 96.8 ± 0.9% ($n = 3$) of control, respectively. Thus, these concentrations of tolbutamide and glibenclamide completely antagonized the action potential shortening produced by the ATP-sensitive K^+ channel-opener, pinacidil.

Effects of tolbutamide and glibenclamide on the action potential shortening produced by hypoxia or metabolic blockade

The baseline values of action potential parameters and twitch tension of papillary muscles stimulated at 1.0 Hz are summarized in Table 1. There were no significant differences in

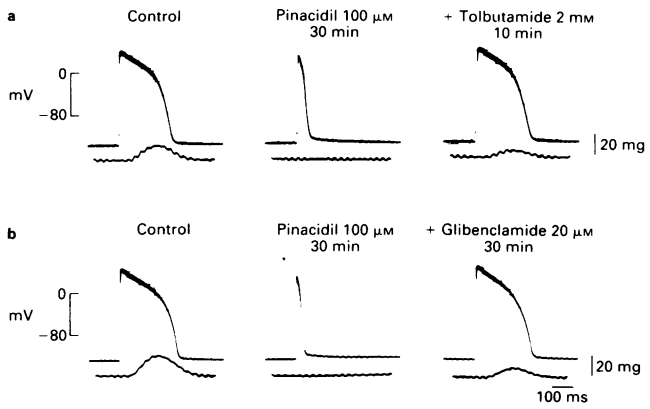

Figure 3 Effects of tolbutamide (2 mM, a) and glibenclamide (20 μM, b) on the pinacidil-induced action potential changes in guinea-pig papillary muscles stimulated at 1.0 Hz. In each pannel upper and lower tracings indicate action potential and twitch wave form, respectively. Note that the action potential shortening and the decrease in twitch tension produced by pinacidil were effectively reversed by tolbutamide and glibenclamide.

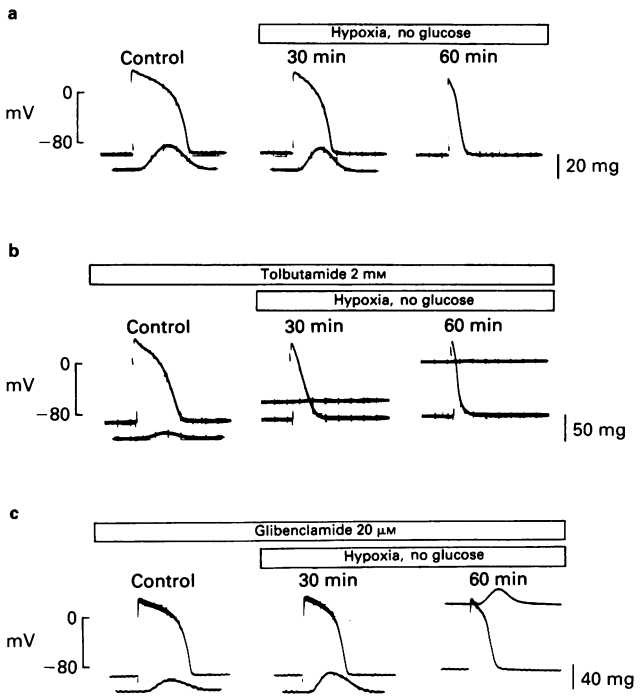

Figure 4 Changes in action potential configuration and twitch wave form of guinea-pig papillary muscles in the absence (a) and presence of 2 mM tolbutamide (b) or 20 μM glibenclamide (c) after exposure to hypoxic, glucose-free solution. Action potential and twitch wave form are depicted in each record except for the record taken after 60 min of hypoxic, substrate-free condition, in which an increase in resting tension was so marked that twitch wave form could not be recorded. Note that glibenclamide, but not tolbutamide, lessened the action potential shortening under the condition.

any of these parameters between subgroups. Pretreatment with 2 mM tolbutamide or 20 μM glibenclamide for 60 min did not significantly affect action potential configuration and twitch tension.

Superfusion of hypoxic, glucose-free solution produced marked changes in action potential configuration and twitch tension, as shown in Figure 4a. Gradual decreases in APD, resting membrane potential (RMP) and action potential amplitude (APA) were observed (Table 2, Figure 5a). An increase in resting tension and a marked decrease in twitch tension also appeared. Twitch tension was significantly decreased to 27.6 ± 13.0% of the control after 60 min hypoxia (Table 2). In the presence of 2 mM tolbutamide, the hypoxia-induced APD shortening was potentiated rather than improved (Figures 4b and 5a). In preparations treated with tolbutamide the hypoxia-induced decrease in twitch tension was more marked than that in control preparations although the difference was not statistically significant (Table 2). In contrast with tolbutamide, glibenclamide retarded the action potential shortening during hypoxia (Figure 4c and 5a). Although the decreases in RMP and APA in glibenclamide-treated preparations were similar to those in untreated preparations, the decrease in twitch tension was not significantly decreased (Table 2). Thus, two sulphonylureas exerted differential influences on the hypoxia-induced APD shortening and contractile failure. Glibenclamide could lessen but not completely prevent the action potential shortening in hypoxic, substrate-free conditions.

Superfusion with a glucose-free solution containing 10 μM dinitrophenol (DNP) produced similar changes in action potential configuration and twitch tension (Table 3). Tolbutamide at a concentration of 2 mM hardly affected the time course of the changes in APD and twitch tension (Table 3 and Figure 5b). Glibenclamide (20 μM) markedly retarded the action potential shortening for up to 20 min. Thereafter, the APD shortening became close to that of the control group, and then it was gradually reversed (Figure 5b). Glibenclamide significantly lessened the decrease in twitch tension at 20 and 45 min (Table 3). Thus, tolbutamide again failed to improve the action potential shortening in glucose-free, DNP-containing solution. Glibenclamide significantly reduced but did not completely prevent the action potential shortening during metabolic blockade.

Effect of glibenclamide on the action potential shortening during experimental ischaemia in isolated canine ventricular myocardium

Since glibenclamide but not tolbutamide improved the action potential shortening during hypoxia or metabolic blockade in guinea-pig isolated papillary muscles, the effect of glibenclamide on the ischaemia-induced APD changes was examined in the canine, coronary-perfused isolated ventricular myocardium. When coronary perfusion was stopped, a progressive decrease in RMP, APA and APD was produced (Figure 6a). In control preparations, RMP was significantly decreased from −83.6 ± 1.6 mV to −70.4 ± 2.6 mV, and APD at the

Table 1 Baseline values of action potential parameters and twitch tension in the absence and presence of tolbutamide or glibenclamide

		RMP (mV)	*APA* (mV)	APD_0 (ms)	APD_{-60} (ms)	$\dot{V}_{max}$ (Vs^{-1})	*TT* (mg)
Untreated ($n = 14$)		−93.8 ± 0.5	130.6 ± 0.7	128.9 ± 6.7	182.6 ± 8.2	204.5 ± 11.2	43.1 ± 13.2
Tolbutamide	pre	−94.3 ± 0.5	130.8 ± 1.1	130.2 ± 7.8	184.5 ± 7.8	227.2 ± 16.1	42.8 ± 7.6
(2 mM, $n = 12$)	post	−94.0 ± 0.4	130.7 ± 1.2	125.6 ± 6.4	178.8 ± 6.6	221.3 ± 17.4	39.7 ± 8.4
Glibenclamide	pre	−92.0 ± 0.6	128.6 ± 1.5	144.5 ± 6.5	200.8 ± 7.9	209.9 ± 18.5	47.1 ± 7.9
(20 μM, $n = 10$)	post	−92.5 ± 0.6	128.5 ± 1.6	145.7 ± 6.9	204.6 ± 8.0	197.3 ± 21.9	45.0 ± 8.4

Values are mean ± s.e. RMP: resting membrane potential; APA: action potential amplitude; APD_0: action potential duration at 0 mV level; APD_{-60}: action potential duration at −60 mV level; $\dot{V}_{max}$: the maximum upstroke velocity of action potential; TT: twitch tension.

Table 2 Changes in action potential parameters and twitch tension during hypoxia in the absence and presence of tolbutamide or glibenclamide

	Control ($n = 7$)			*Tolbutamide* (2 mM, $n = 6$)			*Glibenclamide* (20 μM, $n = 5$)		
	0 min	30 min	60 min	0 min	30 min	60 min	0 min	30 min	60 min
RMP (mV)	−94.1 ± 0.7	−91.4 ± 0.8*	−88.4 ± 3.4	−94.2 ± 0.6	−89.3 ± 2.2	− 88.5 ± 2.1*	−92.8 ± 0.6	− 89.4 ± 2.5	−85.0 ± 0.8*
APA (mV)	131.3 ± 0.8	127.6 ± 1.4*	116.9 ± 5.5*	130.0 ± 1.0	119.3 ± 4.5*	111.3 ± 7.5*	126.8 ± 2.2	120.0 ± 3.0	117.4 ± 3.2*
APD_{-60} (ms)	195.3 ± 5.9	125.0 ± 12.7*	61.4 ± 8.7*	182.8 ± 11.7	74.7 ± 8.8*	43.3 ± 7.1*	222.6 ± 9.9	164.6 ± 17.5	105.4 ± 6.1*†
TT (%)	100	60.1 ± 21.6	27.6 ± 13.0*	100	18.3 ± 4.5*	9.0 ± 4.1*	100	101.6 ± 31.7	65.2 ± 23.5

Values are mean ± s.e. RMP: resting membrane potential; APA: action potential amplitude; APD_{-60}; action potential duration at −60 mV level; TT: twitch tension. TT was expressed as % of the 0 min value. Absolute values of TT at 0 min of the control, tolbutamide and glibenclamide group were 58.1 ± 24.7, 43.0 ± 11.3 and 44.2 ± 8.6 mg, respectively.
* $P < 0.05$ vs 0 min value by paired Student's t test.
† $P < 0.05$ vs control group by unpaired Student's t test for the normalized data.

Table 3 Changes in action potential parameters and twitch tension during metabolic blockade in the absence and presence of tolbutamide or glibenclamide

	Control ($n = 7$)			*Tolbutamide* (2 mM, $n = 6$)			*Glibenclamide* (20 μM, $n = 5$)		
	0 min	20 min	45 min	0 min	20 min	45 min	0 min	20 min	45 min
RMP (mV)	−93.4 ± 0.7	− 90.4 ± 1.3*	−87.0 ± 2.3*	−93.8 ± 0.5	−88.5 ± 1.0*	−89.3 ± 1.0*	−92.2 ± 1.1	−88.2 ± 1.7*	−83.6 ± 3.9
APA (mV)	129.9 ± 1.1	123.7 ± 2.0*	114.9 ± 3.8*	131.3 ± 2.2	121.7 ± 3.6*	113.7 ± 3.5*	130.2 ± 2.3	127.0 ± 2.1	114.6 ± 5.7
APD_{-60} (ms)	170.0 ± 14.3	102.6 ± 22.8*	40.3 ± 4.3*	174.7 ± 7.1	107.5 ± 14.7*	38.7 ± 6.1*	186.5 ± 5.5	172.0 ± 7.7*†	110.4 ± 8.8*†
TT (%)	100	35.8 ± 10.2*	10.5 ± 3.5*	100	24.4 ± 6.1*	12.6 ± 3.4*	100	67.4 ± 8.3*†	68.0 ± 23.1†

Values are mean ± s.e. RMP: resting membrane potential; APA: action potential amplitude; APD_{-60}: action potential duration at −60 mV level; TT: twitch tension. TT was expressed as % of the 0 min value. Absolute values of TT at 0 min of the control, tolbutamide and glibenclamide group were 28.1 ± 8.8, 36.3 ± 13.3 and 45.8 ± 15.6 mg, respectively.
* $P < 0.05$ vs 0 min value by paired Student's t test. † $P < 0.05$ vs control group by unpaired Student's t test for the normalized data.

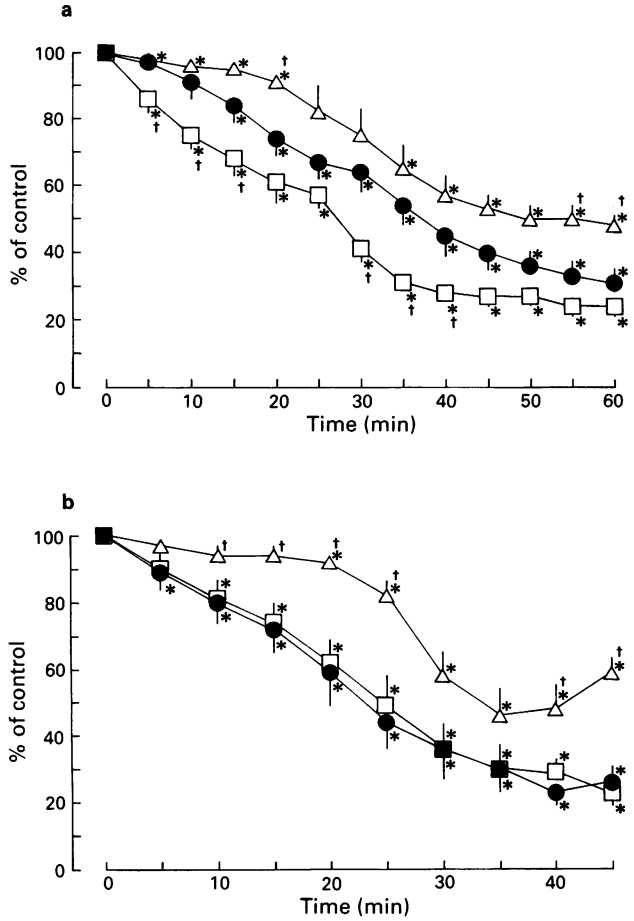

Figure 5 Time courses of changes in action potential duration at −60 mV level (APD_{-60}) during hypoxia (a) and metabolic blockade (b). Closed circles, open squares and open triangles indicate normalized durations of untreated preparations ($n = 7$ for both groups), those treated with 2 mM tolbutamide ($n = 6$ for both groups) and those treated with 20 μM glibenclamide ($n = 5$ for both groups), respectively. The time after the exposure to hypoxic, glucose-free solution (a) or glucose-free, dinitrophenol (10 μM)-containing solution (b) is on the abscissa scale. Values are expressed as mean with s.e. shown by vertical lines. * $P < 0.05$ from the baseline value by paired Student's t test. † $P < 0.05$ from the control group by unpaired Student's t test for the normalized data.

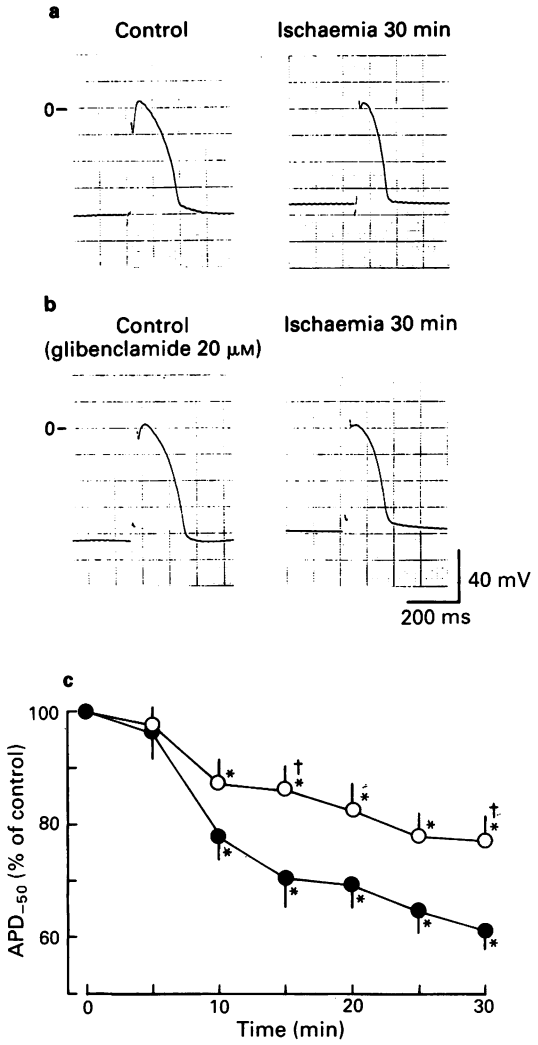

Figure 6 Action potential changes during experimental ischaemia in isolated right ventricular free wall preparations of the dog heart. (a) Ischaemia-induced action potential change in a control preparation. (b) Ischaemia-induced action potential change in a preparation treated with 20 μM glibenclamide. (c) Time courses of changes in APD at −50 mV level (APD_{-50}) during experimental ischaemia in control (●) and glibenclamide-treated preparations (○). Normalized APD_{-50} values are on the ordinate scale. Values are expressed as mean with s.e. shown by vertical lines. * $P < 0.05$ from each control value by paired Student's t test. † $P < 0.05$ from the control group by unpaired Student's t test for the normalized data.

−50 mV level (APD_{-50}) was shortened by 39 ± 3% ($P < 0.001$) from its control value (164 ± 5 ms) after 30 min of myocardial ischaemia (Figure 6c). As shown in Figure 6b, the APD shortening was less in glibenclamide-treated preparations than in control preparations although RMP was decreased to almost the same extent. In the presence of 20 μM glibenclamide, the APD_{-50} shortening at 30 min ischaemia was 23 ± 4% ($n = 5$), which was significantly smaller than that observed in control preparations. Thus, glibenclamide could also lessen the action potential shortening during experimental ischaemia in canine, coronary-perfused isolated myocardium although the drug could not completely abolish it.

Discussion

In cardiac cells it was revealed that metabolic blockade with dinitrophenol (DNP) or cyanide induced time-independent macroscopic outward K^+ current (Noma & Shibasaki, 1985; Escande, 1989) and opened ATP-sensitive K^+ channels (Trube & Hescheler, 1984). From these findings it has been postulated that activation of the K^+ channels is responsible for the shortening of the action potential in hypoxic and ischaemic myocardium. However, confirmation of this hypothesis has been hampered by lack of a suitable experimental method to record the activities of ATP-sensitive K^+ channels from hypoxic or ischaemic cells. In the present study, we used three models to produce the action potential shortening and examined effects of ATP-sensitive K^+ channel blockers on the shortening in attempting to evaluate indirectly an involvement of the K^+ channels. In addition to hypoxic and ischaemic models, we used a DNP, no glucose model since it has been directly proved that this condition activates ATP-sensitive K^+ channels in guinea-pig isolated ventricular cells (Trube & Hescheler, 1984).

Belles *et al.* (1987) reported that tolbutamide at a concentration of 2 mM inhibited the opening of ATP-sensitive K^+ channels in excised patches of guinea-pig ventricular cells in an ATP-free solution. They also showed that this concentration of tolbutamide maximally inhibited the outward current activated by the washout of intracellular ATP in guinea-pig isolated ventricular cells. Accordingly, we chose the concentration of 2 mM tolbutamide in this study because we could not increase its concentration due to its limited solu-

bility. Glibenclamide is a more potent sulphonylurea which is reported to produce the maximal inhibition of the ATP-sensitive K^+ current in pancreatic β-cells in a concentration of 0.03 μM (Zünkler *et al.*, 1988). It has also been reported that glibenclamide in concentrations less than 0.1 μM completely inhibited the opening of ATP-sensitive K^+ channels in excised patches of cardiac myocytes in a solution containing 1 μM ATP (Fosset *et al.*, 1988) and the pinacidil-induced outward current (Arena & Kass, 1989). In the present study we used 20 μM glibenclamide, because preliminary experiments revealed that the inhibitory effect on the action potential shortening during hypoxia or metabolic blockade was saturated at concentrations of 5–10 μM. Consistent with the studies of Belles *et al.* (1987) and Fosset *et al.* (1988), tolbutamide and glibenclamide decreased the opening probability of ATP-sensitive K^+ channels without affecting single channel conductance. We observed that glibenclamide and tolbutamide at these concentrations exerted an inhibitory action of almost the same potency on K^+ channels in the open cell-attached patches exposed to an ATP-free solution. On the other hand, 100 μM pinacidil significantly increased the opening probability in the presence of 0.3 mM ATP, which is consistent with recent whole cell clamp studies (Arena & Kass, 1989a; Martin & Chinn, 1990) and single channel recording studies (Arena & Kass, 1989b; Fan *et al.*, 1990). In this study 100 μM pinacidil produced an action potential shortening and a negative inotropic response in guinea-pig papillary muscles, probably resulting from activation of ATP-sensitive K^+ channels. The action potential shortening produced by pinacidil was completely antagonized by 2 mM tolbutamide or 20 μM glibenclamide, suggesting that the ATP-sensitive K^+ channel blockers can penetrate the cell membrane and inhibit opening of the K^+ channels in intact papillary muscles.

In the present study, oxidative phosphorylation was inhibited in guinea-pig papillary muscles by superfusion with hypoxic solution or DNP-containing solution, concomitantly with glycolytic inhibition by substrate withdrawal. Consistent with a previous report (Hayashi *et al.*, 1987), these insults resulted in a marked shortening of the action potential, a decrease in twitch tension and muscle contracture. When guinea-pig isolated hearts were perfused with hypoxic, glucose-free solution for 60 min and glucose-free, DNP (10 μM)-containing solution for 45 min, myocardial ATP content decreased from 3.0 ± 0.2 to 0.5 ± 0.2 and 1.1 ± 0.2 mmol kg^{-1} wet weight, respectively (Nakaya *et al.*, unpublished observations). The estimated myoplasmic ATP levels would be higher than the apparent K_m (0.5–0.05 mM) for channel inactivation by ATP (Kakei *et al.*, 1985; Thuringer & Escande, 1989; Nichols & Lederer, 1990). Unless the sensitivity of ATP-sensitive K^+ channels to ATP concentration is markedly reduced by some other alterations of the internal environment, it seems unlikely that a decrease in bulk ATP exclusively can explain the action potential shortening during metabolic blockade or hypoxia. Support for this concept derives from a recent study showing that in ferret isolated hearts the shortening of monophasic action potential during metabolic blockade occurred without any large change in myocardial ATP measured by nuclear magnetic resonance (Elliot *et al.*, 1989). However, it may be argued that ATP generated locally at the sarcolemma by membrane-bound glycolytic enzymes may be a determinant factor for the regulation of ATP-sensitive K^+ channels (Weiss & Lamp, 1987; 1989). Therefore, it would be impossible to exclude the possible involvement of ATP-sensitive K^+ channels in the action potential shortening from the biochemical data of myocardial ATP content.

In the present study, tolbutamide and glibenclamide differently influenced the shortening of the action potential induced by hypoxia and metabolic blockade. It is far from clear why tolbutamide aggravated the action potential shortening during hypoxia but hardly affected it during metabolic blockade. It was reported that tolbutamide stimulates glucose utilization and glycolytic flux in the myocardium (Kramer *et al.*, 1983; Tan *et al.*, 1984) and inhibits oxidative phosphorylation in heart mitochondria (Katsumata & Hagiwara, 1973). Therefore, some modifications by tolbutamide of intracellular metabolism might exert different influences on the hypoxia- and DNP-induced APD-shortening, in addition to its inhibitory action on the ATP-sensitive K^+ channels.

Pretreatment with glibenclamide reduced significantly and to the same extent the shortening of the action potential during hypoxia, metabolic blockade and experimental ischaemia. These findings may imply that openings of ATP-sensitive K^+ channels might occur from the early phase of myocardial hypoxia or ischaemia. A similar conclusion was drawn by Sanguinetti *et al.* (1988) who showed that glibenclamide in concentrations of 0.2–10 μM prevented the shortening of the effective refractory period and action potential duration induced by 15 min hypoxia in ferret papillary muscles. However, the sulphonylurea failed to abolish completely the action potential shortening induced by these interventions of longer duration, although the drug at the same concentration effectively antagonized the pinacidil-induced shortening of the action potential. A preliminary report (Rials *et al.*, 1990) has also demonstrated that glibenclamide failed to prevent the shortening of the action potential during myocardial ischaemia *in vivo*. Therefore, changes in membrane current(s) other than the outward current through the ATP-sensitive K^+ channels might also contribute to the action potential shortening during myocardial hypoxia or ischaemia. One possibility may be a decrease in the calcium current, which is also sensitive to intracellular ATP concentration (Irisawa & Kokubun, 1983). Enhancement of the delayed rectifier K^+ current resulting from increased intracellular Ca^{2+} concentration (Tohse, 1990) and activation of the K^+ current induced by an increase in intracellular Na^+ concentration (Kameyama *et al.*, 1984) may also be candidates responsible for the shortening of the action potential in the hypoxic or ischaemic myocardium.

Treatment with glibenclamide, but not tolbutamide, lessened the decrease in twitch tension during hypoxia or metabolic blockade. The protective effect of glibenclamide against the contractile failure was in parallel with the inhibitory effect on the action potential shortening, suggesting that the inflow of the calcium current during the maintained plateau phase is responsible for the effect. Therefore, it is possible that glibenclamide may partially attenuate the contractile failure in ischaemic myocardium. However, it may not be beneficial for the ultimate injury of ischaemic myocardium because the maintained contraction may lead to an increase in energy consumption and calcium overload of ischaemic myocytes. These considerations are only speculative and further studies focused on this point are needed.

In conclusion, the action potential shortening produced by hypoxia, metabolic blockade or experimental ischaemia was lessened, but not completely abolished by the ATP-sensitive K^+ channel blocker, glibenclamide. The underlying mechanisms of the action potential shortening are undoubtedly complex, and changes in membrane currents other than the outward current through the ATP-sensitive K^+ channels may also contribute to the action potential shortening.

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Involvement of capsaicin-sensitive nerves in the bronchomotor effects of arachidonic acid and melittin: a possible role for lipoxin A_4

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1 Functional studies have been performed to evaluate the potential involvement of capsaicin-sensitive nerves in the bronchomotor responses evoked by lipid mediators produced from the metabolic breakdown of arachidonic acid (AA) in the guinea-pig bronchus.

2 In the presence of indomethacin, the exogenous administration of AA (0.01–1 mM) produced a concentration-dependent contractile response in guinea-pig isolated bronchial rings. AA-induced contractions were augmented by epithelium-removal and by thiorphan (10 μM), an inhibitor of tachykinin breakdown. A sustained downward and rightward displacement of the complete concentration-response curve to AA was observed after *in vitro* capsaicin desensitization.

3 BWA4C (1 μM), a selective inhibitor of 5-lipoxygenase, shifted the AA concentration-response curve to the right. In the presence of this inhibitor, capsaicin desensitization did not have any further inhibitory action.

4 A potent, concentration-dependent and capsaicin-sensitive bronchoconstrictor effect was also observed with the polypeptide, melittin (10 nM–1 μM), an activator of phospholipase A_2, which therefore should generate endogenous AA.

5 *In vitro* capsaicin-desensitization produced a significant reduction of the bronchomotor responses evoked by lipoxin A_4 (1–6 μM), but not of those elicited by other lipoxygenases products such as leukotriene D_4 (1–100 nM) or by 15-hydroxyeicosatetraenoic acid (15-HETE, 1–6 μM).

6 These findings indicate that lipoxin A_4 but not leukotriene D_4 or 15-HETE, might be one of the lipoxygenase mediators of excitatory effects of AA on capsaicin-sensitive sensory nerves.

Keywords: Sensory nerves; capsaicin; lipid mediators; sensory neuropeptides; asthma

Introduction

A number of mediators have been implicated in asthma pathophysiology and among them a prominent role has been suggested for lipid mediators, such as platelet-activating factor (PAF) and leukotrienes, and for neuropeptides, such as tachykinins, calcitonin gene-related peptide (CGRP) or vasoactive intestinal peptide (VIP) (Barnes, 1987; 1989a,b). Recently some evidence has been presented which suggests a possible interaction between these two classes of biological mediators. PAF has been shown to release VIP and substance P from guinea-pig isolated lung strip (Rodrigue *et al.*, 1987). In addition, some of the motor effects of leukotriene D_4 may involve the release of sensory neurotransmitters (Stewart *et al.*, 1984; Bloomquist & Kream, 1987). In contrast, CGRP and VIP inhibit the release and/or biosynthesis of peptidoleukotrienes in lung preparations (Di Marzo *et al.*, 1987; 1988). Manzini *et al.* (1989) obtained functional and neurochemical evidence that suggested in guinea-pig, the exogenous administration of arachidonic acid (AA) might produce bronchomotor effects partially through the release of sensory neuropeptides. Oxidation of arachidonic acid leads to the formation of a plethora of eicosanoids, including the cyclo-oxygenase and lipoxygenase products, that have been shown to cause bronchoconstriction, vasoconstriction and increased vascular permeability (Barnes, 1989b). Since some of our previous experiments were performed in the presence of indomethacin, we proposed the hypothesis that products of AA metabolic breakdown via the lipoxygenase pathways might activate the local efferent function of capsaicin-sensitive nerves in guinea-pig airways (Manzini *et al.*, 1989, Manzini & Meini, 1990). In this study we have investigated further the effect of capsaicin-desensitization on the motor response to AA in the presence of inhibitors of cyclo-oxygenase and/or lipoxygenases. Furthermore, we have assessed whether or not the motor effects of AA are affected by epithelium removal or by inhibition of neutral endopeptidases, which increase responses to tachykinins (Maggi *et al.*, 1990). Finally, we have studied the effect of capsaicin desensitization on the bronchomotor actions produced by the polypeptide melittin, a selective activator of phospholipase A_2 (Mollay *et al.*, 1976; Shier, 1979), or by 5 and/or 15-lipoxygenases metabolites, such as leukotriene D_4, 15-hydroxyeicosatetraenoic acid (15-HETE) or lipoxin A_4 (Serhan *et al.*, 1984; Samuelsson *et al.*, 1987; Sigal & Nadel, 1988).

Part of these data have been presented in a preliminary report (Manzini *et al.*, 1989) and communicated at the XIth International Congress of Pharmacology (Manzini & Meini, 1990).

Methods

Male albino guinea-pigs (Rodentia, Torre Pallavicina, Bergamo, Italy) weighing 300–350 g were used throughout the study. Animals were stunned, exsanguinated and the lungs with attached bronchi and trachea were removed quickly. Main bronchi were dissected from the surrounding parenchyma and two (2 mm long) epithelium-free rings were prepared by gently rubbing the luminal surface with a moistened pipe cleaner. In some preparations removal of epithelium was confirmed histologically. Bronchial rings were mounted on two 'L'-shaped stainless steel holders and placed in 5 ml organ baths containing physiological salt solution, (at 37°C and oxygenated with 95% O_2 and 5% CO_2), of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2 2H_2O$ 2.5, $MgCl_2 6H_2O$ 0.5, $NaH_2PO_4 H_2O$ 1.0, $NaHCO_3$ 25 and glucose 10. Contractile responses were recorded with an isometric force transducer and displayed on a Basile Unirecord 7050 polygraph. An optimal resting tension of 750 mg was applied to the preparations. Each preparation was allowed to

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equilibrate for at least 45 min before the start of the experiments. Inhibitors of cyclo-oxygenase and/or lipoxygenase were added to the organ bath at least 45 min before challenge with AA.

In some experiments concentration-response curves (CRC) to AA, melittin and lipoxygenase products were obtained in a cumulative manner, the next concentration being added when the response to the previous one had reached a steady state. Two main bronchi obtained from the same animal were set up in parallel. One was used for the control CRC, the other for CRC after capsaicin-desensitization. Capsaicin-desensitization was performed by incubating the bronchial rings with capsaicin (10 μM) for 15 min followed by several washes before constructing the CRC to the bronchoconstrictor agent.

Analysis and statistical evaluation of results

All data in the text are mean ± standard error of the mean (s.e.mean). Motor effects were measured either as mg of contraction or normalized to the maximal motor response induced, in the same preparation, by KCl (60 mM).

Statistical analyses were performed by Student's t test for paired or unpaired data, when appropriate, with a chosen probability value of 0.05 for significance.

Drugs

Drugs used were the following: arachidonic acid (Sigma), capsaicin, indomethacin and leukotriene D_4 (Serva), carbachol (Merck), lipoxin A_4 and 15(**RS**)-hydroxyeicosatetraenoic acid (15(**RS**)-HETE) (Cascade Biochem), thiorphan (Peninsula), melittin (Bachem) and BW A4C (Wellcome, U.K.).

Solutions of peptides and eicosanoids were made daily in distilled water from stock solutions (kept frozen) and kept on ice during the experiment. Capsaicin (10 mM) and indomethacin (2 mM) were dissolved in absolute ethanol; sodium arachidonate (10 mM) was dissolved as a 10 mM stock solution in water and stored under N_2 at −20°C. Further drug dilutions were made in distilled water. Ethanol did not affect tissue responses at the concentrations used.

Results

Effect of capsaicin desensitization on arachidonic acid-induced bronchomotor response in the presence of indomethacin or BW A4C

In isolated bronchial rings (with epithelium) the administration of exogenous AA (1 mM) produced weak and erratic contractions ($n = 8$). On the other hand, in the presence of the cyclo-oxygenase inhibitor indomethacin (5 μM), AA (1 mM) consistently elicited a sustained contraction with an amplitude, at steady state, of 323 ± 17 mg ($n = 33$) (Figure 1). In the same preparation, such responses were reproducible at 90 min intervals ($n = 6$). Exposure of bronchial rings to capsaicin (10 μM) elicited a substantial contraction (536 ± 46 mg) which could be evoked only once in each preparation, indicating desensitization ($n = 6$). Following capsaicin challenge the response to AA was significantly reduced to 108 ± 34 mg ($n = 6$; $P < 0.05$; Figure 1) (see also Manzini *et al.*, 1989). A similar reduction was not observed when bronchial rings were previously challenged with a KCl concentration (60 mM) equieffective to capsaicin ($n = 3$; data not shown). Furthermore, capsaicin desensitization had no effect on the motor response to carbachol (0.3 μM) ($n = 3$, Figure 1).

In the presence of indomethacin, it is also possible to obtain a cumulative CRC to AA (0.01–1 mM). Previous capsaicin challenge produced a rightward and downward shift of the CRC to AA (Figure 2). The EC_{50} for AA increased from 196 μM (172–229) to 508 μM (435–610) ($n = 6$).

In a further set of experiments we have assessed the effect of capsaicin desensitization on the CRC to AA obtained in the presence of indomethacin and BW A4C, a selective inhibitor of 5-lipoxygenase (Tateson *et al.*, 1988). In the presence of BW A4C (1 μM, incubated 45 min before and during AA administration) the CRC to AA was shifted to the right

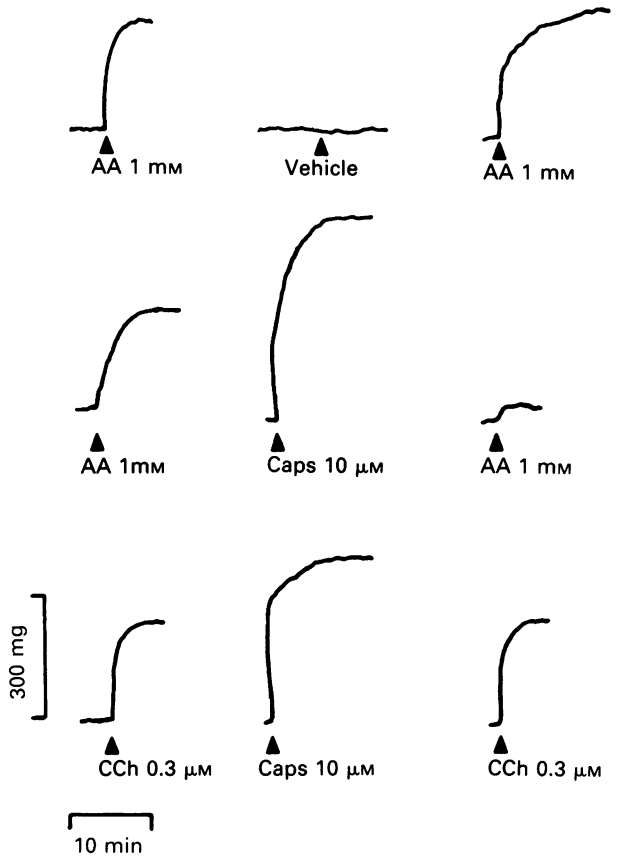

Figure 1 Typical tracings showing the motor response (mg) elicited by arachidonic acid (AA) or carbachol (CCh) in guinea-pig isolated bronchus before or after capsaicin (Caps) challenge. In the same preparations a second application of capsaicin did not exert any contractile effect. Experiments were performed in the presence of indomethacin (5 μM).

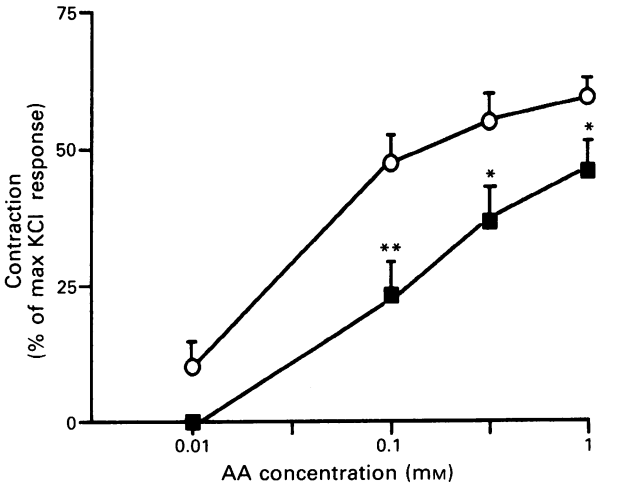

Figure 2 Concentration-response curves to arachidonic acid (AA, 0.01–1 mM) in guinea-pig epithelium-denuded bronchial rings in control conditions (○) and in preparations previously challenged with a desensitizing concentration (10 μM incubated for 15 min) of capsaicin (■). Experiments were performed in the presence of indomethacin (5 μM). Statistical evaluation was performed with Student's t test for unpaired data (* $P < 0.05$; ** $P < 0.01$). Vertical axis: contractile effect expressed as % of maximum contraction produced by KCl (60 mM). Horizontal axis: arachidonic acid concentration.

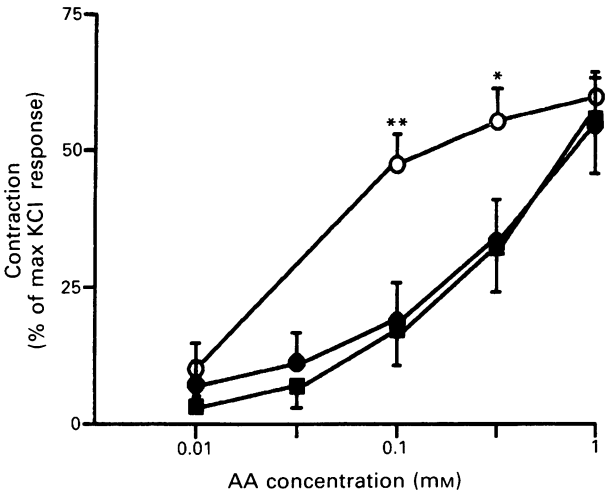

Figure 3 Concentration-response curves to arachidonic acid (AA) in guinea-pig epithelium-denuded bronchial rings in control conditions (circles) and in preparations previously challenged with a desensitizing concentration of capsaicin (10 μM incubated for 15 min) (squares). Experiments were performed in presence of only indomethacin (5 μM) (open symbols) and either indomethacin and BW A4C (1 μM) (solid symbols). Statistical evaluation was performed with Student's t test for unpaired data (* $P < 0.05$; ** $P < 0.01$ versus BW A4C). Vertical axis: contractile effect expressed as % of maximum contraction produced by KCl (60 mM). Horizontal axis: arachidonic acid concentration.

(Figure 3). The EC_{50} increased from 196 μM (172–229) to 389 μM (361–422) ($n = 6$). Interestingly, when 5-lipoxygenase was inhibited, capsaicin-desensitization no longer affected the motor response to exogenous AA [EC_{50}: 389 μM (344–447)] (Figure 3). BW A4C (1 μM) itself had no effect on baseline tone ($n = 12$).

Effect of epithelium removal and inhibition of neutral endopeptidases on arachidonic acid-induced bronchomotor responses

AA (1 mM)-induced motor responses were different in control and in epithelium-denuded preparations (always in the presence of indomethacin). Epithelium removal significantly increased the amplitude of AA-induced bronchomotor responses from 255 ± 27 mg to 368 ± 40 mg ($n = 6$; $P < 0.05$). In a further set of experiments we have investigated the effect of the neutral endopeptidase inhibitor, thiorphan (10 μM) on the motor response elicited by AA (1 mM, in the presence of indomethacin). Thiorphan was used at a concentration previously demonstrated to enhance markedly the motor response to capsaicin and tachykinins (Maggi *et al.*, 1990). Figure 4 shows typical tracings where in the presence of thiorphan, AA-induced contractions were more rapid in onset, similar in amplitude (258 ± 29 mg and 399 ± 94 mg in absence or in presence of thiorphan respectively, $n = 4$, not significant) and were significantly sustained for longer periods. In control preparations the motor responses to AA declined by 44 ± 5% after 80 min, whereas, in presence of thiorphan, responses declined by only 23 ± 5% after 80 min ($n = 4$, $P < 0.05$, Figure 4).

Effect of capsaicin desensitization on bronchomotor responses elicited by melittin

In the presence of indomethacin (5 μM), thiorphan (10 μM) and in the absence of epithelium, melittin (10 nM–1 μM) was an extremely potent and effective bronchoconstrictor agent (Figure 5). It induced contractions starting at a concentration as low as 10 nM and, at the maximal concentration tested (1 μM), it produced a contraction of 688 ± 123 mg ($n = 6$). After capsaicin pretreatment a marked rightward and downward displacement of the CRC to melittin was observed

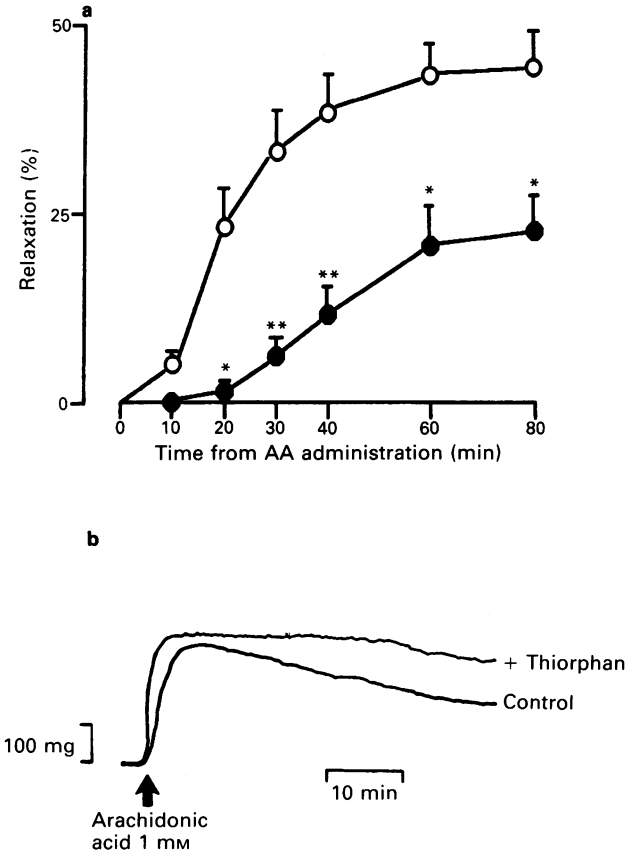

Figure 4 Effect of thiorphan (10 μM) on arachidonic acid (AA, 1 mM)-induced contraction in guinea-pig bronchial rings. Time decay of tonic contraction in control conditions (○) and in presence of thiorphan (●) is shown in (a). Vertical axis: contractile effect expressed as % of relaxation of obtained tone. Horizontal axis: time (min). In (b) the tracings represent two typical motor responses (mg) obtained in two bronchi excised from the same animal. All experiments were carried out in presence of indomethacin (5 μM). Statistical evaluation was performed with Student's t test for unpaired data (* $P < 0.05$; ** $P < 0.01$).

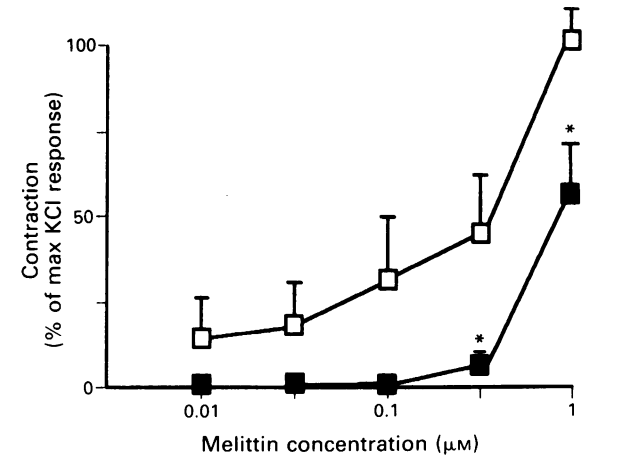

Figure 5 Cumulative concentration-response curves of the bronchomotor responses elicited on guinea-pig bronchial rings by melittin (0.01–1 μM) in control conditions (□) and in preparations pretreated with a desensitizing concentration of capsaicin (10 μM incubated for 15 min) (■). All experiments were performed in epithelium-denuded preparations and in the presence of indomethacin (5 μM) and thiorphan (10 μM). Statistical evaluation was performed with Student's t test for unpaired data (* $P < 0.05$). Vertical axis: contractile effect expressed as % of maximum contraction produced by KCl (60 mM). Horizontal axis: melittin concentration.

(Figure 5). After capsaicin challenge a concentration of melittin as high as 1 μM was required to produce an appreciable contraction (Figure 5).

Effect of capsaicin desensitization on bronchomotor responses elicited by leukotriene D_4, 15-HETE or lipoxin A_4

In this set of experiments we studied the effect of capsaicin desensitization on the motor response produced by three eicosanoids previously found to exert bronchomotor responses in guinea-pigs, i.e. leukotriene D_4 (LTD_4) (Dahlen *et al.*, 1980), 15-HETE (Copas *et al.*, 1982) and lipoxin A_4 (Serhan *et al.*, 1986; Jacques *et al.*, 1988).

LTD_4 (1–100 nM) was a potent and effective bronchospastic agent ($n = 4$; Figure 6). Its EC_{50} was similar in control and in capsaicin-pretreated bronchial preparations being 24 nM (20–29) and 15 nM (12–20), respectively ($n = 4$; Figure 6).

15-HETE was (at least up to the maximum concentration tested) a rather weak contractile agent. At the maximum concentration tested (6 μM) it produced a contraction with an amplitude of 293 $\pm$ 103 mg in control and 220 $\pm$ 46 mg in capsaicin-pretreated preparations (Figure 7a; $n = 4$; not significant).

Lipoxin A_4 produced a fairly rapid and sustained contraction (Figure 8). Albeit less potent than LTD_4 this lipid produced, at the maximum concentration tested (6 μM), a contraction with an amplitude of 512 $\pm$ 33 mg ($n = 4$), similar to that produced by LTD_4 (100 nM) or by KCl (60 mM). Lipoxin A_4-induced contractions were significantly (Figure 7b) reduced in capsaicin-pretreated bronchial rings (Figures 7 and 8). After capsaicin desensitization lipoxin A_4 produced a small response (172 $\pm$ 40 mg, $n = 4$) only at the maximum concentration tested (6 μM).

Discussion

Local release of sensory neuropeptides from capsaicin-sensitive sensory nerve endings can promote plasma protein extravasation, mucus secretion and bronchoconstriction. Such a neurogenic mechanism may play a relevant role in asthma pathophysiology (Barnes, 1986). A number of endogenous substances (bradykinin, histamine, nicotinic agonists) induce at least part of their pulmonary effects through activation of the local efferent function of capsaicin-sensitive nerves (Saria *et al.*, 1988; Ichinose *et al.*, 1990). We have presented evidence

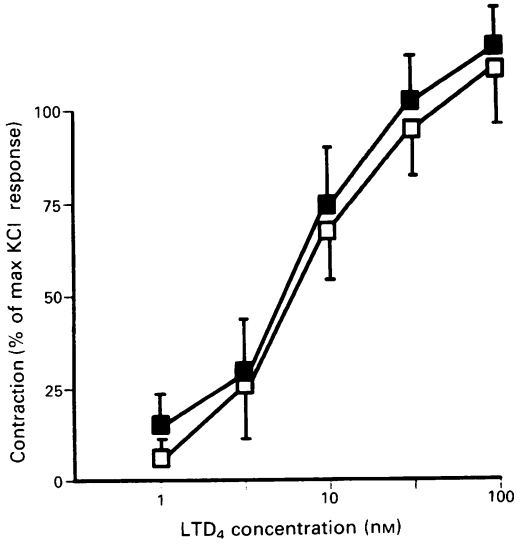

Figure 6 Cumulative concentration-response curves of the bronchomotor responses elicited on guinea-pig bronchial rings by leukotriene D_4 (LTD_4, 1–100 nM) in control conditions (□) and in preparations pretreated with a desensitizing concentration of capsaicin (10 μM incubated for 15 min) (■). All experiments were performed in epithelium-denuded preparations and in the presence of indomethacin (5 μM) and thiorphan (10 μM). No significant statistical difference was observed. Vertical axis: contractile effect expressed as % of maximum contraction produced by KCl (60 mM). Horizontal axis: LTD_4 concentration.

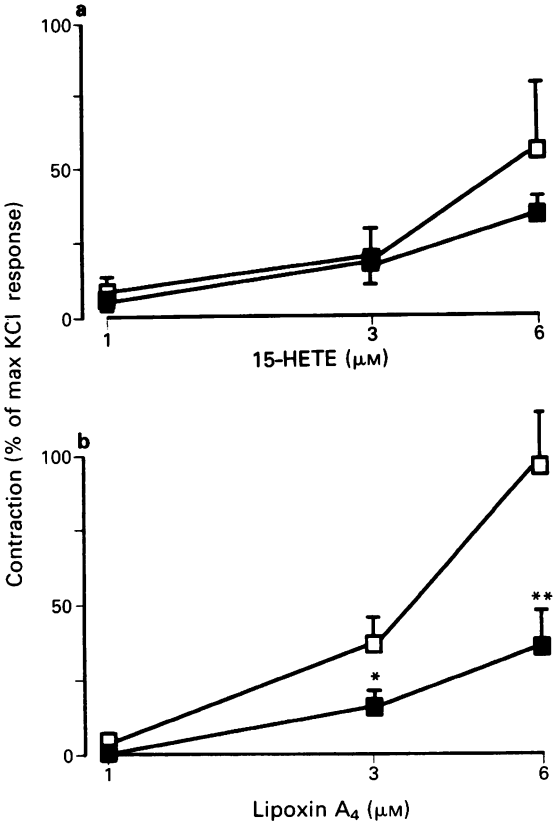

Figure 7 Cumulative concentration-response curves of the bronchomotor responses elicited on guinea-pig bronchial rings by 15-eicosatetraenoic acid (15-HETE, 1, 3 and 6 μM) (a) or lipoxin A_4 (1, 3, 6 μM) (b) in control conditions (□) and in preparations pretreated with a desensitizing concentration of capsaicin (10 μM incubated for 15 min) (■). All experiments were performed in epithelium-denuded preparations and in the presence of indomethacin (5 μM) and thiorphan (10 μM). Statistical evaluation was performed with Student's t test for unpaired data (* $P < 0.05$; ** $P < 0.01$). Vertical axis: contractile effect expressed as % of maximum contraction produced by KCl (60 mM). Horizontal axis: 15-HETE or lipoxin A_4 concentration.

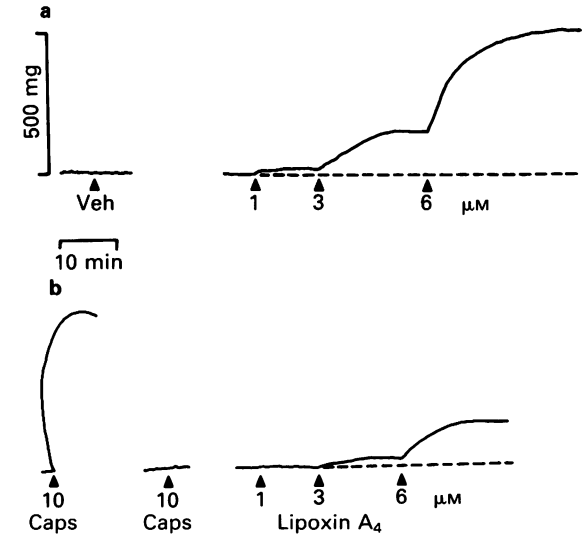

Figure 8 Typical tracings of the motor responses (mg) elicited on guinea-pig bronchial ring by lipoxin A_4 (1, 3 and 6 μM) in control (a) and in a preparation previously desensitized with capsaicin (Caps 10 μM incubated for 15 min) (b). The two bronchial preparations were taken from the same animal. Veh = vehicle.

that some lipid mediators generated by the oxidation (via the lipoxygenase pathways) of arachidonic acid might also act through such a mechanism (Manzini *et al.*, 1989, Manzini & Meini, 1990; this study). This is supported by the following evidence: (a) AA-induced bronchomotor responses are reduced both *in vitro* and *in vivo* by previous capsaicin desensitization; (b) AA induces a capsaicin-sensitive release of sensory neuropeptides from isolated rubbed bronchial rings; (c) AA-induced motor responses are enhanced by interventions, such as epithelium removal or neutral endopeptidase inhibition, which amplify biological responses elicited by local release of sensory neuropeptides (Maggi *et al.*, 1990). It is interesting to note that capsaicin desensitization markedly reduced not only the motor responses produced by exogenously added AA, but also those evoked by melittin, a proposed selective activator of phospholipase A_2 and/or releaser of endogenase AA (Mollay *et al.*, 1976; Shier, 1979). Melittin is a polypeptide extracted from bee venom (Habermann, 1972) which produces endothelium-dependent vasorelaxation (Rapoport *et al.*, 1989), an effect that is prevented by inhibition of phospholipase A_2 and by lipoxygenase, but not by cyclo-oxygenase, inhibitors (Forstermann & Neufang, 1985). It is clear that melittin-induced bronchoconstriction might be attributed not only to AA-derived eicosanoids but also to other lipids the synthesis of which might follow after activation of phospholipase A_2. In this regard it is interesting to note that it has recently been shown that PAF-induced airway hyperresponsiveness in rabbits is abolished by previous *in vivo* treatment with capsaicin (Coyle *et al.*, 1990).

In this paper we have utilized the *in vitro* technique of capsaicin desensitization (Barthò *et al.*, 1987; Maggi *et al.*, 1987) to investigate the capsaicin-sensitive component of AA-induced bronchomotor responses. *In vitro* exposure to high concentrations of capsaicin desensitized a particular subset of primary afferents rendering them resistant to subsequent challenge with capsaisin itself or other stimuli (Maggi & Meli, 1988). Therefore, this procedure permits the identification of agents with actions which might rely upon activation of the efferent function of these nerves. The involvement of capsaicin-sensitive nerves in the response to eicosanoids appears to be rather specific, in fact capsaicin desensitization is effective in the presence of indomethacin but not when the lipoxygenase pathways or even only the 5-lipoxygenase pathway are pharmacologically blocked with BW755C (Manzini & Meini, 1990) or BW A4C (this study). Furthermore, among various eicosanoids, the synthesis of which involves the 5-lipoxygenase pathway, and which have been shown to exert bronchomotor effects, only the motor response to lipoxin A_4, and not those exerted by LTD_4 or 15-HETE, is reduced by previous capsaicin challenge.

These data suggest that lipoxin A_4 might be relevant in lung pathophysiology not only for its direct inflammatory, vasodilator and motor effects (Dahlen *et al.*, 1988) but also as an endogenous activator of sensory nerves. Lipoxin A_4 contracts lung parenchymal strips of various species, including man (Dahlen *et al.*, 1988; Jacques *et al.*, 1988; Sigal & Nadel, 1988). In guinea-pig lung part of its contractile actions are mediated by generation of thromboxane A_2 (Dahlen *et al.*, 1988), but in the present study this is unlikely, since indomethacin was present throughout. Lipoxin A_4 also activates directly protein kinase C (Hansson *et al.*, 1986), and this biochemical action can produce depolarization of nonmyelinated C fibres (Rang & Ritchie, 1988). Further studies are necessary to clarify whether or not lipoxins activate sensory nerves by stimulation of specific receptors on the outer membrane and/or by activation of some intracellular biochemical mechanisms. Interestingly, lipoxin A_4 can be generated by neutrophils (Serhan, 1989), alveolar macrophages, eosinophils and, possibly, epithelial cells (Sigal & Nadel, 1988). Significant amounts of lipoxin A_4 have been detected in bronchoalveolar lavage fluids obtained from patients with lung diseases but not from healthy volunteers (Lee *et al.*, 1990). In view of the above, it is reasonable to speculate that eosinophila and epithelial sheeding, typical hallmarks of airway hyperreactivity and asthmatic bronchial biopsies (Barnes, 1989a), can lead to an increased local production of lipoxins which in turn contributes to the activation of sensory nerves, thus producing neurogenic inflammation.

In conclusion our studies indicate that, in guinea-pig bronchus, some lipid mediators (the synthesis of which involves the 5-lipoxygenase pathway) can interact with capsaicin-sensitive sensory nerves, and therefore, that at least part of the motor effects produced by AA might rely indirectly upon the local release of sensory neuropeptides. Among the eicosanoids, lipoxin A_4 appears to be one of the most likely candidates which activates capsaicin-sensitive nerves in guinea-pig bronchial tissues. The pharmacological control of the interaction between inflammatory mediators and sensory nerves may represent a relevant target for new strategies in treatment of asthma.

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The diverse effects of cromakalim on tension and ^{86}Rb efflux in canine arterial smooth muscle

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1 To characterize further the K^+ channels opened by cromakalim in arterial smooth muscle, the effects of cromakalim on tension and ^{86}Rb efflux were compared in endothelium-denuded strips of coronary, mesenteric and middle cerebral (MC) arteries of the dog.

2 Cromakalim relaxed strips precontracted with 20.9 mM K^+. The maximum relaxation induced by cromakalim varied in the arteries used; 94% in the coronary artery, 60% in the mesenteric artery and only 38% in the MC artery. Cromakalim failed to relax arterial strips precontracted with 65.9 mM K^+.

3 When the effects of cromakalim on ^{86}Rb efflux were determined in 20.9 mM K^+-contracted strips, cromakalim-induced relaxations were accompanied by a large increase in ^{86}Rb efflux in the coronary artery, by a small increase in the mesenteric artery but by an apparent decrease in the MC artery.

4 When 10^{-7} M nifedipine was added to 20.9 mM K^+-contracted strips, to inactivate Ca^{2+}-activated K^+ (K_{Ca}) channels, cromakalim produced a greater increase (measured from the point at which cromakalim was administered) in ^{86}Rb efflux than in the absence of nifedipine, suggesting that the effects of cromakalim on ^{86}Rb efflux from the 20.9 mM K^+-contracted strips may be the resultant of two opposing effects: an increased ^{86}Rb efflux perhaps due to the opening of ATP-sensitive K^+ (K_{ATP}) channels, and a decreased efflux due to the closing of K_{Ca} channels.

5 After the inactivation of K_{Ca} channels in 20.9 mM K^+-contracted strips, the cromakalim-induced increase in ^{86}Rb efflux measured as area under the curve was eight times greater in the coronary artery than in the MC artery. The increase in ^{86}Rb efflux in the mesenteric artery was intermediate between these extremes.

6 Cromakalim also increased the ^{86}Rb efflux from 65.9 mM K^+-contracted strips. This increase was not augmented by the addition of nifedipine. Under these conditions, a similar variation in efflux response (as area under the curve) for cromakalim was noted in the arteries used.

7 The relaxant responses of each artery to cromakalim were competitively antagonized by glibenclamide, a blocker of K_{ATP} channels. The cromakalim-induced increase in ^{86}Rb efflux was also inhibited by glibenclamide.

8 These results suggest that cromakalim-opened K^+ channels in the three arteries may differ in terms of their frequency of occurrence in the plasmalemma, their permeability to ^{86}Rb and their ability to modulate tension development. On the other hand, the activities of K_{Ca} and voltage-sensitive K^+ channels, estimated from the effects of nifedipine, were similar in the three arteries.

Keywords: K^+ channel opener; cromakalim; arterial smooth muscle; arterial relaxation; ^{86}Rb efflux; ATP-sensitive K^+ channels; Ca^{2+}-activated K^+ channels

Introduction

K^+ channels of vascular smooth muscle cells play an important role in determining the resting membrane potential and regulating contractility. Vascular smooth muscle contractions have been demonstrated to be associated with the opening of K^+ channels (Bolton & Clapp, 1984; Casteels & Droogmans, 1985; Aaronson & Jones, 1985). Some of these channels are sensitive to membrane potential (voltage-sensitive K^+ channels), and others are regulated by intracellular Ca^{2+} concentration (K_{Ca} channels). By comparing the effects of noradrenaline and elevated K^+ on the efflux of ^{86}Rb or ^{42}K and on tension in several arterial preparations, it has been demonstrated that these K^+ channels vary in their occurrence and properties from one artery to another (Bolton & Clapp, 1984).

In addition to these K^+ channels, Standen *et al.* (1989) have recently demonstrated the presence of K_{ATP} channels in arterial smooth muscle cells at the single channel level. They have further demonstrated that these channels were opened by the hyperpolarizing vasodilator, cromakalim, and inhibited by the K_{ATP} channel blocker glibenclamide. Cromakalim belongs to a new class of vasodilators, the mechanism of action of which is thought to involve the opening of K^+ channels in vascular smooth muscle. This mechanism of action has been proposed on the basis of the ability of cromakalim to increase ^{86}Rb (or ^{42}K) efflux from preloaded vascular smooth muscle preparations and to hyperpolarize the cell membrane (Hamilton *et al.*, 1986; Weir & Weston, 1986; Quast, 1987; Cook *et al.*, 1988a; Quast & Baumlin, 1988). In vascular smooth muscle preparations, cromakalim inhibits contractions induced by noradrenaline and low concentrations (below 30 mM) of K^+. Under conditions where vascular preparations are contracted with low K^+, the equilibrium potential for K^+ (E_K) is significantly more negative than the actual membrane potential and the threshold for the opening of voltage-dependent Ca^{2+} channels (VDCs). On the other hand, cromakalim is ineffective against contraction induced by high concentrations of K^+, probably because high K^+ reduces E_K to a value less negative than that required to ensure closure of VDCs. Under these conditions, the cromakalim-induced increase in K^+ permeability would not hyperpolarize vascular smooth muscle sufficiently to close VDCs. Recent pharmacological studies have demonstrated that a range of hypoglycaemic sulphonylureas, typified by glibenclamide, antagonize the vascular relaxant responses to cromakalim in a competitive fashion (Winquist *et al.*, 1989; Wilson, 1989; Cavero *et al.*, 1989; Buckingham *et al.*, 1989; Quast & Cook, 1989) and inhibited the cromakalim-induced increase in ^{86}Rb efflux from

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vascular smooth muscle preparations (Quast & Cook, 1989; Masuzawa *et al.*, 1990a). These electrophysiological and pharmacological studies clearly indicate that cromakalim is a potentially useful tool for investigating the properties and populations of K^+ channels in vascular smooth muscle.

The present study was designed to investigate whether cromakalim-opened K^+ channels in different arteries exhibit heterogeneity in their properties and populations. To this end, effects of cromakalim on tension and ^{86}Rb efflux were compared in coronary, mesenteric and middle cerebral (MC) arteries of the dog. Our results indicate that the distribution and properties of cromakalim-opened K^+ channels may be considerably different in the three arteries.

Methods

Preparation of arterial strips

Mongrel dogs of either sex weighing 7–12 kg were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.v.) and exsanguinated. The heart, the brain and the mesenteric artery with an *in situ* outside diameter of 0.6–0.8 mm were removed and placed in the physiological salt solution (PSS) of the following composition (mM): NaCl 115.0, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and dextrose 10.0. The left anterior descending coronary artery (0.6–1.0 mm) was dissected from the heart and the MC artery (0.6–0.8 mm) was dissected from the brain. Helical strips (0.8 mm in width and 7 mm in length) of these arteries were prepared as described previously (Asano *et al.*, 1987; 1988; Masuzawa *et al.*, 1990a, b). The endothelium of the strip was removed by gently rubbing the endothelial surface with cotton pellets.

Strips were mounted vertically between hooks in water-jacketed muscle baths containing 20 ml of the PSS. The PSS was maintained at 37°C and continuously bubbled with a mixture of 95% O_2 and 5% CO_2. The upper end of the strip was connected to a force-displacement transducer (TB-612T, Nihon Kohden Kogyo Co., Tokyo, Japan) for isometric tension recordings. Strips were stretched passively to optimal length by imposing resting tension (coronary, 0.8 g; mesenteric, 0.8 g; MC, 0.7 g). The optimal resting tension was determined by a length-passive tension study (Asano *et al.*, 1987). After application of the resting tension, strips were equilibrated for 90 min. All experiments were conducted on phenoxybenzamine-treated strips to eliminate possible α-adrenoceptor responses to noradrenaline released by the K^+-depolarization. To this end, strips were treated with 2×10^{-6} M phenoxybenzamine during the first 60 min of the 90 min equilibration period (Asano *et al.*, 1988; Masuzawa *et al.*, 1990a, b).

Relaxation of arterial strips

After the equilibration, contractile responses of the strips to the PSS containing 65.9 mM K^+ (K^+ substitution for Na^+) were repeated two or three times until the responses were reproducible. To determine the relaxant responses to cromakalim, strips were contracted by the addition of 15 mM K^+ to the PSS (total K^+ concentration; 20.9 mM) before challenge with this drug. Cumulative concentration-response curves for the relaxant responses to cromakalim were constructed. At the end of each experiment, 10^{-4} M papaverine was added to identify the position of the maximum relaxation. Relaxant responses to cromakalim are expressed as % of the papaverine-induced maximum relaxation.

Effects of glibenclamide on the relaxant responses to cromakalim were determined in 20.9 mM K^+-contracted strips as described previously (Masuzawa *et al.*, 1990a, b). Usually five strips from the same animal were prepared and subjected to different treatments; four strips were treated with glibenclamide (3×10^{-8} M, 1×10^{-7} M, 3×10^{-7} M or 1×10^{-6} M), while the control strip was treated with the vehicle (ethanol) for glibenclamide. Glibenclamide was added 20 min before the K^+-induced contractions. The data were subjected to a Schild plot analysis according to the method of Arunlakshana & Schild (1959), and the pA_2 value for glibenclamide and the slope of the line were determined from the regression analysis.

The relaxant responses to cromakalim were also determined in strips precontracted with 65.9 mM K^+.

Measurements of ^{86}Rb efflux and tension

Strips were mounted vertically on stainless-steel rods and allowed to equilibrate in the PSS for 90 min. Strips were then incubated for an additional 3 h in the PSS to which 14–20 μCi ml^{-1} ^{86}Rb had been added. The final concentration of $^{86}RbCl$ in the PSS was $4–12 \times 10^{-5}$ M. Each strip was then dipped three times (a total of 15 s) into non-radioactive PSS to remove excess radioactivity, and transferred to a temperature-controlled superfusion chamber similar to that described by Su & Bevan (1970). The upper end of the strip was connected to the force-displacement transducer as in the tension experiments. A resting tension of 2.4 g (coronary and mesenteric) or 2.1 g (MC) was applied because larger strips were used. Strips were superfused at a rate of 1 ml min^{-1} with the oxygenated PSS at 37°C. Strips were superfused for the next 30 min before application of the test drugs. The superfusate was sampled by use of a collection period of 2 min and counted for radioactivity in an Aloka autowell gamma counter. The radioactivity remaining in the strip at the end of an efflux sequence was determined by dissolving the strip in 0.5 ml of 1 N nitric acid and the volume of the sample was adjusted to 2.0 ml. The rate constant of ^{86}Rb efflux was then calculated as the radioactivity released from the strip per min at time t divided by the radioactivity remaining in the strip at that time (Imaizumi & Watanabe, 1981; Bolton & Clapp, 1984; Quast, 1987).

Drug effects on the efflux were generally calculated as the peak value of efflux rate constant obtained in the presence of the drug divided by the basal value of efflux rate constant averaged over 6–10 min before drug application. However, in strips of the mesenteric artery, the effect of cromakalim faded during application (see Figure 4) and the area under efflux sequence of the rate constant vs. time plot was chosen as a better measure of the drug effect. Areas were calculated from an increase in the efflux rate constant during the 10 min application time (Quast, 1987; Masuzawa *et al.*, 1990a, b). The efflux rate constant at the time when cromakalim was administered was used as a baseline for the drug effect (Masuzawa *et al.*, 1990a, b).

Statistical analysis

When assessing the EC_{50} value for cromakalim, responses to cromakalim were calculated as % of the maximum response obtained with the drug. The EC_{50} value was obtained from a plot of % response vs. log concentration of cromakalim and expressed as a negative log (pD_2 value).

Unless specified, results shown in the text, tables and figures are expressed as means ± s.e.mean (n = number of preparations). Statistical analysis of the data was performed by use of Student's t test for paired or unpaired data, or by completely randomized design, one-way analysis of variance followed by Newman-Keuls test for a significant F ratio ($P < 0.05$), depending on which test was statistically appropriate. Two groups of data were considered to be significantly different when $P < 0.05$.

Drugs and chemicals

The drugs used were cromakalim (Beecham Pharmaceuticals Research Division), glibenclamide (Sigma Chemical Co.), apamin (Sigma), papaverine hydrochloride (Wako Pure Chemical Industries), nifedipine (Bayer Yakhin Ltd.) and phenoxybenzamine hydrochloride (Nakarai Chemicals). $^{86}RbCl$

(Amersham International) was obtained as a solution containing an initial specific activity of 1.5–2.9 mCi mg^{-1}.

Cromakalim was dissolved in 60% ethanol to make a stock solution of 10^{-2} M with further dilution in distilled water before use. Stock solutions of nifedipine (10^{-3} M) and phenoxybenzamine (10^{-3} M) were prepared in 50% ethanol with further dilution in distilled water before use. Glibenclamide was dissolved in 50% ethanol to make a stock solution of 10^{-3} M with further dilution in the same solvent before use. Aqueous stock solutions were prepared for other drugs. Concentrations of drugs are expressed as final molar concentrations in the muscle bath.

Results

Relaxation of arterial strips induced by cromakalim

Relaxant responses to cromakalim were first determined in 20.9 mM K^+-contracted strips of coronary, mesenteric and MC arteries (Figure 1). In the PSS containing 20.9 mM K^+, strips of these arteries showed a sustained contraction. The magnitude of the K^+-induced contraction in the coronary artery (69.0 ± 3.5% of the 65.9 mM K^+-induced maximum contraction, $n = 22$) and the MC artery (68.6 ± 3.9%, $n = 12$) was significantly ($P < 0.05$) greater than that in the mesenteric artery (47.8 ± 4.9%, $n = 17$). The addition of cromakalim in concentrations ranging from 3×10^{-8} to 1×10^{-5} M caused a concentration-dependent relaxation in these strips (Figure 1a). The maximum relaxation induced by 1×10^{-5} M cromakalim varied in the arteries used; 94.3 ± 0.8% of the papaverine-induced maximum relaxation in the coronary artery ($n = 22$), 59.6 ± 3.8% in the mesenteric artery ($n = 17$) and 38.2 ± 6.5% in the MC artery ($n = 12$) (Figure 1b). The pD_2 value for cromakalim in the coronary (6.53 ± 0.05, $n = 22$) or the mesenteric (6.56 ± 0.05, $n = 17$) artery was significantly larger than that in the MC artery (5.84 ± 0.09, $n = 12$) (Figure 1b).

Antagonism by glibenclamide of the relaxant responses to cromakalim

Glibenclamide in concentrations ranging from 3×10^{-8} to 1×10^{-6} M caused a parallel rightward displacement of the concentration-response curve for cromakalim in strips of these three arteries. At the concentrations used, glibenclamide did not modify the resting tone or K^+-induced contractions. After the 60 min preincubation with 1×10^{-6} M glibenclamide, the resting tone and K^+-induced contractions were not modified. The pA_2 value for glibenclamide and the slope of the regression line obtained from the Schild plot analysis in each artery are shown in Table 1.

Apamin, a blocker of low conductance K_{Ca} channels, in a concentration of 10^{-7} M did not inhibit the relaxant responses to cromakalim in these arteries.

Stimulation by cromakalim of ^{86}Rb efflux from 20.9 mM K^+-contracted strips

To clarify the possible correlation of the cromakalim-induced increase in ^{86}Rb efflux with the tension effect, the effects of cromakalim were determined in 20.9 mM K^+-contracted strips of the coronary artery (Figure 2). Upon stimulation with 20.9 mM K^+, the tension and ^{86}Rb efflux were increased. Under these conditions, the addition of 1×10^{-5} M cromakalim caused a relaxation which was accompanied by a further increase in ^{86}Rb efflux (Figure 2a).

Glibenclamide in a concentration of 10^{-6} M significantly inhibited the cromakalim (10^{-5} M)-induced increase in ^{86}Rb efflux from 20.9 mM K^+-contracted strips of the coronary artery (Figure 3). In the presence of glibenclamide, cromakalim did not relax the strips (Figure 3).

Effects of nifedipine on the cromakalim-induced increase in ^{86}Rb efflux were then determined (Figure 2b). In these experiments, 10^{-7} M nifedipine was added before the application of cromakalim to inactivate the K_{Ca} channels via the inhibition of Ca^{2+} influx. The addition of nifedipine to 20.9 mM K^+-contracted strips caused a relaxation which is accompanied by a decrease in ^{86}Rb efflux. Under these conditions, 10^{-5} M cromakalim increased the ^{86}Rb efflux. When the effect of cromakalim on ^{86}Rb efflux was expressed in terms of area under the curve during the 10 min application time, the effect was significantly ($P < 0.01$) greater in the presence of nifedipine than in

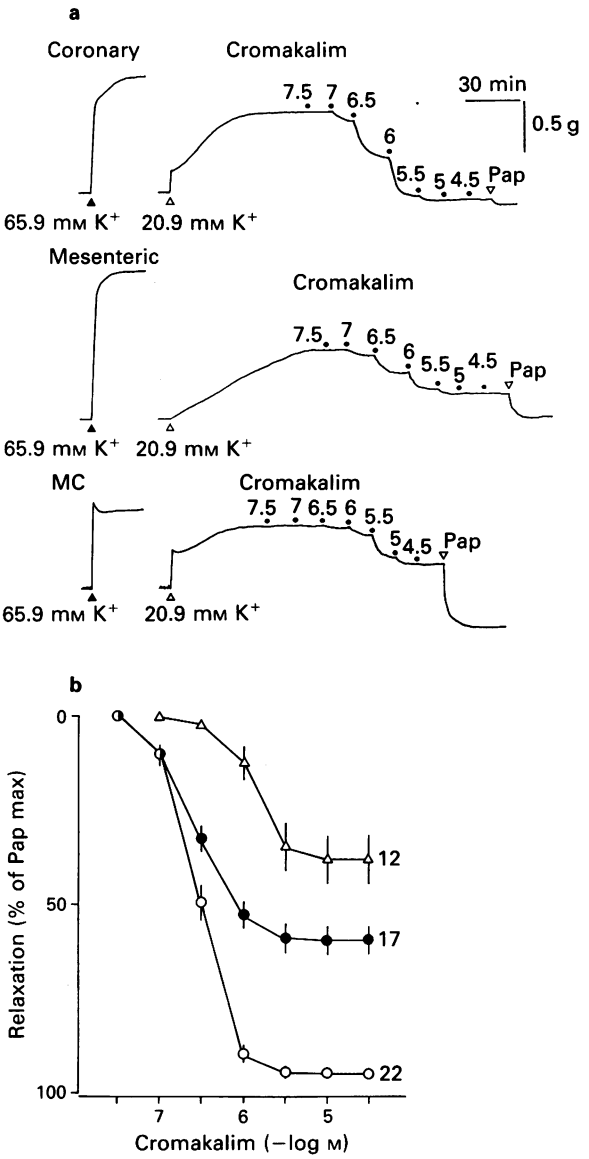

Figure 1 Arterial relaxant responses to cromakalim in 20.9 mM K^+-contracted strips of coronary, mesenteric and middle cerebral (MC) arteries. (a) Typical recordings showing the relaxant responses to cromakalim in 20.9 mM K^+-contracted strips. After the 90-min equilibration, strips were maximally activated by repeated application of the PSS containing 65.9 mM K^+ until the responses were reproducible. Following washout, contraction was subsequently induced by the PSS containing 20.9 mM K^+. After the contraction had reached a plateau, cromakalim was added in a cumulative fashion. At the end of each experiment 10^{-4} M papaverine (Pap) was added to identify the position of maximum relaxation. Concentrations of cromakalim are expressed as a negative log of the molar concentration. The 20.9 mM K^+-induced contractions were well sustained for at least 2 h. (b) Concentration-response curves for the relaxant responses to cromakalim in 20.9 mM K^+-contracted strips of coronary (○), mesenteric (●) and MC (△) arteries. Relaxation induced by 10^{-4} M papaverine was taken as 100%. Data points are means of values from the number of preparations indicated by each curve. Vertical lines represent s.e.mean.

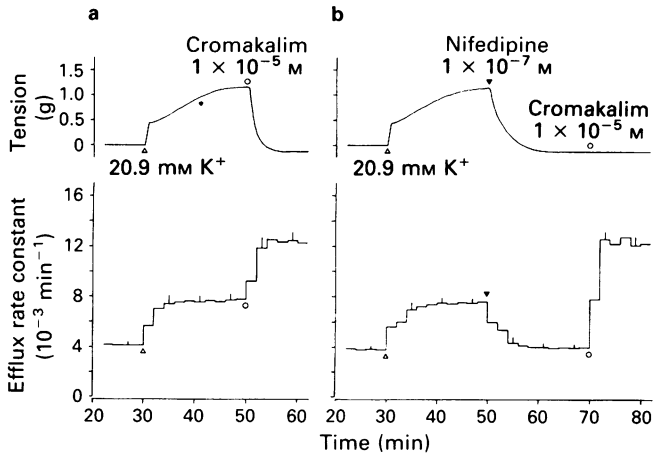

Figure 2 Effects of cromakalim on tension (top panels) and ^{86}Rb efflux (bottom panels) in 20.9 mM K^+-contracted strips of the coronary artery in the absence (a) and presence (b) of nifedipine. Ordinate scale: change in tension expressed as g or ^{86}Rb efflux rate constant expressed as 10^{-3} min^{-1}. Abscissa scale: time (min) after start of the efflux period. In (a), strips preloaded with ^{86}Rb were exposed to 20.9 mM K^+ at the 30th min of the efflux period and then exposed to 10^{-5} M cromakalim at the 50th min. The rate constants are means of 6 determinations. The s.e.mean (representative values) are shown by vertical lines. The ^{86}Rb efflux rate constant in the presence of 20.9 mM K^+ was fairly constant between the 34th and 60th min of the efflux period. In (b), strips preloaded with ^{86}Rb were exposed to 20.9 mM K^+ at the 30th min of the efflux period and then exposed to 10^{-7} M nifedipine at the 50th min. After 20 min, 10^{-5} M cromakalim was applied at the 70th min of the efflux period. Data are plotted as in (a). The rate constants are means of 6 determinations. The ^{86}Rb efflux rate constant in the presence of 20.9 mM K^+ plus 10^{-7} M nifedipine was fairly constant between the 58th and 80th min of the efflux period.

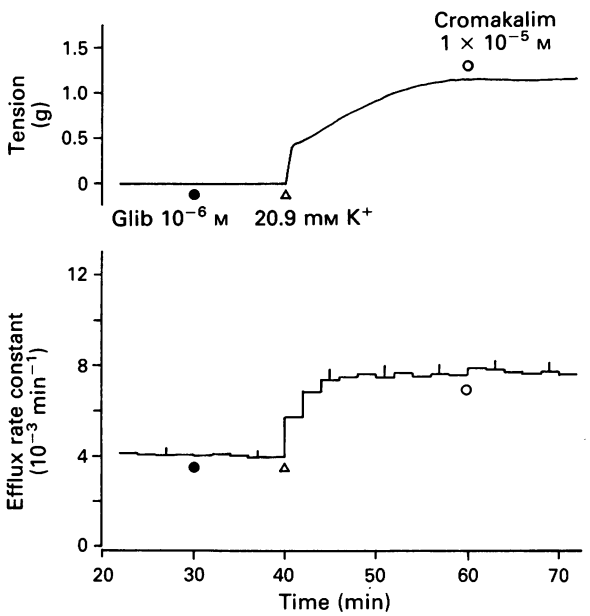

Figure 3 Inhibitory effects of glibenclamide on the cromakalim-induced relaxation (top panel) and increase in ^{86}Rb efflux (bottom panel) in 20.9 mM K^+-contracted strips of the coronary artery. Experimental conditions were the same as in Figure 2a. Strips preloaded with ^{86}Rb were first exposed to 10^{-6} M glibenclamide (Glib) at the 30th min of the efflux period and then exposed to 20.9 mM K^+ and 10^{-5} M cromakalim as indicated. Data are plotted as in Figure 2. The rate constants are the mean of 4 determinations.

the absence of nifedipine (Table 2). The peak values of the ^{86}Rb efflux rate constant during the application of cromakalim were not significantly different between the presence and absence of nifedipine (Figure 2).

Similar experiments were performed in 20.9 mM K^+-contracted strips of the mesenteric artery (Figure 4). The addition of 10^{-5} M cromakalim to the strips caused a relaxation which was accompanied by a small increase in ^{86}Rb efflux (Figure 4a, Table 2). The area under the curve for the cromakalim-induced increase in ^{86}Rb efflux was significantly smaller in the mesenteric artery than in the coronary artery (Table 2). In the presence of nifedipine, 10^{-5} M cromakalim increased the ^{86}Rb efflux (Figure 4b). Again, the area under the curve for the cromakalim-induced increase in ^{86}Rb efflux was significantly greater in the presence of nifedipine than in the absence of nifedipine (Table 2). Even in the presence of nifedipine, the area under the curve for cromakalim was significantly smaller in the mesenteric artery than in the coronary artery (Table 2).

In the MC artery, the addition of 10^{-5} M cromakalim to the 20.9 mM K^+-contracted strips caused a relaxation which was accompanied by a significant decrease in ^{86}Rb efflux (Figure 5a, Table 2). In the presence of nifedipine, cromakalim significantly increased the ^{86}Rb efflux (Figure 5b, Table 2). This increase was significantly smaller than the increase in the coronary or the mesenteric artery (Table 2).

Table 1 The pA_2 value for glibenclamide against cromakalim and the slope of Schild plots in 20.9 mM K^+-contracted strips of coronary, mesenteric and middle cerebral (MC) arteries

Artery	n	pA_2[a]	*Slope*[a]
Coronary	6	7.62 ± 0.12	1.25 ± 0.37
Mesenteric	5	7.20 ± 0.13	1.02 ± 0.20
MC	5	7.53 ± 0.18	0.98 ± 0.19

[a] The pA_2 values and slopes are expressed as means ± s.e.mean and means ± 95% confidence limits, respectively. For details, see Methods.
n indicates the number of preparations used.

Stimulation by cromakalim of ^{86}Rb efflux from 65.9 mM K^+-contracted strips

Further comparison of the effects of cromakalim on ^{86}Rb efflux was made in 65.9 mM K^+-contracted strips of the coronary artery (Figure 6). Upon the stimulation with 65.9 mM K^+, the tension and ^{86}Rb efflux were greatly increased. In these strips, cromakalim failed to relax the strips but increased the ^{86}Rb efflux (Figure 6a). In 65.9 mM K^+-contracted strips, the addition of 10^{-7} M nifedipine also caused a relaxation which was accompanied by a decrease in ^{86}Rb efflux (Figure 6b). Under these conditions, 10^{-5} M cromakalim increased the

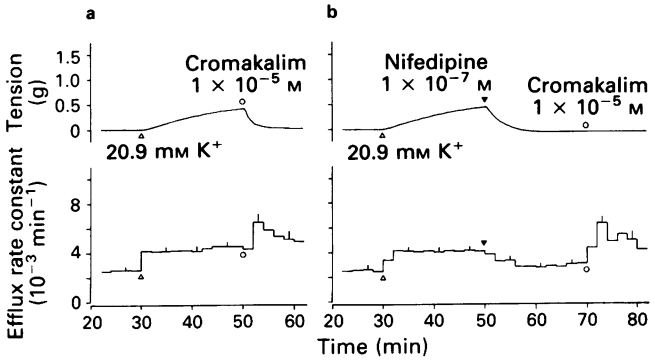

Figure 4 Effects of cromakalim on tension (top panels) and ^{86}Rb efflux (bottom panels) in 20.9 mM K^+-contracted strips of the mesenteric artery in the absence (a) and presence (b) of nifedipine. Experimental conditions were the same as in Figure 2. Data are plotted as in Figure 2. The rate constants are means of 6 determinations for (a) and (b).

Table 2 Effects of cromakalim on ^{86}Rb efflux from K^+-contracted strips of coronary, mesenteric and middle cerebral (MC) arteries in the absence and presence of nifedipine (Nif)

	Effect on ^{86}Rb efflux rate constant (area under curve, 10^{-3} 10 min^{-1})[b]		
Condition[a]	*Coronary*	*Mesenteric*	*MC*
K^+ 20.9 mM	38.2 ± 3.4* (6)	9.6 ± 2.6 (6)	−10.3 ± 2.6* (5)
K^+ 20.9 mM + Nif	74.4 ± 9.8*† (6)	25.9 ± 3.7† (6)	9.2 ± 2.1*† (5)
K^+ 65.9 mM	128.7 ± 16.3* (6)	51.8 ± 6.4 (6)	13.2 ± 2.9* (5)
K^+ 65.9 mM + Nif	132.7 ± 16.0* (6)	51.0 ± 6.1 (6)	13.4 ± 3.1* (5)

[a] Effects of 10^{-5} M cromakalim on ^{86}Rb efflux were determined in 20.9 mM and 65.9 mM K^+-contracted strips in the absence and presence of 10^{-7} M nifedipine.
[b] The effects of cromakalim are expressed as the area of the increase in the ^{86}Rb efflux rate constant during the 10 min application time. For details, see Methods.
Figures in parentheses indicate the number of preparations used.
Data are expressed as means ± s.e.mean.
* Significantly different from the mesenteric artery ($P < 0.05$).
† Significantly different from the value in the absence of 10^{-7} M nifedipine ($P < 0.05$).

^{86}Rb efflux. The areas under the curve for the cromakalim-induced increase in ^{86}Rb efflux were not significantly different between the presence and absence of nifedipine (Table 2).

Similar experiments were then performed in the mesenteric (Figure 7) and MC (Figure 8) arteries. Also in these arteries, cromakalim failed to relax the strips but significantly increased the ^{86}Rb efflux (Figures 7a and 8a). In the presence of 10^{-7} M nifedipine, cromakalim significantly increased the ^{86}Rb efflux in both arteries (Figures 7b and 8b). The areas under the curve for the cromakalim-induced increase in ^{86}Rb efflux from each artery were the same in the presence and absence of nifedipine (Table 2). It is noteworthy that the effects of cromakalim on ^{86}Rb efflux varied in the arteries used (Table 2). The response to cromakalim (measured as area under the curve) in the 65.9 mM K^+-contracted coronary artery was approximately 10 times greater than the equivalent response in the MC artery.

From the results shown in Figures 6b, 7b and 8b, we tried to estimate the properties of K_{Ca} and voltage-sensitive K^+ channels in the three arteries. The estimation was based on the effects of nifedipine. The peak values of the ^{86}Rb efflux rate constant during the stimulation with 65.9 mM K^+ in the coronary, mesenteric and MC arteries were $18.4 \pm 1.5 \times 10^{-3}\,min^{-1}$ ($n = 6$), $17.2 \pm 1.2 \times 10^{-3}\,min^{-1}$ ($n = 6$) and $19.5 \pm 1.8 \times 10^{-3}\,min^{-1}$ ($n = 5$), respectively. Upon the addition of nifedipine, the rate constants were decreased to a steady-state value of $7.9 \pm 0.5 \times 10^{-3}\,min^{-1}$ (coronary, $n = 6$), $7.4 \pm 0.6 \times 10^{-3}\,min^{-1}$ (mesenteric, $n = 6$) and $9.2 \pm 0.7 \times 10^{-3}\,min^{-1}$ (MC, $n = 5$). The nifedipine-sensitive components during the stimulation with 65.9 mM K^+ were calculated as $10.5 \pm 1.3 \times 10^{-3}\,min^{-1}$ (coronary, $n = 6$), $9.8 \pm 1.0 \times 10^{-3}\,min^{-1}$ (mesenteric, $n = 6$) and $10.3 \pm 1.2 \times 10^{-3}\,min^{-1}$ (MC, $n = 5$). These components were regarded as a function of K_{Ca} channels and were similar among the three arteries. Furthermore, the nifedipine-insensitive components (regarded as a function of voltage-sensitive K^+ channels) were also similar among the three arteries. On the other hand, the peak values of the ^{86}Rb efflux rate constant during the application of cromakalim to these strips were $24.0 \pm 3.5 \times 10^{-3}\,min^{-1}$ (coronary, $n = 6$), $15.4 \pm 1.2 \times 10^{-3}\,min^{-1}$ (mesenteric, $n = 6$) and $11.0 \pm 0.8 \times 10^{-3}\,min^{-1}$ (MC, $n = 5$). The cromakalim-sensitive components were then calculated by subtracting the steady-state value before the application of cromakalim from the peak value after the application of cromakalim. This com-

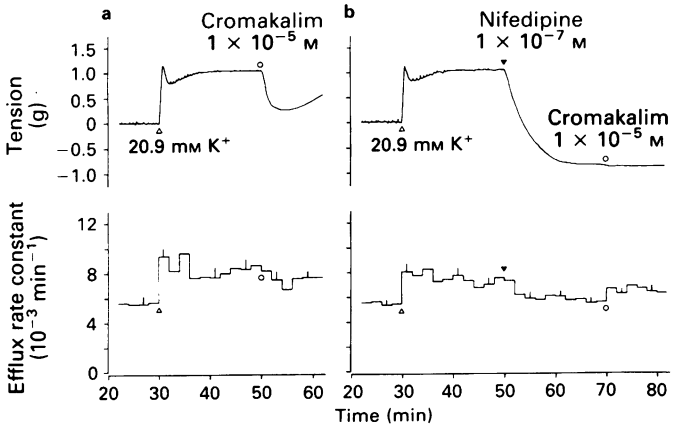

Figure 5 Effects of cromakalim on tension (top panels) and ^{86}Rb efflux (bottom panels) in 20.9 mM K^+-contracted strips of the middle cerebral (MC) artery in the absence (a) and presence (b) of nifedipine. Experimental conditions were the same as in Figure 2. Data are plotted as in Figure 2. The rate constants are means of 5 determinations for (a) and (b).

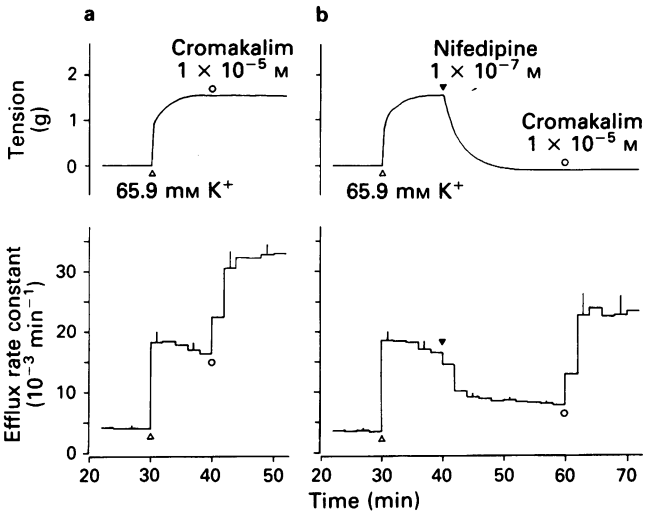

Figure 6 Effects of cromakalim on tension (top panels) and ^{86}Rb efflux (bottom panels) in 65.9 mM K^+-contracted strips of the coronary artery in the absence (a) and presence (b) of nifedipine. In (a), strips preloaded with ^{86}Rb were exposed to 65.9 mM K^+ at the 30th min of the efflux period and then exposed to 10^{-5} M cromakalim at the 40th min. The ^{86}Rb efflux rate constant in the presence of 65.9 mM K^+ was fairly constant between the 40th and 50th min of the efflux period. In (b), strips preloaded with ^{86}Rb were exposed to 65.9 mM K^+ at the 30th min of the efflux period and then exposed to 10^{-7} M nifedipine at the 40th min. After 20 min, 10^{-5} M cromakalim was applied at the 60th min of the efflux period. The ^{86}Rb efflux rate constant in the presence of 65.9 mM K^+ plus 10^{-7} M nifedipine was fairly constant between the 54th and 70th min of the efflux period. Data are plotted as in Figure 2. The rate constants are means of 6 determinations for (a) and (b).

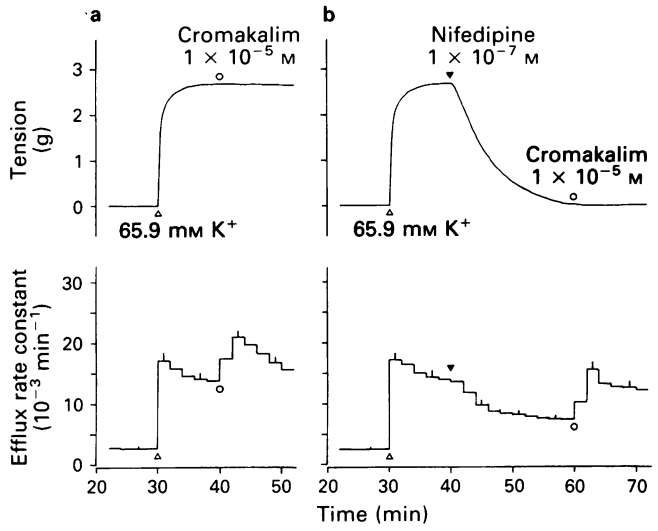

Figure 7 Effects of cromakalim on tension (top panels) and ^{86}Rb efflux (bottom panels) in 65.9 mM K^+-contracted strips of the mesenteric artery in the absence (a) and presence (b) of nifedipine. Experimental conditions were the same as in Figure 6. Data are plotted as in Figure 2. The rate constants are means of 6 determinations for (a) and (b).

ponent varied in the arteries used; $16.2 \pm 2.7 \times 10^{-3}$ min^{-1} for the coronary artery ($n = 6$), $7.9 \pm 0.8 \times 10^{-3}$ min^{-1} for the mesenteric artery ($n = 6$) and $1.7 \pm 0.6 \times 10^{-3}$ min^{-1} for the MC artery ($n = 5$).

Discussion

The experiments described in the present study evaluated the effects of cromakalim on tension and ^{86}Rb efflux in strips without endothelium of coronary, mesenteric and MC arteries of the dog, to compare the properties and populations of cromakalim-opened K^+ channels. The major conclusion is that cromakalim-opened K^+ channels in the three arteries may differ in terms of their frequency of occurence in the plasmalemma, their permeability to ^{86}Rb and their ability to modulate tension development. This conclusion is suggested

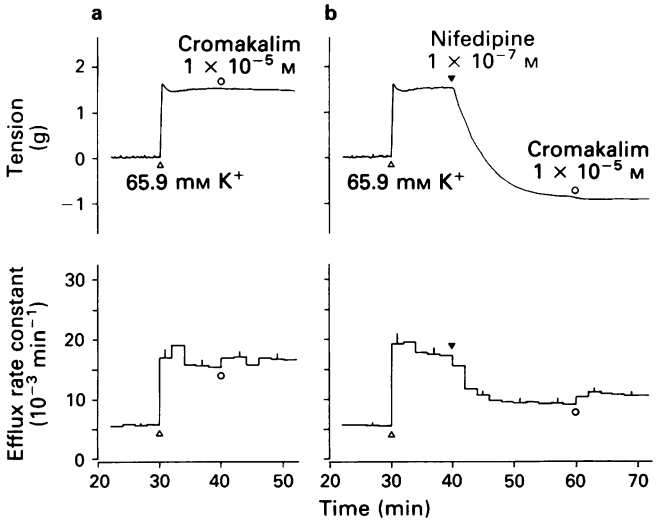

Figure 8 Effects of cromakalim on tension (top panels) and ^{86}Rb efflux (bottom panels) in 65.9 mM K^+-contracted strips of the middle cerebral (MC) artery in the absence (a) and presence (b) of nifedipine. Experimental conditions were the same as in Figure 6. Data are plotted as in Figure 2. The rate constants are means of 5 determinations for (a) and (b).

from the following observations: (1) the maximum relaxant response to cromakalim determined in 20.9 mM K^+-contracted strips was in the order of coronary > mesenteric > MC; (2) the pD_2 value for cromakalim in the MC artery was smaller than that in the coronary or the mesenteric artery; (3) the ability of cromakalim to increase ^{86}Rb efflux from 20.9 mM K^+-contracted strips was in the order of coronary > mesenteric > MC; (4) the same order was observed in ^{86}Rb efflux from the arteries in which the K_{Ca} channels are inactivated by the addition of nifedipine, and (5) the same result was also obtained in ^{86}Rb efflux from 65.9 mM K^+-contracted strips. A likely possibility for the variation in the effects of cromakalim on the three arteries appears to be that the cromakalim-opened K^+ channels in the three arteries vary in their populations and/or conductance.

Glibenclamide is a potent blocker of K_{ATP} channels. A large number of pharmacological studies have demonstrated a competitive antagonism by glibenclamide of the responses to cromakalim in vascular tissues (Winquist *et al.* 1989; Wilson, 1989; Cavero *et al.*, 1989; Buckingham *et al.*, 1989; Quast & Cook, 1989). In the present study, glibenclamide was also demonstrated to antagonize the relaxant responses of the three arteries to cromakalim. The Schild plot analysis for antagonism by glibenclamide of the responses to cromakalim clearly showed a competitive antagonsim in the three arteries. The pA_2 values for glibenclamide in the three arteries were in good agreement with the pA_2 values for this substance obtained in other studies (Wilson, 1989; Cavero *et al.*, 1989; Quast & Cook, 1989). The cromakalim-induced increase in ^{86}Rb efflux was also inhibited by glibenclamide. These observations indicate that cromakalim produced the arterial relaxation through the increase in K^+ permeability via the opening of K_{ATP} channels and these channels were distributed in the three arteries. The relaxant responses to cromakalim were not antagonized by apamin, suggesting that low conductance K_{Ca} channels were not involved in the relaxant responses to this drug. This conclusion has already been proposed in other vascular tissues (Cook *et al.*, 1988b; Hamilton & Weston, 1989; Winquist *et al.*, 1989; Masuzawa *et al.*, 1990a).

The apparent mechanism of action of cromakalim was to increase K^+ permeability, which in turn caused hyperpolarization and subsequent relaxation of arterial smooth muscle (Hamilton *et al.*, 1986; Wier & Weston, 1986; Quast, 1987; Cook *et al.*, 1988a; Quast & Baumlin, 1988). When the arteries were contracted with low concentrations of K^+ (20.9 mM in the present study), the E_K would be more negative than the actual membrane potential and the threshold for VDC opening. Therefore, under these conditions, the increase in K^+ permeability induced by cromakalim results in hyperpolarization and arterial relaxation through the closing of VDCs. On the other hand, cromakalim did not produce the relaxation in strips precontracted with high concentrations of K^+, presumably because the high K^+ reduced the E_K to a value less inside negative than that required to close VDCs. Under these conditions, the cromakalim-induced increase in the K^+ permeability would not hyperpolarize the arterial smooth muscle sufficiently to close VDCs. This assumption is supported by the observation of the present study (Figures 6a, 7a and 8a) that the application of cromakalim to 65.9 mM K^+-contracted strips failed to relax the strips but increased the ^{86}Rb efflux from these strips.

^{86}Rb has been used as a marker of K^+ permeability in smooth muscle (Imaizumi & Watanabe, 1981; Bolton & Clapp, 1984; Smith *et al.*, 1986; Quast & Baumlin, 1988). Upon the application of cromakalim to 20.9 mM K^+-contracted strips, ^{86}Rb efflux was increased in the coronary and mesenteric arteries but was decreased in the MC artery. However, after the addition of nifedipine to the 20.9 mM K^+-contracted strips, cromakalim increased the ^{86}Rb efflux from these arteries. The extent of the increase in ^{86}Rb efflux from each artery was unexpectedly greater in the presence than in the absence of nifedipine. In the coronary and mesenteric arteries, the peak value of efflux rate constant induced by cro-

makalim was the same with and without nifedipine. These findings strongly suggest that the effects of cromakalim on the 20.9 mM K^+-contracted strips seem to be the resultant of two opposing effects; an increased ^{86}Rb efflux due to the opening of K_{ATP} channels, and a decreased efflux due to the secondary inactivation of other K^+ channels which were previously activated by 20.9 mM K^+. Arterial smooth muscle contraction induced by K^+ is accompanied by an increase in ^{86}Rb efflux, presumably due to an opening of K_{Ca} channels (Bolton & Clapp, 1984; Casteels & Droogmans, 1985; Aaronson & Jones, 1985; see also this paper, Figures 2–8). On the other hand, the inhibition by drugs of transmembrane Ca^{2+} influx (either directly or indirectly) resulted in the closing of K_{Ca} channels (Casteels & Droogmans, 1985; Aaronson & Jones, 1985; see also this paper, Figures 2, 4–8). Therefore, the increase in ^{86}Rb efflux from 20.9 mM K^+-contracted strips of the coronary and mesenteric arteries may be explained by the assumption that the amount of the increased ^{86}Rb efflux due to the opening of K_{ATP} channels is greater than the amount of the decreased efflux due to the closing of K_{Ca} channels, while the reverse is true for the MC artery in which the cromakalim-induced relaxation was accompanied by the decreased efflux. The assumption of two opposing effects of cromakalim is supported by the following observations: (1) relaxant responses to nifedipine were accompanied by the decreased ^{86}Rb efflux in 20.9 mM K^+-contracted strips, and (2) effects of cromakalim on ^{86}Rb efflux from 65.9 mM K^+-contracted strips, where cromakalim failed to cause a relaxation, were the same for the presence and absence of nifedipine. The latter observation clearly indicates the possibility that the relaxation induced by cromakalim was not due to a direct action on VDCs which are the site of action of nifedipine. From these results, it is likely that the cromakalim-induced increase in K^+ permeability causes hyperpolarization, which in turn inactivates VDCs in K^+-contracted arteries. The inactivation of VDCs decreases the influx of Ca^{2+}, which results in the closing of K_{Ca} channels. Therefore, under the conditions in which the K_{Ca} channels are inactivated, ^{86}Rb efflux due to the opening of K_{ATP} channels induced by cromakalim can be compared among the arteries. The present study also provided an analysis of the effects of cromakalim on ^{86}Rb efflux from K^+-contracted arteries.

It has been demonstrated that cromakalim increased the tissue permeability to ^{42}K more than to ^{86}Rb, the difference being more marked at low cromakalim concentrations (Quast & Baumlin, 1988). Furthermore, the basal efflux and K_{Ca} channel-activated efflux have been demonstrated to be selective for ^{42}K over ^{86}Rb (Smith *et al.*, 1986). Therefore, it is possible that the ^{86}Rb efflux quantitatively underestimated that of ^{42}K. However, it is also possible that the extent of the underestimation does not differ in the arteries used. Because we used a relatively high concentration of cromakalim (1×10^{-5} M) in the present study, the effects of this drug on ^{86}Rb and ^{42}K efflux may be essentially the same as long as the effects were compared.

In the present study, we have examined the efflux of ^{86}Rb through K^+channels opened by cromakalim and found that stimulation of efflux was in the order, coronary > mesenteric > MC (Table 2). We also examined the functions of K_{Ca} and voltage-sensitive K^+ channels during the stimulation with 65.9 mM K^+ in the three arteries. In the presence of nifedipine, the transmembrane influx of Ca^{2+} induced by high K^+ is greatly inhibited, and therefore the K_{Ca} channels are not opened. The results obtained in the presence of nifedipine suggest that in the absence of a substantial influx of Ca^{2+}, high K^+-induced depolarization still causes an effect on ^{86}Rb efflux. The increased ^{86}Rb efflux that persists with nifedipine results from the effect of high K^+-induced depolarization on K^+ exchange (Casteels & Droogmans, 1985; Aaronson & Jones, 1985). The functions of K_{Ca} channels, estimated as nifedipine-sensitive components, were similar among the three arteries. Not only the K_{Ca} but also the voltage-sensitive K^+ channels functioned similarly among the three arteries.

Compared with the effects of cromakalim on ^{86}Rb efflux from 20.9 mM K^+-contracted strips, the effects of this drug on 65.9 mM K^+-contracted strips were augmented by approximately two fold in each artery, indicating that the opening of K_{ATP} channels induced by cromakalim is modulated by membrane potential. The opening of these channels is more pronounced when the smooth muscle membrane is depolarized. Therefore, it is likely that the binding of cromakalim to a specific site located within the K_{ATP} channel is influenced by the state of the channel and that this state is determined by the membrane potential.

In conclusion, the present study compared the properties of K_{ATP} channels in coronary, mesenteric and MC arteries of the dog. The K_{ATP} channels in the three arteries exhibit considerable heterogeneity in their distribution or functions, while the activities of both K_{Ca} and voltage-sensitive K^+ channels are similar among the three arteries.

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Putative neurotrophic factors and functional recovery from peripheral nerve damage in the rat

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1 In rats, recovery of sensory-motor function following a crush lesion of the sciatic or tibial nerve was monitored by measuring foot reflex withdrawal from a local noxious stimulation of the foot sole.

2 Putative neurotrophic compounds were tested on this functional recovery model: melanocortins (peptides derived from ACTH (corticotropin) and α-MSH (melanotropin)), gangliosides and nimodipine were effective whereas isaxonine and TRH (thyrotropin releasing hormone) were not.

3 Structure-activity studies with melanocortins revealed a similar effectiveness of α-MSH, [N-Leu^4, D-Phe^7]-α-MSH, desacetyl-α-MSH and the $ACTH_{4-9}$ analogue ORG 2766, questioning the validity of the previously suggested notion that the melanotrophic properties of these peptides are responsible for their neurotrophic effect.

4 As recovery of function after peripheral nerve damage follows a similar time course in hypophysectomized (five days post operation) and sham-operated rats, effective melanocortin therapy does not mimic an endogenous peptide signal in the repair process from pituitary origin.

5 Subcutaneous treatment with ORG 2766 ($7.5\,\mu g\,kg^{-1}\,48\,h^{-1}$) facilitates recovery of function following peripheral nerve damage in young (6–7 weeks old), mature (5 month old) and old (20 month old) rats.

6 In view of the diversity in structure of the effective neurotrophic factors and the complexity of nerve repair, the present data support the notion that peripheral nerve repair may be facilitated by different humoral factors likely to be active on different aspects of the recovery process.

Keywords: Peripheral nerve regeneration; sciatic crush lesion; adrenocorticotrophic hormone (ACTH); melanotropin (α-MSH); ORG 2766; neurotrophic factors; gangliosides; nimodipine

Introduction

Although progress has been made in the surgical repair of peripheral nerve trauma, it is clear that the development of new pharmacotherapeutics is a prerequisite for further advancement of repair of damaged nerves (Gelijns *et al.*, 1987). For many years neurologists turned to vitamin B treatment to improve peripheral nerve function with questionable results (Jennekens, 1984). Following the initial discovery of Nerve Growth Factor (NGF), more and more protein- and peptide-factors have been isolated and characterized that enhance neurite outgrowth in cultured foetal neurones (Varon, 1985; Dekker *et al.*, 1987a). As the molecular processes that underlie postlesion axonal repair in the adult in part resemble a replay of those processes that govern neurite outgrowth and network formation in the developing nervous system (Bär *et al.*, 1990), developmentally active factors are often screened for their efficacy in nerve repair. However, until now no clinically proven new pharmacotherapy has emerged. In the present study using a crush lesion of the rat sciatic nerve we evaluated the efficacy of various factors claimed to have beneficial effects on functional recovery in experimental regeneration and reinnervation. Although these factors are of a completely different chemical nature and would affect the neurone via different mechanisms of action, they have all been shown to accelerate maturation and neurite outgrowth in cultured foetal neurones (corticotropin/melanotropin (ACTH/MSH) like peptides: rat spinal cord and cerebral neurones, Richter-Landsberg *et al.*, 1987, Van der Neut *et al.*, 1988; gangliosides: various primary neurone cultures, see Ledeen 1984; nimodipine: medial raphe 5-hydroxytryptaminergic neurones, Azmitia, 1989; isaxonine: mouse spinal ganglion, Hugelin *et al.*, 1977). In the present experiments, detailed structure-activity studies were performed to characterize further the active site in ACTH/MSH-like peptides that may be responsible for their neurotrophic activity. The data show that not all factors that are reported to promote neurite outgrowth *in vitro* are effective in an *in vivo* nerve repair model and that there are multiple message sites encoding for the modulating influence of ACTH/MSH-like peptides on functional recovery following peripheral nerve lesion.

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Methods

Animals and surgery

Female rats of an inbred Wistar strain (TNO CpB, Zeist, The Netherlands), 6–7 weeks of age, weighing approximately 120–140 g, were used. Rats were anaesthetized with Hypnorm (Duphar, Weesp, NL) containing fluanisone ($10\,mg\,ml^{-1}$) and phentanylcitrate ($0.2\,mg\,ml^{-1}$) in a dose of $0.08\,ml\,kg^{-1}$ body weight. Crush lesioning of the peripheral nerve was performed as described in detail by De Koning *et al.* (1986). The right sciatic nerve was crushed for 30 s, with a haemostatic forceps. In one experiment male rats of different ages were used: rats of 6–7 weeks, 5 months and 20 months of age (weighing 150, 450 and 500–600 g, respectively) received a crush lesion for 30 s of the right tibial nerve. Hypophysectomy was performed under light anaesthesia by transauricular route as described previously (Gispen *et al.*, 1970). Five days after hypophysectomy ($n = 8$) or sham operation ($n = 16$), rats were either subjected to crush lesioning or to sham operation of the right sciatic nerve (see also legend to Figure 4). Reduction of body weight, adrenal atrophy to a wet weight of less than 10 mg and absence of tissue in the sella tursica upon postmortem macroscopic inspection, documented the efficacy of the surgery.

Functional recovery

The return of sensorimotor function was measured by the foot reflex withdrawal test, described previously (De Koning *et al.*,

1986; Van der Zee et al., 1988). The rat was immobilized by hand presenting the sole of its foot to the examiner. A small electric current was applied to the sole of the foot at a given place (position 3, see De Koning et al., 1986) through two stimulation poles. A normal rat retracted its paw immediately when the skin of the foot sole closes the electric circuit. Rats bearing a crush lesion initially failed to do so. A response was scored as positive if the rat retracted its paw from the noxious stimulus instantaneously. A range of six brief pulses of current (0.1–0.6 mA) was used to ascertain the lowest strength of current required to elicit paw retraction. A rat was considered fully recovered (100% recovery) when it retracted its paw at a stimulus of 0.1 mA. Failure to retract at a 0.6 mA current stimulus indicated 0% recovery. Intermediate % recoveries were 17, 33, 50, 66 and 83 at 0.6, 0.5, 0.4, 0.3 and 0.2 mA respectively. The examiner performing the reflex withdrawal test was not aware of the treatment a given rat had received.

Data analysis

The treatment code was partially broken at the end of the experiment to allow analysis of the data obtained from the various treatment groups. The final treatment code indicating which group had received what treatment was broken only after analysis of the data was completed. The data obtained by the functional recovery test were expressed as the mean % recovery per day (±s.e.) and group differences were analyzed by an analysis of variance for repeated measurements (ANOVA), followed by a supplemental t test, using the raw data prior to transformation to percentage recovery.

Drugs

Peptides were synthesized and donated by Organon International B.V., Oss, The Netherlands. α-MSH (melanotropin), desacetyl-α-MSH, [N-Leu^4,D-Phe^7]-α-MSH, MSH_{11-13}, β-MSH, γ_2-MSH, $ACTH_{6-9}$, $ACTH_{7-16}$ and ORG 2766, an $ACTH_{4-9}$ analogue (H-Met(O_2)-Glu-His-Phe-D-Lys-Phe-OH). The peptides were freshly dissolved in saline and administered s.c. in a dose of 0.01, 0.05, 0.1, 1.0 or 10 μg 0.5 ml^{-1} 48 h^{-1}. The male rats of different ages and weights received ORG 2766 in a s.c. dose of 7.5 μg kg^{-1} 48 h^{-1}. Thyrotrophin-releasing hormone (TRH) was dissolved in saline and administered i.v. in a dose of 0.1 mg (bolus injection of 0.05 mg at 1 h and 3 h following surgery). Isaxonine (N-isopropyl-amino-2 pyrimidine orthophosphate, a gift from Organon International B.V.) was freshly dissolved in saline and administered i.p. daily in a dose of 35 mg. The ganglioside mixture (GA mixt = GM1, GD1a, GD1b, GT) was a gift from Fidia Research Laboratories (Abano Terme, Italy). The gangliosides were freshly dissolved in saline and administered i.p. in a dose of 0.7 mg per day. Nimodipine (a gift from Tropon GmbH, Köln, F.R.G.) was freshly dissolved in polyethyleneglycol (PEG) and administered i.p. daily in a dose of 20 mg kg^{-1} in 0.1 ml PEG per 100 g body weight. Control rats received vehicle s.c., i.v., i.p. or orally.

Results

Effect of putative neurotrophic compounds

In the first experiment different groups of rats, bearing a crush lesion in the right sciatic nerve, were treated with different putative neurotrophic compounds. The dose, route of administration and treatment schedule for each of the compounds was chosen from the literature and was reported to be optimally effective for the given drug (see legend to Figure 1). Routinely, rats treated with vehicle begin to respond to the noxious stimulus approximately at day 16–18 post operation and are considered fully recovered in this test at approximately 23–24 days (see control groups in Figure 1a,b,c,d).

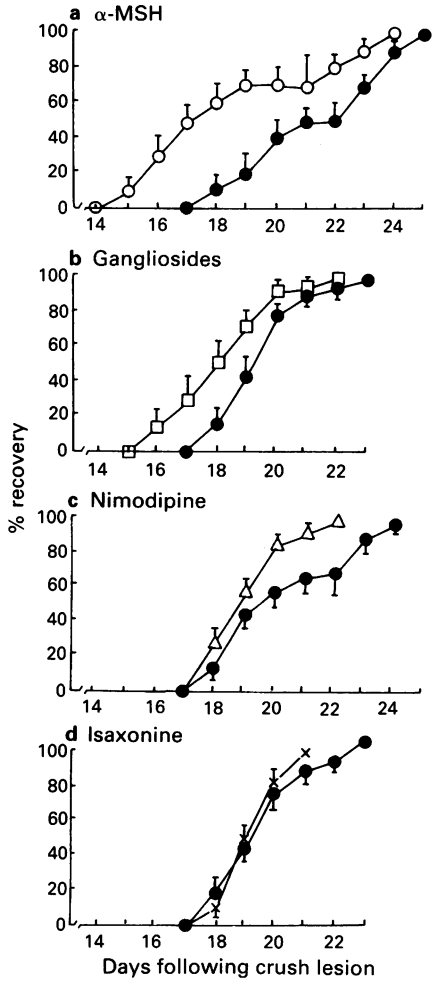

Figure 1 The effect of neurotrophic compounds on functional recovery following crush lesion of the sciatic nerve in the rat. The return of sensorimotor function was measured by the foot reflex withdrawal reaction after foot sole stimulation with a range of six current strengths of 0.1–0.6 mA. The data are expressed as the mean percentage (%) receovery per day (s.e.mean shown by vertical bars). In each group $n = 10$. (a) Rats receiving s.c. α-MSH (○), dose 10 μg 48 h^{-1}, showed a significantly enhanced recovery compared to rats receiving saline (●); $F(1, 18) = 14.5$, $P < 0.005$. (b) Treatment i.p. with gangliosides (□), 0.7 mg per day, significantly enhanced the functional recovery compared to saline treatment (●); $F(1, 18) = 4.7$, $P < 0.05$. (c) Nimodipine i.p., treatment (△), 20 mg kg^{-1} per day, resulted in a significantly enhanced recovery of function compared to treatment with the vehicle polyethylene glycol (●); $F(1, 18) = 5.6$, $P < 0.05$. (d) Isaxonine i.p., (x), 35 mg per day, showed no effect on the functional recovery following rat sciatic nerve crush, compared to saline (●). For the statistical evaluation an analysis of variance for repeated measurements (ANOVA) was performed using the raw data.

Treatment with α-MSH, gangliosides or nimodipine accelerated return of function substantially (Figure 1a,b,c), whereas treatment with isaxonine (Figure 1d) or TRH (data not shown) was ineffective.

Structure-activity studies with melanocortins

In order to gain insight into how the neurotrophic information is encoded within the α-MSH/ACTH molecule, various fragments and analogues were tested in the foot reflex withdrawal test as described above. Most peptides were tested at least in a dose of 1 or 10 μg per rat or both. In Figure 2 the '% recovery above control' at day 17 post surgery is presented. As can be seen, α-MSH, [N-Leu^4,D-Phe^7]-α-MSH, desacetyl-α-MSH and β-MSH were active whereas γ_2-MSH, containing the 4–9 sequence with one modification (Gly^5), was not. Interestingly, the short sequence $MSH/ACTH_{11-13}$ inhibited the

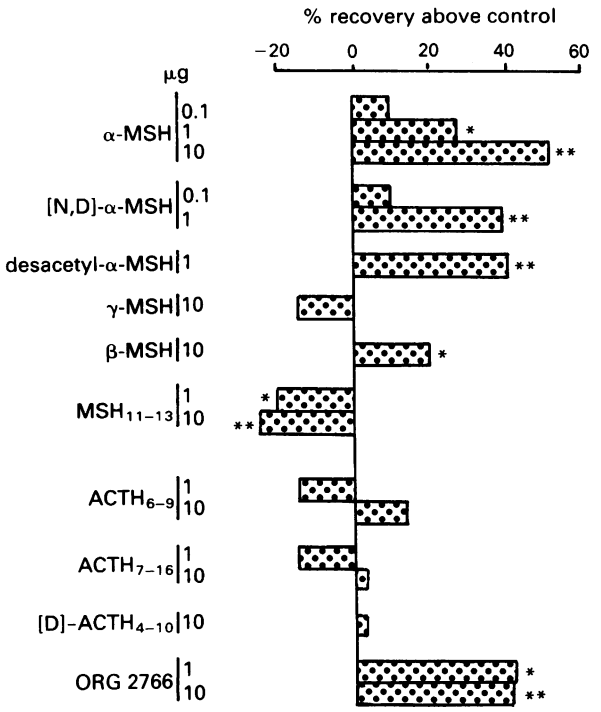

Figure 2 The effect of α-MSH/ACTH-fragments and -analogues on functional recovery following crush lesion of the rat sciatic nerve. The functional recovery was measured with the foot reflex withdrawal test (stimulus range 0.1–0.6 mA), and expressed as the mean percentage recovery. Each peptide-fragment or -analogue, in the dose(s) indicated in the Figure, was tested in a group of 10 rats and compared with its own saline-treated control group. The statistical evaluation (ANOVA) was performed on the raw data of all experimental days, comparing the peptide treatment values with the saline treatment values (as in Figure 1). In this diagram for each peptide-treated group ($n = 10$) the % recovery on day 17, with the % recovery of their own control group subtracted from it, is depicted as '% recovery above control'. Significantly enhanced functional recovery following crush lesion of the rat sciatic nerve is shown for α-MSH 1 μg ($F(1, 18) = 5.0$, $P < 0.05$) and 10 μg ($F(1,18) = 14.5$, $P < 0.005$); [NLeu^4,D-Phe^7]-α-MSH 1 μg ($F(1, 18) = 14.2$, $P < 0.005$); desacetyl-α-MSH 1 μg ($F(1, 18) = 13.4$, $P < 0.005$); β-MSH 10 μg ($F(1, 18) = 4.5$, $P < 0.05$); ORG 2766 1 μg ($F(1, 18) = 4.6$, $P < 0.05$) and 10 μg ($F(1, 18) = 11.1$, $P < 0.005$). The fragment MSH_{11-13} (doses 1 and 10 μg) inhibited significantly the functional recovery following crush lesion, $F(1, 18) = 4.5$, $P < 0.05$ and $F(1, 18) = 4.9$, $P < 0.05$, respectively. [N,D]-α-MSH = [N-Leu^4,D-Phe^7]-α-MSH; [D]-$ACTH_{4-10}$ = [D-Phe^7]-$ACTH_{4-10}$.
Significance: * $P < 0.05$; ** $P < 0.005$.

recovery of function, whereas $ACTH_{7-16}$ was without effect. The short sequence $ACTH_{6-9}$ and [D-Phe^7]-$ACTH_{4-10}$ were both inactive in this test paradigm whereas the $ACTH_{4-9}$ analogue ORG 2766 was active (Figure 2).

Neurotrophic activity of ORG 2766 at different ages

Male rats of 6–7 weeks, 5, 20 and 34 months were used. Crush lesioning of the right tibial nerve was performed on all rats at the same position. Rats of the first three age groups, were divided at random over two groups one of which was treated s.c. with ORG 2766 (7.5 μg kg^{-1} 0.5 ml^{-1} 48 h^{-1}) and the other treated with saline according to the same schedule. The oldest rats of 34 months of age ($n = 7$) received no treatment. As shown in Figure 3, control rats displayed return of sensorimotor function in an age-dependent manner. The younger the rat the earlier the return of sensorimotor function. The main effect of age between the saline group C and group D (oldest rats) was not significant. However, the interaction between age and time following surgery reached significance indicating a slower speed of recovery in group D (see Figure 3). In the three age groups treated with peptide (A,B,C), the neurotrophic influence of ORG 2766 on the recovery is evident, shown by a significantly enhanced functional recovery (see legend Figure 3).

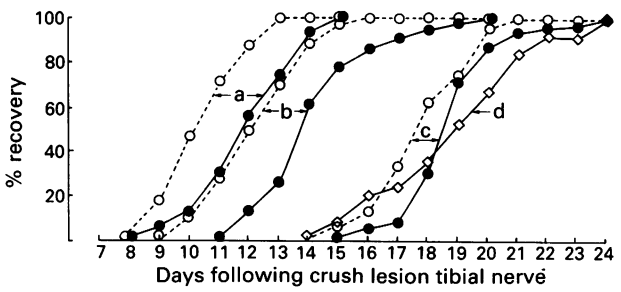

Figure 3 Effect of ORG 2766 administration following crush lesion of the tibial nerve in rats of different ages. The return of sensorimotor function following a crush lesion of the tibial nerve was measured with the foot reflex withdrawal test (stimulus range 0.1–0.6 mA), and expressed as the average % recovery (±s.e.mean < 20%, not shown). Rats were treated s.c. with ORG 2766 7.5 μg kg^{-1} 48 h^{-1} (○) or saline (●). (a) In 6–7 weeks old ORG 2766-treated rats ($n = 17$) the functional recovery was significantly enhanced compared to saline-treated age-matched rats ($n = 16$), $F(1, 31) = 4.4$, $P < 0.05$. (b) In 5 months old ORG 2766-treated rats ($n = 8$) a significantly enhanced functional recovery was apparent compared to saline-treated rats ($n = 8$) of the same age, $F(1, 14) = 20.2$, $P < 0.001$. (c) ORG 2766-treated rats of 20 months of age ($n = 5$) showed a significantly enhanced recovery of sensorimotor function compared to age-matched saline-treated rats ($n = 9$), $F(1, 12) = 4.7$, $P < 0.05$. In addition, both saline- and ORG 2766 peptide-treated groups showed an age-dependent regeneration rate; the young adult rats (a) reached 100% recovery earlier than the mature adults (b), and the latter regenerated faster than the old rats (c) ($P < 0.001$). (d) Non-treated rats of 34 months of age ($n = 7$) (□). Comparison between saline group (c) and (d): no main effect; interaction between age and time following operation $F(4, 56) = 2.7$, $P < 0.05$. For the statistical evaluation, an analysis of variance for repeated measurements (ANOVA) was performed using the raw data.

As a consequence of difference in bodyweight the distance between the distal border of the crush and the position of the footsole where the return of sensorimotor function was measured differed per control group. After completion of the experiments the exact measurements were taken and ranged from approximately 29 mm (6–7 weeks) to 32 mm (34 months).

Recovery of function following sciatic nerve crush in hypophysectomized rats

Five days after hypophysectomy or sham operation, rats were either subjected to crush lesioning or to sham surgery of the right sciatic nerve. Subsequently, the return of sensorimotor

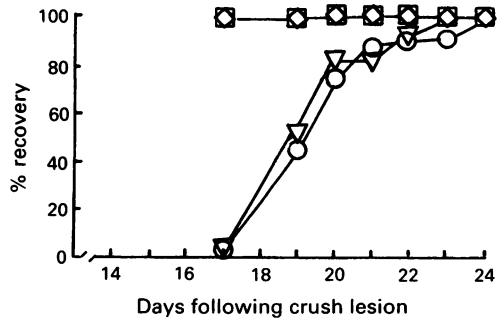

Figure 4 Recovery of function following sciatic nerve crush in hypophysectomized rats. Five days after hypophysectomy or sham operation, rats were subjected to sciatic crush lesion or sham lesion. The return of sensorimotor function was determined with the foot reflex withdrawal test (stimulus range 0.1–0.6 mA), and depicted as the mean percentage (%) recovery (± s.e.mean < 10%, not shown). The functional recovery following nerve crush in hypophysectomized rats (○, $n = 4$) was not different from sham-operated rats (▽, $n = 4$). Both sham-lesioned hypophysectomized (□, $n = 8$) and sham-operated (◇, $n = 8$) rats showed an intact sensorimotor function (reaction 100%).

function, as assessed by the foot reflex withdrawal test, was monitored. All sham operated and hypophysectomized rats that did not bear a crush lesion in their right sciatic nerve displayed full sensorimotor function. Furthermore, in hypophysectomized rats the recovery period following the sciatic nerve crush was not different from that observed in sham-operated, crush-lesioned rats (Figure 4).

Discussion

In the present study, we examined the efficacy of various putative neurotrophic compounds in a rat model of functional recovery following peripheral nerve damage. As shown previously in this model, the pattern of recovery over time is similar when responsiveness to a given stimulus rather than to a graded stimulus is measured or when the data are expressed as % recovery or as stimulus number of animals recovered (De Koning *et al.*, 1986; Van der Zee *et al.*, 1988). For the different treatment groups, the age and sex of the rats, the surgery and the test procedure were the same. However, the dose, route and schedule of treatment differed per drug and were identical to reported optimal conditions for the neurotrophic activity of the drugs (Hugelin *et al.*, 1979; Gorio *et al.*, 1983; Faden & Jacobs, 1985; Dekker *et al.*, 1987b; Van der Zee *et al.*, 1987; 1988). Indeed these optimal conditions resulted for α-MSH, gangliosides and nimodipine treatment in a facilitation of recovery of sensorimotor function following a crush lesion of the sciatic nerve. The precise mechanism of the neurotrophic action of these compounds is not yet fully understood. It should be kept in mind that neurotrophic activity as measured in an *in vivo* model might result from a direct neuronal effect or an indirect effect via non-neuronal elements of the nerve involved in the repair process.

Neurotrophic peptides of the ACTH/MSH family might exert their neurotrophic action by mimicking an endogenous signal early in the repair process originating from pro-opiomelanocortin (POMC) expression in the cell bodies or from neurofilament breakdown in the distal portion of damaged axons (for review see Bär *et al.*, 1990). Both melanocortins and gangliosides have been shown to improve neurite outgrowth into the distal nerve portion (Gorio *et al.*, 1983; Dekker, 1987; 1988), however, the peptides exclusively increase the number of newly formed sprouts not influencing their rate of outgrowth (Verhaagen *et al.*, 1987; Gerritsen van der Hoop *et al.*, 1988). Thus it is unlikely that an effect on denervation supersensitivity may account for the effect of melanocortins on peripheral nerve regeneration (see also Strand *et al.*, 1986). Ganglioside enhancement has been suggested to originate from a beneficial effect of NGF-receptor efficacy (Gorio *et al.*, 1983; Sebille, 1984; Ledeen, 1985). The effectiveness of nimodipine may involve fine regulation of intracellular calcium in outgrowing sprouts (Kater *et al.*, 1988), but very little in fact is known of the neurotrophic mechanism of this drug. The differences in chemical nature and presumed mechanisms of action suggest that the enhanced recovery of function is the result of drug effects at different levels of the repair process. It should be realized that all three drugs affect development and maturation in tissue culture (see Introduction). The data therefore underscore the following notions: (i) the *in vivo* test system as used here is a useful tool in the study of different putative neurotrophic agents; (ii) it is likely that various endogenous factors (at different times following surgery) affecting different steps in the repair process govern the recovery process; (iii) optimal recovery may be obtained by a therapy based on the combination of various neurotrophic agents (see also Varon, 1985). Development of new effective pharmacotherapy of peripheral nerve damage should take into account the consequences of these latter two notions.

The fact that isaxonine, although reported by some to be active in both development and repair of neurones (Hugelin *et al.*, 1977; 1979; Legrain, 1977; Sebille & Hugelin, 1982), was inactive in the present test study is in line with the poor results obtained with this compound by others (LeQuesne *et al.*, 1985). Although Faden & Jacobs (1985) reported that TRH improved functional neurological recovery after experimental spinal trauma in cats, we found no effect of TRH treatment on functional recovery after sciatic crush lesion.

The structure-activity studies with various peptides revealed some interesting new insight in the neurotrophic properties of melanocortins (ACTH/MSH-like peptides). Clearly, the structure-activity relationship of this phenomenon differs from that observed for other ACTH/MSH nervous system interactions (De Wied & Jolles, 1982; Gispen & De Wied, 1984), notably also from that observed in studies on vestibular compensation in frogs (Lüneburg & Flohr, 1988) and on recovery of function following brain damage in rats (Wolterink & Van Ree, 1990). Previously it was concluded that the melanotrophic features of these peptides were most probably responsible for their effect on peripheral nerve regeneration (Bijlsma *et al.*, 1983). Although N-acetylation is of crucial importance to various biological activities of α-MSH (Gispen *et al.*, 1975; O'Donohue *et al.*, 1981; Spruijt *et al.*, 1985), the equal effectiveness of α-MSH and desacetyl-α-MSH in facilitating recovery following peripheral nerve damage indicates no importance of N-acetylation to the neurotrophic property of the peptide (this study, Dekker, 1987). In fact, the data obtained with other fragments suggest that the neurotrophic activity is brought about by a mechanism different from that of the classical melanotrophic activity. For, the potentiated MSH peptide [N-Leu^4,D-Phe^7]-α-MSH, extremely potent in the melanophore assay (Sawyer *et al.*, 1980) and in the excessive grooming test (Spruijt *et al.*, 1985), is as effective as the parent molecule α-MSH in enhancing peripheral nerve regeneration. β-MSH containing the same 4–10 sequence is also active, but γ_2-MSH with one substitution (Gly^5) in the 4–9 sequence is inactive. This latter peptide was also inactive in facilitating functional recovery in rats with bilateral parafascicular lesions (Nyakas *et al.*, 1985) and in vestibular compensation rate after labyrinthectomy in frogs (Lüneburg & Flohr, 1988). The sequence (11–13) which is known to represent a dormant information site additional to the region (4–10) (Eberle & Schwyzer, 1975; Schwyzer, 1980; Eberle *et al.*, 1985), was shown to inhibit rather than to stimulate the recovery of function following peripheral nerve damage (Figure 2). To our knowlege the only other reported inhibition by neuropeptides is the observation by the group of Strand which demonstrated that the deleterious changes in neuromuscular function (muscle action potential and muscle contraction) in hypophysectomized rats were exacerbated by high doses of $ACTH_{4-10}$, although low doses had the opposite effect (Gonzalez & Strand, 1981); subsequently they observed in 14-day-old peptide-treated rats, that high but not low doses of ORG 2766 lead to impoverished rather than enriched nerve terminal branching during maturation of the motor endplates (Frischer & Strand, 1988). Finally, the ACTH4–9 analogue, ORG 2766, originally developed to diminish melanotrophic activity and to be more stable than the parent sequence (Greven & De Wied, 1973), was as active as α-MSH. Thus at present it remains uncertain what the precise localization of the neurotrophic activity is, although the region 4–10 is critical. As observed previously (Bijlsma *et al.*, 1983), changes in the region 4–10, the ultimate message region for the biological activity of ACTH/MSH (Schwyzer, 1980; Eberle *et al.*, 1985) often affect the neurotrophic activity of these peptides. Thus γ_2-MSH, $ACTH_{6-9}$, $ACTH_{7-16}$ and [D-Phe^7]- $ACTH_{4-10}$ with an incomplete or modified 4–10 sequence are ineffective, whereas in contrast $ACTH_{6-10}$ and the modified 4–9 analogue ORG 2766 are effective (see also Bijlsma *et al.*, 1982; De Koning *et al.*, 1986; Dekker, 1987; Van der Zee *et al.*, 1988).

In attempting to understand why exogenous administered fragments or analogues of endogenous circulating peptide hormones facilitate the repair of damaged peripheral nerve, the question arises whether these fragments mimic peptides

secreted by the pituitary. For, it is well established that hypophysectomy leads to a severe interference of macromolecule metabolism including a dramatic drop in RNA and protein synthesis in rat brain stem and spinal cord (Gispen *et al.*, 1970; Dunn & Schotman, 1986), which can be counteracted by supplementing ACTH-like peptides (Dunn & Schotman, 1986). Kanje *et al.* (1988) showed that peripheral nerve regeneration, as evaluated by the 'pinch-test', was slightly impaired in hypophysectomized rats. However, the present experiment clearly shows that after five days of hypophysectomy, when there is no pituitary source from which these peptides might reach the damaged nerve or spinal cord, the recovery period from nerve damage is similar to that seen in sham-operated rats. These data suggest that pituitary born peptides are of less importance to the repair process than those presumably found in the damaged nerve itself (Edwards *et al.*, 1984). As noted by others (Black & Lasek, 1979; Pestronk *et al.*, 1980), regeneration speed is dependent on the age of the subject. Indeed the present study confirms and extends this notion. The recovery period for a given lesion in old rats is longer than that in young rats. Comparing the oldest rats (34 months) to those of 20 months of age, the significance interaction between age and time is taken to indicate a further impairment of the recovery capacity of nerves of very old rats. Functional recovery takes longer following a proximal than following a distal lesion of the sciatic nerve (De Koning *et al.*, 1986). However, the increase in distance between lesion site and point of measurement on the foot sole in old rats (maximally 10%) is too small to account for this age-related slowing of the recovery process. The efficacy of ORG 2766 treatment in peripheral nerve regeneration is evident not only in young adult but also in 5 months and in 20 months old rats (this study). Bijlsma *et al.* (1983) previously reported a beneficial, though small, peptide effect in one year old rats.

In conclusion, the present study demonstrates that neurotrophic factors of different nature and mechanism of action may facilitate the recovery of function of damaged peripheral nerve *in vivo*. Furthermore, the data indicate that ACTH/MSH-like peptides are effective in young adult, mature and old rats. If they are effective by mimicking an endogenous peptide signal, that peptide is not of pituitary origin. Collectively the structure activity studies on the neurotrophic activity of ACTH/MSH-like peptides do question the conclusion that melanotrophic activity is responsible for the effect.

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Evidence that an L-arginine/nitric oxide dependent elevation of tissue cyclic GMP content is involved in depression of vascular reactivity by endotoxin

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1 The aim of this investigation was to study the relationship between contractile responsiveness, activation of the L-arginine pathway and tissue levels of guanosine 3' : 5'-cyclic monophosphate (cylic GMP) in aortic rings removed from rats 4 h after intraperitoneal administration of bacterial endotoxin (*E.coli.* lipopolysaccharide, LPS, 20 mg kg^{-1}).

2 LPS-treatment resulted in a reduction of the sensitivity and maximal contractile response to noradrenaline (NA).

3 Depression of the maximal contractile response was restored to control by 6-anilo-5,8-quinolinedione (LY 83583, 10 μM), which prevents activation of soluble guanylate cyclase.

4 Cyclic GMP levels in tissue from LPS-treated rats were 2 fold greater than cyclic GMP levels detected in tissue from control (saline-treated) rats. The LPS-induced increase in cyclic GMP content was observed both in the presence and absence of functional endothelium.

5 Addition of L-arginine (1 mM) to maximally contracted aortic rings produced significant relaxation of rings from LPS-treated rats but not rings from control animals. In the LPS-treated group, addition of L-arginine was also associated with a significant increase in cyclic GMP content. L-Arginine had no effect on the cyclic GMP content of control rings. D-Arginine (1 mM) was without effect.

6 In rings from LPS-treated rats, N^G-nitro-L-arginine methyl ester (L-NAME, 300 μM), an inhibitor of nitric oxide (NO) production, increased the contractile response to NA and prevented the LPS-induced increase in cyclic GMP content. In control rings, L-NAME increased the NA sensitivity only when the endothelium remained intact and reduced the cyclic GMP content of these rings to that of control endothelium-denuded rings.

7 These results demonstrate that LPS-induced hyporeactivity to NA occurs secondarily to activation of the L-arginine pathway and subsequent activation of soluble guanylate cyclase in vascular tissue. In addition they suggest that LPS induces the production of an NO-like relaxing factor in non-endothelial cells.

Keywords: Nitric oxide; cyclic GMP; N^G-nitro-L-arginine methyl ester (L-NAME); endotoxin (LPS); isolated arteries

Introduction

Administration of *E.coli* lipopolysaccharide (LPS) to rats causes hyporesponsiveness to several contractile agents such as noradrenaline (NA), vasopressin and angiotensin II (Fink *et al.*, 1985; Schaller *et al.*, 1985; Wakabayashi *et al.*, 1987) which persists *ex vivo* in tissue removed from rats treated with LPS.

Recently an important role in determining vascular function and reactivity has been attributed to nitric oxide (NO) formed in endothelial cells by oxidation of the amino acid L-arginine (Palmer *et al.*, 1988a). Modification of vascular reactivity by endothelium-derived NO is based on an increase in the guanosine 3' : 5'-cyclic monophosphate (cyclic GMP) content of smooth muscle (Holzmann, 1982; Rapoport & Murad, 1983) produced by direct stimulation of soluble guanylate cyclase (Arnold *et al.*, 1977; Ignarro *et al.*, 1986). The exact mechanisms by which cyclic GMP evokes smooth muscle relaxation are not entirely clear but increased smooth muscle cyclic GMP content is associated with reduced intracellular calcium levels (Kai *et al.*, 1987) and dephosphorylation of myosin light chain kinase (Draznin *et al.*, 1986).

Recent results from our laboratory have suggested that LPS-induced vascular hyporeactivity results from activation of an L-arginine-dependent pathway producing NO or an NO-like relaxing factor in non-endothelial cells (Fleming *et al.*, 1990a; Julou-Schaeffer *et al.*, 1990). The present study further investigates this hypothesis by the use of L-arginine, a substrate for the production of NO (Palmer *et al.*, 1988a), and N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthesis (Hobbs & Gibson, 1990). The effects of these substances on contractile responsiveness to NA and tissue cyclic GMP content were compared in parallel. In addition NO-induced activation of soluble guanylate cyclase was prevented with 6-anilo-5,8-quinolinedione (LY 83583, Mulsch *et al.*, 1988).

A preliminary account of these results has been presented to the International Congress of Pharmacology (Fleming *et al.*, 1990b).

Methods

Preparation of tissue

Male Wistar rats (12 to 15 weeks, 250–300 g) were injected intraperitoneally with either LPS (20 mg kg^{-1} in 0.15 ml 100 g^{-1} saline) or saline. After 4 h the rats were killed by stunning and cervical dislocation. Thoracic aortae were removed, cleared of adherent connective tissue and cut into rings of approximately 2 mm in length for contractile studies or divided into five large rings, four of which were used for determination of cyclic GMP. In the latter case, 2 mm rings cut from the remaining aortic segment were also used to assess contractile responsiveness to NA and the role of the L-arginine pathway. In some rings the vascular endothelium was mechanically removed by rubbing gently with blunt forceps.

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Contraction studies

Rings were mounted, under 2 g tension, in organ baths containing physiological salt solution (PSS) of the following composition (mM): NaCl 118.4, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.3, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 11.7, at 37°C and bubbled with 95% O_2: 5% CO_2 (pH 7.4). All experiments were carried out in the presence of indomethacin (10 μM). Developed tension was measured with an isometric force transducer (Celaster, Celle l'Evescault, Lusignan, France) and visualized by computer. After an equilibration period of 90 min, during which time the PSS was changed at 15 min intervals, the basal tension was re-adjusted to 2 g. The presence of functional endothelium was verified by addition of acetylcholine (ACh, 1 μM) in arteries precontracted with noradrenaline (NA, 1 μM). The ability of ACh to induce relaxation of unrubbed rings was taken as an indication of the presence of functional endothelium. The use of this criterion has previously been verified in tissues precontracted with submaximal concentrations of NA (Julou-Schaeffer *et al.*, 1990). After a washing period of 60 min contractile responses were monitored during the stepwise cumulative addition of NA (1 nM to 10 μM). To investigate the effect of NO-induced stimulation of soluble guanylate cyclase, LY 83583 (10 μM) or solvent was added to tissues 3 to 5 min after a stable maximum response to NA was obtained and the contractile response monitored over a further 15 min. To study the effect of inhibition of NO synthesis from L-arginine, tissues were incubated with either L-NAME (300 μM) or its solvent (water, 100 μl) for 10 min prior to the cumulative addition of NA. When the stable maximum response was obtained, either L- or D-arginine (1 mM) or solvent (water, 100 μl) was added to each bath. Rings were removed, blotted dry, placed in a dessicator overnight and then weighed. Results are expressed as tension developed (g) per mg dried tissue.

Determination of tissue cyclic GMP content

Aortic segments were incubated in PSS at 37°C and oxygenated with a gas mixture of 95% O_2 and 5% CO_2. After a 90 min period during which the PSS was changed at 15 min intervals the tissues were either rapidly frozen in an aluminium clamp pre-cooled in liquid nitrogen (non-stimulated) or treated as described for contractile studies and frozen following cumulative addition of NA (NA-stimulated). Aortic segments were thawed in 400 μl of perchloric acid (1N), homogenized with a Potter glass/glass homogenizer for 30 s followed by sonication (Ultrason-Annemasse, Type 75TS, France) for 15 s. Following centrifugation at 10,000 *g* for 5 min, cyclic GMP content of the supernatant was determined by radioimmunoassay (Immunotech S.A.). DNA content was measured as described previously (Brunk *et al.*, 1976). Cyclic GMP content was expressed as fmol μg^{-1} DNA.

Since the cyclic GMP content of control, endothelium-intact, tissues varied substantially between experiments, each experiment was performed with its own internal controls.

Drugs

Noradrenaline bitartrate (Sigma), was stored as a 1 μM stock solution in buffer containing Na_2SO_3 7.9 mM and HCl 34 mM and diluted as required. Acetylcholine chloride (Sigma), was stored as a 10 mM stock solution in NaH_2PO_4 buffer (pH 4) and diluted as required. All other substances were freshly prepared prior to each experiment. N^G-nitro-L-arginine methyl ester (L-NAME, Sigma), L-arginine hydrochloride (Calbiochem) and D-arginine hydrochloride (Sigma) were dissolved in deionised water, 6-anilo-5,8-quinolinedione (LY 83583, Lilly) was prepared as a 10 mM solution in dimethylsulphoxide (DMSO) and diluted in water, indomethacin (Sigma) was dissolved in 4% $NaHCO_3$ solution and LPS (*E.coli* 055 : B5, Difco) was dissolved in saline (0.9% NaCl).

Parameters and statistical analysis

The concentrations of agonist causing half-maximal contraction (EC_{50}) were calculated by logit-log regression and are expressed as pD_2 values ($-\log EC_{50}$). pD_2 values, maximal contractile responses and changes in tension induced by L- or D-arginine (expressed as a percentage of the maximal contractile response to NA) were compared by Student's *t* test for unpaired data. Statistical comparisons of cyclic GMP content were made by analysis of variance (ANOVA). If significant differences were detected by ANOVA, individual means were compared with an *a posteriori* Student-Newman-Keuls test. *P* values of less than 0.05 were considered significant. Results are expressed throughout as the mean ± standard error of the mean (s.e.mean) from *n* experiments.

Results

Effect of LPS-treatment on aortic ring contractility

Compared with the response of tissues from control animals, LPS-treatment induced a rightward shift of the concentration-response curve to NA represented by a decrease in the pD_2 values (Table 1). LPS-treatment also induced a reduction of the maximal contractile response (by approximately 35%, $P < 0.05$). In rings from control rats, contractile responsiveness was modified by the presence of functional endothelium (Table 1). In contrast, contractile responsiveness in rings from LPS-treated rats was not altered by removal of the endothelium (Table 1). This is in agreement with results obtained previously (Julou-Schaeffer *et al.*, 1990).

Effect of 6-anilo-5,8-quinolinedione

LY 83583 (10 μM), which destroys NO and prevents activation of soluble guanylate cylase (Mulsch *et al.*, 1988), caused a significant increase in recorded tension of rings from control animals only when the endothelium remained intact (the maximum contractile response being 3.71 ± 0.44 before and 4.48 ± 0.30 g mg^{-1} tension, NS, after LY 83583 in the absence of endothelium and 3.07 ± 0.39 before and 4.42 ± 0.30 g mg^{-1} tension, $P < 0.05$, after LY 83583 in the presence of endothelium, Figure 1).

In aortic rings from rats given LPS, LY 83583 increased the contractile response to NA towards that of control tissues both in the absence and presence of endothelium (the

Table 1 Effects of *E.coli* lipopolysaccharide (LPS)-treatment and N^G-nitro-L-arginine methyl ester (L-NAME) on contractile responses to noradrenaline

	Treatment	Maximum contractile response (g mg^{-1} dried tissue)	pD_2
Control +E	Solvent	3.20 ± 0.28	7.34 ± 0.16
Control −E	Solvent	2.79 ± 0.18	8.04 ± 0.08*
Control +E	L-NAME	4.07 ± 0.43††	8.19 ± 0.11††
Control −E	L-NAME	3.44 ± 0.52	8.62 ± 0.17
LPS-treated +E	Solvent	2.05 ± 0.30†	6.85 ± 0.16†
LPS-treated −E	Solvent	2.24 ± 0.21††	7.20 ± 0.16††
LPS-treated +E	L-NAME	4.45 ± 0.45	7.89 ± 0.13
LPS-treated −E	L-NAME	4.49 ± 0.45	8.27 ± 0.13

Representation of maximal contractile responses and pD_2 values obtained to noradrenaline (1 nM to 10 μM) in aortic rings with (+E) and without (−E) endothelium, from control and LPS-treated rats, in the presence or absence of L-NAME (300 μM). Results are presented as mean ± s.e.mean, $n = 9$–12. * Indicates a significant difference from the absence of endothelium ($P < 0.05$). † Indicates a significant difference from respective control (saline-treated) groups not treated with LPS or L-NAME: †$P < 0.05$; ††$P < 0.01$.

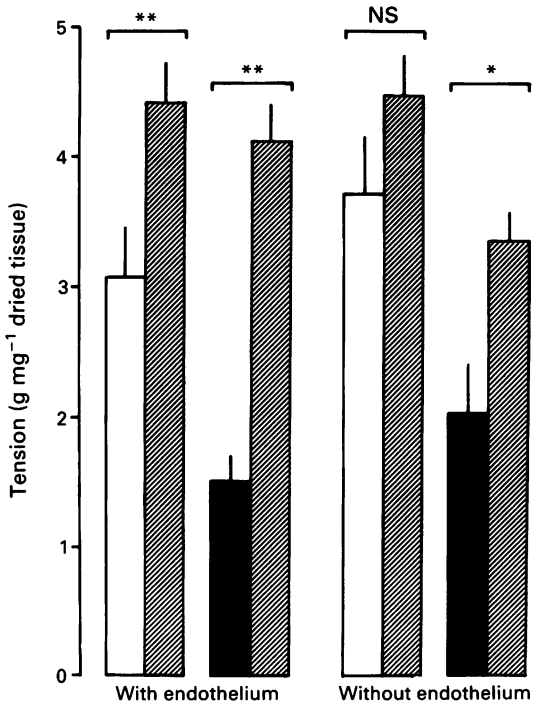

Figure 1 Histogram depicting the effect of 6-anilo-5,8-quinolinedione (10 μM, hatched columns) in aortic rings, with and without endothelium, from control (open columns) and *E.coli* lipopolysaccharide (LPS) treated rats (solid columns) maximally contracted with noradrenaline (10 μM). Results are presented as mean of $n = 9$, vertical lines show s.e.mean. Significant difference between groups: $^{*}P < 0.05$; $^{**}P < 0.01$; NS, not significant.

maximum contractile response being increased from 1.51 ± 0.20 to $4.12 \pm 0.29\,g\,mg^{-1}$ tension, $P < 0.0001$, in the presence of endothelium and from 2.04 ± 0.37 to $3.36 \pm 0.21\,g\,mg^{-1}$ tension, $P < 0.005$, in the absence of endothelium, Figure 1).

Similar results were obtained when activation of soluble guanylate cyclase was inhibited with methylene blue (Fleming *et al.*, 1990a; Julou-Schaeffer *et al.*, 1990).

Effect of LPS-treatment on cyclic GMP content

Administration of LPS to rats was associated with a 2 fold ($P < 0.005$) increase in the cyclic GMP content of endothelium intact, NA-stimulated aortic tissue (Figure 2). In control tissues the level of cyclic GMP was modified by the presence of endothelium, detected levels of cyclic GMP being 9 fold greater ($P < 0.001$) in the presence compared with the absence of endothelium. In contrast, removal of the endothelium in rings from LPS-treated rats did not significantly alter cyclic GMP content (Figure 2).

Contraction studies on the fifth segment of aortae in which cyclic GMP was determined, verified that LPS-treatment resulted in a decrease in the maximal contractile response to NA, maximal contractile responses being, $2.97 \pm 0.31\,g\,mg^{-1}$ tension in control and $1.49 \pm 0.23\,g\,mg^{-1}$ tension in the LPS-treated group in the presence of endothelium ($n = 4$–5) and $3.81 \pm 0.27\,g\,mg^{-1}$ tension in control and $1.76 \pm 0.17\,g\,mg^{-1}$ tension in the LPS-treated group in the absence of functional endothelium ($n = 4$–5).

LPS-treatment produced similar results in aortic rings in the absence of any stimulation by NA (not shown).

Role of the L-arginine pathway

Effect of L- and D-arginine In NA-stimulated tissues from control and LPS-treated rats, neither the presence of solvent nor D-arginine had any significant effect on the contractile response (Figure 3) or tissue levels of cyclic GMP (Figure 4).

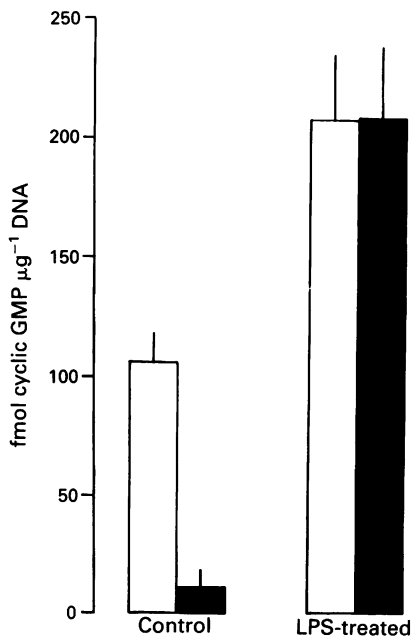

Figure 2 Histogram showing the cyclic GMP content in aortic rings maximally contracted with noradrenaline (10 μM), from control and *E.coli* lipopolysaccharide (LPS)-treated rats, in the presence (open columns) and absence (solid columns) of functional endothelium. Results are presented as mean of $n = 6$; vertical lines show s.e.mean.

Addition of L-arginine did not cause relaxation or increase cyclic GMP levels in control tissue. However, in rings from LPS-treated rats, L-arginine produced significant relaxation ($72 \pm 7\%$, $P < 0.005$ in the presence and $72 \pm 10\%$, $P < 0.005$ in the absence of endothelium, Figure 3) and caused a 5 to 6

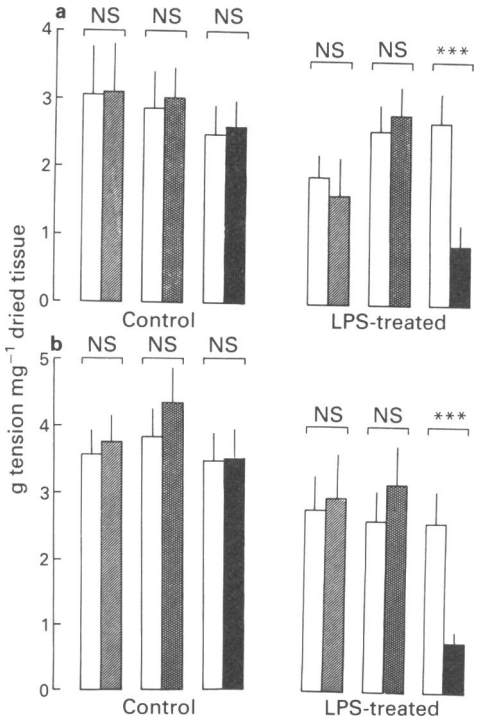

Figure 3 Comparative effects of solvent (hatched columns), D-arginine (1 mM, crosshatched columns) and L-arginine (1 mM, solid columns) on the contractile response of aortic rings from control and *E.coli* polysaccharide (LPS)-treated rats maximally contracted with noradrenaline (10 μM, open columns) (a) in the presence and (b) in the absence of endothelium. Results are presented as mean of $n = 6$; vertical lines show s.e.mean. Significant difference between groups: $^{***}P < 0.005$; NS, not significant.

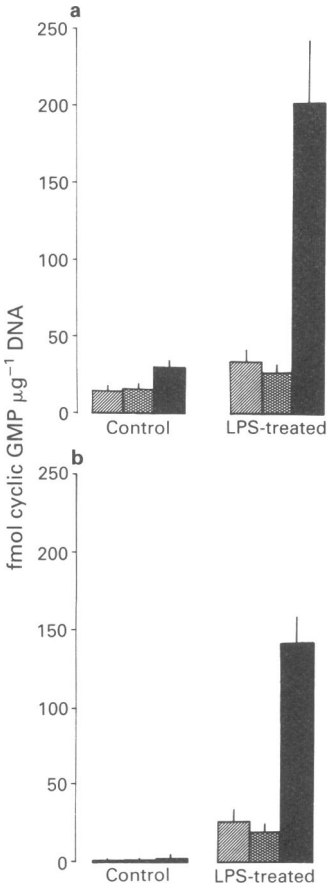

Figure 4 Histogram depicting the cyclic GMP content in aortic rings, (a) with and (b) without endothelium, from control and *E.coli* lipopolysaccharide (LPS)-treated rats maximally contracted with noradrenaline (10 μM) in the presence of either solvent (hatched columns), D-arginine (1 mM, crosshatched columns) or L-arginine (1 mM, solid columns). Results are presented as mean of $n = 6$; vertical lines show s.e.mean.

fold increase ($P < 0.005$) in the cyclic GMP content (from 33.4 ± 8.9 and 26.0 ± 8.2 fmol μg^{-1} DNA to 201.5 ± 41.0 and 141.2 ± 17.6 fmol μg^{-1} DNA in the presence and absence of endothelium respectively, Figure 4).

Effect of L-NAME Inhibition of NO production with L-NAME increased the NA-sensitivity and maximum contraction of aortic rings from control animals only when the endothelium remained intact (Table 1). This is consistent with a basal production of NO by endothelial cells. In the presence of L-NAME, addition of either L- or D-arginine (1 mM) to arteries maximally contracted with NA was without significant effect on contractile tension (not shown). In control tissues L-NAME (300 μM, Figure 5) reduced the cyclic GMP content of endothelium intact rings to that of endothelium-denuded rings (from 26.4 ± 9.65 fmol μg^{-1} DNA to 3.28 ± 0.12 fmol μg^{-1} DNA, $P < 0.001$).

In tissues from LPS-treated rats, L-NAME (300 μM, Table 1) restored NA-sensitivity and maximal contractile responses to control levels. Addition of L-arginine (1 mM) in the presence of L-NAME induced a relaxation in tissues maximally contracted with NA (by $46 \pm 7\%$ in the presence and $60 \pm 10\%$ in the absence of endothelium respectively, $P < 0.01$). D-Arginine failed to produce any relaxation (not shown). L-NAME also abolished LPS-induced increases in cyclic GMP content (cyclic GMP content being reduced from 136.6 ± 27.5 and 109.4 ± 28.2 fmol μg^{-1} DNA to 3.51 ± 0.67 and 5.23 ± 0.73 fmol μg^{-1} DNA, in the presence and absence of endothelium, respectively, $P < 0.001$, Figure 5).

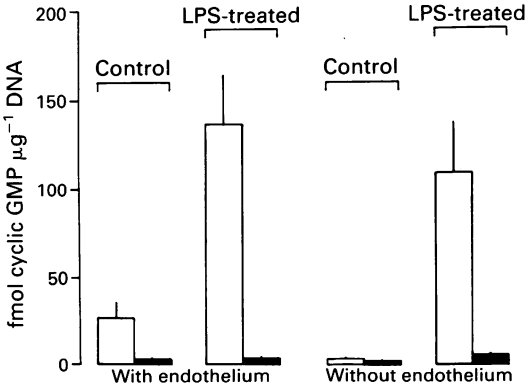

Figure 5 Histogram depicting the cyclic GMP content in aortic rings, with and without endothelium, from control and *E.coli* lipopolysaccharide (LPS)-treated rats maximally contracted with noradrenaline (10 μM) in the presence (solid columns) and absence (open columns) of N^G-nitro-L-arginine methyl ester (300 μM). Results are presented as mean of $n = 5$; vertical lines show s.e.mean.

Discussion

The present study demonstrates a reduced responsiveness to NA in aortic rings removed from LPS-treated rats. This is in agreement with previously published results (Wakabayashi *et al.*, 1987; Bigaud *et al.*, 1990).

Modification of vascular reactivity by endothelium-derived NO is based on an increase in the cyclic GMP content of smooth muscle (Rapoport & Murad, 1983) produced by direct stimulation of soluble guanylate cyclase (Arnold *et al.*, 1977; Ignarro *et al.*, 1986; Mulsch *et al.*, 1987). In the present study LY 83583, which at the concentration used has been reported to destroy NO and inhibit soluble guanylate cyclase (Mulsch *et al.*, 1988; 1989a), increased contractile tension in aortic tissue from control rats only when the endothelium remained intact. This result is consistent with activation of soluble guanylate cyclase by NO basally released from the endothelium. In contrast, in aortic rings from LPS-treated rats, restoration of contractile responsiveness by LY 83583 was independent of the presence of functional endothelium. The hypothesis that soluble guanylate cyclase was activated following administration of LPS is supported by direct measurement of tissue cyclic GMP content. In endothelium-intact aortic rings from LPS-treated rats the cyclic GMP content was 2 fold greater than that of control rings.

In both non-stimulated and NA-stimulated rings from control rats, the presence of a functional endothelium was associated with a 4 to 5 fold difference in tissue cyclic GMP content. In contrast, the cyclic GMP content of tissue from LPS-treated rats was elevated to comparable levels in both the presence and absence of endothelium. Taken together, the cyclic GMP measurements and results obtained with LY 83583 indicate that, activation of soluble guanylate cyclase by a substance of non-endothelial origin precedes induction of vascular hyporeactivity by LPS.

In accordance with the results presented here, McKenna (1988) reported that inhibition of endogenous cyclic GMP production by methylene blue improved responses of aortae from septic rats to both NA and KCl. However, an elevated vascular content of cyclic GMP was not associated with this observation. The reasons for this contradictory finding are not clear but may reflect a difference between models of endotoxic and septic shock.

Since NO is known to activate soluble guanylate cyclase in smooth muscle (Arnold *et al.*, 1977) the role of the oxidative L-arginine/NO pathway in induction of the LPS-induced hyporeactivity and elevation of cyclic GMP was investigated. L-Arginine, substrate for the production of NO (Palmer *et al.*, 1988a), induced relaxation and a 5 to 6 fold increase of cyclic GMP in rings from LPS-treated but not control animals. The stereospecific nature of the pathway was demonstrated by the

lack of response to D-arginine. These observations indicate that LPS-treatment induces activation of the L-arginine pathway in vascular tissue. Continued activation of this pathway in vascular tissue *ex vivo* would result in depletion of endogenous substrate and account for the relaxation and elevated cyclic GMP observed upon exogenous addition of L-arginine. L-NAME, an inhibitor of NO biosynthesis, produced effects on control tissue that are consistent with basal production of NO by the endothelium and restored the contractile responsiveness of LPS-treated rings to control. The effect of L-NAME was reversed by L- but not D-arginine. Restoration of vascular responsiveness was concomitant with a reduction of cyclic GMP to a level comparable with that of endothelium-denuded control tissue. Thus the restoration of vascular reactivity in tissue from LPS-treated animals produced by inhibition of the L-arginine pathway, was associated with a decrease in the cyclic GMP content.

The L-arginine pathway exhibits a stereospecific requirement for L-arginine (Palmer *et al.*, 1988b; Sakuma *et al.*, 1988), is inhibited by L-arginine analogues such as N^G-monomethyl-L-arginine (Rees *et al.*, 1989) or L-NAME (Hobbs & Gibson, 1990) and usually acts as a transduction mechanism for stimulation of soluble guanylate cyclase (Ignarro *et al.*, 1986; Mulsch *et al.*, 1987; Moncada *et al.*, 1989). Using these criteria the presence of the L-arginine pathway has been demonstrated in a number of tissues that may be activated by LPS namely, mast cells (Salvemini *et al.*, 1990a), platelets (Radomski *et al.*, 1990), neutrophils (Rimele *et al.*, 1988), macrophages (Hibbs *et al.*, 1988; Marletta *et al.*, 1988; Stuehr *et al.*, 1989) and vascular endothelium (Mulsch *et al.*, 1989b; Mayer *et al.*, 1989; Salvemini *et al.*, 1989; 1990b). Within vascular tissue removed from LPS-treated rats the cellular source of NO is unlikely to be the endothelium since the L-arginine-dependent NO producing pathway was activated in endothelium-denuded tissue from LPS-treated rats. The efficiency of our technique of endothelium removal was demonstrated in aortic tissue from control animals both by the low level of cyclic GMP detected and by lack of responsiveness to acetylcholine.

We and others, have recently reported that incubation of endothelium-denuded rat aortae with LPS results in a hyporeactivity to NA, identical to that described in vessels, from LPS-treated rats and is associated with an increased tissue content of cyclic GMP (Fleming *et al.*, 1990a; Beasley, 1990). This observation would tend to exclude as the NO source all cell types not indigenous to vascular tissue, with the exception of blood constituents that remain attached to or which infiltrate the vessel wall on removal from the rat. Resident populations of lymphocytes and macrophages have been described within the aortic adventitia (Rhodin, 1980) and intima (Freudenberg & Riese, 1976) of untreated rats. Therefore, although the number of these cells in vascular tissue may be depleted during the endothelium denuding process, they remain possible sources of NO production. Additionally, the possibility cannot be excluded that cells such as fibroblasts or smooth muscle can be activated by LPS to produce NO. Indeed, results obtained from endothelium-denuded arterial tissue have recently been proposed as evidence for the existence of an NO-like vascular smooth muscle-derived relaxing factor (Wood *et al.*, 1990).

The results of this investigation indicate that within endothelium-denuded vascular tissue there exists an LPS-sensitive or inducible enzyme that converts L-arginine to a metabolite which activates soluble guanylate cyclase. This metabolite exhibits the characteristic features of NO generated by the oxidative L-arginine pathway.

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P_2 purinoceptor regulation of mucin release by airway goblet cells in primary culture

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1 The effects of adenine analogues on mucin release by airway goblet cells have been examined in a hamster primary tracheal epithelial cell culture.

2 Adenosine, a P_1 receptor agonist, had no effect on mucin release even at 2 mM, while ATP, a P_2 receptor agonist, stimulated mucin release in a dose-dependent fashion with an apparent EC_{50} of 20 μM. The relative potency order among adenine nucleotides was ATP > ADP > AMP = adenosine.

3 $ATP_\gamma S$, a non-hydrolyzable analogue of ATP, was equipotent with ATP in stimulating mucin release. The potency order among some ATP analogues was ATP > 2-methylthio ATP > α,β-methylene ATP > β,γ-methylene ATP. Reactive blue 2, a putative P_{2y}-purinoceptor antagonist, did not block the ATP-induced mucin release.

4 The present results indicate that mucin release by airway goblet cells is stimulated by extracellular ATP via P_2 receptor-mediated mechanism. We suggest that this mechanism may be important in the physiological regulation of airway goblet cell mucin release *in vivo*.

Keywords: P_2-purinoceptor; airway goblet cells; mucin release

Introduction

Mucus lining the airway luminal surface plays an important role in host defence against airborne particles and chemicals. The protective function of mucus is due mainly to the physicochemical properties of constituent mucous glycoproteins (mucins). Abnormalities in either the quality or the quantity of airway mucins not only affects removal of respired particles and bacteria, but may also impair host defences leading to further pathology.

In man, airway mucins are derived from two different cell types; globlet cells of the surface epithelium and mucous cells of the submucosal gland. Although much is known about the regulation of airway mucin secretion by submucosal glands, very little is known about goblet cell mucin regulation. Release of airway goblet cell mucins can be stimulated by two classes of agents: (a) inhaled chemical 'irritants' such as sulphur dioxide (Spicer *et al.*, 1974), ammonia vapour (Gallagher *et al.*, 1986), and tobacco smoke (Jones *et al.*, 1973) and (b) various kinds of proteases released during airway inflammation (Klinger *et al.*, 1984; Adler *et al.*, 1986; Niles *et al.*, 1986; Kim *et al.*, 1987). These agents are not present in normal airways.

Recently, we have characterized a primary cell culture system derived from hamster tracheal surface epithelium (HTSE) (Kim *et al.*, 1985; Wasano *et al.*, 1988; Kim *et al.*, 1989b). Confluent cultures of HTSE cells become highly enriched (>90%) with secretory cells, resembling goblet cells in their morphology, and synthesize and secrete mucin-like glycoproteins (MLGP). Judged by their carbohydrate structure, these MLGP are indistinguishable from *in vivo* mucins. Use of tracheal surface epithelial cell cultures as an *in vitro* model for studying the regulation of goblet cell mucin release has recently been reviewed (Kim & Brody, 1989). Using this primary cell culture system, our laboratory has tested a number of agents for their possible effects on mucin release from airway goblet cells. We have identified four different conditions under which mucin release is stimulated; increases or decreases of pH of the cell culture medium (Kim *et al.*, 1989a), neutrophil proteases (Kim *et al.*, 1987; 1989a), hypoosmolarity (Kim *et al.*, 1989a), and retraction of collagen gels on which secretory cells are maintained (Kim & Brody, 1987). Both the pH change and the presence of proteases in the airway luminal fluids are conditions which can be caused by inhalation of 'irritant gases' and during airway inflammation, respectively, and have been shown to stimulate release of goblet cell mucins *in vivo*. However, no endogenous agents which can influence mucin release from primary TSE cell cultures have been identified yet.

In this paper, we show that mucin release from cultured airway goblet cells can be stimulated by ATP via a P_2 receptor-mediated mechanism. These observations provide for the first time a secretory control mechanism for airway goblet cell mucins that could be physiological in nature and probably involves a cell surface receptor.

Methods

Hamster primary tracheal surface epithelial (HTSE) cell culture

Tracheae were obtained from male golden syrian hamsters eight to ten weeks of age (Harlan Sprague Dawley, Indianapolis, Indiana, U.S.A.). HTSE cells were harvested and cultured as previously described (Kim *et al.*, 1989a). Dissociated cells were plated on a thick collagen gel prepared inside 24 well tissue culture dishes (Falcon) as previously described (Kim *et al.*, 1989a).

Metabolic labelling of mucins and treatment of cultures

Mucins were metabolically radiolabelled by incubating confluent cultures (24 well plates) with 0.2 ml/well of the complete growth medium containing 10 μC ml^{-1} of [^{3}H]-glucosamine for 24 h, and at the end of the labelling period the spent media (the pretreatment or pulsed sample) was collected. After collecting the spent media, the cultures were washed twice with Dulbecco's phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS) in order to remove both remaining radioactive materials and serum components. The washed cultures were then chased for 30 min in PBS containing test materials in appropriate concentrations, and the chased media (the treatment sample) collected. In the case of reactive blue 2, the drug was added 10 min prior to the 20 min treatment with ATP. Both pretreatment and treatment samples were centrifuged to remove floating cells and stored at −60°C until assayed for their ^{3}H-mucin content.

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Quantitation of ^{3}H-mucins

High molecular weight glycoconjugates excluded from Sepharose CL-4B (Pharmacia) and resistant to proteoglycan-digesting enzymes were defined as mucins (Kim *et al.*, 1985). Details of the mucin assay have previously been described (Kim *et al.*, 1987). 'Mucin release' from each dish during treatment was expressed as a ratio of the amount of ^{3}H-mucin released during the treatment period to the amount of ^{3}H-mucin released during the pretreatment period in order to compensate for variations in basal release rate among dishes.

Detection of cell membrane damage

The presence or absence of cytoplasmic leak due to cell membrane damage following treatments was identified by measuring lactic acid dehydrogenase (LDH) in the culture medium with a commercial LDH assay kit (Sigma) as previously described (Kim *et al.*, 1989a). Thus, the term 'cell membrane damage' throughout the text refers to cytoplasmic leak due to cell membrane damage. Briefly, the spent medium was collected, centrifuged, and a 50 μl aliquot of the supernatant immediately added to the reaction mixture. At the end of the reaction, OD_{500} of each sample was read in a spectrophotometer. For a positive control, LDH from *Leuconostoc mesenteroidas* (Sigma) was used; the presence of 0.1 u of the enzyme in 50 μl of the medium resulted in a decrease of OD_{500} by 0.2.

Materials

The sources of chemicals used were as follows: D-[6-^{3}H]-glucosamine HCl (39.2 Ci mmol^{-1}; New England Nuclear, Boston, MA, U.S.A.); adenosine, adenosine 5'-monophosphate (AMP), adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), α,β-methylene ATP, β,γ-methylene ATP, ATP$_\gamma$S, and reactive blue 2 (60% pure) from Sigma Chemical Co. (St. Louis, MO, U.S.A.); 2-methylthio ATP from Research Biochemicals Inc. (Natick, MA, U.S.A.).

Statistics

Means of 'mucin release' values of each group were converted to percentage of the control and expressed as mean values $\pm$s.e. The difference between groups was assessed by Student's t test for unpaired samples. $P > 0.05$ was considered as not significantly different.

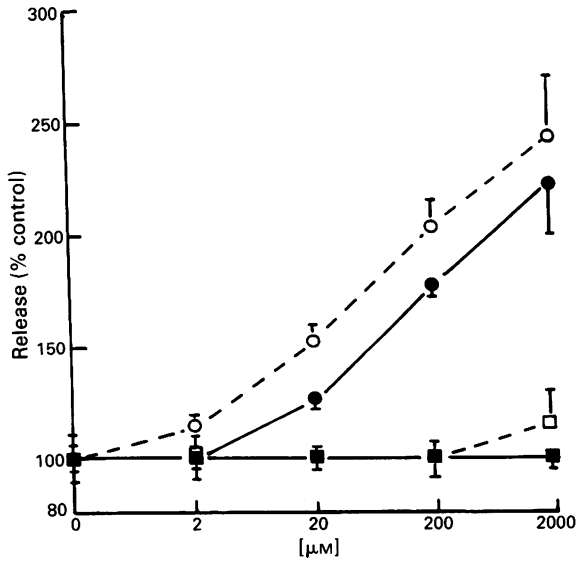

Figure 1 Effect of ATP, ADP, AMP and adenosine on ^{3}H-mucin release in hamster tracheal epithelial cell culture. Confluent cultures (16 mm) were pulsed with [^{3}H]-glucosamine for 24 h and chased for 30 min in the presence of the adenine analogues: ATP (○), ADP (●), AMP (□), and adenosine (■). Data represent means of four culture dishes with s.e. shown by vertical bars.

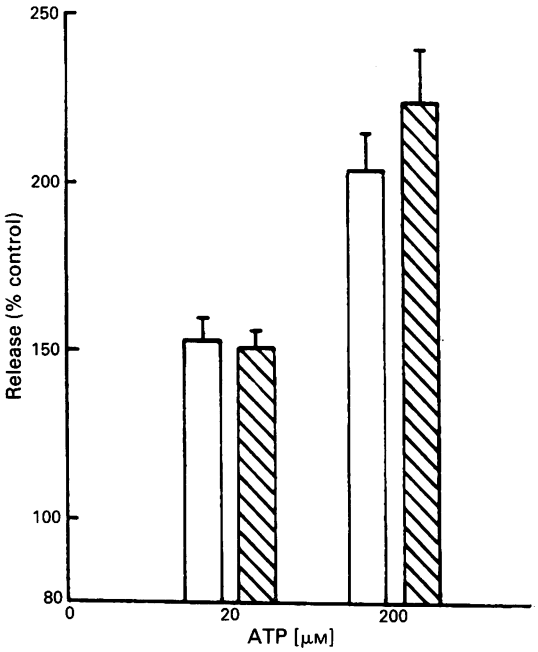

Figure 2 Effect of ATP$_\gamma$S on ^{3}H-mucin release in hamster tracheal epithelial cell culture. Confluent cultures (16 mm) were pulsed with [^{3}H]-glucosamine for 24 h and chased for 30 min in the presence of ATP (open columns) or ATP$_\gamma$S (hatched columns). Data represent means of four culture dishes; s.e. shown by vertical bars. There were no significant ($P > 0.05$) differences between ATP- and ATP$_\gamma$S-treated groups.

Results

Effect of adenine nucleotides on mucin release

ATP caused a concentration-dependent increase of mucin release with an apparent EC_{50} of 20 μM (Figure 1), whereas neither adenosine nor AMP influenced the rate of mucin release even at 2 mM concentration. Mucin release was stimulated also by ADP in a dose-dependent fashion; 28% by 20 μM and 78% by 200 μM. Thus, the mucin-releasing potency among these adenine nucleotides was in the order of ATP > ADP > AMP = adenosine (Figure 1). ATP$_\gamma$S, a non-hydrolyzable analogue of ATP, was equipotent with ATP (Figure 2). Other ATP analogues tested also showed mucin releasing effects and their potency order was; ATP > 2-methylthio ATP > α,β-methylene ATP > β,γ-methylene ATP (Figure 3). No detectable cytotoxicity was observed with 2 mM ATP based on LDH release (Table 1).

Effects of reactive blue 2 and Mg^{2+} on ATP-induced mucin release

As shown in Figure 4, reactive blue 2 itself caused significant mucin release (52% by 30 μM and 79% by 100 μM). Addition of 40 μM ATP to the cultures which had been pretreated with

Table 1 Effect of ATP on lactic acid dehydrogenase (LDH) release in hamster tracheal epithelial cell culture

Treatment	*OD* ($n = 4$)
PBS	0.537 ± 0.006
ATP (2 mM)	0.550 ± 0.007

Confluent cultures (16 mm wells) were treated for a 30 min period, and at the end of the treatment period, aliquots of spent media were collected and assayed for the LDH activity as described in Methods. The OD_{500} value for the control (PBS) group corresponds to about 0.6% of the total cellular LDH activity. Each value represents a mean ± s.e. of four wells. There were no significant differences ($P > 0.05$) among the above treatment groups.

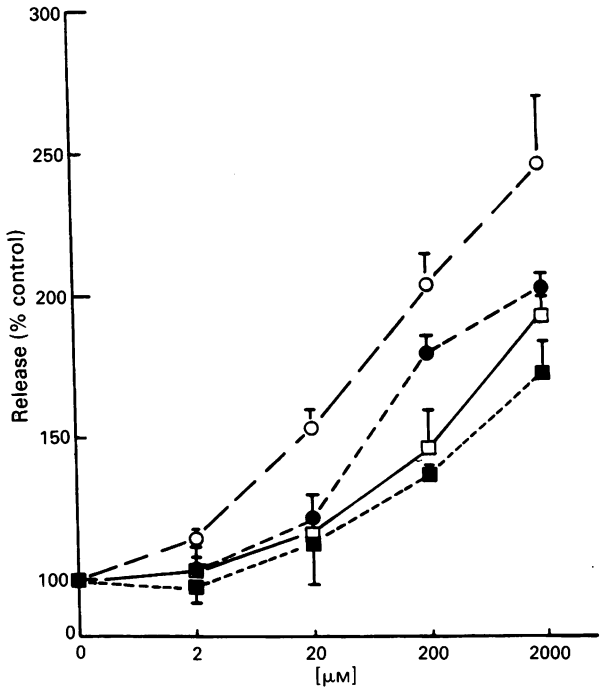

Figure 3 Effect of ATP, 2-methylthio ATP, α,β-methylene ATP, and β,γ-methylene ATP on ^{3}H-mucin release in hamster tracheal epithelial cell culture. Confluent cultures (16 mm) were pulsed with [^{3}H]-glucosamine for 24 h and chased for 30 min in the presence of the ATP analogues: ATP (○), 2-methylthio ATP (●), α,β-methylene ATP (□), and β,γ-methylene ATP (■). Data represent means of four culture dishes; vertical bars show s.e.mean.

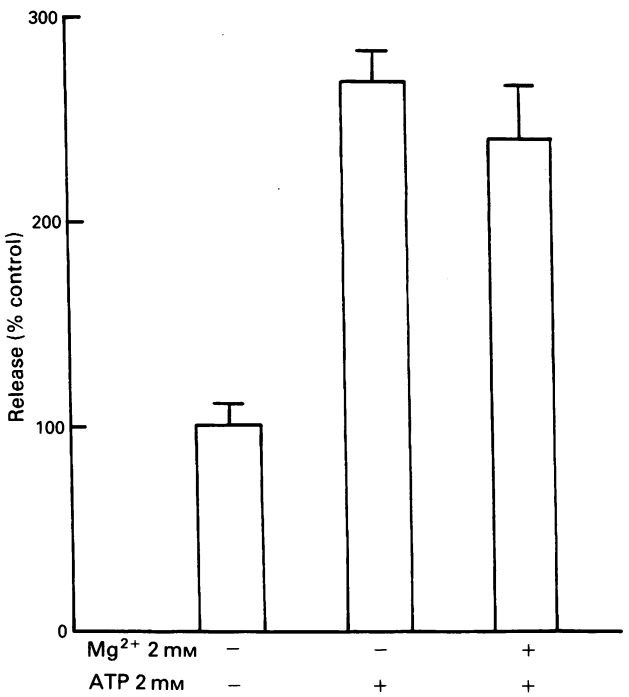

Figure 5 Effect of Mg^{2+} on ATP-induced ^{3}H-mucin release in hamster tracheal epithelial cells in culture. Confluent cultures (16 mm) were pulsed with [^{3}H]-glucosamine for 24 h and chased for 30 min in the presence of a combination of 2 mM ATP and 2 mM $MgCl_2$. ATP was prepared in Ca^{2+}, Mg^{2+} free PBS (see Methods) which was used as a chase medium. Data represent means of four culture dishes; s.e. shown by vertical bars. Mucin release by ATP was not significantly different ($P > 0.05$) in the presence or absence of Mg^{2+}.

30 μM or 100 μM reactive blue 2 resulted in greater increases in mucin release than either ATP or reactive blue 2 alone; increases in mucin release by 40 μM ATP in cultures pretreated with 0, 30 and 100 μM reactive blue 2 were 54%, 104%, and 121%, respectively. The presence or absence of 2 mM Mg^{2+} in the treatment solution did not affect the mucin releasing effect of 2 mM ATP (Figure 5).

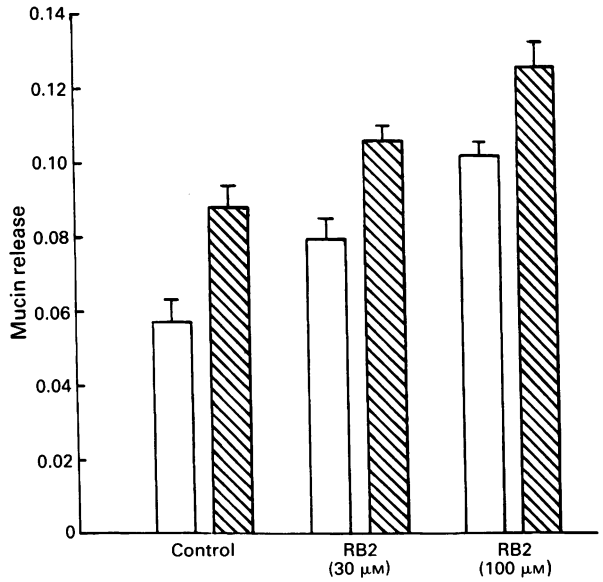

Figure 4 Effect of ATP on ^{3}H-mucin release in the presence of reactive blue 2 in hamster tracheal epithelial cell culture. Confluent cultures (16 mm) were pulsed with [^{3}H]-glucosamine for 24 h and chased for 30 min in the presence of a combination of reactive blue 2 and ATP. Reactive blue 2 was added 10 min prior to the addition of ATP. The ordinate scale (mucin release) represents the ratio of ^{3}H-mucin released during the chase period to ^{3}H-mucin released during the pulse period: without ATP (open columns) and with ATP (hatched columns). Data represent means of four culture dishes; s.e. shown by vertical bars. Addition of ATP caused significant ($P < 0.01$) increases, regardless of the presence of reactive blue 2.

Discussion

It is well known that extracellular purine nucleotides interact with their specific cell surface receptors or purinoceptors. Purinoceptors have been divided into two classes based on relative pharmacological potency order of agonists, specific antagonists, and their effects on adenosine 3′ : 5′-cyclic monophosphate (cyclic AMP) levels (Burnstock, 1978): P_1 purinoceptors induce responses to adenosine > AMP > ADP > ATP, and are antagonized by the xanthines, and affect cyclic AMP levels. In contrast, P_2 purinoceptor-mediated events occur in response to ATP > ADP > AMP > adenosine, they are not antagonized by xanthines, and they do not influence cyclic AMP levels. As can be seen in Figure 1, the potency order of mucin release by these analogues indicates that mucin release by purine nucleotides involves a P_2 purinoceptor-mediated mechanism. In addition, the fact that both ATP and $ATP_\gamma S$ showed the same potency (Figure 2) seems to suggest that the ATP-induced mucin release may not involve the hydrolysis of ATP. Recently, P_2 purinoceptors were subdivided into at least two subtypes based purely on potency order (Burnstock & Kennedy, 1985): P_{2x} purinoceptors respond to α,β-methylene ATP = β,γ-methylene ATP > ATP = 2-methylthio ATP, while P_{2y} purinoceptors are stimulated by 2-methylthio ATP ≫ ATP > α,β-methylene ATP = β,γ-methylene ATP. Figure 3 shows that the mucin-releasing potency among these four analogues is in the order of ATP > 2-methylthio ATP > α,β-methylene ATP > β,γ-methylene ATP. Thus, interestingly, the P_2 purinoceptor responsible for mucin release does not appear to belong to

either P_{2x} or P_{2y}. It is important to note that the original classification of these P_2 purinoceptors was based on the potency order of these ATP analogues on smooth muscle contractility (Burnstock & Kennedy, 1985), but there are considerable variations among tissues and cell types in their relative potency order (Gordon, 1986).
1986).

Recently, the type II pneumocyte, another population of airway epithelial secretory cells, has been shown to release phospholipids in response to ATP presumably via a P_{2y} receptor-mediated mechanism, again purely based on the pharmacological potency order (Rice & Singleton, 1986). The ATP-induced phospholipid release by cultured type II pneumocytes was blocked by reactive blue 2 in a dose-dependent manner (Rice & Singleton, 1989). Reactive blue 2 was previously found to be a specific P_{2y} receptor blocker in smooth muscle preparations (Burnstock & Warland, 1987). In the present HTSE cell culture system, the concentrations of reactive blue 2 which caused phospholipid release in type II pneumocytes did not block the ATP-induced mucin release, but rather increased mucin release by itself (Figure 4). Therefore, it appears that the P_2 purinoceptor on HTSE cells is different from the one on type II pneumocytes. However, the difference may not be the receptor itself but the different micromilieu on the cell surface; we have recently shown that the cell surface of HTSE cells contains mucins tightly bound to the external plasma membrane (Kim *et al.*, 1987). It is possible that these cell surface mucins or the mucin layer serve as a barrier to these ligands, thus altering their effective concentrations at the receptor site.

Lastly, some evidence exists for the presence of another purinoceptor on the cell membrane, specific only for ATP^{-4} (a tetravalent form of ATP) (Tatham *et al.*, 1988). Stimulation of this receptor causes the permeabilization of the cell membrane (Cockcroft & Gomperts, 1979) and the subsequent Ca^{2+}-dependent release of histamine from mast cells (Bennett *et al.*, 1981). However, in HTSE cells, ATP-induced mucin release does not seem to involve such a mechanism judging from the following data: (1) ATP induced mucin release even in a Ca^{2+}-free PBS solution, and (2) addition of a high concentration of Mg^{2+} which should dramatically reduce the actual concentration of ATP^{-4} did not significantly ($P > 0.05$) influence the effect of ATP (Figure 5).

We conclude that mucin release from cultured airway goblet cells can be stimulated by ATP and other purine nucleotides via a P_2 receptor-mediated mechanism. This may be a physiological mechanism involved in the regulation of mucin release by airway goblet cells *in vivo*. Understanding of the pharmacology of the P_2-purinoceptor-induced mucin release may provide useful strategies for development of new drugs controlling airway mucin secretion. We are currently working on the biochemical mechanisms involved in the ATP-mediated mucin release.

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Pulse exposure to protein synthesis inhibitors enhances vascular responses to des-Arg^9-bradykinin: possible role of interleukin-1

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1 The modulation of the spontaneous increase in contractile responses to des-Arg^9-bradykinin (des-Arg^9-BK) of rabbit aortic strips incubated *in vitro* was studied. Rapid hypotensive responses to exogenous kinins were also measured in rabbits anaesthetized 5 h following pretreatment.

2 Continuous exposure to the protein synthesis inhibitors cycloheximide (71 μM) or anisomycin (3.8 μM) profoundly inhibited the sensitization to des-Arg^9-BK in incubated aortic strips. However, temporary (3 h) inhibition of protein synthesis *in vitro* followed by further incubation (3 h) of tissues without inhibitor, paradoxically enhanced both the maximal contractile responses to des-Arg^9-BK (1.7 μM) and the apparent affinity of the kinin without affecting contractions to noradrenaline (NA, 100 nM) at 6.5 h.

3 The stimulatory activity of the short treatment (pulse) with cycloheximide was abolished in the presence of dexamethasone sodium phosphate (100 μM throughout the incubation). The function of receptors for kinins did not appear to be altered directly by the steroid treatment.

4 Interleukin-1β (IL-1β), applied at low concentrations (100–250 pg ml^{-1}) on aortic strips between 3 h and 6.5 h of incubation time, mimicked the selective stimulatory effect of the cycloheximide pulse on responses to des-Arg^9-BK. Higher concentrations of IL-1β (0.5–5 ng ml^{-1}) did not further amplify the responses to des-Arg^9-BK but decreased the contractile responses to NA.

5 The modulation by IL-1β of vascular sensitivity to des-Arg^9-BK and to NA was prevented by blockade of protein synthesis.

6 The induction *in vivo* by IL-1β (5 μg kg^{-1}) or by cycloheximide (10 mg kg^{-1}) of cardiovascular responsiveness to des-Arg^9-BK was demonstrated with a blood pressure assay of exogenous kinins or with tissues isolated *ex vivo* 5 h after pretreatment of animals. Evidence of active disposition of cycloheximide *in vivo* was also obtained.

7 We propose the production of endogenous IL-1 as a possible mechanism for the enhancement of responsiveness to des-Arg^9-BK observed in tissues pulsed with a protein synthesis inhibitor and for the inducing effect of cycloheximide or *E. coli* lipopolysaccharide *in vivo*. These results suggest that effects mediated by the BK_1 receptor for kinins are potentially present in pathological conditions associated with IL-1 production.

Keywords: Des-Arg^9-bradykinin, biological activity; rabbit aortic strips; sensitization to kinins, modulation; cycloheximide, vascular effects; interleukin-1

Introduction

The generation of vasoactive kinins, such as bradykinin (BK), from circulating blood kininogen is characteristic of the early phase of inflammatory reactions following tissue injury or Gram negative infection (Marceau *et al.*, 1983; Mason *et al.*, 1970). The vascular effects of BK include vasodilatation and increased vascular permeability (Elliot *et al.*, 1960). These effects are thought to be mediated by BK_2 receptors for kinins which are prevalent in vascular tissues under non-pathological conditions (Regoli & Barabé, 1980; Marceau *et al.*, 1983). BK is rapidly converted to its relatively stable metabolite without the C-terminal arginine residue, des-Arg^9-BK, by kininase I present in plasma and exudates (Erdös & Sloane, 1962; Proud *et al.*, 1987). This conversion is often considered as an inactivation because, in contrast to BK, the affinity of des-Arg^9-BK for BK_2 receptors is very low (Regoli & Barabé, 1980).

However, des-Arg^9-BK may become biologically active following the appearance of a distinct type of receptor, termed BK_1, selective for kinin fragments without the C-terminal arginine residue (Regoli & Barabé, 1980). The rabbit aorta incubated in Krebs solution is an *in vitro* model of vascular sensitization specific to kinins such as des-Arg^9-BK (Regoli *et al.*, 1977). The vascular tissue, which is initially insensitive either to BK or to des-Arg^9-BK, shows a selective increase in contractile responses to kinins as a function of incubation time *in vitro* (Bouthillier *et al.*, 1987; deBlois *et al.*, 1988). In this system, the effect of kinins is thought to be mediated through the BK_1 receptor for kinins because des-Arg^9-BK is ten times more potent than BK and because the effects of all kinins are blocked by the selective BK_1 receptor antagonist [Leu^8]-des-Arg^9-BK (for review see Regoli & Barabé, 1980). The sensitization process occurs even in endothelium-denuded aortae (Bouthillier *et al.*, 1987). However, it is dependent on the *de novo* synthesis of protein as it is blocked by continuous application of the protein synthesis inhibitor (PSI) cycloheximide (Bouthillier *et al.*, 1987). Finally, the increase in responsiveness to kinins in the incubated rabbit aorta is almost completely inhibited by the anti-inflammatory steroid dexamethasone sodium phosphate (DSP) (deBlois *et al.*, 1988).

Other smooth muscle preparations in which spontaneous sensitization to the contractile action of des-Arg^9-BK has been observed include strips of human colon (Couture *et al.*, 1981), rat isolated duodenum (Boschcov *et al.*, 1984) and urinary bladder (Marceau *et al.*, 1980), and rabbit basilar artery (Whalley *et al.*, 1983). In addition, des-Arg^9-BK elicits vasorelaxant responses mediated by cyclo-oxygenase products in the rabbit coeliac artery (Ritter *et al.*, 1989) and mesenteric artery (Churchill & Ward, 1986; deBlois & Marceau, 1987). In the rabbit mesenteric artery, the increase in responsiveness to

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des-Arg^9-BK is cycloheximide-sensitive as in tissues contracted by the kinin, and it is reduced but not abolished by the complete removal of the endothelium (deBlois *et al.*, 1987).

Selective induction of cardiovascular sensitivity to des-Arg^9-BK is also observed *in vivo* in rabbits injected with *E. coli* lipopolysaccharide (LPS) (Regoli *et al.*, 1981; deBlois *et al.*, 1989). Unlike control animals, where it has no apparent effect, an intra-arterial bolus of des-Arg^9-BK causes a rapid, transient hypotensive response in anaesthetized rabbits 5 h after pretreatment with LPS. In hearts obtained from LPS-treated animals and perfused *ex vivo* at constant flow, des-Arg^9-BK elicits a BK_1 receptor-mediated decrease in peripheral vascular resistance (Regoli *et al.*, 1981). The precise mechanism of the hypotensive effect of des-Arg^9-BK in the rabbit is not known. Kinins may act on endothelial BK_1 receptors to release vasodilator agents such as endothelium-derived relaxing factor or prostacyclin as shown for cultured bovine endothelial cells (D'Orleans-Juste *et al.*, 1989). Alternatively, des-Arg^9-BK may act on other cell types (e.g. smooth muscle) to produce vasorelaxation as suggested by the significant residual responses to des-Arg^9-BK observed in endothelium-denuded mesenteric arteries (deBlois & Marceau, 1987).

Rabbits injected with LPS produce interleukin-1β (IL-1β) *in vivo* (Cannon *et al.*, 1989). Cultured vascular smooth muscle and endothelial cells also produce IL-1 in response to LPS (Libby *et al.*, 1986a,b). Moreover, LPS and IL-1β are potent stimulants of the sensitization to des-Arg^9-BK in rabbit aortae incubated *in vitro* (Bouthillier *et al.*, 1987; deBlois *et al.*, 1988). This raises the possibility that the vascular sensitization to des-Arg^9-BK may be associated with the endogenous production of IL-1. In addition to LPS, inhibition of protein synthesis also induces the production of IL-1 mRNA in vascular smooth muscle (Warner *et al.*, 1987) and endothelial cells (Libby *et al.*, 1986a). Furthermore, removal of the PSI may lead to the increased production of protein(s) corresponding to the accumulated mRNA(s) (Warner & Libby, 1989). Thus, we speculated that a transient inhibition of protein synthesis might increase vascular responsiveness to des-Arg^9-BK. The experiments described here provide evidence for the association between IL-1 and vascular sensitivity to des-Arg^9-BK. We found that vascular responses to des-Arg^9-BK are enhanced by a transient inhibition of protein synthesis *in vitro* or *in vivo*, and by IL-1β injected *in vivo* into rabbits. The induction by IL-1 of BK_1 receptors for relatively stable metabolites of kinins may accentuate and prolong the hypotensive effect of kinins in pathologies associated with a dramatic fall in blood pressure, such as the septic shock (Wilson *et al.*, 1989).

Methods

Isolated blood vessels

The thoracic aorta was isolated from New Zealand White rabbits of either sex (1.2 to 1.8 kg). The vessels were prepared, suspended under a basal tension of 2 g in 5 ml organ chambers containing Krebs solution, and their responses to agents were recorded as previously described (Bouthillier *et al.*, 1987). The level of responsiveness to des-Arg^9-BK was monitored by applying the kinin fragment at a concentration of 1.7 μM, which is approximately the EC_{95} (Regoli & Barabé, 1980). The upper panel of Figure 1 represents the schematic diagram for the application of vasoactive agents during the incubation of the smooth muscle preparation. Des-Arg^9-BK was routinely applied 1, 3 and 6 h after the beginning of the tissue incubation, and noradrenaline (NA, 100 nM) after 1.5 and 6.5 h of incubation. The repeated stimulations with des-Arg^9-BK were done in order to monitor the progressive increase of responsiveness of aortic strips to the kinin metabolite. NA was applied to monitor the response to an unrelated contractile agent. Tissues were allowed to respond for 10 min. At the end of this period, the vasoactive agents were removed by washing the tissues twice with fresh Krebs solution. The maximal level of contraction observed during the 10 min observation period is presented and is expressed in g of isometric tension. The longest period between washings with fresh Krebs solution was 90 min.

Pharmacological modulation of in vitro *sensitization to des-Arg^9-bradykinin*

The lower panel of Figure 1 is a summary of the various types of treatment applied on the rabbit aortic strips during the *in vitro* incubation. Tissues were exposed to a PSI (solid bars), namely cycloheximide (71 μM) or anisomycin (3.8 μM), for various periods of time *in vitro*: (a) for the first 1 h, (b) for the first 3 h, or (c) for the whole incubation period. In strips temporarily treated (pulsed) with a PSI, the fluid bathing the tissues was changed every hour after the end of the exposure to the inhibitor. This protocol was followed to investigate the possible stimulating effect of a temporary blockade of protein synthesis on the vascular sensitization to des-Arg^9-BK. In some experiments, incubated strips were exposed continuously to the inhibitory steroid DSP (100 μM, hatched bars) which was applied (d) alone or (e) in combination with a PSI for the first 3 h. Some strips were treated for the last 3.5 h of incubation with (f) DSP (100 μM) or with (g) IL-1β (0.1–5 ng ml^{-1}, open bars). IL-1β (5 ng ml^{-1}) was also applied for the first 3 h (h) alone or (i) in combination with a high concentration of a PSI (anisomycin, 38 μM).

In vivo *sensitization to des-Arg^9-bradykinin*

Five hours prior to any other experimental procedure, rabbits were given an i.v. injection of one of the following agents: cycloheximide (1 or 10 mg kg^{-1}), *E. coli* LPS (25 μg kg^{-1}), IL-1β (5 μg kg^{-1}) or tumor necrosis factor α (TNFα, 5 μg kg^{-1}). Control animals received 1 ml of physiological saline (1 ml) or bovine serum albumin (BSA, 1 mg kg^{-1}) dissolved in physiological saline which was the vehicle for IL-1β and TNFα.

Two experimental approaches were used to monitor the vascular sensitization to des-Arg^9-BK that occurred *in vivo* following the pretreatments. First, vascular responses to des-Arg^9-BK were measured *ex vivo* in aortic strips isolated from pretreated rabbits. Strips were prepared and suspended in organ chambers as described above. The sensitization to des-Arg^9-BK occurs spontaneously *in vitro* in aortae isolated from normal rabbits (Bouthillier *et al.*, 1987). However, it is both absent at 1 h and repressed by continuous exposure to cycloheximide. Thus, the following procedure was adopted to measure specifically the degree of sensitization to des-Arg^9-BK occurring *in vivo* without interference from the *in vitro* sensitization. The aortic strips were routinely incubated in the presence of cycloheximide (71 μM) and stimulated with des-Arg^9-BK (1.7 μM) at 1 h. The contractile response to NA (100 nM) at 1.5 h was also measured.

Second, rapid hypotensive responses to exogenous kinins were measured in rabbits anaesthetized 5 h following pretreatment. Rabbits were anaesthetized with lignocaine and sodium pentobarbitone and ventilated as described previously (Regoli *et al.*, 1981). Immediate blood pressure changes in response to intra-arterial injection of BK or of des-Arg^9-BK were recorded from the left carotid artery as described by Bouthillier *et al.* (1987).

In a separate set of experiments, rabbits were injected with the angiotensin converting enzyme inhibitor captopril (5 or 10 mg kg^{-1}) or enalapril (5 or 10 mg kg^{-1}) and anaesthetized 20 h later for blood pressure measurements. This longer delay between the pretreatments and the anaesthesia was chosen to reproduce an experimental procedure previously described (Nwator & Whalley, 1989) which reportedly induced in 20 h a state of cardiovascular sensitivity to des-Arg^9-BK. In addition,

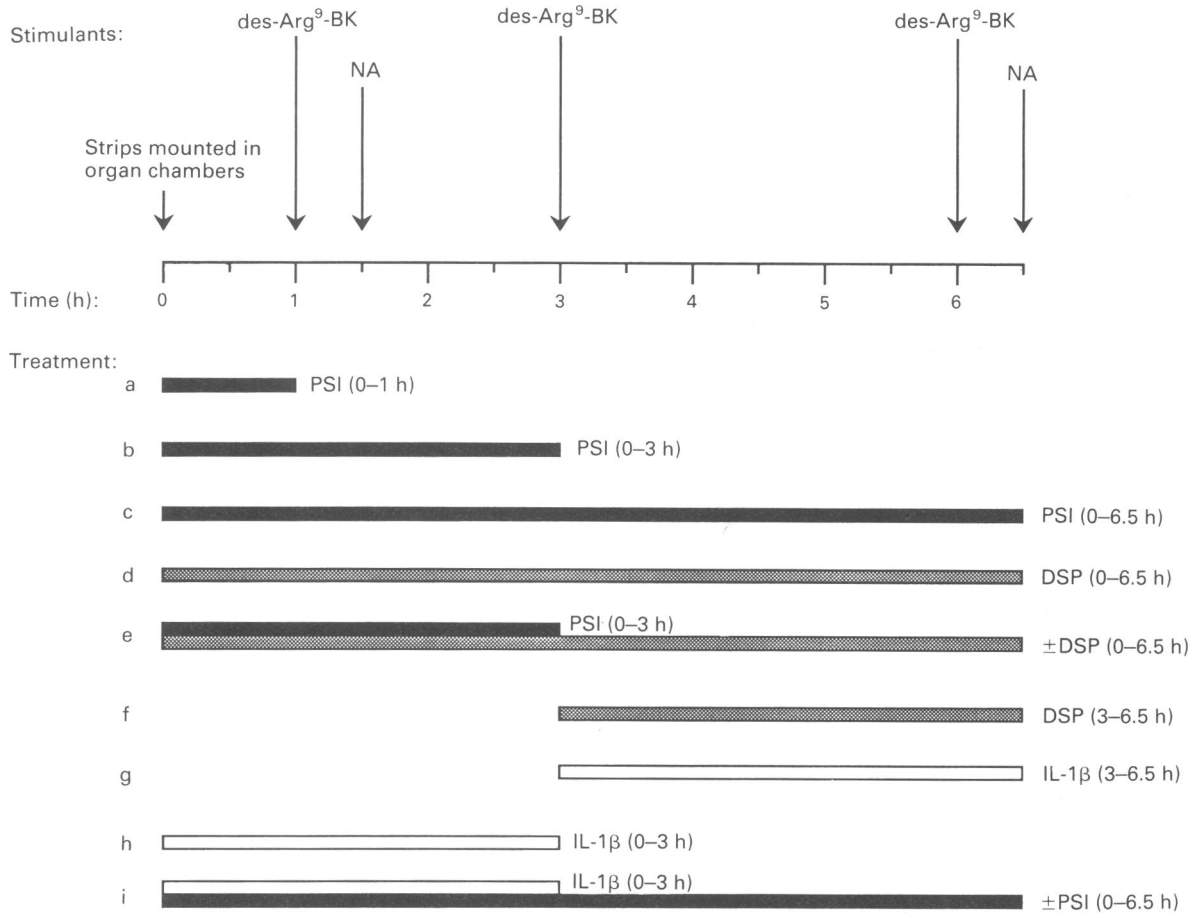

Figure 1 Upper panel: schematic diagram for the application of vasoactive agents during the incubation of the rabbit aortic strips. Des-Arg^9-bradykinin (des-Arg^9-BK, 1.7 μM) was routinely applied 1, 3 and 6 h after the beginning of the tissue incubation, and noradrenaline (NA, 100 nM) after 1.5 and 6.5 h of incubation. Tissues were allowed to respond for 10 min. At the end of this period, the vasoactive agents were removed by washing the tissues twice with fresh Krebs solution. Lower panel: summary of the various types of treatment applied on the rabbit aortic strips during the *in vitro* incubation. Tissues are exposed to a protein synthesis inhibitor (PSI, solid bars), namely cycloheximide (71 μM) or anisomycin (3.8 μM), for various periods of time *in vitro*: (a) for the first 1 h, (b) for the first 3 h, or (c) for the whole incubation period. In some experiments, incubated strips were exposed continuously to the inhibitory steroid dexamethasone sodium phosphate (DSP, 100 μM, hatched bars) which was applied (d) alone or (e) in combination with a PSI for the first 3 h. Some strips were treated for the last 3.5 h of incubation with (f) DSP (100 μM) or with (g) interleukin-1β (IL-1β, open bars) at increasing concentrations (0.1–5 ng ml^{-1}). IL-1β (5 ng ml^{-1}) was also applied for the first 3 h (h) alone or (i) in combination with a high concentration of PSI (anisomycin, 38 μM).

a group of rabbits was anaesthetized for blood pressure measurements 5 h after an i.v. injection of captopril (5 mg kg^{-1}).

Time course of protein synthesis inhibition by cycloheximide in vivo

The transient nature of vascular cells exposure to cycloheximide *in vivo* was investigated by measuring the inhibition of [^{35}S]-methionine incorporation into cultured fibroblasts by plasma from a cycloheximide-treated rabbit. At various intervals following pretreatment, a rabbit injected with cycloheximide (10 mg kg^{-1}) was bled through the central ear artery into tubes containing a final heparin concentration of 18 u ml^{-1}. The plasma was collected by centrifugation at 4°C and stored frozen at −20°C. Rabbit dermal fibroblasts were derived from subcutaneous explants, cultured in Dulbecco's Modified Eagle Medium (DMEM, from Gibco, Grand Island, NY, U.S.A.) supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 2% non-essential amino-acids, 100 u ml^{-1} penicillin and 100 u ml^{-1} streptomycin as described (Marceau & Tremblay, 1986; all reagents from Gibco). Cells (1.0×10^5) were seeded in each well of a 12-well plate (Linbro, Flow Labs, Mclean, VA, U.S.A.; well diameter, 24 mm) and further cultured for 2 days. Then the culture medium was replaced with DMEM containing 5% FCS, 5% rabbit plasma from the cycloheximide-treated animal and [^{35}S]-methionine (10 μCi ml^{-1}; specific activity 200 mCi mmol^{-1}, Amersham, Oakville, Canada). At the end of the incubation, the cells were washed extensively with phosphate buffered (78 mM, pH 7.4) saline and solubilized in 1 ml NaOH (0.15 N). Scintillation cocktail (Scintiverse, Fisher Scientific, Fairlawn, NJ, U.S.A.) was added and the radioactivity incorporated into the cells was measured.

Drugs

Cycloheximide, anisomycin and (−)-noradrenaline were purchased from Sigma (St Louis, MO, U.S.A.). Bradykinin and des-Arg^9-BK were from Bachem (Torrance, CA, U.S.A.). Dexamethasone sodium phosphate was from Sabex (Montréal, Québec, Canada), heparin sodium U.S.P. was from Organon (Toronto, Ontario, Canada). Lipopolysaccharide from *E. coli*, 0111:B4, was from DIFCO Lab. (Detroit, MI, U.S.A.). Interleukin-1β (recombinant, human sequence) was a gift from Biogen S.A. (Geneva, Switzerland) and tumor necrosis factor α (recombinant, human sequence) a gift from Genetics Institute (Boston, MA, U.S.A.). Pentobarbitone sodium was from Abbot Laboratories (Toronto, Ontario, Canada) and lignocaine hydrochloride from Astra (Mississauga, Ontario, Canada). Captopril and enalapril were gifts from the Squibb and Merck companies, respectively. Endotoxin content of the cycloheximide and IL-1β solutions

that were injected *in vivo* in some experiments, was verified by the *Limulus* amoebocyte lysate assay (Sigma).

Statistical analysis

Results are expressed as means ± standard errors (s.e.) of the mean. In most *in vitro* protocols, responses obtained in treated tissues were matched with those from paired controls from the same rabbit; results were then analysed by means of one way analysis of variance (ANOVA) followed by Student's *t* test for paired samples in order to minimize the effect of inter-animal variation. ANOVA followed by Dunnett's test was used when more than three comparisons were made between treated groups or with the control group (Tallarida & Murray, 1987).

Results

Effect of protein synthesis inhibitors on responses to des-Arg^9-bradykinin in vitro

Initially insensitive to a near maximal concentration of des-Arg^9-BK (1.7 μM), the rabbit isolated aorta selectively increased its contractile response to the kinin as a function of incubation time *in vitro* (Figure 2a). The effect of PSIs on this sensitization process varied according to the time schedule of exposure *in vitro* (Figure 2, typical tracings; Figure 3a and b, statistical analyses). Whereas application of cycloheximide (71 μM) throughout the incubation period completely inhibited the development of vascular sensitivity to des-Arg^9-BK, a short application of the PSI during the first hour significantly increased the level of maximal response to des-Arg^9-BK recorded after 6 h of incubation. Tissues exposed to cyclo-

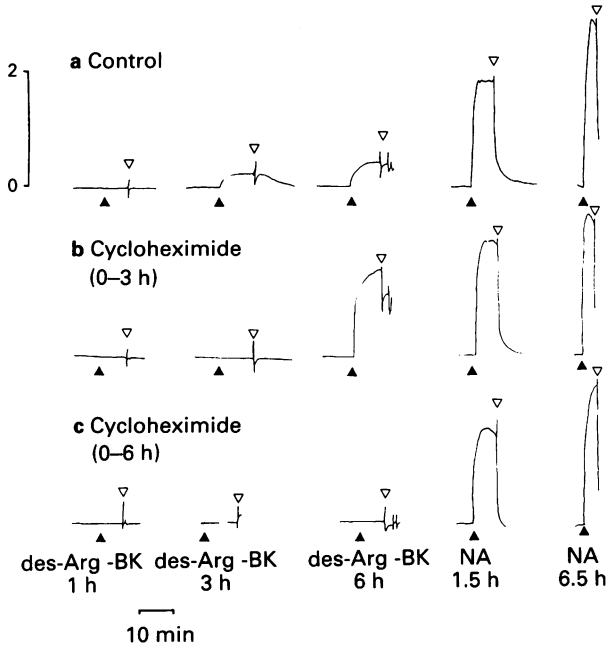

Figure 2 Contractile effect of des-Arg^9-bradykinin (des-Arg^9-BK, 1.7 μM) and of noradrenaline (NA, 100 nM) on the rabbit isolated aorta as a function of incubation time *in vitro*. Three aortic strips isolated from the same animal were stimulated with des-Arg^9-BK for 10 min after 1, 3 and 6 h of incubation and with NA after 1.5 and 6.5 h of incubation. Exposure to NA was ended after the peak contractile response was reached. Cycloheximide (71 μM) was applied to paired strips from the same aorta (b) for the first 3 h of incubation or (c) was present continuously in the bathing fluid. One tissue (a) was left untreated as a control for spontaneous sensitization. Ordinate scale: isometric contraction in g; abscissa scale: time in min. Closed symbols indicate the time of the application of the vasoactive agents and open symbols indicate the end of the stimulation period. The spontaneous increase in responses to des-Arg^9-BK of incubated rabbit aortic strips was selectively amplified following a temporary blockade of protein synthesis. Continuous exposition to cycloheximide abolished the sensitization to the kinin.

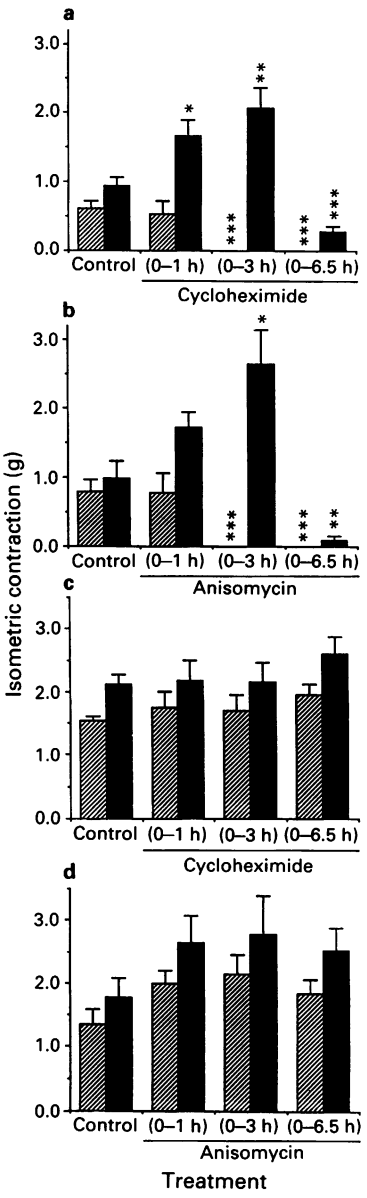

Figure 3 Selective increase in responses to des-Arg^9-bradykinin (des-Arg^9-BK, 1.7 μM) of the incubated rabbit aorta transiently exposed to a protein synthesis inhibitor (PSI). Responses to the kinin (a and b) and to noradrenaline (NA, 100 nM, c and d) are shown. The vasoactive agents were applied on tissues for 10 min. Adjacent hatched and solid columns refer to measurements in the same set of tissue at different times during the incubation: des-Arg^9-BK was applied after 3 h (hatched columns) and 6 h (solid columns) and NA after 1.5 h (hatched columns) and 6.5 h (solid columns). Some tissues were exposed to cycloheximide (71 μM, $n = 8$–10, a and c) or anisomycin (3.8 μM, $n = 5$–7, b and d) for the first 1 or 3 h of incubation. Another set of tissues were exposed continuously (0–6.5 h) to the PSI. Vertical lines represent s.e. of the mean. Statistically significant differences between responses of treated and control tissues at 3 h or at 6 h were determined by one way analysis of variance followed by Student's *t* test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

heximide for the first 3 h of incubation did not respond to des-Arg^9-BK at the end of this period. However, when the same tissues were incubated for an additional 3 h without cycloheximide and then stimulated with the kinin, a markedly higher level of contraction was observed (Figures 2b and 3a). Contractions at 6 h were significantly greater following a long exposure (first 3 h of incubation) than a short exposure (first 1 h) to cycloheximide. Anisomycin, a PSI structurally unrelated to cycloheximide, behaved similarly: it prevented or increased the development of responses to des-Arg^9-BK depending whether it was applied continuously or transiently

to the incubated rabbit aorta (Figure 3b). Contractions to NA (100 nM) were not affected by any of the treatments (Figure 3c and d).

Concentration-effect curves of des-Arg^9-bradykinin

Complete cumulative concentration-effect curves for des-Arg^9-BK were established at 6 h as previously described (Regoli *et al.*, 1978) in tissues exposed to cycloheximide for the first 3 h of incubation. As already shown in Figure 3a, this treatment resulted in an increase in the maximal response of aortic strips to des-Arg^9-BK (Figure 4a). In addition, the concentration-effect curve expressed as a percentage of the maximal response was shifted to the left in comparison with the paired control curve (Figure 4b).

Inhibition by dexamethasone of the stimulant effect of cycloheximide in vitro

Continuous exposure of aortic strips to DSP was shown to inhibit specifically the sensitization to des-Arg^9-BK even in the presence of known stimulants of the sensitization process (deBlois *et al.*, 1988). We investigated whether the stimulating effect of cycloheximide in this system was sensitive to inhibition by DSP (100 μM). The stimulant effect of the cycloheximide pulse was virtually abolished by the steroid (Figure 5a). Moreover, the two groups of tissues incubated in the presence of DSP had similar levels of responsiveness to des-Arg^9-BK at 6 h. We investigated the possibility that a steroid-induced protein could inhibit the contractile response to des-Arg^9-BK by acting on the receptor or at a post-receptor level. In tissues responsive to des-Arg^9-BK at 3 h, a subsequent treatment with DSP from 3 h to 6.5 h did not reduce responsiveness to des-Arg^9-BK at 6 h as compared to 3 h in the same tissue (Figure 5b). This suggests that the receptor function was not altered directly by the steroid treatment.

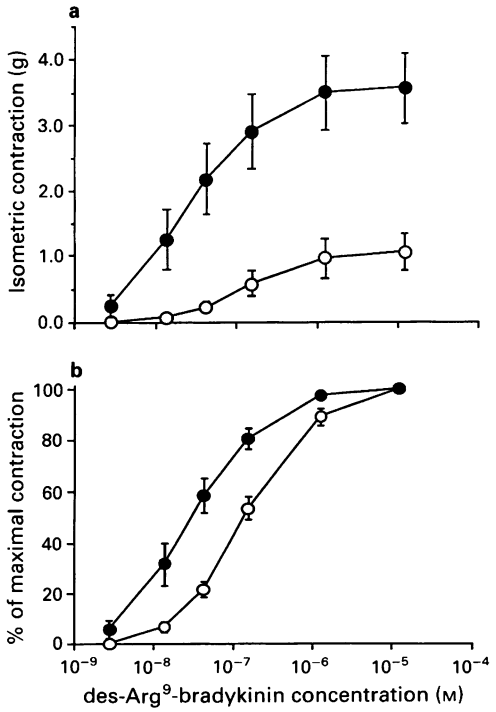

Figure 4 Concentration-effect curves for des-Arg^9-bradykinin on (○) control rabbit aortic strips or on tissues exposed to cycloheximide (71 μM) for the first 3 h of incubation (●). The points represent the mean of 4 determinations made after 6 h of incubation and vertical lines show s.e. mean. The same data are presented (a) as g of contraction or (b) as a % of the maximal contraction. The maximal response to the kinin was increased and the half-maximal concentration was lower in tissues pulsed with cycloheximide as compared to control.

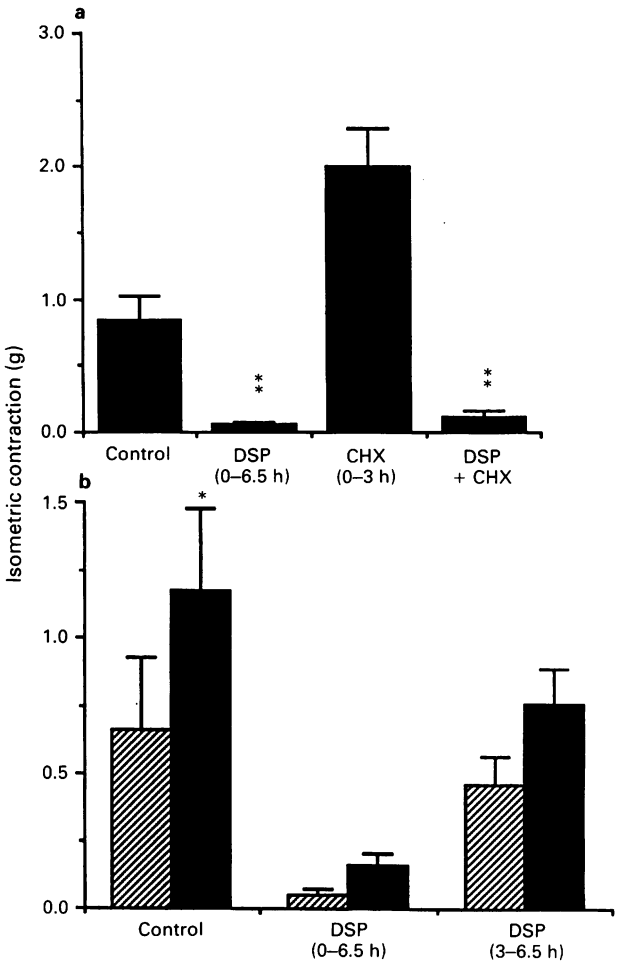

Figure 5 Effect of the glucocorticoid, dexamethasone sodium phosphate (DSP, 100 μM) on the response to des-Arg^9-bradykinin (des-Arg^9-BK, 1.7 μM) of incubated strips of rabbit aorta. In (a) paired tissues were treated with DSP throughout the incubation period (0–6.5 h) or with cycloheximide (CHX, 71 μM) for the first 3 h (0–3 h) or with both treatments combined. One group of tissues was left untreated as a control for the spontaneous increase in responsiveness to des-Arg^9-BK. Responses to the kinin at 6 h are shown as the mean of 6 determinations; s.e. shown by vertical bars. DSP markedly inhibited the increase in responses to des-Arg^9-BK of rabbit aortic strips even in tissues exposed temporarily to cycloheximide. Student's *t* test for paired samples was used to compare tissues treated with DSP to the corresponding tissue incubated without the steroid. In (b), tissues were incubated with DSP (100 μM) throughout the incubation period (0–6.5 h), for the last 3.5 h (3–6.5 h) or without the steroid (control). Responses to the kinin in the same set of tissues at 3 h (hatched columns) and 6 h (adjacent solid columns) are shown as the mean of 5 determinations with s.e. shown by vertical bars. In tissues exposed to DSP for the last 3.5 h, the steroid did not reduce the magnitude of the response at 6 h as compared to 3 h. Student's *t* test for paired samples was used to compare the levels of response to des-Arg^9-BK at 3 h and 6 h within each group of tissues. Responses to noradrenaline were not affected by any of those treatments (not shown). Levels of significance: * $P < 0.05$; ** $P < 0.01$.

Effect of interleukin-1β on tissue responsiveness to des-Arg^9-bradykinin

To test whether exogenous IL-1β could reproduce the stimulatory effect of cycloheximide on the responses to des-Arg^9-BK in the incubated rabbit aorta, the cytokine was added at various concentrations to the bathing fluid for the last 3.5 h of incubation (3–6.5 h). This schedule of application was chosen to reproduce the putative production of endogenous IL-1 by cells in the vascular wall exposed to cycloheximide for the first 3 h of incubation. Application of IL-1β at low concentrations (100–250 pg ml^{-1}) for the last 3.5 h of incubation had a selective stimulatory effect on the responses to des-Arg^9-BK of

aortic strips (Figure 6a and b). Tissues exposed to IL-1β at these concentrations had a level of response to the kinin (at 6 h) or to NA (at 6.5 h) that was not significantly different from those of tissues exposed to cycloheximide for the first 3 h. In contrast, tissues left untreated as controls for spontaneous sensitization had significantly weaker contractile responses to des-Arg^9-BK as indicated by ANOVA and Dunnett's test. At higher concentrations of IL-1β (0.5–5 ng ml^{-1}), the level of responses to the kinin was not further enhanced but the level of contractions in response to NA was significantly reduced (82% inhibition in the presence of 5 ng ml^{-1} IL-1β).

Continuous exposure to a high concentration of the PSI anisomycin (38 μM) prevented the sensitization to des-Arg^9-BK even in tissues incubated with IL-1β (5 ng ml^{-1}; Figure 7a) suggesting that the synthesis of one or more proteins different from IL-1 is necessary for the sensitization to the kinin. In addition, the desensitization to NA in tissues exposed to the IL-1β was prevented by blockade of protein synthesis (Figure 7b).

In vivo *induction of vascular sensitivity to des-Arg^9-bradykinin*

Since cycloheximide was shown to enhance the spontaneous development of responsiveness to des-Arg^9-BK *in vitro*, we administered the agent to rabbits in order to verify whether it could induce *in vivo* a state of vascular responsiveness to the kinin metabolite. LPS, a known inducer of cardiovascular

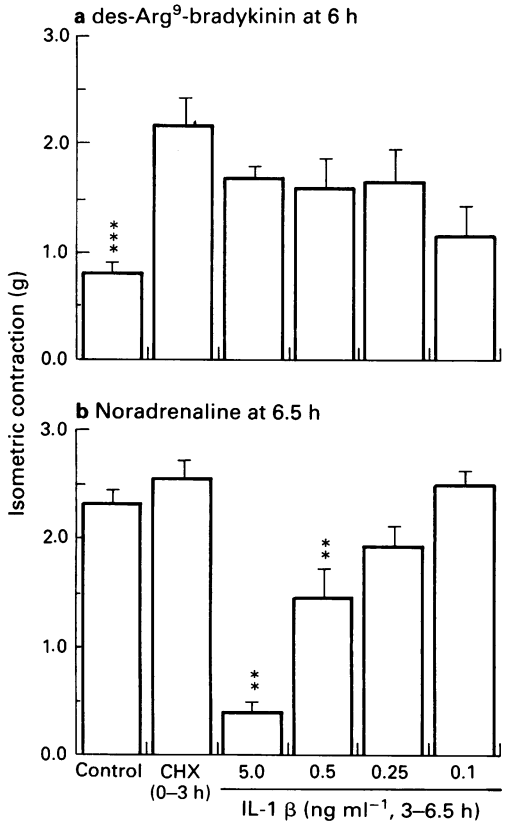

Figure 6 Effects of a temporary exposure to cycloheximide or to interleukin-1β on the contractile response of rabbit aortic strips to des-Arg^9-bradykinin (des-Arg^9-BK, 1.7 μM) and to noradrenaline (100 nM). Strips were exposed to cycloheximide (CHX, 71 μM) for the first 3 h of incubation (0–3 h) or to interleukin-1β (IL-1β) at various concentrations for the last 3.5 h (IL-1β, 3–6.5 h). Responses to the kinin at 6 h are shown as the mean of 7–19 determinations; vertical bars show s.e. IL-1β, applied at low concentrations for the last 3.5 h of incubation, mimicked the selective stimulatory effect of the pulse of cycloheximide on the contractile response to des-Arg^9-BK. The control and IL-1β-treated groups were compared to the cycloheximide-treated group by use of one-way analysis of variance followed by Dunnett's test. Levels of significance: ** $P < 0.01$; *** $P < 0.001$.

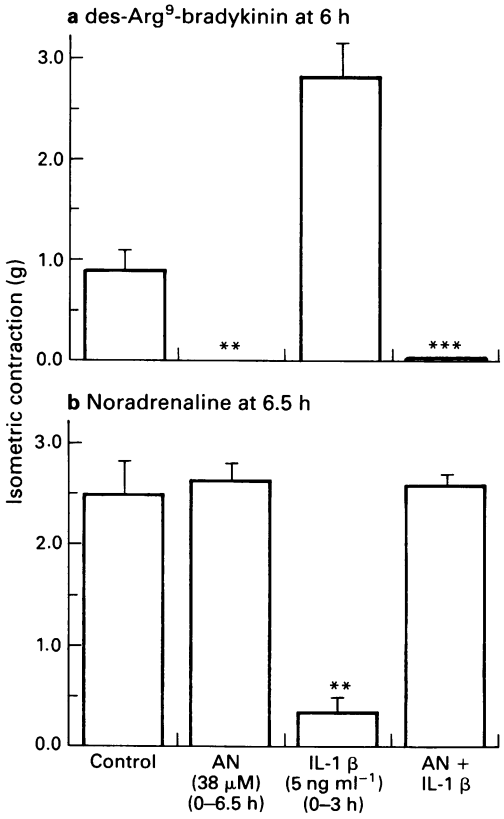

Figure 7 Inhibitory effect of anisomycin in the presence of interleukin-1β (IL-1β) on the sensitization of the incubated rabbit aorta to des-Arg^9-bradykinin (des-Arg^9-BK). Paired aortic strips were incubated in the presence of a high concentration of anisomycin (38 μM, 0–6.5 h) or IL-1β (5 ng ml^{-1}, 0–3 h) or a combination of the two agents. One tissue was left untreated as a control for spontaneous sensitization to the kinin. Responses (a) to des-Arg^9-BK (1.7 μM) at 6 h and (b) to noradrenaline (NA, 100 nM) at 6.5 h are shown as the mean of 5 determinations; vertical bars show s.e. Anisomycin (AN) prevented the sensitization to des-Arg^9-BK in tissues incubated with or without IL-1β as well as the desensitization to NA in tissues exposed to the cytokine. In (a), strips exposed to anisomycin were compared to the corresponding tissue incubated without the inhibitor by use of Student's t test for paired samples. In (b), Dunnett's test was used to compare all groups to the control group. Levels of significance: ** $P < 0.01$; *** $P < 0.001$.

sensitivity to des-Arg^9-BK (Regoli *et al.*, 1981) and IL-1β, the mRNA of which is induced in cultured vascular cells by LPS or by cycloheximide (Warner *et al.*, 1987; Libby *et al.*, 1986b), were also injected separately *in vivo*.

Thoracic aortae from rabbits treated *in vivo* with cycloheximide (10 mg kg^{-1} i.v., 5 h before isolation) had a significantly higher level of contraction in response to des-Arg^9-BK after a short period (1 h) of incubation *in vitro* than tissues isolated from saline-treated rabbits (Figure 8a). The effect of cycloheximide was dose-dependent and did not reach statistical significance at the lower dose tested (1 mg kg^{-1}). LPS (25 μg kg^{-1}) was a more potent *in vivo* inducer of vascular sensitivity to des-Arg^9-BK than cycloheximide. The pro-inflammatory cytokine IL-1β (5 μg kg^{-1}) was also an inducer *in vivo* whereas its BSA vehicle (1 mg kg^{-1}) was not. None of these pretreatments affected the *in vitro* response to NA (100 nM) recorded at 1.5 h (Figure 8b). Only 1 ng of endotoxin per mg of cycloheximide was detected in the solution of inhibitor administered *in vivo* by the *Limulus* amoebocyte lysate assay. The solution of IL-1β contained no detectable endotoxin by this assay (detection limit: 10 pg ml^{-1}).

The induction *in vivo* of cardiovascular sensitivity to des-Arg^9-BK was also demonstrated by a blood pressure assay for exogenous kinins. Rabbits injected with cycloheximide (10 mg kg^{-1}), IL-1β (5 μg kg^{-1}) or LPS (25 μg kg^{-1}), but not

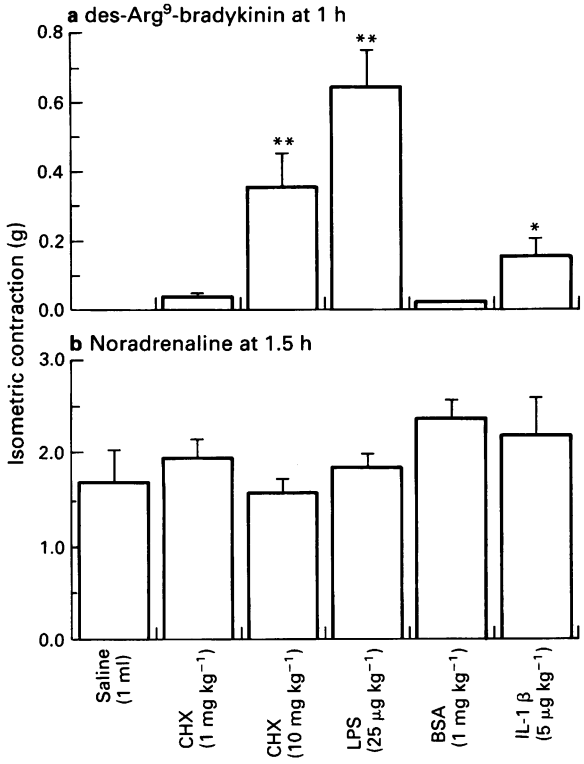

Figure 8 Selective induction *in vivo* of vascular responsiveness to des-Arg^9-bradykinin (des-Arg^9-BK) by cycloheximide (CHX, 10 mg kg^{-1}), *E. coli* lipopolysaccharide (LPS, 25 μg kg^{-1}) or interleukin-1β (IL-1β, 5 μg kg^{-1}) as assessed *in vitro* by the contractile effects of (a) des-Arg^9-BK (1.7 μM) after 1 h of incubation or (b) noradrenaline (100 nM) at 1.5 h in strips of rabbit aorta isolated 5 h after the i.v. injection of the agent. Aortic tissues were incubated *in vitro* in the presence of cycloheximide (71 μM). Vertical bars represent s.e.mean of 8 determinations. Values were compared to control (saline) by use of one way analysis of variance followed by Dunnett's test, except for IL-1β which was compared to the bovine serum albumin (BSA)-treated group by the use of Student's t test for unpaired samples. Levels of significance: * $P < 0.05$; ** $P < 0.01$.

with the saline or the BSA (1 mg kg^{-1}) vehicles, developed significant hypotensive responses to exogenous des-Arg^9-BK given as an intra-arterial bolus 5 h after the pretreatment (Table 1). LPS was the most potent inducer at doses tested. Rabbits pretreated with TNFα (5 μg kg^{-1}, $n = 2$), a pro-inflammatory cytokine which is induced by LPS in cultured smooth muscle cells (Warner & Libby, 1989), did not respond to exogenous des-Arg^9-BK (data not shown). Responses to exogenous bradykinin were present in the saline-treated group (25 ng: 5.1 ± 0.4 mmHg; 100 ng: 13.8 ± 3.1 mmHg; 250 ng: 20.1 ± 1.5 mmHg) and were not affected by any of the pretreatments (data not shown).

In addition, it has been reported that captopril (5 mg kg^{-1}) or enalapril (5 mg kg^{-1}), which are widely used inhibitors of the angiotensin converting enzyme, could induce a state of cardiovascular responsiveness to des-Arg^9-BK when administered i.v. to rabbits 20 h before anaesthesia (Nwator & Whalley, 1989). These results could not be reproduced in our laboratory even with a double dose of the agent (Table 1). Moreover, responses to exogenous des-Arg^9-BK were also monitored in rabbits that had received captopril (5 mg kg^{-1}) 5 h only before anaesthesia. This experiment was undertaken because of a possible 'fading away' of the vascular sensitivity during the 20 h experimental period. No significant response to exogenous des-Arg^9-BK could be observed under these conditions (Table 1).

Time course of protein synthesis inhibition by i.v. cycloheximide

[^{35}S]-methionine incorporation into cultured fibroblasts was inhibited by 69% following incubation with 5% (v/v) plasma sampled 5 min after i.v. injection of cycloheximide (10 mg kg^{-1}) (Table 2). The inhibition of [^{35}S]-methionine incorporation was less extensive with plasmas obtained later

Table 2 [^{35}S]-methionine incorporation into cultured rabbit dermal fibroblasts exposed for 6 h to rabbit plasma (5% v/v) sampled following i.v. injection with cycloheximide (10 mg kg^{-1})[a]

Time of blood sampling	[^{35}S]-*methionine incorporated*[c] (c.p.m. × 10^{-2})
Control[b]	708 ± 51
5 min	232 ± 17[d]
30 min	283 ± 7[d]
60 min	347 ± 17[d]
120 min	473 ± 23[d]
180 min	551 ± 38[d]
240 min	645 ± 27

[a] Rabbit dermal fibroblasts were incubated for 6 h in Dulbecco's Modified Eagle Medium in the presence of 5% FCS (v/v) and 5% plasma sampled at various times following injection of cycloheximide (10 mg kg^{-1}) to a rabbit.
[b] Rabbit plasma sampled before cycloheximide injection.
[c] Results are expressed as c.p.m. of [^{35}S]-methionine incorporated per 10^5 cells and represent the mean ± s.e. of quadriplicate estimates.
[d] Value significantly different from control ($P < 0.01$ as calculated by Dunnett's test).

Table 1 Effect of des-Arg^9-bradykinin on the mean arterial blood pressure of anaesthetized rabbits subjected to various treatments *in vivo*

Treatment (dose)[a]	n	*Basal blood pressure* (mmHg) Systolic	Diastolic	*Hypotensive effect of des-Arg^9-bradykinin* 0.25 μg	1.0 μg	2.5 μg
Saline (1 ml)	4	116 ± 1	97 ± 1	0[b]	0	0
Cycloheximide (10 mg kg^{-1})	9	109 ± 5	91 ± 4	4 ± 1	8 ± 2*	13 ± 2**
LPS (25 μg kg^{-1})[c]	4	105 ± 9	80 ± 8	6 ± 1*	15 ± 5*	19 ± 6*
BSA (1 mg kg^{-1})	8	110 ± 3	87 ± 6	0	0	0
IL-1β (5 μg kg^{-1})	12	105 ± 4	78 ± 4	3 ± 1***	5 ± 1***	8 ± 1***
Captopril (5 mg kg^{-1})	3	113 ± 8	76 ± 1	0	0	0
Captopril (5–10 mg kg^{-1})[d]	6	106 ± 6	78 ± 5	0	0	0
Enalapril (5–10 mg kg^{-1})[d]	4	109 ± 11	77 ± 5	0	0	0

[a] Treatments were in the form of a single i.v. injection 5 h before anaesthesia, except for captopril (5–10 mg kg^{-1}) and for enalapril which were given 20 h before anaesthesia.
[b] Fall of mean arterial blood pressure, mmHg. Results are compared with the control group (saline) by use of Dunnett's test, except for IL-1β, which was compared to BSA by use of Student's t test. Significance levels are expressed as follows: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.
[c] Abbreviations used: LPS: lipopolysaccharide; BSA: bovine serum albumin; IL-1β: interleukin-1β.
[d] Pool of results obtained with an equal number of animals treated with a single or a double dose of the agent.

on. Plasma sampled 4 h after cycloheximide injection did not significantly inhibit [^{35}S]-methionine incorporation as compared to control (Table 2). These results suggest that an active disposition of cycloheximide occurs in rabbits *in vivo* within the 5 h experimental period used in this study.

Discussion

Des-Arg^9-BK is hypotensive in rabbits injected with LPS and a vasodilator in perfused hearts from these treated animals (Regoli *et al.*, 1981). By contrast, it is a vasoconstrictor in the rabbit aortic preparation (Regoli *et al.*, 1981; deBlois *et al.*, 1989). The fall in mean arterial blood pressure in response to des-Arg^9-BK is probably not functionally related to the contractile response of the aorta. Nevertheless, this tissue was used to study the process of vascular sensitization to des-Arg^9-BK *in vitro* for three reasons. First, the myotropic response of the rabbit aorta to kinins is mediated exclusively by BK_1 receptors and has been extensively characterized (for review see Regoli & Barabé, 1980). Secondly, the rabbit aorta being insensitive to vasoactive prostaglandins which may interfere with the contractile response (Förstermann *et al.*, 1984), responds to des-Arg^9-BK with a monophasic contraction (Bouthillier *et al.*, 1987). This makes the sensitization easier to study than, for instance, that occurring in the mesenteric artery in which a contraction is followed by a prostaglandin-mediated relaxation (deBlois & Marceau, 1987). Finally, in rabbits sensitized *in vivo* with LPS, the contractile response of the isolated aorta to des-Arg^9-BK is a good indicator of the systemic cardiovascular sensitivity to the kinin observed by use of the blood pressure assay (Regoli *et al.*, 1981; deBlois *et al.*, 1989; present results).

The *de novo* synthesis of BK_1 receptors for kinins has been postulated to account for the increased responses to des-Arg^9-BK observed in smooth muscle preparations incubated *in vitro* and in the cardiovascular system of endotoxaemic rabbits (Regoli *et al.*, 1978; Marceau *et al.*, 1983). Although the precise mechanism of sensitization is still unknown, the dependence on protein synthesis of the *in vitro* process is well established. In the present study, we have documented a paradoxical stimulant effect of PSIs on the development of vascular responses to des-Arg^9-BK *in vitro* and *in vivo*. We also observed the *in vivo* induction in the rabbit of cardiovascular sensitivity to des-Arg^9-BK by the pro-inflammatory cytokine IL-1β. Our results suggest a possible association between the endogenous production of IL-1 and vascular sensitivity to agonists of the BK_1 receptor for kinins.

Stimulating effect of protein synthesis inhibitors in vitro

Temporary inhibition of protein synthesis with two structurally unrelated inhibitors, namely cycloheximide and anisomycin, enhanced both the maximal response of the rabbit aorta to des-Arg^9-BK and the apparent affinity of the kinin. This phenomenon has been reported previously with IL-1β (deBlois *et al.*, 1988) and epidermal growth factor (EGF; Bouthillier *et al.*, 1987). Although stimulation of EGF production in aortic strips pulsed with cycloheximide *in vitro* cannot be ruled out, it is known that the synthesis of mRNA for IL-1 is induced by cycloheximide in cultured vascular smooth muscle cells (Warner *et al.*, 1987) or endothelial cells (Libby *et al.*, 1986a). Moreover, inhibition of IL-1 synthesis by glucocorticoids (Smith, 1980) may partially account for the inhibitory effect of DSP on responses to des-Arg^9-BK in tissues pulsed with cycloheximide. The induction but not the subsequent function of receptors for kinins appears to be sensitive to the inhibitory effect of DSP because incubation of kinin-sensitive aortic strips with the glucocorticoid for a period of time sufficient for the synthesis of steroid-induced proteins did not result in a decreased level of response to the kinin.

Low concentrations of IL-1β mimicked the stimulating effect of cycloheximide on responses to des-Arg^9-BK. These *in vitro* concentrations were similar to those used to stimulate prostaglandin synthesis in human fibroblasts (Dinarello *et al.*, 1986) and those found in the plasma of children suffering from severe infection (1.5 ng ml^{-1}, Giarardin *et al.*, 1989). This suggests that the production of IL-1 may be a major event regulating the vascular sensitivity to des-Arg^9-BK.

The inhibition by anisomycin of the spontaneous sensitization to des-Arg^9-BK that occurs *in vitro* was not reversed by a high concentration of IL-1β suggesting that the *de novo* synthesis of one or more proteins distinct from IL-1β is required. This may include *de novo* BK_1 receptor synthesis, the postulated mechanism for the increase in vascular sensitivity to des-Arg^9-BK (Regoli *et al.*, 1978).

The potent inhibition of contractile responses to NA produced by higher IL-1β concentrations was prevented by inhibition of protein synthesis. This confirms previous observations made in the rat aorta with α-adrenoceptor agonists (Beasley *et al.*, 1989; McKenna, 1990). A major discrepancy between the rabbit and the rat preparations is the effect of IL-1β on the apparent contractility of the smooth muscle preparations. Beasley *et al.* (1989) and McKenna (1990) suggested that vascular contractility was depressed non specifically following incubation of rat aortic strips with IL-1β because it also decreased contractions by KCl. In this study, the enhanced responses to des-Arg^9-BK in rabbit aortic strips showing a decreased sensitivity to NA suggest that the intrinsic smooth muscle contractility was not depressed.

Induction in vivo

As shown with tissues *ex vivo* and with blood pressure measurements in anaesthetized rabbits, a transient inhibition of protein synthesis *in vivo* with i.v. cycloheximide induced an increase in vascular responsiveness to des-Arg^9-BK. Vascular sensitivity to the kinin metabolite also was induced by injection of IL-1β (5 μg kg^{-1}). IL-1 occurs in the plasma of rabbits injected with LPS (Cannon *et al.*, 1989) and LPS injection produces sensitization to des-Arg^9-BK *in vivo* (Regoli *et al.*, 1981). Furthermore, cultured vascular cells produce IL-1 in response to LPS (Libby *et al.*, 1986a,b) and IL-1 mRNA in response to cycloheximide (Libby *et al.*, 1986a; Warner *et al.*, 1987). Finally, TNFα (5 μg kg^{-1}), a cytokine also induced by LPS but not by cycloheximide in vascular cells, failed to induce a state of vascular sensitivity to des-Arg^9-BK (not shown). Thus, IL-1 may be the common mediator responsible for the inducing effect of both cycloheximide and LPS on the vascular sensitivity to des-Arg^9-BK in the rabbit.

It is unlikely that the activity of IL-1β or cycloheximide *in vivo* is due to a possible contamination by LPS because the *Limulus* amoebocyte lysate assay did not detect any endotoxin in the IL-1β preparation and only approximately 1 ng endotoxin per mg in the cycloheximide preparation. The stronger *in vivo* inducing effect of cycloheximide (10 mg kg^{-1}) or of LPS (25 μg kg^{-1}) as compared to IL-1β (5 μg kg^{-1}) may reflect the ability of these agents to stimulate a sustained endogenous production of cytokines, e.g. IL-1 itself (Dinarello *et al.*, 1987) or IL-2, another *in vitro* stimulant of the sensitization to des-Arg^9-BK in the rabbit aorta preparation (deBlois *et al.*, 1988). Since IL-1 is frequently synergistic with other cytokines such as IL-2 (Le *et al.*, 1986), the possibility exists that IL-1 action is a necessary but not sufficient step leading to vascular sensitivity to des-Arg^9-BK. This hypothesis will be tested on rabbit vascular tissues and cells in culture.

Alternatively, it is possible that PSIs stimulate the expression of gene(s) for factors involved in the sensitization to des-Arg^9-BK but different from IL-1. For example, inhibition of protein synthesis reportedly induces the mRNA for β-interferon (Ringold *et al.*, 1984) and TNFα (Warner & Libby, 1989), and for the nuclear transcription factors c-*myc* (Makino *et al.*, 1984) and c-*fos* (Sariban *et al.*, 1988). Also, a direct tran-

scriptional stimulation of synthesis of membrane receptors for des-Arg^9-BK (B_1-type) in the presence of PSIs is a possibility that cannot be ruled out at this time.

Nwator & Whalley (1989) reported cardiovascular responses of the BK_1-type in rabbits injected with the angiotensin converting enzyme inhibitors captopril or enalapril 20 h before anaesthesia. The rationale for such an experiment was that the inflammatory side-effects seen with long term use of this group of antihypertensive drugs in man may be related to elevated endogenous kinin and B_1 receptor levels. However, we have been unable to reproduce these results in rabbits injected with a similar or double dose of captopril or enalapril 20 h or 5 h (captopril group) before anaesthesia. The very low baseline blood pressure of anaesthetized animals used in the study of Nwator & Whalley (1989), or traces of endotoxin contaminating the drug preparations (which were administered in appreciable quantity: 5 mg kg^{-1}) may explain the discrepant results.

In summary, we have demonstrated that the spontaneous sensitization to des-Arg^9-BK of the isolated rabbit aorta was enhanced following short treatments with PSIs. DSP *in vitro* was inhibitory even in tissues pulsed with cycloheximide. The selective induction *in vivo* by cycloheximide and by IL-1β of cardiovascular responsiveness to des-Arg^9-BK was demonstrated using tissues *ex vivo* and a blood pressure assay of exogenous kinins. We propose that the production of endogenous IL-1 is a possible mechanism for the stimulated sensitization to des-Arg^9-BK observed in tissues pulsed with PSI *in vitro* and for the inducing effect of cycloheximide or LPS *in vivo*. These results suggest that effects mediated by the B_1 kinin receptor may be present in pathological conditions associated with IL-1 production. For example, the sensitization to the hypotensive action of des-Arg^9-BK observed in rabbits injected with LPS or IL-1β raises the intriguing possibility that some of the effects of kinins in the septic shock (Wilson *et al.*, 1989) may be mediated by receptors of the BK_1-type.

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Investigation into the 5-hydroxytryptamine receptor mediating smooth muscle relaxation in the rat oesophagus

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1 An investigation has been made into the 5-hydroxytryptamine (5-HT) receptor mediating relaxation of rat oesophagus in preparations precontracted with carbachol.

2 In tissues treated with pargyline (100 μM) and in the presence of corticosterone (30 μM) and cocaine (30 μM) the potency of 5-HT and 5-methoxytyramine (5-MeOT) was not changed but the maximum response to these agonists was reduced. Thus there was no evidence of metabolism and/or uptake through an amine depleting mechanism.

3 The relaxant concentration-effect curves to 5-HT were shifted to the left in a concentration-related manner by isobutylmethylxanthine (1 and 10 μM), suggesting the involvement of adenosine 3':5'-cyclic monophosphate in these responses.

4 5-HT produced concentration-related relaxations of rat oesophagus with an EC_{50} value of 0.24 μM. Several indole agonists were tested and the following rank order of potency of key agonists obtained: 5-HT > α-methyl-5-hydroxytryptamine = 5-carboxamidotryptamine (5-CT) > 5-MeOT. In contrast, 2-methyl-5-hydroxytryptamine, sumatriptan and 8-hydroxy-2-(di-n-propylamino) tetralin were weak or inactive.

5 The substituted benzamides, metoclopramide, cisapride, renzapride and **R,S**-zacopride acted as partial agonists, producing 60–70% of the 5-HT maximum.

6 The relaxation responses to 5-HT were neither inhibited by antagonists selective for 5-HT_1 or 5-HT_2 receptors nor by the 5-HT_3 receptor antagonists, ondansetron, granisetron or MDL 72222.

7 The relaxation responses induced by 5-HT, 5-CT, 5-MeOT and renzapride were selectively inhibited by high concentrations of ICS 205-930 with pK_B values of approximately 6.

8 The 5-HT receptor mediating relaxation in rat oesophagus cannot be designated 5-HT_1, 5-HT_2 or 5-HT_3 under the current 5-HT classification, but the observed effects are consistent with stimulation of the putative 5-HT_4 receptor.

Keywords: 5-hydroxytryptamine receptor; relaxation; rat oesophagus

Introduction

It has been observed that 5-hydroxytryptamine (5-HT) produces relaxation of rat isolated oesophageal muscularis mucosa precontracted with muscarinic agonists (Bieger & Triggle, 1985). This relaxation was not antagonized by methysergide, metergoline or ketanserin suggesting that 5-HT_1 and 5-HT_2 receptors, as classified by Bradley *et al.* (1986), were not involved in the response. Additionally, Bieger & Triggle (1985) reported that the relaxation induced by 5-HT was not affected by tetrodotoxin suggesting that it was unlikely that neuronal 5-HT_3 receptors were involved in this response. However, in contrast with this latter observation it has recently been found that the selective 5-HT_3 receptor antagonists, MDL 72222, granisetron and ICS 205-930, inhibit the effect of 5-HT in this preparation (Triggle *et al.*, 1988).

The aim of the present study was to characterize further the receptor type mediating the 5-HT-induced relaxation of rat oesophagus by use of selective agonists and antagonists. A preliminary account of this work has been presented to the British Pharmacological Society (Reeves *et al.*, 1989).

Methods

Female Wistar rats weighing between 120–180 g were killed by cervical dislocation, the abdomen opened and the most distal 2 cm of the oesophagus removed. The segments of oesophagus (one from each animal), with muscle layers and mucosa intact, were suspended in the longitudinal plane under an initial tension of approximately 0.5 g in a modified Krebs-Henseleit solution at 32°C gassed with 95% O_2/5% CO_2. The ionic composition of the Krebs-Henseleit solution (in mM) was NaCl 118.5, $NaHCO_3$ 25.0, KCl 4.7, $MgSO_4$ 0.6, KH_2PO_4 1.2, $CaCl_2$ 1.3 and glucose 11.1. This solution routinely contained indomethacin (3 μM) and except in experiments specifically aimed at investigating the effects of ketanserin, it contained ketanserin (1 μM).

Effects of agonists and antagonists

The oesophageal preparations were contracted by addition of a submaximal concentration of carbachol (1 μM) to the bathing solution, and on establishing a stable contractile response, a concentration of 5-HT was added. Once the maximum relaxation response to that concentration of 5-HT was obtained, higher concentrations were added in a cumulative manner to produce cumulative concentration-effect curves to 5-HT.

In agonist studies, a cumulative concentration-effect curve to 5-HT was constructed followed either by a second curve to 5-HT in control experiments, or by a curve to the test agonist. The relative potencies of the agonists were compared with 5-HT at the 50% response level for the 5-HT control concentration-effect curve. In antagonist studies, a control concentration-effect curve to an agonist was constructed followed by a test curve in the presence of the antagonist. Having established a stable contraction to carbachol (1 μM), antagonists were equilibrated for 30 min before construction of the test agonist concentration-effect curve.

In some experiments, following the construction of control curves to 5-HT or 5-methoxytryptamine (5-MeOT) as described above, the tissues were treated with pargyline (100 μM) for 30 min and then washed several times in fresh Krebs solution before the second application of carbachol. In these pargyline-treated preparations the effect of a mixture of

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corticosterone (30 μM) and cocaine (30 μM) on the responses mediated by 5-HT or 5-MeOT was investigated (as described above for antagonist studies). In separate experiments, the effect of isobutylmethylxanthine (IBMX, 1 and 10 μM) on the relaxant responses to 5-HT was investigated.

In all experiments, following the construction of the control concentration-effect curve, the preparations were washed with fresh Krebs solution and allowed to recover for at least 1 h, with further washes, before re-contracting with carbachol prior to the construction of the test curve.

Selectivity of antagonists

Antagonists that inhibited 5-HT-induced relaxations were also tested against isoprenaline-induced relaxations. Control cumulative concentration-effect curves to isoprenaline were constructed in carbachol-contracted preparations, followed by test curves in the presence of the antagonist, as described above for 5-HT.

Analysis of results

The relaxant responses are expressed as a percentage (arithmetic mean ± s.e.mean) of the maximum response obtained in the appropriate control concentration-effect curve. Equipotent molar ratios and EC_{50} values were calculated graphically for each preparation from the 50% response level and expressed as geometric means with 95% confidence limits in parentheses. The negative logarithm of the apparent dissociation constant for an antagonist (pK_B) was estimated by calculation of the mean (+ 95% confidence limits) of the individual results: $pK_B = \log$ (dose-ratio − 1) − log (antagonist concentration). The number of observations is indicated by n.

Drugs

Drugs obtained from commercial sources were 5-methoxytryptamine hydrochloride (5-MeOT, Sigma), 5-hydroxytryptamine hydrochloride (5-HT, Sigma), 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT, Research Biochemicals Incorporated), carbachol chloride (BDH Chemicals), cisapride (Janssen), cocaine hydrochloride (May & Baker Ltd), cyanopindolol (Sandoz), (3α-tropanyl)-1H-indole-3-carboxylic acid ester (ICS 205-930, Research Biochemicals Inc.), corticosterone (Sigma), ketanserin tartrate (Janssen), 1αH,3α,5αH-tropan-3yl-3,5-dichloro-benzoate (MDL 72222, Research Biochemicals Inc), 3-isobutyl-1-methylxanthine (IBMX, Aldrich Chemical Company Ltd), isoprenaline bitartrate dihydrate (Ward Blenkinsop), mesulergine (Sandoz), metergoline (Farmitalia), methiothepin maleate (Roche), methysergide hydrogen maleate (Sandoz), metoclopramide hydrochloride (Sigma), pargyline hydrochloride (Abbott Laboratories Ltd), spiperone (Janssen) and tetrodotoxin (Sigma).

Drugs synthesized by Glaxo Group Research Ltd were α-methyl-5-hydroxytryptamine maleate (α-Me-5-HT), 2-methyl-5-hydroxytryptamine hydrochloride monohydrate (2-Me-5-HT), 5-carboxamidotryptamine maleate (5-CT), 1,2,3,9-tetrahydro-9-methyl-3-[-2-methyl-1H-imidazol-1-yl) methyl]-4H-carbazole-4-one hydrochloride (ondansetron; GR38032F), 3-[2-dimethyl-amino]ethyl-N-methyl-1H-indole-5-methane sulphonamide (sumatriptan; GR43175), endo-4-amino-5-chloro-2-methoxy-N-(1-azabi-cyclo[3.3.1] non-4-yl) benzamide hydrochloride (renzapride; BRL24924), endo-4-(9-methyl-9-azabicyclo[3.3.1]non-3-yl)-1-methyl-1H-indazole-3-carboxamide hydrochloride (granisetron; BRL43694), and (R,S)-zacopride hydrochloride.

Results

Effects of 5-hydroxytryptamine receptor agonists

A submaximal concentration of carbachol (1 μM) produced a well maintained contraction (of between 1–2 g) for at least 60 min. 5-HT (0.01–10 μM) produced concentration-related relaxations with a mean EC_{50} value of 0.24 (0.19–0.29) μM and a mean maximum reduction of the carbachol tone of 52.3 ± 3.2% at 10 μM (n = 16); concentrations of 5-HT above 10 μM produced no further relaxation. The 5-HT responses were reproducible and two similar consecutive concentration-effect curves could be obtained in each preparation (Figure 1); thus the response to 5-HT did not change with time.

The effects of the combination of corticosterone (30 μM) and cocaine (30 μM) on responses to 5-HT or 5-MeOT in pargyline (100 μM)-treated preparations are shown in Figure 2. Treatment with pargyline, corticosterone and cocaine failed to shift the concentration-effect curves to 5-HT (ED_{50} values of 0.29(0.12–0.67) and 0.26(0.17–0.40) μM) or 5-MeOT (EC_{30} values of 7.3(4.8–11.2) and 7.2(4.3–12.5) μM) for control and test curves respectively, however, these compounds did produce marked reductions in the maximum responses to 5-HT and 5-MeOT of 27.2 ± 6.0% and 56.5 ± 2.8% (each n = 4) respectively. These reductions in carbachol tone complicated the analysis of the responses to 5-HT and 5-MeOT, and indeed this necessitated comparison of EC_{30} values for 5-MeOT since the maximum response to 5-MeOT after the drug additions did not achieve 50% of the control curve. In addition, although treatment with pargyline did not affect the size of the contraction to carbachol, the administration of corticosterone and cocaine resulted in an inhibition of 36.9 ± 2.7% (n = 8) of the carbachol tone. Preliminary experiments investigating the effects of cocaine alone indicated that the effects observed with the aforementioned mixture of compounds were predominantly due to the action of cocaine. Cocaine (10 μM) alone produced a reduction in carbachol tone of 20.5 ± 3.4% and caused a 27.2 ± 4.0% decrease in the 5-HT (10 μM) maximum (n = 4).

The effects of IBMX on the relaxant response to 5-HT are shown in Figure 3. IBMX alone (1 and 10 μM) had no direct relaxant effect but produced a concentration-related potentiation of the responses to 5-HT resulting in leftward shifts of 3.6(1.2–11.2) at 1 μM and 7.6(1.3–43.4) at 10 μM of the 5-HT concentration-effect curves.

The effects of 5-HT and other 5-HT receptor agonists are shown in Figure 4 and are summarised in Table 1. The effects of 5-HT were mimicked by 5-CT (0.1–100 μM) and α-Me-5-HT (0.1–100 μM) which exhibited equipotent molar ratios of approximately 20, and also by 5-MeOT with an equipotent

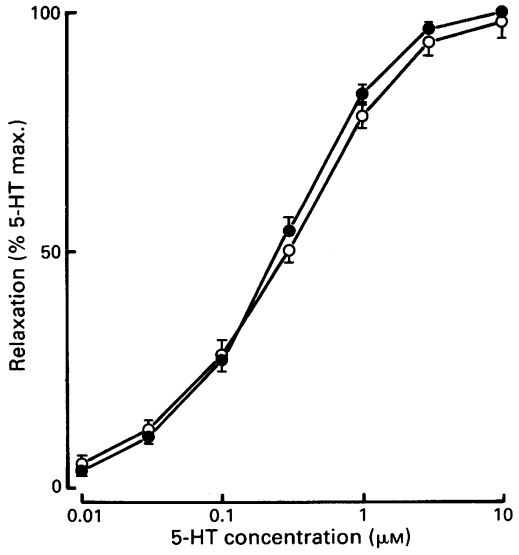

Figure 1 The relaxant effect of repeated cumulative concentration-effect curves to 5-hydroxytryptamine (5-HT) in carbachol-contracted rat oesophagus. (●) Control curve 1; (○) control curve 2. Each point is the mean of 6 observations; s.e.mean shown by vertical bars. Results expressed as % of curve 1 maximum.

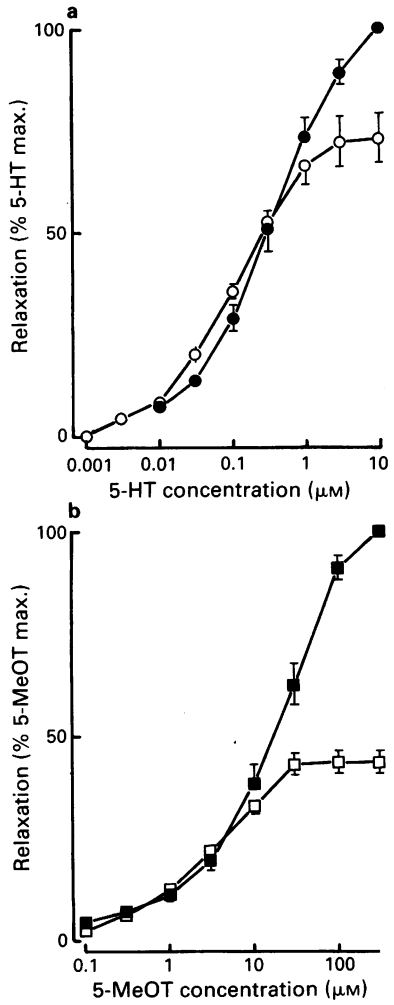

Figure 2 The effect of the combination of 100 μM pargyline (Par), 30 μM corticosterone (Cor) and 30 μM cocaine (Coc) on the relaxant responses to (a) 5-hydroxytryptamine (5-HT) and (b) 5-methoxytryptamine (5-MeOT). (●) Control concentration-effect curve to 5-HT and (○) in the presence of Par + Cor + Coc. (■) Control curve to 5-MeOT and (□) in the presence of Par + Cor + Coc. Each point is the mean of 4 observations; s.e.mean shown by vertical bars. Results are expressed as % of respective control maxima.

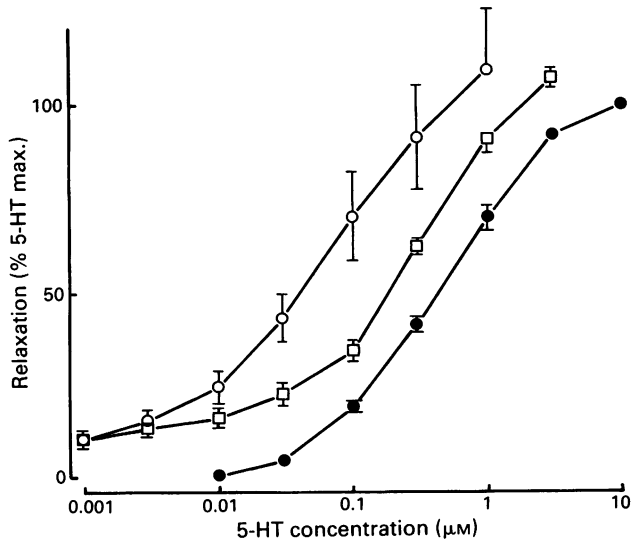

Figure 3 The effect of isobutylmethylxanthine (IBMX) on 5-hydroxytryptamine (5-HT) responses in rat oesophagus. (●) Control 5-HT responses ($n = 6$) and the effect of 5-HT in the presence of (□) 1 μM IBMX and (○) 10 μM IBMX (each $n = 3$). Each point is the mean with s.e.mean shown by vertical bars.

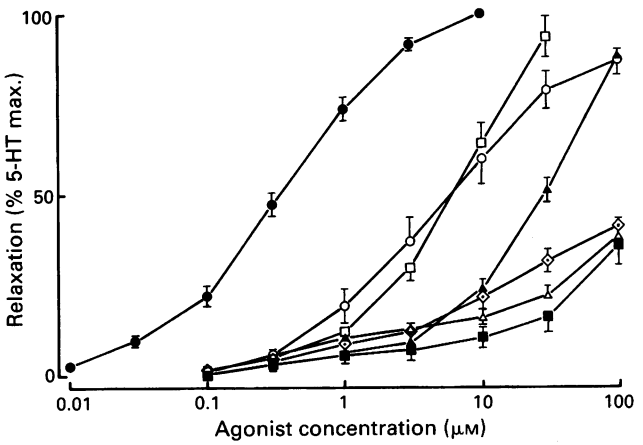

Figure 4 The effect of 5-hydroxytryptamine (5-HT) and other 5-HT receptor agonists (indole derivatives) in the rat oesophagus. (●) Control 5-HT ($n = 24$) and the agonist effect of (○) 5-carboxamidotryptamine; (□) α-methyl-5-HT; (▲) 5-methoxy-tryptamine; (■) 2-methyl-5-HT; (△) sumatriptan and (◇)8-hydroxy-2-(di-n-propylamino)tetralin. Each point is the mean of 4 observations (s.e.mean shown by vertical bars) calculated as a percentage of the control 5-HT maximum.

molar ratio of 90. The maximum responses to these agonists were close to that produced by 5-HT. The other agonists tested, 2-Me-5-HT, 8-OH-DPAT and sumatriptan were weak or inactive at concentrations up to 100 μM. The rank order of potency of the 5-HT agonists tested was: 5-HT > α-Me-5-HT = 5-CT > 5-MeOT > 2-Me-5-HT = sumatriptan = 8-OH-DPAT.

In addition to the effect of the indole analogues of 5-HT, the agonist effects of four substituted benzamides, metoclopramide, cisapride, renzapride and **R,S**-zacopride were investigated and the results obtained are shown in Figure 5 and summarised in Table 1. Each of the benzamides acted as a partial agonist producing approximately 60–70% of the 5-HT maximum, although it must be pointed out that the apparent partial agonist activity of metoclopramide and cisapride could not be examined thoroughly because high concentrations (> 100 μM) produced a non-specific relaxation of the carbachol tone that could not be inhibited by ICS205-930 (see below).

Effects of 5-hydroxytryptamine receptor antagonists

A wide range of 5-HT receptor antagonists with some degree of selectivity for the different 5-HT receptor subtypes have

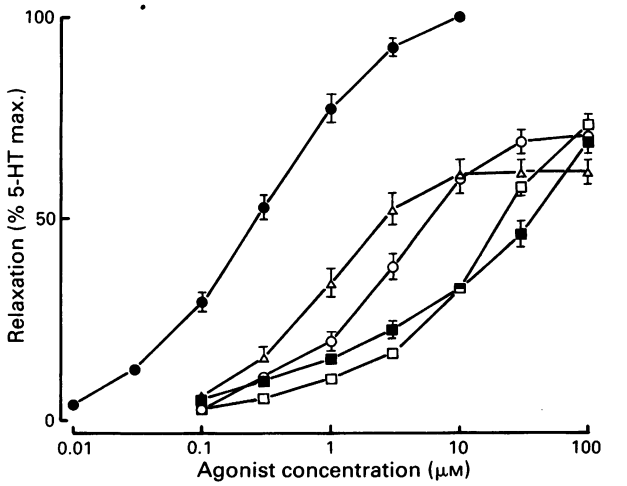

Figure 5 The agonist effect of 5-hydroxytryptamine (5-HT) and certain substituted benzamides in rat oesophagus. (●) Control 5-HT responses ($n = 16$) and the agonist responses of (△) renzapride; (○) **R,S**-zacopride; (■) cisapride and (□) metoclopramide ($n = 4$). Each point is the mean (s.e.mean shown by vertical bars) calculated as a percentage of the 5-HT maximum.

Table 1 Summary of the effects of 5-hydroxytryptamine (5-HT) receptor agonists in rat isolated oesophagus

Agonist	*5-HT receptor selectivity as agonists*	*Equipotent molar ratio where 5-HT = 1* (95% confidence limits)	*% 5-HT maximum responses**
5-HT	Non-selective	1.0	100 ± 0
5-CT	5-HT_1	20.1 (7.9–51.5)	87.1 ± 4.1
α-Me-5-HT	5-HT_2	15.6 (12.6–19.4)	93.6 ± 5.6
5-MeOT	Non-selective (but inactive at 5-HT_3)	90.4 (72.9–112.1)	88.3 ± 1.7
Renzapride	5-HT_4 ?	16.5 (6.8–40.2)	61.5 ± 3.1
R,S-zacopride	5-HT_4 ?	31.7 (17.5–56.5)	70.5 ± 3.5
Cisapride	5-HT_4 ?	90.9 (61.6–134.2)	73.3 ± 2.6
Metoclopramide	5-HT_4 ?	84.2 (16.8–175.0)	69.0 ± 2.9
Sumatriptan	5-HT_1	>300	38.6 ± 7.4
8-OH-DPAT	5-HT_{1A}	>300	41.2 ± 2.3
2-Me-5-HT	5-HT_3	>300	36.0 ± 5.4

5-CT: 5-carboxamidotryptamine; α-Me-5-HT: α-methyl-5-HT; 5-MeOT: 5-methoxytryptamine; 8-OH-DPAT: 8-hydroxy-2-(di-n-propylamino)tetralin; 2-Me-5-HT: 2-methyl-5-HT.
* at 100 μM, except for 5-HT (10 μM), α-Me-5-HT (30 μM) and 5-MeOT (300 μM).

been tested against the 5-HT-induced relaxation response in rat oesophagus. Those antagonists exhibiting no inhibitory effect are shown in Table 2. High concentrations of antagonists with affinity for 5-HT_1-like, 5-HT_2 and 5-HT_3 receptors were without effect, as was the sodium ion channel blocker, tetrodotoxin (Narahashi, 1972).

In contrast to the results obtained with the three 5-HT_3 receptor antagonists, ondansetron, MDL 72222 and granisetron (Table 2), a fourth compound, ICS 205-930, did produce antagonist effects. ICS 205-930 (1 and 10 μM) produced concentration-related, rightward shifts of the 5-HT concentration-effect curves with no depression of the maximum response to 5-HT. From these data pK_B values of approximately 6 were determined (Table 3), although it must be emphasised that the data were not consistent with a Schild slope of unity and thus ICS 205-930 did not appear to behave as a truly competitive antagonist under these conditions. ICS 205-930 (at 3 μM) was also tested against cumulative concentration-effect curves to 5-MeOT, 5-CT and renzapride. ICS 205-930 produced rightward shifts of these agonist curves with no depression of their maxima. From these data mean pK_B values of approximately 6 were again calculated (Table 3).

Isoprenaline (0.01–3 μM) elicited concentration-related and reproducible relaxations with mean EC_{50} values (μM) of 0.19 (0.13–0.29) and 0.28 (0.24–0.32) respectively for consecutive curves, and at 3 μM produced a complete reversal of the carbachol-induced contraction. ICS 205-930 (10 μM) produced no significant inhibition of the responses to isoprenaline with a mean concentration-ratio of only 1.2 (0.8–1.9) compared to the control curve, a value not significantly different from unity.

Table 2 Summary of antagonists with no inhibitory effect on 5-hydroxytryptamine (5-HT)-induced relaxation in rat isolated oesophagus ($n = 4$ throughout)

Compound	*Antagonist selectivity*	*Concentration tested* (μM)	*Concentration ratio* (95% confidence limits)
Spiperone	$5\text{-HT}_{1A}/5\text{-HT}_2$	1.0	1.0 (0.5–2.0)
Cyanopindolol	$5\text{-HT}_{1A}/5\text{-HT}_{1B}$	1.0	1.4 (0.9–2.3)
Mesulergine	$5\text{-HT}_{1C}/5\text{-HT}_2$	1.0	0.9 (0.7–1.3)
Methysergide	5-HT_1-like/5-HT_2	10	1.0 (0.4–2.9)
Methiothepin	$5\text{-HT}_1/5\text{-HT}_2$	0.3	1.0 (0.5–1.8)
Metergoline	$5\text{-HT}_1/5\text{-HT}_2$	1.0	0.5 (0.2–1.3)
Ketanserin	5-HT_2	1.0	0.4 (0.3–0.5)
Ondansetron	5-HT_3	1.0	1.1 (0.5–2.4)
Granisetron	5-HT_3	10	1.8 (0.9–3.6)
MDL 72222	5-HT_3	1.0	1.0 (0.8–1.2)
Tetrodotoxin	Na^+ channel blocker	0.3	0.8 (0.3–1.6)

Table 3 Mean pK_B values for ICS 205-930 against 5-hydroxytryptamine (5-HT) receptor agonists in rat oesophagus

Agonists used	*Concentration ICS 205-930 tested* (μM)	n	*Concentration ratio* (95% confidence limits)	pK_B value
5-HT	1.0	6	3.1 (1.7–5.5)	6.3 ± 0.1
	10	6	7.5 (2.6–21.1)	5.8 ± 0.1
5-CT	3.0	3	6.4 (1.1–38.4)	6.2 ± 0.2
5-MeOT	3.0	3	3.6 (2.6–5.1)	5.9 ± 0.1
Renzapride	3.0	4	4.2 (1.3–13.1)	6.0 ± 0.2

5-CT: 5-carboxamidotryptamine; 5-MeOT: 5-methoxytryptamine.

At the concentrations tested, none of the antagonists, with the exception of ICS 205-930, had any obvious effect on the carbachol-induced contractions. ICS 205-930 at 10 μM produced a small inhibition of 19.0 ± 1.3% of the carbachol tone, but had no effect at 1 and 3 μM.

Discussion

The present study has shown that 5-HT produces concentration-related and reproducible relaxations of rat oesophagus precontracted with carbachol, and this is in agreement with the work of Bieger & Triggle (1985). This response is apparently mediated through a direct effect on the smooth muscle, a neuronal component being unlikely since tetrodotoxin had no effect.

Since 5-HT can be a substrate for amine uptake processes and can be degraded by monoamine oxidase, the rank order of potency of 5-HT receptor agonists might have been affected by the degree of uptake and/or metabolism of each agonist. Therefore the effect of the irreversible monoamine oxidase inhibitor, pargyline, and the amine uptake inhibitors corticosterone and cocaine on the relaxant responses to both 5-HT and 5-MeOT was investigated. The addition of high concentrations of pargyline, corticosterone and cocaine produced no potentiation of the responses to either 5-HT or 5-MeOT in this study suggesting that if uptake did occur, it did not significantly modify relaxation under the conditions of these experiments. However, the mixture of these inhibitors did cause a reduction in the maximum response obtained to 5-HT, and more markedly to 5-MeOT (Figure 2), an effect that could be due in part to the large inhibition of the carbachol-induced tone produced by the addition of corticosterone and cocaine although, why the maximum response to 5-MeOT was reduced more than that to 5-HT cannot be explained. This reduction in carbachol tone complicated the analysis of the responses to 5-HT and 5-MeOT, but with this caveat in mind there was no evidence of potentiation of the relaxant responses to these agonists. Preliminary experiments suggested that these inhibitory effects were predominantly caused by cocaine, possibly through its local anaesthetic activity. These inhibitors were not used in subsequent experiments.

The relaxant responses to 5-HT were mimicked by 5-CT, α-Me-5-HT and 5-MeOT which produced responses close to the 5-HT maximum whereas 2-Me-5-HT, sumatriptan and 8-OH-DPAT were weak or inactive. As shown in Table 4 the rank order of potency of the key agonists was not typical of a 5-HT_1, 5-HT_2 or 5-HT_3-receptor. In addition, the very weak activity of sumatriptan and 8-OH-DPAT confirms that the 5-HT_1-like receptor characteristic of the dog saphenous vein (Feniuk *et al.*, 1985) and the 5-HT_{1A} receptor (Hoyer *et al.*, 1985) were not involved. Furthermore, these deductions derived from agonist data are confirmed by the lack of effect of high concentrations of 5-HT_1, 5-HT_2 and most of the 5-HT_3 receptor antagonists (Table 2).

The finding that the substituted benzamides, metoclopramide, **R,S**-zacopride, cisapride and renzapride were partial agonists in rat oesophagus was of particular interest, although high concentrations (100 μM) of metoclopramide and cisapride could not be used since non-specific relaxant responses occurred which were not inhibited by ICS 205-930, 10 μM (unpublished observations). Preliminary studies in our laboratory (Reeves *et al.*, 1989) and by others (Triggle *et al.*, 1988) have previously found that the benzamides were unsurmountable antagonists of 5-HT in rat oesophagus. Although we cannot comment on the work of Triggle *et al.* (1988), we believe that our preliminary results (Reeves *et al.*, 1989) with the benzamides were misleading. In these early experiments the benzamides were administered prior to carbachol and only tested as antagonists using an experimental protocol that would not have clearly identified any direct agonist effects. In the present studies the carbachol-induced tone was established before the addition of the benzamides, and this has allowed a quantitative study of agonist effects. The antagonist effects of the benzamides previously observed (Reeves *et al.*, 1989), were probably attributable to the affinity of the benzamides for the 5-HT receptor in rat oesophagus which then restricted the access of, and responses to, 5-HT.

The lack of involvement of 5-HT_1 and 5-HT_2 receptors in the relaxant response to 5-HT in the present study is in agreement with the results reported by Bieger & Triggle (1985). However, particularly interesting observations are derived from closer inspection of the effects of 5-HT_3 receptor antagonists. In our study, the 5-HT_3 antagonists MDL 72222

Table 4 Rank order of agonist potency of selective compounds at 5-hydroxytryptamine (5-HT) receptor subtypes

Receptor subtype	*Agonist potency*	*References*
5-HT_1-like (contraction)	5-CT > 5-HT > αMe-5-HT > 2-Me-5-HT = 0	Feniuk *et al.* (1985)
5-HT_1-like (relaxation)	5-CT ≫ 5-HT ≫ 2-Me-5-HT = α-Me-5-HT = 0	Humphrey (1984)
5-HT_2	5-HT = α-Me-5-HT > 5-CT > 2-Me-5-HT	Humphrey (1984)
5-HT_3	5-HT = 2-Me-5-HT > α-Me-5-HT ≫ 5-CT	Humphrey (1984)
Rat oesophagus	5-HT > α-Me-5-HT = 5-CT ≫ 2-Me-5-HT > 0	This study

5-CT: 5-carboxamidotryptamine; α-Me-5-HT: α-methyl-5-HT; 2-Me-5-HT: 2-methyl-5-HT.

(Fozard, 1974) and granisetron (Sanger & Nelson, 1989) did not affect the response to 5-HT. These results contrast with those of Triggle *et al.* (1988) who found that these compounds were potent antagonists of 5-HT in the rat isolated oesophageal muscularis mucosa. This discrepancy is difficult to explain; the present study used whole sections of rat oesophagus whereas Triggle *et al.* (1988) used only the muscularis mucosa, a preparation where the external striated muscle layers are removed. However, preliminary work in our laboratory would show no difference between the two isolated oesophagus preparations in this respect (unpublished observations). Indeed, 2-Me-5-HT was a weak agonist in both the present study and that of Triggle *et al.* (1988) and this result, taken with the lack of effect of ondansetron (Table 2), makes it unlikely that 5-HT_3 receptors are involved in the relaxant responses to 5-HT in rat oesophagus.

In contrast to the results obtained for ondansetron, granisetron and MDL 72222, high concentrations of the 5-HT_3 receptor antagonist ICS 205-930 (Richardson *et al.*, 1985) produced concentration-related antagonism of the 5-HT-induced relaxations. From these data pK_B values of approximately 6 were calculated, and although it did not appear to behave as a truly competitive antagonist against 5-HT, this is much lower than the reported affinity constant for ICS 205-930 at 5-HT_3 receptors in rat tissues where a pA_2 value of approximately 8.5 was obtained (Butler *et al.*, 1988). It therefore appears that ICS 205-930 interacts with a non-5-HT_3 receptor site in rat oesophagus. In addition to the effect of ICS 205-930 against 5-HT, a high concentration of ICS 205-930 also inhibited the relaxant responses to 5-CT, 5-MeOT and the benzamide, renzapride, again with pK_B values of approximately 6. The effects of ICS 205-930 were selective, since relaxant responses to isoprenaline were unaffected. The consistency of these pK_B values for ICS 205-930 suggests that both the indole and benzamide agonists were interacting with the same single population of receptors. As in the present study, high concentrations of ICS 205-930 also inhibit responses to 5-HT and the benzamides in mouse colliculi neurones (Dumuis *et al.*, 1988; 1989), guinea-pig ileum (Craig & Clarke, 1990; Eglen *et al.*, 1990) and guinea-pig hippocampus (Bockaert *et al.*, 1990) with affinity constants ranging from 6.0 to 6.5, a result which is considered to be indicative of the presence of the putative 5-HT_4 receptor (Clarke *et al.*, 1989).

The observation that the benzamides are partial agonists in rat oesophagus is also consistent with the presence of 5-HT_4 receptors in this tissue; similar results with these compounds have been reported in other tissues exhibiting 5-HT_4 receptors (Dumuis *et al.*, 1989; Bockaert *et al.*, 1990; Eglen *et al.*, 1990). With respect to the indoles, comparison of data from the present study (Table 1) with the results of Craig & Clarke (1990) in guinea-pig ileum shows that although 5-HT, 5-CT, α-Me-5-HT and 5-MeOT were agonists in both preparations (with 2-Me-5-HT being inactive), their rank orders of potency were different in these tissues. These findings could be indicative of different receptor types, although the results with ICS 205-930 (see above) do not support this contention; clearly these differences in agonist relative potency need to be resolved.

The suggestion that 5-HT-induced relaxation of rat oesophagus is mediated by 5-HT_4 receptors is also corroborated by the studies with IBMX which potentiated the responses to 5-HT. The latter result is consistent with the intracellular mediation by adenosine 3':5'-cyclic monophosphate (cyclic AMP) of smooth muscle relaxation in rat oesophagus, and indeed 5-HT_4 receptors have been shown to be positively coupled to adenylate cyclase in both mouse colliculi neurones (Dumuis *et al.*, 1988; 1989) and guinea-pig hippocampus (Bockaert *et al.*, 1990).

In conclusion, the 5-HT receptor type eliciting relaxation of rat oesophagus cannot be identified as 5-HT_1-like, 5-HT_2 or 5-HT_3, and the data presented here strongly suggest that this effect is mediated via 5-HT_4 receptors.

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Separation of two pathways for calcium entry into chromaffin cells

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1 The effects of various drugs on $^{45}Ca + {}^{40}Ca$ uptake into cultured bovine adrenal chromaffin cells evoked by 1,1-dimethyl-4-phenylpiperazinium (DMPP) or high K, were studied. In the presence of 1 mM external ^{40}Ca, with ^{45}Ca as a radiotracer, unstimulated cells took up an average of 0.13 fmol/cell ^{40}Ca and 772 c.p.m./10^6 cells of ^{45}Ca ($n = 76$). Upon stimulation with DMPP (100 μM for 60 s) or K (59 mM for 60 s), Ca uptake increased to 0.92 and 1 fmol/cell, respectively.

2 Flunarizine behaved as a potent blocker of both DMPP- and K-evoked Ca uptake (IC_{50} of 1.76 and 1.49 μM, respectively for DMPP and K). A similar picture emerged with Cd ions, though Cd exhibited an IC_{50} against K (1.86 μM) slightly lower than the IC_{50} against DMPP (8.14 μM).

3 Clear cut differences were observed with amiloride, guanethidine, nimodipine and nisoldipine which behaved as selective blockers of DMPP-mediated Ca uptake responses: IC_{50} values to block DMPP effects were 290, 27, 1.1 and 1.63 μM respectively for amiloride, guanethidine, nimodipine and nisoldipine. Amiloride blocked K-evoked Ca uptake by only 35% and guanethidine did not affect it. Nisoldipine inhibited K-evoked Ca uptake only partially at low concentrations (about 30%); a second blocking component was observed at the highest concentration used (10 μM). At 10 μM, nimodipine blocked K-evoked Ca uptake by 50%.

4 Thus, it seems that the nicotinic receptor mediated Ca uptake pathway can be pharmacologically separated from the K-activated pathway. The results are compatible with the hypothesis that in cultured bovine adrenal chromaffin cells, stimulation of nicotinic receptors recruits a single type of Ca channel which is sensitive to flunarizine, Cd, amiloride, guanethidine, nimodipine and nisoldipine. The results also suggest that K depolarization might be recruiting in addition to this channel, another Ca channel which is highly sensitive to Cd and flunarizine, resistant to nisoldipine, nimodipine and amiloride, and insensitive to guanethidine.

Keywords: Chromaffin cells; calcium channels; flunarizine; guanethidine; amiloride; nisoldipine; nimodipine; cadmium

Introduction

Douglas & Rubin (1961) first demonstrated that acetylcholine or high K concentrations triggered adrenal medulla catecholamine release by activating a Ca channel located on the chromaffin cell plasmalemma. Since both nicotinic stimulation and direct depolarization activate Ca uptake and secretion in a parallel manner (Douglas & Poisner, 1962; Kilpatrick *et al.*, 1982; Holz *et al.*, 1982; Artalejo *et al.*, 1986), it seemed likely that with both types of stimuli, voltage-dependent Ca channels were used for Ca entry. These channels were demonstrated to be present in chromaffin cells by means of whole-cell recordings of Ca currents by use of patch-clamp techniques (Fenwick *et al.*, 1982).

On pharmacological grounds (i.e. inhibition of secretion by specific Ca channel antagonists) it seemed to us that both nicotinic and high-K-mediated catecholamine release were triggered by the same type of Ca channels (Ceña *et al.*, 1983). This is supported by the observation that (+)-isradipine (a 1,4-dihydropyridine derivative) blocks the nicotinic response only in the presence of Na (Cárdenas *et al.*, 1988; Abajo *et al.*, 1989). Therefore, it is likely that Na entering through the acetylcholine receptor ionophore (Amy & Kirshner, 1982; Wada *et al.*, 1985) causes cell depolarization and opening of Ca channels. Up to now, this picture has implied that external Ca entering chromaffin cells stimulated via nicotinic receptors or high K uses the same end pathway, i.e. a voltage-sensitive Ca channel of the L-subtype.

We present pharmacological data in this paper that call for a reconsideration of this widely accepted picture. By using flunarizine (a piperazine derivative), Cd (an inorganic Ca channel blocker), amiloride (a K-sparing diuretic), guanethidine (a sympathetic neuronal blocker), nimodipine and nisoldipine (1, 4-dihydropyridine derivatives) we demonstrate here that Ca channels recruited by nicotinic- or high-K stimulation can be pharmacologically separated.

Methods

Preparation of chromaffin cells

Bovine adrenomedullary chromaffin cells were isolated following standard methods (Livett, 1984) with the following modifications: (a) once in the cell culture unit, adrenal glands were washed three times with Ca- and Mg-free Locke buffer (mM: NaCl 154, KCl 5.6, $NaHCO_3$ 3.5, glucose 11 and HEPES buffer 10, at pH 7.2) at room temperature; (b) digestion of adrenal medulla was carried out by injecting 5 ml of a solution containing 0.25% collagenase, 0.5% bovine serum albumin and 0.01% soybean trypsin inhibitor in Ca-Mg-free Locke buffer, and incubating the glands at 37°C for 15 min; this procedure was repeated thrice; (c) collagenase was washed out with a large volume of Ca-Mg-free Locke buffer and then cells were filtered first with a 217 μm and thereafter with a 80 μm nylon mesh; (d) cells resuspended in Ca-Mg-free Locke buffer were placed on self-generated Percoll gradients containing 19 ml Percoll (17.1 ml Percoll plus 1.9 ml 10 fold concentrated Ca-Mg-free Locke buffer at pH 5), plus 21 ml of cell suspension (about 50–100 × 10^6 cells); the final pH of the mixture was 7.2. The mixture was centrifuged at 13,000 r.p.m. for 20 min (rotor SS-34, Sorvall centrifuge Model RC-5) at 22°C. Then, the lower band of the gradient (enriched in adrenaline-containing cells, Moro *et al.*, 1990) was taken, washed once with Ca-Mg-free Locke buffer and a second time with Dul-

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becco's modified Eagle's medium (DMEM). Finally, cells were resuspended in DMEM supplemented with 10% foetal calf serum, 10 μM cytosine arabinoside, 10 μM fluorodeoxiuridine, 50 u ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. Cells were plated at a density of 1 × 10^6 cells/well in 24 multiwell Costar plates and incubated at 37°C in a water-saturated, 5% CO_2/95% air atmosphere. Medium was changed 24 h later with fresh DMEM. Viability of the cells (usually greater than 90%) was estimated by Trypan blue exclusion.

Calcium uptake into chromaffin cells

Calcium uptake studies were carried out in cells after 2–3 days in culture. Before the experiment, cells were washed twice with 0.5 ml Krebs-HEPES solution (composition (mM): NaCl 140, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 1.0, glucose 11, HEPES 10, at pH 7.2) at 37°C. ^{45}Ca uptake into chromaffin cells was studied by incubating the cells at 37°C with $^{45}CaCl_2$ at a final concentration of 4 μCi ml^{-1} in the presence of Krebs-HEPES (basal uptake), high K solution (Krebs-HEPES containing 59 mM KCl with isosmotic reduction of NaCl) or DMPP solution (Krebs-HEPES with 100 μM final concentration of the nicotinic receptor agonist, 1,1-dimethyl-4-phenyl-piperazinium, DMPP). This incubation was carried out during 1 min and at the end of this period the test medium was rapidly aspirated and the uptake reaction was ended by adding 0.5 ml of a cold Ca-free Krebs-HEPES containing 10 mM $LaCl_3$. Finally, cells were washed 5 times more with 0.5 ml of cold Ca-free Krebs-HEPES containing 10 mM $LaCl_3$ and 2 mM EGTA, at 15 s intervals.

To measure radioactivity retained by chromaffin cells, the cells were scraped with a plastic pipette tip while adding 0.5 ml 10% trichloroacetic acid, 2 ml of scintillation fluid (Ready Micro, Beckman) was added and the samples counted in a Packard beta counter. Results are expressed as counts min^{-1} per 10^6 cells, fmol of total Ca taken up by a single cell or normalized as % of Ca taken up by control cells.

When possible, IC_{50}s for each drug to block DMPP or K-evoked ^{45}Ca uptake were estimated through non-linear regression analysis of inhibition curves, using a GraphPAD programme from ISI software, for a PC computer.

Materials and solutions

The following materials were used: collagenase from *Clostridium histolyticum* (Boehringer-Mannheim); bovine serum albumin fraction V, soybean trypsin inhibitor, cytosine arabinoside, fluorodeoxiuridine, guanethidine, amiloride, DMPP, EGTA (Sigma); Percoll (Pharmacia); DMEM, foetal calf serum, penicillin and streptomycin (GIBCO); scintillation fluid Ready micro (Beckman); ^{45}Ca (Specific activity 10–40 mCi mg^{-1} calcium, Amersham). All other chemicals were reagent grade. Amiloride, nimodipine, nisoldipine and flunarizine were dissolved in ethanol, and diluted in saline solutions to the desired concentrations. Experiments were performed under sodium lighting.

Results

Cell calcium uptake: signal to noise ratio

Figure 1 shows the increase in ^{45}Ca uptake by cells stimulated with increasing concentrations of DMPP or K. The threshold concentration for DMPP was 3 μM and for K, 17,7 mM; the maximum effect was obtained with 100 μM DMPP and 59 mM K. Therefore, these concentrations were selected to perform the following experiments.

We wished to compare the effects of various drugs on DMPP- and K-evoked Ca uptake. Thus, concentrations of the stimulants provoking maximal signals were selected (Figure 2). DMPP (100 μM for 60 s) induced ^{45}Ca uptake (in the presence of 1 mM ^{40}Ca) of 5663 ± 407 c.p.m./10^6 cells (data from 42 individual wells from 14 different batches of cells). This figure represents 0.92 ± 0.07 fmol of total Ca taken up by a single chromaffin cell upon stimulation of its nicotinic receptors during 60 s.

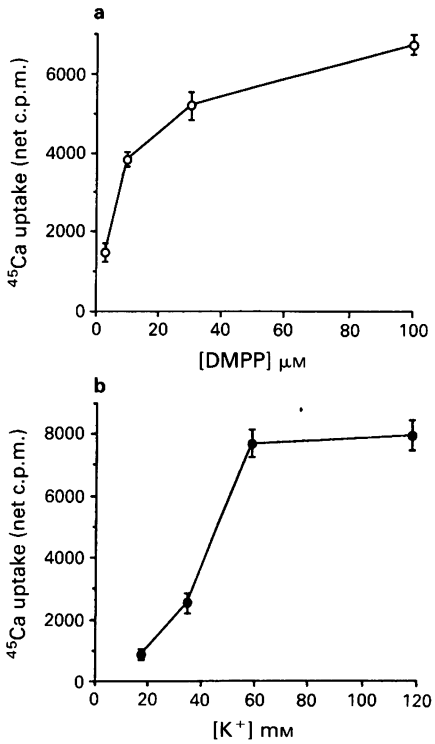

Figure 1 ^{45}Ca uptake into chromaffin cells stimulated with increasing concentrations of 1,1-dimethyl-4-phenylpiperazinium (DMPP) (a) or K (b). Cells in different wells were exposed to different concentrations of DMPP or K (abscissa scale) for 60 s. ^{45}Ca uptake is expressed in net c.p.m./well (ordinates); basal ^{45}Ca taken up by resting cells was subtracted from evoked ^{45}Ca uptake. Data are means of 8 wells from two separate batches of cells; s.e.mean shown by vertical bars.

High K concentrations (59 mM for 60 s) produced comparable signals. The average ^{45}Ca taken up amounted to 6145 ± 483 c.p.m./10^6 cells (data from 42 individual wells from 14 different batches of cells). This figure represents

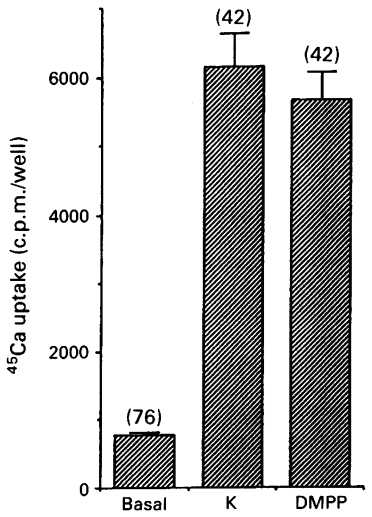

Figure 2 ^{45}Ca uptake into resting or stimulated chromaffin cells. ^{45}Ca retained by unstimulated cells (Basal) or cells stimulated with 1,1-dimethyl-4-phenylpiperazinium (DMPP, 100 μM for 60 s) or K (59 mM for 60 s) is expressed in c.p.m./10^6 cells (ordinate scale). The ratio DMPP/Basal was 7.3 and K/Basal, 7.9. Data are means of the number of experiments shown in parentheses; vertical bars show s.e.mean.

1.00 ± 0.08 fmol of total Ca taken up by a single chromaffin cell depolarized for 60 s with 59 mM K.

In basal conditions (1 min incubation in Krebs-HEPES solution containing 4 μCi ml^{-1} ^{45}Ca plus 1 mM ^{40}Ca) cells retained 771 ± 35 c.p.m. (data from 76 individual wells from 25 different batches of cells). In individual experiments, stimulated cells took up about 5–10 fold the amount of ^{45}Ca retained by unstimulated cells. Thus, signal to noise ratio was adequate to perform studies with increasing concentrations of drugs to block Ca uptake.

Flunarizine blocks equally well DMPP- and K-evoked Ca uptake into chromaffin cells

Flunarizine (Figure 3) inhibited DMPP- and K-evoked Ca uptake into chromaffin cells in a concentration-dependent manner. Full blockade was achieved at a concentration of 10 μM. The IC_{50} to inhibit the DMPP response was 1.76 μM; the K response exhibited an IC_{50} of 1.49 μM.

Effects of cadmium on calcium uptake by chromaffin cells

Cd ions blocked both DMPP- and K-evoked Ca uptake into chromaffin cells. The IC_{50} for the K response was 1.86 μM and that for DMPP 8.14 μM (Figure 4). At 30 μM Cd, blockade of ^{45}Ca uptake was complete with both stimuli.

Selective block by amiloride of the nicotinic receptor-evoked calcium uptake into chromaffin cells

K-evoked Ca uptake into chromaffin cells was affected little by increasing concentrations of amiloride; only at 1 mM, could a 35% blockade be seen. In contrast, DMPP-evoked Ca uptake was gradually inhibited by increasing concentrations of this drug (Figure 5). The IC_{50} was 290 μM, and full blockade of Ca uptake was achieved at 1 mM.

Selective inhibition by guanethidine of nicotinic receptor-evoked calcium uptake into chromaffin cells

Guanethidine did not affect K-evoked Ca uptake into chromaffin cells; the Ca uptake mechanism was depressed by only 20% at a concentration of 10 μM. This concentration fully blocked DMPP-evoked Ca uptake. In contrast to amiloride, the inhibition curve was very steep (Figure 6). Guanethidine had little effect on the Ca uptake mechanism in concentrations up to 10 μM; at 30 μM, 70% blockade was achieved. The IC_{50} was 27 μM.

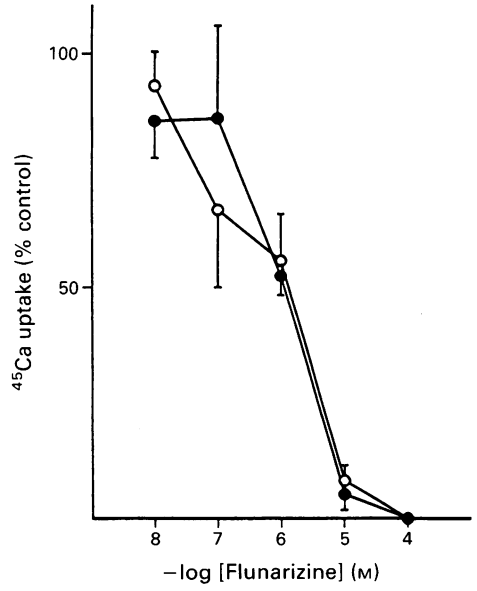

Figure 3 Flunarizine blocks equally well the uptake of Ca into chromaffin cells stimulated with (○) 1,1-dimethyl-4-phenylpiperazinium (DMPP, 100 μM for 60s) or (●) K (59 mM for 60 s). Before stimulation, cells were preincubated with each concentration of flunarizine for 10 min. ^{45}Ca uptake (ordinate scale) was normalized to 100% (^{45}Ca taken up by cells in the absence of flunarizine). Data are means of 4 triplicate experiments from different batches of cells; s.e.mean shown by vertical bars.

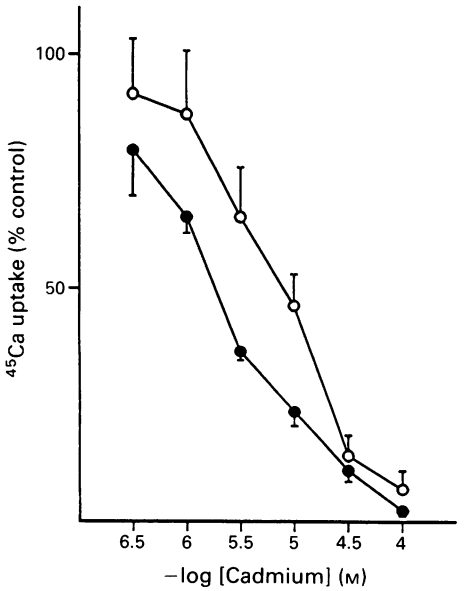

Figure 4 Effects of Cd ions on ^{45}Ca uptake into chromaffin cells evoked by (○) 1,1-dimethyl-4-phenylpiperazinium (DMPP, 100 μM for 60 s) or (●) K (59 mM for 60 s). Experimental protocol as in Figure 3 but here, Cd instead of flunarizine was used as blocking agent. Data (normalized to 100% of control ^{45}Ca uptake) are means of 4 triplicate experiments done with cells from different batches; s.e.mean shown by vertical bars.

Effects of nimodipine and nisoldipine on nicotinic receptor and K-evoked calcium uptake into chromaffin cells

Blockade of K-evoked ^{45}Ca uptake by nimodipine and nisoldipine exhibited a biphasic pattern. At submicromolar concentrations, a 10–30% blockade was observed. Then, a second

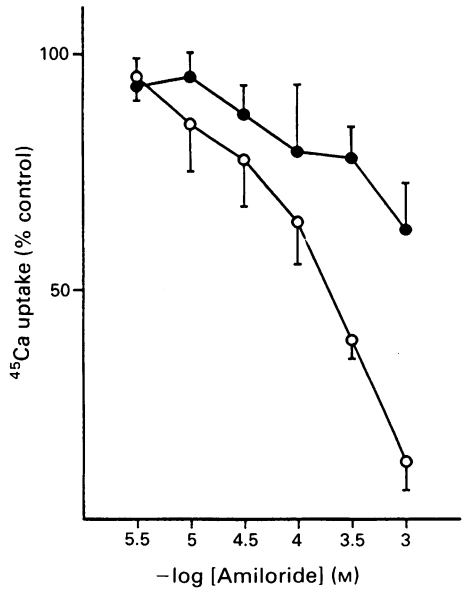

Figure 5 Effects of amiloride on ^{45}Ca uptake into chromaffin cells stimulated with (○) 1,1-dimethyl-4-phenylpiperazinium (DMPP, 100 μM for 60 s) or (●) K (59 mM for 60 s). Experimental protocol as in Figure 3. Data (normalized to 100% of control ^{45}Ca uptake) are means of 4 triplicate experiments made with cells from different batches; s.e.mean shown by vertical bars.

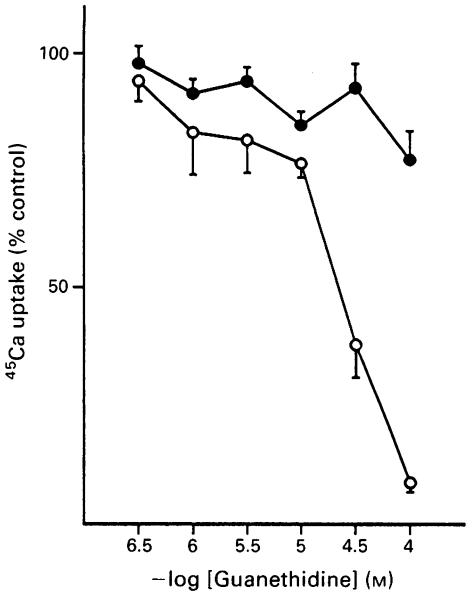

Figure 6 Effects of guanethidine on ^{45}Ca uptake into chromaffin cells stimulated with (○) 1,1-dimethyl-4-phenylpiperazinium (DMPP, 100 μM for 60 s) or (●) K (59 mM for 60 s). Experimental protocol as in Figure 3. Data (normalized to 100% of control Ca uptake) are means of 4 triplicate experiments made with cells from different batches; s.e.mean shown by vertical bars.

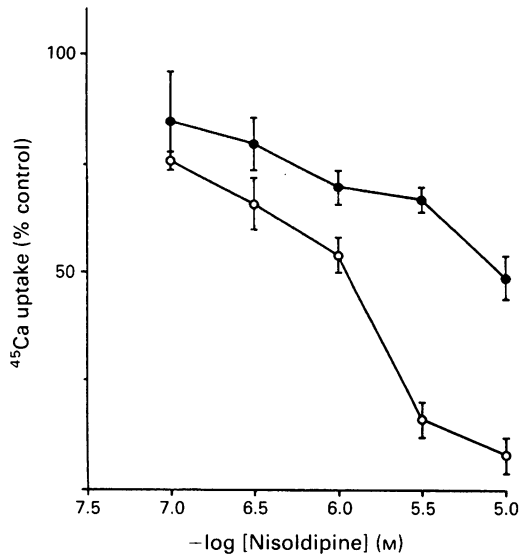

Figure 8 Effects of nisoldipine on ^{45}Ca uptake into chromaffin cells evoked by (○) 1,1-dimethyl-4-phenylpiperazinium (DMPP, 10 μM for 60 s) or (●) K (59 mM for 60 s). The experimental protocol was as in Figure 7, but here each concentration of nisoldipine was present for 90 min before and during stimulation of the cells with DMPP or K. Data (normalized to 100% of control ^{45}Ca uptake) are means of 2 triplicate experiments made with cells from different batches; s.e.mean shown by vertical bars.

component seemed to arise at concentrations between 3 and 10 μM (Figure 7). Because nisoldipine is a slowly acting dihydropyridine derivative and these experiments were performed with preincubation times of 10 min, we tested its effects on Ca uptake after exposure of the cells to this drug for 90 min. Figure 8 shows that the blocking effects of nisoldipine on K- and DMPP-evoked Ca uptake were similar to those seen when cells were pre-exposed to the drug for only 10 min. The IC_{50} to block DMPP-evoked Ca uptake in these conditions was 1.63 nM, about 3 fold lower than with a 10 min preincubation. However, K-evoked Ca uptake was affected similarly with a 10- or 90-min pre-incubation period with nisoldipine.

In the case of nimodipine, another dihydropyridine derivative, a partial blockade of K-evoked Ca uptake (about 50% at 10 μM) was obtained. DMPP-evoked Ca uptake was completely blocked by nimodipine following a biphasic pattern (Figure 9). At 30 nM 30–40% blockade was obtained; then, at μmolar concentrations, nimodipine blocked DMPP-evoked Ca uptake completely (IC_{50} = 1.1 μM).

Discussion

The various Ca^{2+} channel blockers used in this study behave quite differently when their effects on ^{45}Ca uptake into chro-

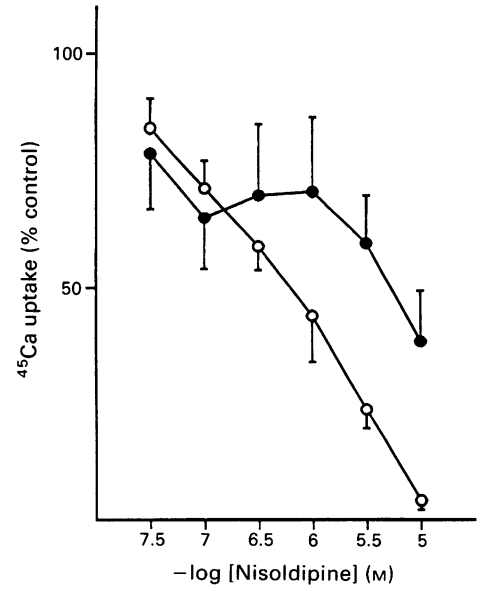

Figure 7 Effects of nisoldipine on ^{45}Ca uptake into chromaffin cells evoked by (○) 1,1-dimethyl-4-phenylpiperazinium (DMPP, 100 μM for 60 s) or (●) K (59 mM for 60 s). Nisoldipine was present for 10 min before and during stimulation of the cells with DMPP or K. Experimental protocol as in Figure 3. Data (normalized to 100% of control ^{45}Ca uptake) are means of 7 triplicate experiments made with cells from different batches; s.e.mean shown by vertical bars.

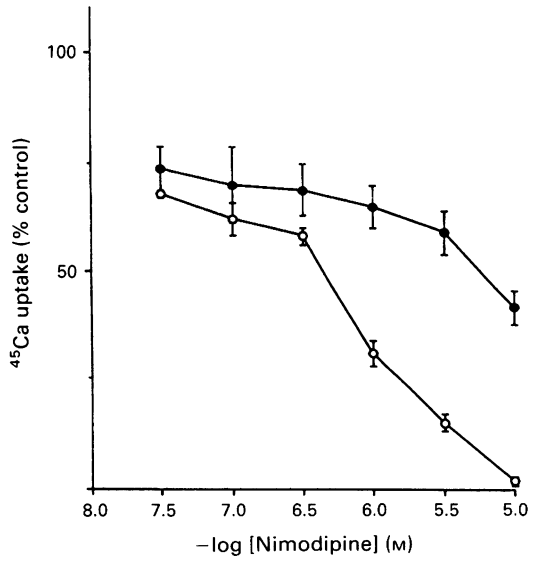

Figure 9 Effects of nimodipine on ^{45}Ca uptake into chromaffin cells stimulated by (○) 1,1-dimethyl-4-phenylpiperazinium (DMPP, 100 μM for 60 s) or (●) K (59 mM for 60 s). Nimodipine was present for 90 min before and during stimulation of the cells with DMPP or K^+. The rest of the experiment followed a protocol similar to that described in Figure 3. Data are means of 2 triplicate experiments; s.e.mean shown by vertical bars.

Table 1 IC_{50}s for various agents to block ^{45}Ca uptake into chromaffin cells stimulated with 1,1-dimethyl-4-phenylpiperazinium (DMPP) or K

	n	*DMPP*	*K*
Flunarizine	4	1.76	1.49
Cadmium	4	8.14	1.86
Amiloride	4	290	—
Guanethidine	4	27	—
Nisoldipine (10 min)	7	5	—
Nisoldipine (90 min)	2	2.63	—
Nimodipine	2	1.1	—

Values were calculated from the data in Figures 3–9, by a non-linear regression analysis computer programme (see Methods). IC_{50}s are expressed in μM; *n* shows the number of experiments in triplicate from different batches of cells.

maffin cells, stimulated with DMPP or K, are studied (see summary of IC_{50}s in Table 1). Bovine chromaffin cells may contain two different populations of Ca channels; in our experimental conditions, DMPP may recruit only one population of those channels but high K may activate all available channels. With this hypothesis in mind, our results could be explained as follows.

Flunarizine blocks L and T channels in cardiac cells (Tytgat *et al.*, 1988); thus it seems to be a rather non-specific Ca channel blocker, justifying in this manner its ability to block DMPP- and K-evoked Ca uptake. The same applies to Cd, an inorganic cation with little selectivity for Ca channel subtypes (Tsien *et al.*, 1988; Kostyuk, 1989). Though Cd seemed to block better K-evoked Ca uptake, DMPP was also highly sensitive to the cation.

A different picture can be drawn with the other drugs. Amiloride is reported to be a selective T-type Ca channel blocker in cardiac cells (Tang *et al.*, 1988). This might explain why this molecule fully blocks DMPP-evoked Ca uptake, yet K effects are affected little. A similar reasoning applies for nimodipine and nisoldipine, two 1,4-DHP derivatives selectively blocking L-type Ca channels in several excitable cells (Tsien *et al.*, 1988; Kostyuk, 1989). The drugs blocked DMPP effects much more efficiently than the effects induced by K depolarisation.

A particular case can be made with guanethidine, a potent blocker of noradrenaline release from sympathetic nerve terminals (Kirpekar, 1975) which, as far as we know, is not yet catalogued as a Ca channel blocker. Guanethidine dissociated the K from the DMPP response, blocking the nicotinic effects but leaving intact the Ca pathway activated by direct depolarization. Since adrenal chromaffin cells are quite similar, structurally and functionally, to sympathetic neurones, it might be that guanethidine is blocking N-type Ca channels which are probably mediating transmitter release at sympathetic nerve terminals (Lipscombe *et al.*, 1989). If so, the Ca channel recruited by nicotinic stimulation in bovine chromaffin cells may be pharmacologically, similar to the N channel in sympathetic neurones; and guanethidine might block these channels. Guanethidine blocks high-threshold Ca currents in bovine adrenal chromaffin cells (personal communication of Gandía, Lacinova & Morad). Of course, a direct blockade of nicotinic receptors by guanethidine cannot be discarded at present.

Heterogeneity of Ca channels in bovine chromaffin cells is a polemic issue. Radioligand binding studies with [^{3}H]-isradipine (Castillo *et al.*, 1989), [^{3}H]-nitrendipine and [^{3}H]-ω-conotoxin (Ballesta *et al.*, 1989; 1990) suggest the presence of various binding sites associated with different Ca channels. Measurements of ^{45}Ca uptake (Ballesta *et al.*, 1989) and Ca_i transients (Rosario *et al.*, 1989) also indicate the presence of heterogeneous populations of Ca channels. However, direct measurement of Ca currents with whole-cell or single-channel recording patch-clamp techniques favour the presence of homogeneous (Fenwick *et al.*, 1982; Hoshi *et al.*, 1984; Ceña *et al.*, 1989) or heterogeneous (Artalejo *et al.*, 1989) populations of Ca channels. The results of our present experiments are in line with the view that different Ca channel subtypes might be present in cultured bovine adrenal chromaffin cells. This is not the case for the cat adrenal gland which seems to be equipped mostly with Ca channels highly sensitive to DHPs and controlling very efficiently the secretory process (see review by Artalejo *et al.*, 1988).

In conclusion, by using a pharmacological approach to dissect Ca entry pathways in cultured bovine adrenal chromaffin cells, we have demonstrated that nicotinic- and high-K stimulation recruit different subtypes of voltage-dependent Ca channels.

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The role of endogenous thromboxane in contractions to U46619, oxygen, 5-HT and 5-CT in the human isolated umbilical artery

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1 The effects of selective thromboxane antagonists and a thromboxane synthase inhibitor on the contraction to 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619) and oxygen in the human umbilical artery (HUA) were examined. The effect of the antagonists on contractions to both 5-hydroxytryptamine (5-HT) and 5-carboxamidotryptamine (5-CT) were also examined.

2 U46619 (0.3 nM–10 μM) contracted the HUA. This contraction was antagonized by two selective thromboxane receptor antagonists EP092 (10 nM–1 μM) and GR32191B (10 nM–1 μM). The contraction was not affected by the selective thromboxane synthase inhibitor, dazoxiben (10 nM–1 μM).

3 When the oxygen tension was increased from 16 mmHg to 120 mmHg, the HUA transiently contracted. Both thromboxane antagonists inhibited this contraction in a concentration-dependent manner with 1 μM almost completely abolishing the response (the oxygen-induced contraction of the control preparation normally increases with a second exposure to 120 mmHg oxygen).

4 In low (16 mmHg) oxygen, responses to both 5-HT and 5-CT were unaffected by both thromboxane receptor antagonists at concentrations up to 1 μM. In high oxygen (120 mmHg) responses to both 5-HT and 5-CT were biphasic in nature, with an additional initial high sensitivity phase, which was abolished by a cyclo-oxygenase inhibitor. In high oxygen, EP092 and GR32191B blocked this initial phase in a concentration-dependent manner, returning sensitivity to 5-HT and 5-CT to that seen in low oxygen.

5 The thromboxane synthase inhibitor, dazoxiben, at concentrations greater than 10 nM inhibited the contraction to 120 mmHg oxygen and at 1 μM, dazoxiben almost abolished the response. In low oxygen, the response to 5-HT was unaffected by dazoxiben at concentrations up to 10 μM. In high oxygen, the initial phase of the contraction to 5-HT was inhibited by concentrations greater than 10 nM, with no effect on the maximum response.

6 The results show that thromboxane receptor antagonism or blockade of thromboxane synthesis selectively attenuates oxygen-induced contractions and those responses to 5-HT and 5-CT which are dependent on high oxygen for their expression. This suggests that the contractions caused by high oxygen tension, and the enhancement of the contractile effects of low concentrations of 5-HT and 5-CT in the presence of high oxygen tension are mediated by endogenously released thromboxane A_2.

Keywords: Human umbilical artery; oxygen tension, 5-HT; thromboxane synthesis; 5-carboxamidotryptamine; U46619

Introduction

Investigation into the reactivity of the human umbilical artery (HUA) has shown that increasing the level of oxygen above 16 mmHg will induce a transient contraction which can be blocked by various cyclo-oxygenase inhibitors (MacLennan *et al.*, 1988). This confirmed earlier work (Lewis, 1968; Bor & Guntheroth, 1970; Oberhansli-Weiss *et al.*, 1972; McGrath *et al.*, 1986) as to the contractile effect of oxygen but also suggested that oxygen's effect was mediated via prostaglandins.

Other studies have shown that the endoperoxides and thromboxane A_2 (TxA_2) are very potent constrictors of the isolated artery (Tuvemo *et al.*, 1976; Svensson *et al.*, 1977), while the principal products of arachidonic acid metabolism in the artery are prostacyclin (PGI_2) and prostaglandin E_2 (PGE_2) (Bjoro *et al.*, 1986). However Benedetto *et al.* (1987), have shown that TxA_2 is produced along the whole length of the umbilical artery, while Sernieri *et al.* (1983) have demonstrated that in human arteries most of the TxA_2 production takes place in the media of the smooth muscle. Also Bjoro *et al.* (1986) showed that 5-hydroxytryptamine (5-HT) increased TxA_2 production in HUA but depressed that of PGI_2, whereas angiotensin I and angiotensin II stimulated formation of PGI_2 and depressed that of TxA_2. Furthermore MacLennan & McGrath (1986) and MacLennan *et al.* (1989) have characterized the receptors mediating contraction to 5-HT in the HUA and have also shown that increasing the oxygen to 120 mmHg induces the expression of a previously quiescent population of 5-HT_1-like receptors.

Subsequently, we have studied the vasoconstrictor effects of 9,11-dideoxy-11α,9α-epoxymethano-$PGF_{2\alpha}$ (U46619, TxA_2 stable analogue), oxygen and 5-HT on the HUA and in a preliminary communication reported that the selective TxA_2 antagonist, EP092, could inhibit the contractions to U46619 and to increasing oxygen levels to 120 mmHg. We also found that the antagonist had no effect on the 5-HT-induced contraction in low oxygen (16 mmHg) but at high oxygen levels (120 mmHg) the 5-HT_1-like receptor response was blocked with no effect on the maximum response (McGrath *et al.*, 1989).

We now describe a quantitative investigation of the effects of two thromboxane antagonists and a thromboxane synthase inhibitor; EP092, GR32191B and dazoxiben respectively, in inhibiting the oxygen-induced contraction of the HUA. In addition we have examined the effects of these drugs on contractions to both 5-HT and the relatively selective 5-HT_1-receptor agonist 5-carboxamidotryptamine (5-CT) (Engel *et al.*, 1983) in either low or high oxygen levels.

Methods

All experiments were carried out on isolated preparations of the HUA obtained from normal term pregnancies via various methods of delivery. Method of delivery has no effect on the

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reactivity of the artery. The collection and storage of the cords is described extensively elsewhere (MacLennan et al., 1988).

Experimental protocol

Arteries were dissected free of the surrounding Wharton's jelly in de-oxygenated Krebs solution, to reduce the oxygen tension as far as possible. Longitudinal strips of artery 1–1.5 cm in length, were suspended within 40 ml organ baths containing Krebs saline at 37°C under a force of 1 g wt. The tissues were equilibrated for 2 h before any experimentation, gassed with 2.5% O_2, 8% CO_2 balance N_2 to produce an oxygen tension of 16 mmHg. The Po_2 was measured continuously from an oxygen electrode inserted in one of the organ baths (IL O_2 electrode, meter model 787, Strathkelvin Instruments). These conditions were maintained except when the oxygen was increased to 120 mmHg; 16% O_2, 8% CO_2 balance N_2; to examine the oxygen response and also those to 5-HT and 5-CT in high oxygen. Isometric tension was recorded with Grass FT03c transducers and a Linseis recorder (Typ 2065).

Oxygen To examine the oxygen response all the tissues were exposed to 120 mmHg oxygen (after the initial equilibration period) for 5 min during which time a transient contraction was produced (this contraction peaked within 3–4 min and returned to baseline within 5–10 min), then the gassing was returned to 16 mmHg oxygen, and a single concentration of the test agent was added to each tissue, with one acting as a time control. After 45 min the preparations were again exposed to 120 mmHg oxygen and the response measured. These second responses were expressed as a percentage of the response to the initial exposure to 120 mmHg for each tissue.

Cumulative concentration-response curves (CCRCs) to agonists were constructed by increasing the bathing solution concentration by 0.5 $\log_{10}$ increments at intervals, when the preceding response had reached a plateau; this was at approximately 3–5 min intervals for all agonists. Response as % of the maximum was plotted against log (agonist concentration). pD_2 values were calculated as $-\log(EC_{50})$, where EC_{50} is the concentration of agonist which gives 50% of the maximum response.

U46619 Cumulative concentration-response curves (CCRC) were carried out (0.3 nM–10 μM) to U46619 in low oxygen only (16 mmHg). Antagonists and the synthase inhibitor were studied as follows: one strip acted as a control and agent(s) were added to other strips from the same artery (10 nM–1 μM). CCRCs to the agonist were constructed simultaneously on all strips. For each preparation the concentration-ratio (CR) was calculated as the ratio of the EC_{50} of U46619 (+antagonist), over the EC_{50} for U46619 from the control curve. A Schild plot was constructed with the CRs: log(CR − 1) (ordinate scale) was plotted against log(antagonist concentration) (abscissa scale) (Arunlakshana & Schild, 1959). A line of best fit was found for the points by linear regression (least squares) which gave the slope and an estimate of the pA_2 as the intercept of the regression line with the x-axis. The mean values of the slope of the regression line and estimated pA_2 are presented together with their respective 95% confidence limits.

5-Hydroxytryptamine and 5-carboxamidotryptamine CCRCs were constructed to either 5-HT or 5-CT (0.3 nM–10 μM) in each preparation in both low (16 mmHg) and high (120 mmHg) oxygen. Only one concentration of either GR32191B, EP092 or dazoxiben was used in each preparation. The agent was present 45 min before construction of the CCRC in low oxygen. After completion of the curve, the Krebs solution was exchanged four times until the baseline returned to normal. After a further 20 min, the oxygen tension was increased to 120 mmHg and left for another 20 min before the agent was added again, each preparation receiving the same concentration in both low and high oxygen. The agent was present again for 45 min before carrying out a second CCRC. The effect of reversing the process, i.e. high oxygen CCRC before the low oxygen CCRC was not significant and did not contribute to the changes seen in the response.

The physiological salt solution was Krebs-bicarbonate saline of composition (mM): NaCl 119, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0 and glucose 11.1.

Drugs used were: 5-hydroxytryptamine creatinine sulphate (5-HT, Sigma), 5-carboxamidotryptamine (Glaxo), 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619, UpJohn), (rac) 9α,11α-ethano-1-methyl-13(N-phenylthio-carbamoyl) hydrazono-ω-heptanor-prosta-5 2-enoic acid (EP092), and dazoxiben, gifts from Dr R.L. Jones Edinburgh University, (1α-(6'-carboxyhex-3'2-enyl)-2β-(N-piperidino)-3α-hydroxy-5α-(4''-biphenylyl methoxy)-cyclopentane (GR32191B), gift from Dr P. Lumley, Glaxo Group Research.

All drugs except U46619 and EP092 were dissolved in distilled water. U46619 was initially dissolved in methyl acetate with subsequent dilutions made in distilled water. EP092 was initially dissolved in high performance liquid chromatography grade absolute ethanol with dilutions made in distilled water.

Statistics

Statistical comparisons of the means of groups of data were made by use of Student's t test for paired or unpaired data, where appropriate. A level of probability of $P < 0.05$ was taken to indicate statistical significance. In all experiments $n = 6$ unless otherwise stated.

Results

Contractions to U46619

An initial comparison was made of the effects of the two thromboxane antagonists versus the contraction of the HUA to U46619. This served to determine the potency of the drugs for subsequent examination of the responses to oxygen, 5-HT and 5-CT. Figure 1a and b show the effects of GR32191B and EP092 respectively. Both control groups show a concentration-dependent contraction to U46619 with threshold of 0.3 nM, the maximum obtained at 1 μM and pD_2 values of 8.45 ± 0.21 and 8.55 ± 0.19. GR32191B 10 nM, 100 nM, and 1 μM caused parallel rightward shifts of the CCRC to U46619 with no significant change in the maximum response (Figure 1a). These shifts are consistent with a competitive antagonism yielding a slope 0.96 (0.85–1.07) and a pA_2 value of 8.04 (Figure 2a). EP092 10 nM, 100 nM and 1 μM produced similar rightward shifts in the U46619 CCRC (Figure 2b). However, with 1 μM of EP092 the largest practicable concentration of U46619 (10 μM) failed to produce a contraction equal to the control maximum: and for this reason only an estimated pA_2 value of 8.05, slope 1.35, could be obtained, based on the two lower antagonist concentrations (Figure 2b). Dazoxiben at concentrations 10 nM–10 μM had no effect on either the pD_2 or the maximum response to U46619 (results not shown).

Contractions to increased oxygen tension

The two antagonists and the synthase inhibitor were examined further in separate experiments. In these experiments, one strip acted as a control, while different concentrations of the agents were added to other strips from the same artery. The second contractions are expressed as a percentage of the first contraction to 16% oxygen. Re-exposure of the artery after 45 min to the same oxygen tension, resulted in a greatly increased response (Figure 3). This was taken into account when assessing the action of the antagonists and the synthase inhibitor by correcting the second responses for the percentage change of the second response in the time control preparation. An average percentage inhibition was then calculated for each concentration of antagonist and inhibitor (Figure 4a,b and c). The potency of each agent in reducing the

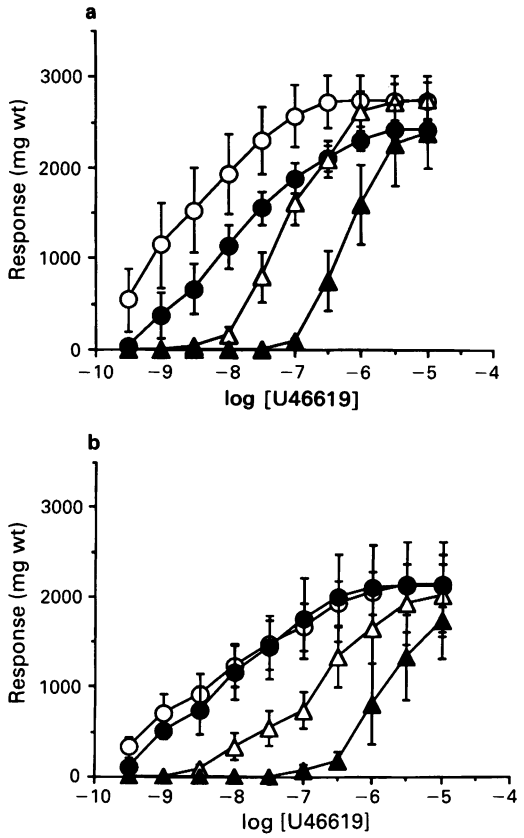

Figure 1 The effects of (a) GR32191B and (b) EP092; (○) control, (●) 10 nM, (△) 100 nM, (▲) 1 μM, on contractions to U46619 in the human umbilical artery. Responses are expressed in absolute terms. Points represent mean ($n = 6$); vertical bars show s.e.mean.

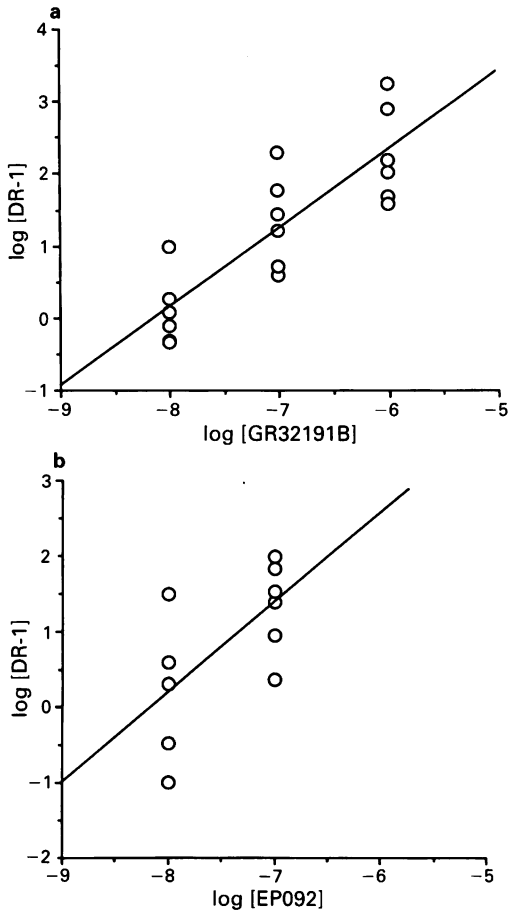

Figure 2 This figure represents a Schild plot of the effects of (a) GR32191B and (b) EP092 against contractions to U46619. Each point on the graph represents the log[CR − 1] for each individual preparation. A linear regression line has been fitted through the points to determine the slope and pA_2 values. With EP092, only the two antagonist concentrations have been used to construct the linear regression line. ($n = 6$).

O_2-induced contraction was assessed as the IC_{50}, the concentration of the antagonist or inhibitor that reduced the response to oxygen by 50%. The IC_{50} was interpolated from the graph of log(antagonist concentration) against response (% of the second response of the control tissue to 16% oxygen). IC_{50}s for GR32191B, EP092 and dazoxiben were 28 nM, 120 nM and 88 nM respectively. Thus GR32191B is at least 3 fold more potent than either EP092 or dazoxiben at inhibiting the oxygen-induced contraction.

Contractions to 5-hydroxytryptamine and 5-carboxamidotryptamine

At physiological Po_2, the two antagonists and the synthase inhibitor at concentrations up to 1 μM had no effect on either the pD_2 or the maximum response to either 5-HT or 5-CT at concentrations up to 1 μM (results not shown).

However, when the Po_2 was increased to 120 mmHg the two antagonists and the synthase inhibitor all exhibited some antagonist activity against both 5-HT and 5-CT.

5-Hydroxytryptamine In all three control groups, when the Po_2 was raised to 120 mmHg the CCRC became biphasic, with lower concentrations (0.3 nM–1 nM) producing responses (these concentrations were subthreshold for producing contraction in low oxygen). All three agents blocked this increased responsiveness at the low 5-HT concentrations, suggesting a thromboxane-dependent response, since both the receptor antagonists and the synthase inhibitor had similar effects (Figure 5a,b and c). A representative trace is illustrated in Figure 6. This component of the 5-HT CCRC is ketanserin-resistant but methysergide-sensitive (McGrath & MacLennan, 1986). In no case was there a significant effect on the maximum response to 5-HT or on the position of the upper

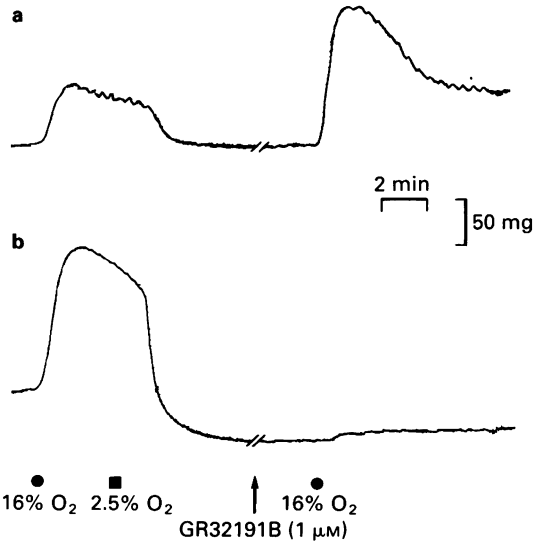

Figure 3 Representative traces of the response to increased oxygen tension. Panel (a) shows the time control response (note the increase in absolute size of the contraction) with the panel (b) showing the second response to oxygen in the presence of GR32191B (1 μM): (●) indicates change in the oxygen to 120 mmHg; (■) denotes return to 16 mmHg oxygen. ↑ indicates addition of GR32191B (1 μM) to (b) with 30 min contact time.

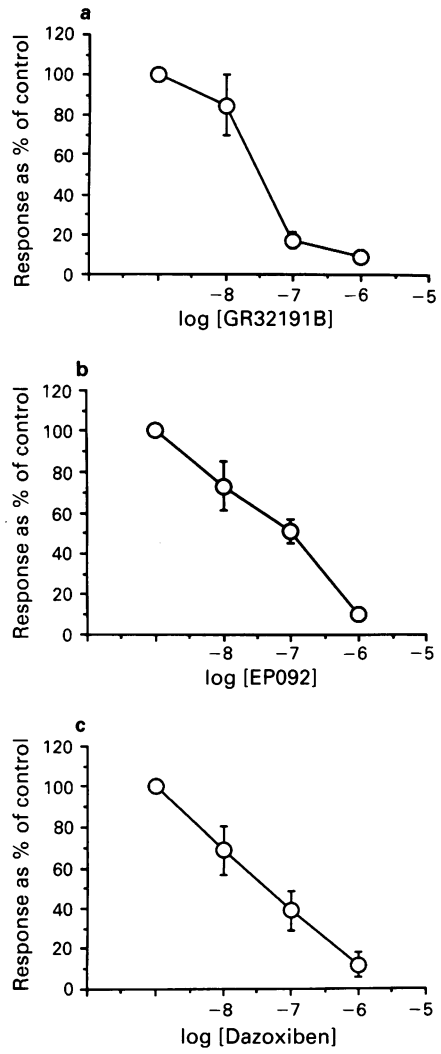

Figure 4 Inhibition of the 120 mmHg O_2-induced contraction in the human umbilical artery by (a) GR32191B, (b) EP092 and (c) dazoxiben. The results are expressed as a percentage of the control values (see methods for details). The first point on each curve represents the control response. Values represent mean ($n = 6$); s.e.mean shown by vertical bars.

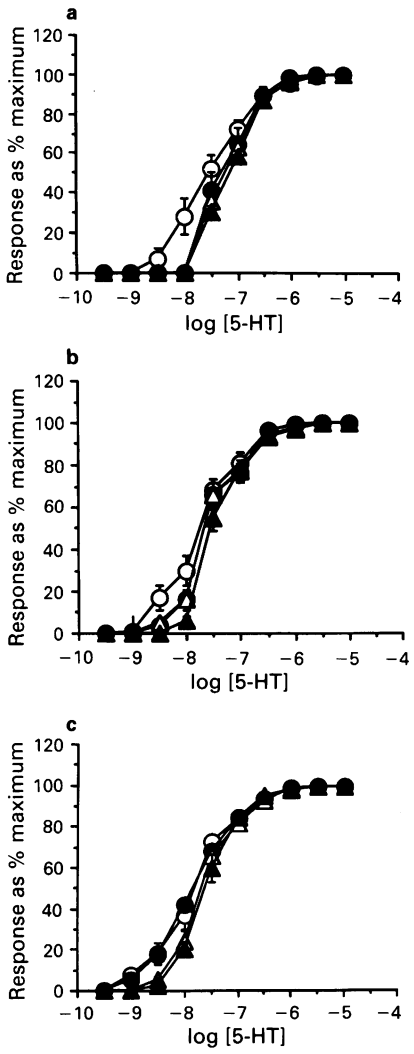

Figure 5 The effects of (a) GR32191B, (b) EP092 and (c) dazoxiben on the cumulative concentration-response curves to 5-hydroxytryptamine (5-HT) in high oxygen (120 mmHg) in the human umbilical artery. Responses are expressed as a percentage of the maximum response for each curve: (○) control; (●) 10 nM; (△) 100 nM; (▲) 1 μM. Points represent mean ($n = 6$); s.e.mean shown by vertical bars.

part of the curve (>70% of the maximum), indicating a selective effect on the 5-HT_1-like receptor-mediated response.

5-Carboxamidotryptamine As with 5-HT, when the level of oxygen was raised to 120 mmHg, a response became apparent at the lower concentrations (0.03 nM–3 nM) of 5-CT. 5-CT is believed to be more potent and selective at 5-HT_1 receptors (Engel *et al.*, 1983) and for this reason the actions of the antagonists were also examined using this agonist. (These are the receptors which are dependent on oxygen in order to become functional). Both receptor antagonists inhibited the oxygen-induced increase in responsiveness to 5-CT with 1 μM transforming the curve to that in low oxygen (Figure 7a and b). Again, the maximum response was not altered from that of the control, indicating a selective inhibition of the oxygen-dependent part of the response.

Discussion

The results suggest that oxygen contracts the HUA via production of thromboxane. That the thromboxane produced acts through specific thromboxane receptors is indicated by the effects of the receptor antagonists. This showed that the receptor antagonists and the synthase inhibitor had no blocking affinity for the 5-HT_2 receptor or depression of the excitation-contraction coupling processes subsequent to such receptor activation. The effects of the synthase inhibitor suggest that the umbilical artery actually synthesises the thromboxane in response to an elevation of oxygen above 16 mmHg. The likely dominance of thromboxane rather than, say, PGE_2, in determining tone in this vessel is supported by some previous observations.

Although Bjoro *et al.* (1986) showed that the principal products of arachidonic acid metabolism in this vessel are PGI_2 and PGE_2, Benedetto *et al.* (1986) have demonstrated considerable TxA_2 production along the whole length of the umbilical artery; both groups used 95% oxygen, 5% carbon dioxide. Our observations indicate that exaggerated production of thromboxane is likely to be observed at such high levels of O_2 and the same may apply to PGE_2, reducing the physiological significance of concentrations monitored in high O_2. Furthermore, PGE_2 seems an unlikely physiological modulator since it will contract the umbilical artery (when the level of oxygen is at 16 mmHg) only at very high concentrations (>1 μM). These contractions can be inhibited by thromboxane antagonists but not the synthase inhibitor, suggesting an action on thromboxane receptors (unpublished

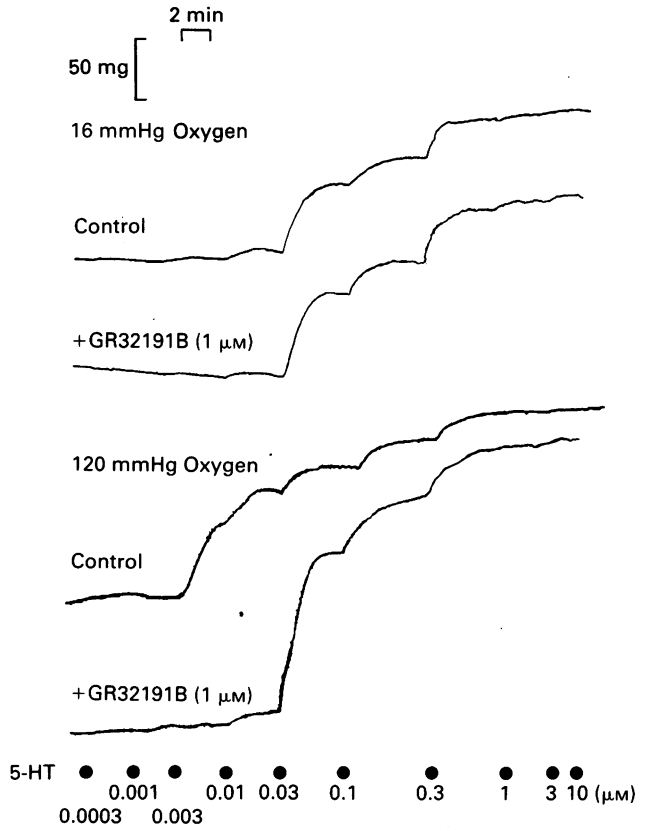

Figure 6 Representative traces showing the effects of 120 mmHg oxygen on contractions to 5-hydroxytryptamine (5-HT) and the subsequent effect of GR32191B (1 μM) on this response. The upper two traces illustrate 5-HT cumulative concentration-response curves in 16 mmHg oxygen, the lower two traces being in 120 mmHg oxygen, the lower trace in each of the two groups being in the presence of GR32191B (1 μM). Note the lack of effect in 16 mmHg with the increase in sensitivity in high oxygen being blocked. Each (●) represents addition of 5-HT cumulatively in 0.5 log units range (0.3 nM–3 μM) the unlabelled values are the intermediate concentrations.

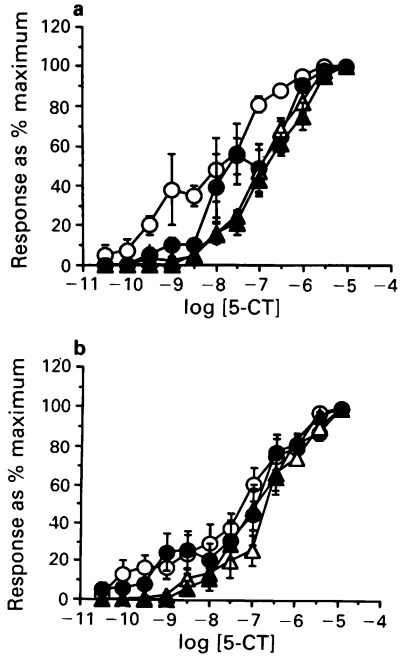

Figure 7 The effects of (a) GR32191B and (b) EP092 on cumulative concentration-response curves to 5-carboxamidotryptamine (5-CT) in high oxygen (120 mmHg) in the human umbilical artery. Responses are expressed as a percentage of the maximum response for each curve: (○) control; (●) 10 nM; (△) 100 nM; (▲) 1 μM. Points represent mean (n = 6); s.e.mean shown by vertical bars.

observations). Since the synthase inhibitor inhibits the oxygen-induced contraction but does not block PGE_2 synthesis, the oxygen-induced contraction cannot be due to PGE_2 release. Furthermore Jones *et al.* (1982), have demonstrated that at concentrations greater than 1 μM, PGE_2 will act on TxA_2 receptors which parallels the present results.

The potencies of the antagonists in blocking the oxygen-induced contraction are within the range for blocking thromboxane receptors, when their inhibition of the U46619 contraction is taken into consideration, and are similar to those obtained in other preparations (Armstrong *et al.*, 1985; Jones *et al.*, 1989; Lumley *et al.*, 1987). Tymkewycz *et al.* (1989) used 80 μM dazoxiben to inhibit TxA_2 production in platelets but in the umbilical artery, concentrations greater than 1 μM were maximal for inhibition of the oxygen contraction. In a previous study the oxygen-induced effects in the HUA were shown to be particularly sensitive also to cyclo-oxygenase inhibitors (MacLennan *et al.*, 1988).

The lack of effect of the two antagonists and the synthase inhibitor on responses to either 5-HT or 5-CT in low oxygen, together with their effectiveness in high oxygen, indicates a selective blocking action for thromboxane-dependent responses. The 'extra' response in elevated oxygen is resistant to blockade by ketanserin (MacLennan & McGrath, 1986; MacLennan *et al.*, 1989) indicating the introduction by thromboxane of a functional receptor sensitive to low concentrations of agonists, which is distinct from the $5\text{-}HT_2$-receptor-mediated response to higher concentrations of both 5-HT and 5-CT. However, the response is dependent on oxygen for its expression. These results therefore add to the observations of MacLennan *et al.* (1988), that cyclo-oxygenase inhibitors blocked the oxygen contraction. We have gone further along the synthetic pathway to determine which cyclo-oxygenase-dependent product is responsible for the oxygen effect.

The implications from these results may be important when we consider pre-eclamptic pregnancies. In this condition, there is thought to be an imbalance between placental production of PGI_2 and TxA_2, with TxA_2 levels being significantly increased (Walsh, 1985). In a normal pregnancy, the levels of both will be approximately equivalent, so that their biological actions may be balanced. An imbalance may be responsible for the increased vasoconstriction, platelet aggregation and reduced uteroplacental blood flow characteristic of pre-eclampsia (Pritchard *et al.*, 1976; Chesley, 1978; Dennis *et al.*, 1982).

Schiff *et al.* (1989) tested the effect of aspirin on the risk of development of pregnancy-induced hypertension (PIH) in a double blind study. They showed that the number of women who developed PIH or pre-eclamptic toxaemia was significantly lower among the aspirin-treated than among placebo-treated women. The ratio of serum levels of TxA_2 to PGI_2 after three weeks of treatment was decreased by 34.7% in the aspirin-treated group but increased by 51% in the placebo-treated group. Benigni *et al.* (1989) have shown that low doses of aspirin will selectively inhibit the increase in production of maternal thromboxane normally associated with PIH and pre-eclampsia while sparing vascular prostacyclin, but will only partially suppress neonatal platelet thromboxane. They assume that the mechanism of action of aspirin is to suppress the production of thromboxane by platelets, although this has not been established. Vascular smooth muscle could also be responsible for the production of some of the thromboxane since its release can be stimulated from both umbilical arteries and placental arteries (unpublished observations). It may also be of benefit to these patients to give a specific thromboxane receptor antagonist or a thromboxane synthase inhibitor, as such treatments would have no effect on the production of PGI_2. The present study certainly shows that the umbilical vascular thromboxane production is far more sensitive to inhibitors than is production from platelets.

In conclusion, we have demonstrated that the contraction of the umbilical artery to increased levels of oxygen is due to

production and release of a thromboxane synthase product, most likely TxA_2. Also, the increase in sensitivity to both 5-HT and 5-CT observed in high oxygen can be selectively inhibited by these antagonists.

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Bioassay of nitric oxide released upon stimulation of non-adrenergic non-cholinergic nerves in the canine ileocolonic junction

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1 The release and the nature of the inhibitory non-adrenergic non-cholinergic (NANC) neurotransmitter was studied in the canine ileocolonic junction. A circular muscle strip of the canine ileocolonic junction served as donor tissue in a superfusion bioassay in which rings of rabbit aorta with the endothelium removed served as detector tissue.

2 The ileocolonic junction released a labile factor with vasodilator activity upon stimulation of non-adrenergic non-cholinergic (NANC) nerves in response to electrical impulses and the nicotinic receptor agonist 1,1-dimethyl-4-phenylpiperazinium (DMPP). This release was respectively frequency- and concentration-dependent.

3 The release was reduced by the blocker of neuronal conductance, tetrodotoxin, and by the inhibitor of the nitric oxide (NO) biosynthesis N^G-nitro-L-arginine. The biological activity was enhanced by superoxide dismutase and eliminated by haemoglobin. Hexamethonium abolished only the release in response to DMPP.

4 Injection of adenosine 5'-triphosphate (ATP) or vasoactive intestinal polypeptide (VIP) onto the cascade induced relaxations of the rabbit aorta but they were different from those induced by NO or the transferable factor.

5 Based on organ bath experiments in which the reactivity of different parts of the circular smooth muscle layer of the ileocolonic junction was investigated, a muscle strip of superficial circular muscle with submucosa was chosen as the detector strip in the bioassay cascade.

6 The ileocolonic junction dose-dependently relaxed in response to nitroglycerin and NO. NO was much more potent in the rabbit aorta than in the canine ileocolonic junction. Equivalent amounts of the transferable factor, endothelium-derived relaxing factor and NO, as assessed by the relaxation of the rabbit aorta, failed to affect the ileocolonic junction.

7 In conclusion, our results demonstrate the release of a transferable vasorelaxant factor in response to NANC nerve stimulation which behaves pharmacologically like NO but not like ATP or VIP. Therefore, we suggest that NO or a NO releasing substance is the inhibitory NANC neurotransmitter in the canine ileocolonic junction.

Keywords: NO bioassay; endothelium-derived relaxing factor (EDRF); nitric oxide (NO); neurotransmission; non-adrenergic non-cholinergic nerves (NANC); ileocolonic junction

Introduction

Non-adrenergic non-cholinergic (NANC) nerves are believed to provide the main inhibitory autonomic nerve supply to gut smooth muscle (Burnstock & Costa, 1973; Burnstock, 1981). The exact nature of the transmitter(s) released by these nerves however is still debated. Adenosine 5'-triphosphate (ATP) (Burnstock, 1972) and vasoactive intestinal polypeptide (VIP) (Goyal & Rattan, 1980) are considered as the two main candidates depending on the tissue and/or species studied. Recently evidence has been presented for nitric oxide (NO) as an NANC inhibitory transmitter in some regions of the gut including the anococcygeus (Gillespie *et al.*, 1989; Ramagopal & Leighton, 1989), the ileocolonic junction and the stomach (Boeckxstaens *et al.*, 1990a,b; 1991a,b).

Previously, we demonstrated that electrical impulses, nicotinic receptor stimulation, ATP and γ-aminobutyric acid (GABA) stimulate NANC nerves resulting in relaxation of the canine ileocolonic junction (Pelckmans *et al.*, 1989; Boeckxstaens *et al.*, 1990c,d,e). These relaxations were mimicked and mediated by NO, suggesting NO as inhibitory NANC neurotransmitter (Boeckxstaens *et al.*, 1990a,b; 1991b). Using a superfusion bioassay cascade, we also demonstrated the release of a NO-like factor in response to stimulation of the NANC nerves (Boeckxstaens *et al.*, 1990a; Bult *et al.*, 1990).

In the present study, the release of this transferable factor in response to electrical impulses and nicotinic receptor stimulation by 1,1-dimethyl-4-phenylpiperazinium (DMPP) was further characterized. In addition, we investigated the responsiveness of the canine ileocolonic junction placed in a bioassay cascade to the transferable factor, the endothelium-derived relaxing factor (EDRF), NO and nitroglycerin.

Methods

Tissue preparation

The ileocolonic junction as donor tissue in the bioassay cascade
Mongrel dogs of either sex (body weight 10–30 kg) were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.v.) and a laparotomy was performed. The ileum and the colon were resected 5 cm above and 3 cm below the ileocolonic junction respectively. After cleaning and rinsing of the resection specimen, the mucosa was removed (Pelckmans *et al.*, 1989)

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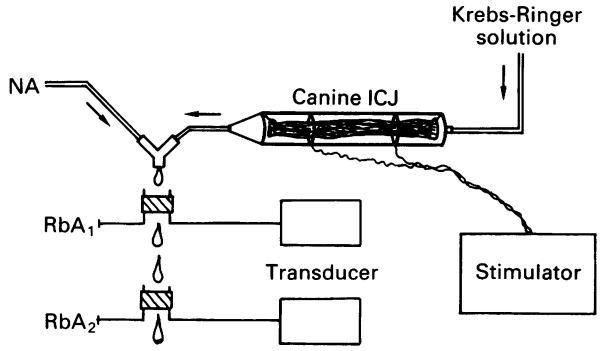

Figure 1 Schematic illustration showing the experimental set-up of the superfusion bioassay (ileocolonic junction: ICJ; noradrenaline: NA; rabbit aorta: RbA).

and the submucosa was peeled off from the ileocolonic junction. A circular muscle strip (about 4 mm wide and 2 cm long) was prepared and mounted in a perfusion chamber with a volume of about 0.5 ml and was perfused (3 ml min^{-1}) with a modified Krebs-Ringer solution (mM: NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, CaEDTA 0.026 and glucose 11.1) maintained at 37°C. This solution contained superoxide dismutase (SOD, 20 units ml^{-1}), L-arginine (50 μM) and guanethidine (3 μM) and was aerated with a mixture of 80% N_2, 15% O_2 and 5% CO_2. The perfusion chamber contained two platinum ring electrodes, through which the muscle strip was pulled (Figure 1). Electrical impulses (rectangular waves, 25 mA, 9 V) were delivered by a GRASS stimulator and a direct current (d.c.) amplifier at an interval of at least 15 min resulting in reproducible responses.

Reactivity of the ileocolonic junction in organ baths After removal of the mucosa, strips (about 3 mm wide and 2 cm long) containing different tissue layers were cut by means of sharp dissection: muscle strips with (type A) or without submucosal layer (type C), muscle strips of superficial circular muscle with submucosa (type B) and muscle strips from which the superficial circular muscle and submucosa were removed (type D) (Figure 2). The muscle strips were mounted in organ baths containing a modified Krebs-Ringer solution aerated with 95% O_2 and 5% CO_2 at 37°C. They were connected to a strain gauge transducer (Statham UC2) for continuous measurement of isometric tension and used at the optimal point of the length-tension relationship (Pelckmans *et al.*, 1989).

The ileocolonic junction as detector tissue in the bioassay cascade Based on the organ bath experiments, a type B strip was chosen as detector in the bioassay cascade. This strip was mounted vertically immediately above a ring of rabbit abdominal aorta denuded of its endothelium and was connected to a strain gauge transducer for continuous measurement of isometric tension. After the muscle strip was placed at the optimal point of the length-tension relationship (Pelckmans *et al.*, 1989), the detector tissues were superfused

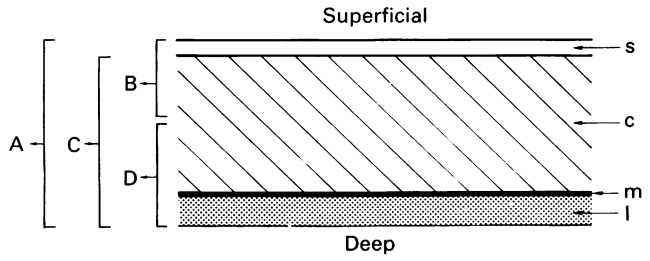

Figure 2 Schematic illustration showing the different layers (s = submucosa; c = circular muscle; m = myenteric plexus; l = longitudinal muscle) of the circular muscle strips (type A, B, C and D) of the canine ileocolonic junction.

by the effluent of the canine ileocolonic junction or by the perfusate of a segment of 5 cm of rabbit thoracic aorta and were contracted by an infusion of noradrenaline (10 μM). The sensitivity of the bioassay tissues was standardized by a bolus injection of nitroglycerin (1 nmol).

Rabbit aortic rings as detector tissue in the bioassay cascade New Zealand white rabbits (2000–2500 g) were killed by an overdose of sodium pentobarbitone. After a laparotomy, the abdominal aorta was removed and placed in modified Krebs-Ringer solution. Rings of abdominal aorta (3 mm wide) were cut and denuded of their endothelium by gentle rubbing. They were arranged either in a cascade with a delay of 2–3 s between the two rings or in parallel. The tissues were mounted under 8 g resting tension and their isometric tension was recorded. The aortic rings were superfused with the effluent of the canine ileocolonic junction and were contracted by an infusion of noradrenaline (0.1 μM) (Figure 1). A bolus of acetylcholine (3 nmol) was injected directly onto the aortic rings to verify the absence of endothelium (Furchgott & Zawadzki, 1980). Subsequently, atropine (0·3 μM) was introduced into the perfusate and the sensitivity of the bioassay tissues was standardized by a bolus injection of nitroglycerin (0.1 nmol).

The rabbit thoracic aorta as donor of endothelium-derived relaxing factor The rabbit thoracic aorta was removed and placed in modified Krebs-Ringer solution as described (Bult *et al.*, 1988). One cannula was inserted in the rostral opening, red blood cells were gently flushed away with modified Krebs-Ringer solution and the second cannula was placed in the caudal outlet. The aorta was positioned on a perspex support in an organ chamber filled with 400 ml modified Krebs-Ringer solution and perfused with modified Krebs-Ringer solution (3 ml min^{-1}).

Experimental protocols

Organ bath experiments After 45 min of equilibration, the muscle strips were stimulated with acetylcholine (2 μM) and after incubation with atropine (0.3 μM), the reactivity to noradrenaline (30 μM) was tested. Next, the effects of electrical stimulation (4 and 16 Hz, 1 ms, pulse train lasting 10 s), acetylcholine (30 μM), ATP (100 μM), GABA (100 μM) and nitroglycerin (1 μM) were studied on type B strips in the presence of atropine (0.3 μM) and compared with the responses obtained in type A strips. After each experiment, the preparations were placed in 4% formaldehyde, and sections were made for light microscopy.

Superfusion bioassay cascade In a first series of experiments, the effect of a transferable factor released by the canine ileocolonic junction in response to electrical stimulation (0.5–16 Hz, 0.5–2 ms, 25 mA, 9 V, pulse trains lasting 5–30 s) and infusion of the nicotinic agonist DMPP (3–100 μM, during 1 min) was investigated on rabbit aortic rings in the presence of atropine (0.3 μM), guanethidine (3 μM), L-arginine (50 μM) and SOD (20 units ml^{-1}). The effects of N^G-nitro-L-arginine (L-NNA, 100 μM), tetrodotoxin (1 μM), L-arginine (50 μM), SOD (20 units ml^{-1}), haemoglobin (0.1 μM) and hexamethonium (100 μM) were studied on the release of the factor induced by electrical stimulation (16 Hz, 2 ms, pulse train lasting 20 s) or DMPP (30 μM). In addition, two aortic rings were arranged in a cascade to evaluate the stability of the transferable factor released in response to electrical stimulation or DMPP and of NO (0.1–0.5 nmol), ATP (1 μmol) and VIP (0.5 nmol) directly injected onto the cascade.

In a second series of experiments, the effect of the transferable factor released in response to electrical stimulation (16 Hz, 2 ms) was studied on the canine ileocolonic junction and the rabbit aorta. The latter was mounted in the bioassay cascade immediately under the ileocolonic junction. In addition, dose-respones curves to nitroglycerin (0.03–1 nmol) and

NO (0.1–100 nmol) directly injected onto the cascade were constructed and the effect of EDRF released by a perfused rabbit aorta in response to acetylcholine (30 nmol) was investigated.

Drug used

The following drugs were used: acetylcholine chloride, adenosine 5'-triphosphate (ATP), γ-aminobutyric acid (GABA), L-arginine, bovine haemoglobin, bovine serum albumin, 1-1-dimethyl-4-phenylpiperazinium (DMPP) (Sigma Chemical Co., St. Louis, MO, U.S.A.), atropine sulphate (Federa, Brussels, Belgium), guanethidine monosulphate (Ciba-Geigy, Switzerland), N^G-nitro-L-arginine (L-NNA), tetrodotoxin (Janssen Chimica, Beerse, Belgium), nitroglycerin (Merck, Darmstadt, Germany), noradrenaline hydrogentartrate (Fluka AG, Buchs SG, Switzerland), vasoactive intestinal polypeptide (VIP, UCB Bioproducts, Brain-l'Alleud, Belgium).

All drugs were administered as aqueous solutions except L-NNA which was dissolved in 0.065 M HCl. Ascorbic acid (0.57 mM) was added to the solution of noradrenaline. All solutions were prepared on the day of experimentation, except VIP (0.1 nM), haemoglobin (1 mM) and tetrodotoxin (1 mM), in sodium citrate, pH 4.8) which were stored at −20°C between experiments.

NO solutions and haemoglobin were prepared as described (Kelm *et al.*, 1988). Bovine recombinant superoxide dismutase (SOD) was a gift of Grünenthal GmbH (Aachen, Germany).

Presentation of results and statistical analysis

The results are expressed as g contraction or as percentage decrease of the noradrenaline-induced contraction of the rabbit aortic rings or of the muscle strip of the canine ileocolonic junction.

Values are shown as mean ± s.e.mean for the number of dogs indicated. For statistical analysis, a two tailed Student's *t* test for paired or unpaired observations was used. *P* values of less than 0.05 were considered to be significant.

Results

Detection of the transferable factor by rabbit aortic rings

When the effluent of the unstimulated canine ileocolonic junction (mucosa and submucosa removed) was directed onto the cascade, the top aortic ring relaxed by 5 ± 3% ($n = 7$) of the noradrenaline (0.1 μM)-induced contraction. This relaxation was significantly increased to 18 ± 6% ($n = 7$) by SOD (20 units ml^{-1}). In the presence of atropine (0.3 μM), guanethidine (3 μM), L-arginine (50 μM) and SOD (20 units ml^{-1}), the tissue of the ileocolonic junction released more of this vasorelaxant factor upon electrical stimulation (0.5–32 Hz, 2 ms, pulse trains lasting 20 s) (Figures 3 and 4). The release was frequency-dependent (Figure 4) and varied with the pulse duration (0.5–2 ms) and the duration of the pulse train (5–30 s) (Table 1). In addition, infusion of the nicotinic agonist DMPP (0.3–100 μM) concentration-dependently induced the release of a transferable factor by the ileocolonic junction (Figures 3 and 4). However, when the submucosal layer was still present, the release of vasorelaxant activity was not detected.

The relaxations of the rabbit aortic rings induced by the factor had the same morphology as those induced by nitroglycerin (0.1 nmol), NO (0.1 nmol) or ATP (1 μmol) directly injected onto the cascade (Figure 3). On the other hand, injection of VIP (0.5 nmol) over the bioassay tissues resulted in a slowly developing and long lasting relaxation (Figure 3). The biological activity of the factor declined to the same extent (electrical stimulation, 44 ± 6%, $n = 7$; DMPP, 41 ± 15%, $n = 4$) as the effect of NO (42 ± 5%, $n = 4$) during passage down the cascade, whereas that of ATP and VIP remained unchanged (Figure 3).

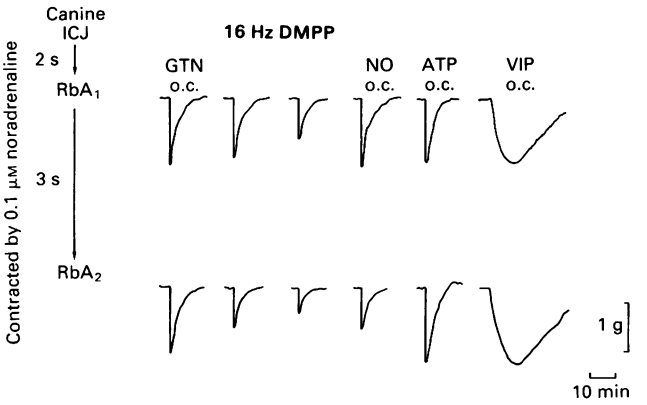

Figure 3 Relaxation of rabbit aortic (RbA) rings arranged in a cascade by a transferable factor released by the canine ileocolonic junction (ICJ) in response to electrical impulses (16 Hz, 2 ms) and 1,1-dimethyl-4-phenylpiperazinium (DMPP, 30 μM) and by injection of nitric oxide (NO, 0.1 nmol), ATP (1 mol) and vasoactive intestinal polypeptide (VIP, 0.5 nmol) onto the cascade (o.c.). The biological activity of the transferable factor and of NO, but not of ATP and VIP, decreased to the same extent during the delay of 3 s between upper and lower aortic ring. Breaks in the tracings represent periods of tissue equilibration. The experiments were performed in the presence of atropine (0.3 μM), guanethidine (3 μM), L-arginine (50 μM) and superoxide dismutase (20 units ml^{-1}). The rings of rabbit aorta were denuded of endothelium and contracted submaximally by a continuous infusion of noradrenaline (0.1 μM). The sensitivity of the bioassay was standardized by injection of nitroglycerin (GTN, 0.1 nmol). Similar results were obtained in 3 other experiments.

The relaxation of the aortic rings in response to electrical stimulation (16 Hz, 2 ms) or infusion of DMPP (30 μM) was significantly augmented from 5 ± 5% ($n = 7$) and 2 ± 1% ($n = 5$) to 28 ± 8% and 20 ± 6% respectively by SOD (20 units ml^{-1}) and from 9 ± 4% ($n = 4$) and 9 ± 2% ($n = 4$) to 23 ± 3% and 26 ± 5% respectively by L-arginine (50 μM). On the other hand, these relaxations were inhibited by haemoglobin (0.1 μM), L-NNA (100 μM) and tetrodotoxin (1 μM)

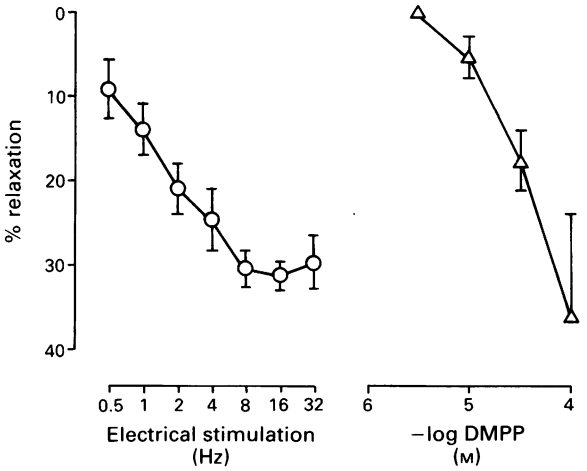

Figure 4 Relaxation of the top rabbit aortic ring induced by the frequency/concentration-dependent release of a vasorelaxant factor by the canine ileocolonic junction in response to electrical impulses (○, 0.5–32 Hz, 2 ms) and 1,1-dimethyl-4-phenylpiperazinium (DMPP, △, 3–100 μM) respectively. The experiments were performed as shown in Figure 3 in the presence of atropine (0.3 μM), guanethidine (3 μM), L-arginine (50 μM) and superoxide dismutase (20 units ml^{-1}). Results are shown as mean for 4 experiments (with s.e.mean shown by vertical bars) and are expressed as percentage decrease of the noradrenaline (0.1 μM)-induced contractions.

Table 1 Effect of pulse duration (0.5–2 ms, 20 s) and duration of pulse train (2 ms, 5–30 s) on the relaxation of the rabbit aorta to the transferable factor released by electrical stimulation (16 Hz)

	% *relaxation*	n
(a) Pulse duration (ms)		
0.5	19 ± 6	5
1	25 ± 4	5
2	30 ± 5	5
(b) Duration of pulse train (s)		
5	14 ± 5	4
10	27 ± 7	4
20	29 ± 6	4
30	36 ± 9	4

The experiments were performed as shown in Figure 3. Atropine (0.3 μM), guanethidine (3 μM), L-arginine (50 μM) and superoxide dismutase (20 units ml^{-1}) were present. Results are shown as mean ± s.e.mean for the number of experiments (n) indicated and are expressed as percentage decrease of the noradrenaline (0.1 μM)-induced contraction of the top aortic ring.

(Table 2). Hexamethonium (100 μM) abolished the release of the factor induced by DMPP but had no effect on the release in response to electrical stimulation (Table 2). The relaxations induced by nitroglycerin (0.1 nmol), ATP (1 μmol) or VIP (0.5 nmol) were not affected by SOD or haemoglobin (results not shown).

The canine ileocolonic junction as bioassay tissue

Organ bath experiments Histology confirmed the composition of the type A muscle strips, as schematically illustrated in Figure 2. Type B strips indeed only contained the submucosa and superficial circular muscle. The submucosa or the submucosa and the superficial circular muscle layer had been removed in type C or type D strips respectively, as indicated in Figure 2.

After equilibration, all different types of strips contracted to the same extent to acetylcholine (2 μM, type A: 3.8 ± 0.6 g; type B: 4.0 ± 0.5 g; type C: 3.7 ± 0.8 g; type D: 3.7 ± 0.3 g, n = 7). However, only type A and type B strips contracted in a stable manner to noradrenaline (30 μM) (3.2 ± 0.6 g and 2.9 ± 0.4 g respectively, n = 7). Removal of the submucosa

Table 2 Effect of haemoglobin, N^G-nitro-L-arginine (L-NNA), tetrodotoxin and hexamethonium on the relaxation of the rabbit aorta to the transferable factor released in response to electrical stimulation (16 Hz, 2 ms) and 1,1-dimethyl-4-phenylpiperazinium (DMPP, 30 μM)

	Electrical stimulation (16 Hz, 2 ms) (% relaxation)	*DMPP* (30 μM) (% relaxation)
Control	30 ± 8	21 ± 4
Haemoglobin 0.1 μM	1 ± 1*	0*
L-NNA 0.1 mM	9 ± 4*	0*
Tetrodotoxin 1 μM	2 ± 1*	3 ± 3*
Hexamethonium 0.1 mM	30 ± 7	0*

The experiments were performed as shown in Figure 3. Atropine (0.3 μM), guanethidine (3 μM), L-arginine (50 μM) and superoxide dismutase (20 units ml^{-1}) were present. Results are shown as mean ± s.e.mean for 4–7 experiments and are expressed as percentage decrease of the noradrenaline (0.1 μM)-induced contraction of the top aortic ring.
* $P < 0.05$, significantly different from control, Student's t test for paired observations.

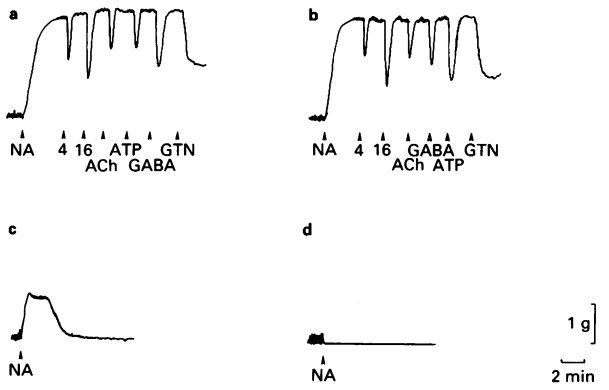

Figure 5 Isometric tension recordings showing the reactivity of (a) type A, (b) type B, (c) type C and (d) type D circular muscle strips of the canine ileocolonic junction to noradrenaline (NA, 30 μM). The different tissue layers of these muscle strips are schematically illustrated in Figure 2. In (a) and (b), the effect of electrical stimulation (4 and 16 Hz, 1 ms), acetylcholine (ACh, 30 μM), ATP (100 μM), γ-aminobutyric acid (GABA, 100 μM) and nitroglycerin (GTN, 1 μM) are shown during a noradrenaline-induced contraction. The experiments were performed in the presence of 0.3 μM atropine. Similar results were obtained in 6 additional experiments.

(type C) resulted in a transient contraction to noradrenaline whereas the spontaneous activity was abolished by noradrenaline in type D muscle strips (Figure 5).

During a noradrenaline (30 μM)-induced contraction in the presence of atropine (0.3 μM), the NANC responses to electrical stimulation (4 and 16 Hz, 1 ms), acetylcholine (30 μM), ATP (100 μM), GABA (100 μM) and nitroglycerin (1 μM) were identical in the type A and type B muscle strips (Figure 5).

Superfusion bioassay cascade Type B muscle strips were superfused more homogeneously than type A strips, resulting in more reproducible, stable and sustained contractions to acetylcholine (2 μM) or noradrenaline (10 μM). Since the organ bath experiments demonstrated that these strips reacted in the same way as type A strips, all further experiments were performed on type B strips.

Infusion of 0.1 μM noradrenaline on the detector tissues had no effect on the motor activity of the ileocolonic junction but contracted the rabbit aorta submaximally (3.7 ± 0.5 g, n = 7). During this contraction in the presence of atropine (0.3 μM), guanethidine (3 μM), L-arginine (50 μM) and SOD (20 units ml^{-1}), EDRF released by the perfused rabbit aorta in response to acetylcholine (30 nmol), and the transferable factor released in response to electrical stimulation (16 Hz, 2 ms) of the ileocolonic junction induced a relaxation of 73 ± 9% (n = 4) and 38 ± 5% (n = 5) of the rabbit aortic ring respectively. The spontaneous activity of the ileocolonic junction was not affected.

Infusion of 10 μM noradrenaline contracted both detector tissues (ileocolonic junction: 1.8 ± 0.2 g, n = 9; rabbit aorta: 4.7 ± 0.4 g, n = 7). Nitroglycerin (0.03–1 nmol) and NO (0.1–100 nmol), directly applied onto the cascade, dose-dependently relaxed both the canine ileocolonic junction and the rabbit aorta (Figures 6 and 7). However, NO relaxed the rabbit aorta more potently than the ileocolonic junction (Figure 7). In both tissues, nitroglycerin was more potent than NO (Figure 7). EDRF and the transferable factor now relaxed the rabbit aorta by 38 ± 5% (n = 4) and 12 ± 3% (n = 5) respectively, but failed to relax the ileocolonic junction (Figure 6). In the presence of 1 mM L-arginine, the relaxation of the aortic ring to the transferable factor was significantly increased to 37 ± 8% (n = 3); however, the factor did not relax the ileocolonic junction (Figure 6). Injection of 0.1–0.3 nmol nitroglycerin or 0.1–0.3 nmol NO produced relaxations equal to those of EDRF and the ileocolonic factor on the aortic ring, but only nitroglycerin induced relaxation of the ileocolonic junction (Figure 6).

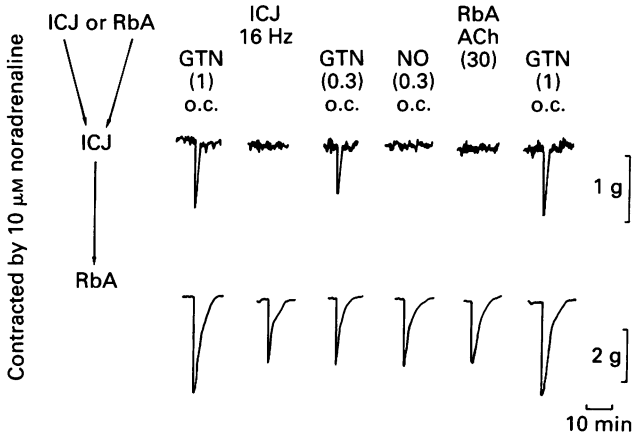

Figure 6 Isometric tension recordings of the canine ileocolonic junction (ICJ) and the rabbit aorta (RbA) showing the effect of the transferable factor (electrical stimulation; 16 Hz, 2 ms), or nitroglycerin (GTN, 0.3 nmol) and NO (0.3 nmol) injected onto the cascade (o.c.). Endothelium-derived relaxing factor (EDRF) released in response to acetylcholine (ACh, 30 nmol) from a perfused rabbit aorta relaxed the aortic ring to the same degree. Breaks in the tracing represent periods of tissue equilibration. The numbers in parentheses are the doses expressed in nmol. The experiments were performed in the presence of atropine (0.3 μM), guanethidine (3 μM), L-arginine (1 mM) and superoxide dismutase (20 units ml^{-1}). If the effect of EDRF was tested, atropine was infused only onto the detector tissues. The ring of rabbit aorta was denuded of endothelium and both tissues were contracted by a continuous infusion of noradrenaline (10 μM). The sensitivity of the bioassay was standardized by injection of nitroglycerin (1 nmol). Similar results were obtained in 2 other experiments.

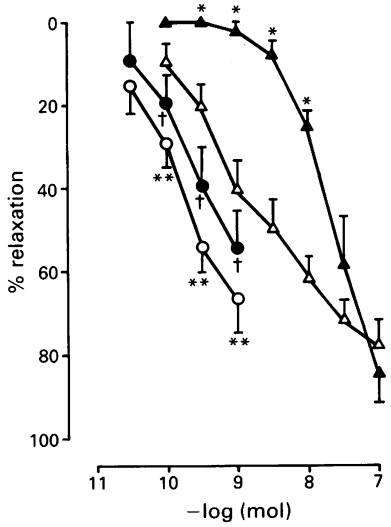

Figure 7 Relaxation of the canine ileocolonic junction (filled symbols) and the rabbit aorta (open symbols) in response to nitroglycerin (○, 0.03–1 nmol) and NO (△, 0.1–100 nmol) injected onto the cascade. The experiments were performed as shown in Figure 6 in the presence of atropine (0.3 μM), guanethidine (3 μM), L-arginine (50 μM) and superoxide dismutase (20 units ml^{-1}). Results are shown as mean for at least 5 experiments with s.e.mean shown by vertical bars and are expressed as percentage decrease of the noradrenaline (10 μM)-induced contraction.

* $P < 0.05$, significantly different, sensitivity of the canine ileocolonic junction versus rabbit aorta to NO, Student's t test for unpaired observations.

† $P < 0.05$, significantly different, sensitivity to NO versus nitroglycerin in the canine ileocolonic junction, Student's t test for unpaired observations.

** $P < 0.05$, significantly different, sensitivity to NO versus nitroglycerin in the rabbit aorta, Student's t test for unpaired observations.

Discussion

Endothelium-derived relaxing factor (EDRF) is synthesized and released by the vascular endothelium in response to diverse substances (Furchgott & Zawadzki, 1980; Furchgott, 1984). This vasorelaxant factor is now thought to be nitric oxide (NO) (Palmer *et al.*, 1987) and/or S-nitrosocysteine which spontaneously decomposes with the liberation of NO (Myers *et al.*, 1990; Ignarro, 1990). NO is enzymatically synthesized from its precursor L-arginine (Palmer *et al.*, 1988a; Schmidt *et al.*, 1988), a pathway which has been demonstrated in several cell types underlying a variety of biological actions (Moncada *et al.*, 1989). In particular, formation of NO from L-arginine has been reported in the rat cerebellum (Garthwaite *et al.*, 1988; Dickie *et al.*, 1990) and forebrain (Knowles *et al.*, 1989). In addition, in the peripheral nervous system, evidence is available favouring a role of NO in NANC neurotransmission (Gillespie *et al.*, 1989; Ramagopal & Leighton, 1989; Boeckxstaens *et al.*, 1990a,b; 1991a,b; Bult *et al.*, 1990; Gibson *et al.*, 1990; Tucker *et al.*, 1990). Exogenous NO directly relaxes the smooth muscle and mimics the relaxations induced by stimulation of the intramural NANC nerves. Furthermore, inhibition of NO biosynthesis by L-arginine analogues reduces the NANC responses without affecting the postjunctional effector cells. Finally, we here demonstrate the release of NO in response to NANC nerve stimulation which is a fundamental criterion for NO to be considered as putative neurotransmitter (Burnstock *et al.*, 1986).

Stimulation of the NANC nerves in the canine ileocolonic junction by electrical impulses or the nicotinic agonist DMPP triggered the release of a labile transferable factor in a frequency/concentration-dependent manner. This stimulated release was tetrodotoxin-sensitive, indicating that neuronal structures were activated, rather than other cell populations such as endothelial or mononuclear cells, or micro-organisms. Recently, evidence has indeed been obtained that the enzyme which catalyzes NO formation, NO synthase, is present in the myenteric plexus and associated neuronal processes in the intestine and is present in particularly large amounts in neuronal axons (Bredt *et al.*, 1990). In addition, it has also been demonstrated that intact neuronal tissue can release NO upon stimulation (Dickie *et al.*, 1990). Although we cannot exclude the possibility that an as yet unidentified neurotransmitter induced the release of the transferable factor from some non-neuronal cell, e.g. the smooth muscle, we favour the view that NO or a NO releasing substance is the transmitter itself and is released by the NANC nerves.

The nature of the factor released is indeed non-adrenergic non-cholinergic since both atropine and guanethidine were present. In addition, the aortic rings failed to relax to acetylcholine or noradrenaline. As illustrated by the decrease of the biological activity down the cascade, the factor released by the ileocolonic junction is unstable, a feature which is not compatible with peptides like VIP, amines, vasodilator prostanoids or ATP, but rather with a substance like NO. Relaxations induced by NO indeed declined to the same extent as those in response to the transferable factor. In addition, the biological activity of both NO and the factor, but not of VIP and ATP, was increased by SOD, a substance that prolongs the half-life of NO through suppression of its inactivation by superoxide anion (Gryglewski *et al.*, 1986). Furthermore, L-arginine, the precursor of NO (Palmer *et al.*, 1988a; Schmidt *et al.*, 1988), increased the release of the factor whereas the latter was decreased by inhibition of NO biosynthesis. The enhanced release after L-arginine supplementation, which is generally not observed when vascular endothelial cells are used as the source of NO (Palmer *et al.*, 1989a,b), could be in agreement with the previous reported hypothesis of a continuous release of NO in the canine ileocolonic junction (Boeckxstaens *et al.*, 1990b). This was confirmed by the finding that the effluent of the unstimulated ileocolonic junction relaxed the aortic rings, an effect that was enhanced by

SOD. Previously, comparable evidence has been obtained illustrating basal release of EDRF from unstimulated endothelial cells (Griffith *et al.*, 1984; Gryglewski *et al.*, 1986). Finally, haemoglobin, known to trap NO avidly (Martin *et al.*, 1985), abolished the biological activity of the factor, but not that of ATP and VIP. These results indicate that NO, but not ATP or VIP, accounts for the vasorelaxant effect of the transferable factor, suggesting NO or a NO releasing substance as the inhibitory NANC neurotransmitter. Similar results were obtained with the NANC innervation of the canine lower oesophageal sphincter (unpublished observations) and the rat gastric fundus (Boeckxstaens *et al.*, 1991a) indicating that this mechanism is not confined to the tissue and/or species studied.

We examined the effect of the transferable factor on the ileocolonic junction itself and compared it with that of NO, nitroglycerin and EDRF. Preliminary organ bath experiments were performed to determine which part of the ileocolonic junction was most suitable as detector tissue in the bioassay cascade. These experiments revealed spatial differences in reactivity to noradrenaline in the circular muscle layer: the superficial part contracted to noradrenaline whereas the spontaneous activity of the deeper part of the circular muscle layers was abolished by noradrenaline. Spatial organisation of the spontaneous electrical activity and of the innervation has been reported previously in the circular smooth muscle of the canine colon (Smith *et al.*, 1987a,b; 1989). It was suggested that different transmitters and/or different receptors may be present through the thickness of the circular layer (Smith *et al.*, 1989), which might also explain our results. However, it should also be considered that differences in response to noradrenaline might result from injury to the smooth muscle cells due to tissue manipulation. However, all strips contracted to acetylcholine, indicating that the contractile apparatus of the smooth muscle cells was still functional. In addition, the type B strips behaved similarly to the type A strips. Placed in the bioassay cascade, only the type B strip contracted in a sustained manner to noradrenaline, justifying its choice as detector strip.

Submaximal contraction of the ileocolonic junction was achieved by an infusion of 10 μM noradrenaline (Pelckmans *et al.*, 1990), a concentration which maximally contracted the rabbit aorta. Although this reduced the sensitivity of the rabbit aorta to nitroglycerin (Herman *et al.*, 1971) and the transferable factor, this experimental condition was optimal for investigation of the effect of different substances on the ileocolonic junction. However, even maximal stimulation of the release of the factor, as judged from the response of the aortic ring, was not sufficient to cause relaxation of the ileocolonic junction. In addition, amounts of NO or EDRF that matched the vasodilator activity of the factor also failed to affect the ileocolonic junction. This could be explained by a low sensitivity of this nonvascular smooth muscle to NO compared to the rabbit aorta. Indeed, only high amounts of NO relaxed the ileocolonic junction, suggesting that greater amounts of the transferable factor and EDRF are also required to be effective. In contrast, nitroglycerin, a stable substance from which NO is liberated in the smooth muscle cell, was almost equipotent in the ileocolonic junction and in the rabbit aorta under these experimental conditions. This great difference in responsiveness to exogenous NO and to NO liberated from nitroglycerin in the canine ileocolonic junction may therefore rather indicate that a significant proportion of the exogenous NO is inactivated before it reaches the smooth muscle cells. The submucosa for example may trap or act as a biological barrier to NO. This is illustrated by the finding that release of the transferable factor was only detected from tissue from which the submucosa has been removed. High tissue concentrations of superoxide anions (Gryglewski *et al.*, 1986), quick oxidation or binding to the submucosa may all account for a higher degree of inactivation of NO compared to nitroglycerin. This might also explain the previously reported low sensitivity to NO and EDRF in other nonvascular smooth muscle (Shikano *et al.*, 1987; Dusting *et al.*, 1988; Gillespie & Sheng, 1988; Buga *et al.*, 1989; Cocks & Angus, 1990), since these bioassay tissues generally still contained their mucosa and submucosa. Vascular rings on the other hand were without endothelium and most probably lack such possible barriers to NO. Alternatively, the transferable factor, EDRF and NO may represent more selective and potent relaxants of vascular than of nonvascular smooth muscle.

In conclusion, we were able to demonstrate the release of an unstable vasorelaxant factor in response to NANC nerve stimulation, which behaves pharmacologically like NO and is synthesized from L-arginine. Since we previously also demonstrated that NO mimics and mediates the NANC relaxations in the canine ileocolonic junction (Boeckxstaens *et al.*, 1990a,c, d), we propose NO or a NO releasing substance as the putative NANC neurotransmitter in this tissue. In contrast to classical neurotransmitters, the instability of NO makes the classical inactivation by uptake or enzyme systems unnecessary. Furthermore, since NO is a highly lipophilic substance, it will diffuse easily through the postjunctional cellular membrane without binding to an extracellular receptor. Inside the cell, the haem group of guanylate cyclase will bind NO which will induce the synthesis of guanosine 3':5'-cyclic monophosphate (cyclic GMP) resulting in smooth muscle relaxation (for review, see Waldman & Murad, 1987). Therefore, if future experiments confirm the role of NO in NANC neurotransmission, it will be an odd and primitive neurotransmitter which is also reflected by its chemical composition.

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The role of nitric oxide in inhibitory non-adrenergic non-cholinergic neurotransmission in the canine lower oesophageal sphincter

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1 The role of nitric oxide (NO) in non-adrenergic non-cholinergic (NANC) neurotransmission was studied on circular muscle strips of the canine lower oesophageal sphincter (LOS). Electrical field stimulation evoked frequency-dependent relaxations, which were resistant to adrenergic and cholinergic blockade and abolished by tetrodotoxin.

2 Exogenous administration of NO induced concentration-dependent and tetrodotoxin-resistant relaxations which mimicked those in response to electrical stimulation.

3 N^G-nitro-L-arginine (L-NNA), a stereospecific inhibitor of NO-biosynthesis, inhibited the relaxations induced by electrical stimulation but not those by exogenous NO or vasoactive intestinal polypeptide (VIP).

4 The effect of L-NNA was prevented by L-arginine, the precursor of the NO biosynthesis but not by its enantiomer D-arginine.

5 Haemoglobin abolished the NO-induced responses and reduced those evoked by electrical stimulation.

6 Cumulative administration of VIP induced concentration-dependent relaxations, which were slow in onset and sustained. A complete relaxation to VIP was not achieved and the relaxations were not affected by L-NNA.

7 In conclusion, our results provide evidence that NANC relaxations are mediated by NO, suggesting NO or a NO releasing substance as the final inhibitory NANC neurotransmitter in the canine LOS.

Keywords: Electrical stimulation (ES); lower oesophageal sphincter (LOS); nitric oxide (NO); non-adrenergic non-cholinergic nerves (NANC); vasoactive intestinal polypeptide (VIP); N^G-nitro-L-arginine (L-NNA)

Introduction

The lower oesophageal sphincter (LOS) is a specialized segment of the most distal part of the oesophagus. It plays an important role in preventing gastroesophageal reflux by maintaining an intraluminal pressure, higher than the fundus pressure. To allow the passage of food and liquids into the stomach, the LOS relaxes upon swallowing. These relaxations are mediated by a non-adrenergic non-cholinergic (NANC) neurotransmitter, since the inhibitory innervation of the gastrointestinal tract, including the LOS, is mainly provided by NANC neurones (Burnstock & Costa, 1973; Abrahamsson, 1986). Although the precise nature of the NANC neurotransmitter in the LOS is still debated, vasoactive intestinal polypeptide (VIP) is believed to be the most likely candidate. VIP is present in nerve terminals in the LOS region (Alumets *et al.*, 1979; Zimmerman *et al.*, 1989), it relaxes the LOS by direct action on the smooth muscle cells and increases intracellular adenosine 3':5'-cyclic monophasphate (cyclic AMP) contents (Bitar & Makhlouf, 1982). Furthermore, VIP is released by neural stimulation (Goyal & Rattan, 1980) and VIP antiserum partially inhibits the neurally induced relaxation of the feline LOS (Biancani *et al.*, 1984; Behar *et al.*, 1989).

However, a role for nitric oxide (NO) as neurotransmitter has been suggested in rat and mouse anococcygeus muscle (Gillespie *et al.*, 1989; Ramagopal & Leighton, 1989; Gillespie & Sheng, 1990; Gibson *et al.*, 1990), in rat cerebellum (Garthwaite *et al.*, 1988) and forebrain (Knowles *et al.*, 1989).

In previous studies, we provided evidence for the proposal that NO is a NANC neurotransmitter in the canine ileocolonic junction (Boeckxstaens *et al.*, 1990; 1991b,c; Bult *et al.*, 1990) and rat gastric fundus (Boeckxstaens *et al.*, 1991a).

The present study was designed to investigate the role of NO in the canine LOS by means of haemoglobin, which is known to trap NO avidly (Martin *et al.*, 1985) and N^G-nitro-L-arginine (L-NNA), a stereospecific inhibitor of NO biosynthesis (Ishii *et al.*, 1990; Moore *et al.*, 1990; Mülsch & Busse, 1990).

Methods

Tissue preparation

Mongrel dogs of either sex (body weight 10–20 kg) were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.v.) and a laparotomy was performed. Five cm of tissue above and below the LOS-region was removed, cut open longitudinally and rapidly rinsed in Krebs-Ringer buffer. The squamocolumnar border was located (Huizinga & Walton, 1989) and marked with steel pins and the mucosa was removed from the region below the border. Two muscle strips, 3 to 10 mm distal to the squamocolumnar border, were cut and each strip was again cut into 4 or 5 segments resulting in 8 to 10 circular muscle strips of approximately 2 mm wide and 10 mm long. The strips were tied at each end with silk thread and mounted in organ baths (25 ml, except for the VIP experiments: 5 ml) filled with modified Krebs-Ringer solution (mM: NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, CaEDTA 0.026 and glucose 11.1), maintained at 37°C and continuously aerated with 95% O_2 and 5% CO_2.

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Isometric tension recording

Each muscle strip was placed between two platinum plate electrodes for field stimulation of the intramural nerves. Next they were connected to a strain gauge transducer (STATHAM UC2) for continuous measurement of isometric tension and an initial passive tension of 4 g was applied on each strip. After an equilibration period of 30 min, the strips were stimulated electrically with a GRASS-stimulator (8 Hz, 0.5 ms, 9 V, 100 mA) and a direct current amplifier in stimuli-trains of 10 s. Only tissue that gained tone spontaneously and relaxed on electrical stimulation was considered to be LOS tissue (Christensen *et al.*, 1973; Barnette *et al.*, 1990). These strips were allowed to equilibrate for at least 60 min before experimentation.

Experimental protocols

All experiments were performed during a 5-hydroxytryptamine (5-HT, 3 μM)-induced contraction and in the presence of atropine (1 μM) and guanethidine (3 μM). After each 5-HT-induced contraction the muscle strips were washed four times every 5 min.

First, dose-response curves to NO (0.1–30 μM, non-cumulative) and nitroglycerin (0.1–30 μM cumulative) were constructed on circular muscle strips of the LOS. Next, the effect of haemoglobin (10–30 μM), tetrodotoxin (1 μM), L-NNA (3–100 μM), hexamethonium (100 μM), phentolamine (10 μM) and propranolol (1 μM) was studied on the relaxations induced by electrical stimulation (ES, 0.25–16 Hz, 0.5 ms), NO (3 μM), isoprenaline (100 μM) and nitroglycerin (10 μM). The effect of L-NNA was re-examined in the presence of L-arginine (5 mM) and D-arginine (5 mM). Finally, we examined the inhibitory effect of L-NNA (30 μM) on a cumulative dose-response curve to VIP. All VIP experiments were performed in the presence of 0.1% bovine serum albumin.

All antagonists were added at least 10 min prior to the 5-HT-induced contraction.

Drugs used

The following drugs were used: L-arginine, D-arginine, bovine haemoglobin, bovine serum albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.); atropine sulphate (Federa, Brussels, Belgium); isoprenaline hydrochloride, 5-hydroxytryptamine creatinine sulphate monohydrate, N^G-nitro-L-arginine, tetrodotoxin (Janssen Chimica, Beerse, Belgium); phentolamine, guanethidine monosulphate (Ciba-Geigy, Switzerland); nitroglycerin (Merck, Darmstadt, Germany), propranolol hydrochloride (ICI-Pharma, Belgium); VIP (UCB bioproducts, Braine-l'Alleud, Belgium).

All drugs were dissolved or diluted in distilled water and solutions were made on the day of experimentation. Ascorbic acid (570 μM) was added to the solutions of 5-hydroxytryptamine and isoprenaline. The stock solution of tetrodotoxin (1 mM in sodium citrate, pH 4.8) was stored at −20°C. VIP aliquots were stored at −20°C and further diluted on the day of experimentation. NO solutions and haemoglobin were prepared as described by Kelm *et al.* (1988). All drugs were added in volumes less than 0.5% of the bath volume.

Statistical analysis

Relaxations are expressed as percentage of the maximal relaxation induced by electrical field stimulation (16 Hz, 0.5 ms) at the beginning of experimentation (Barnette *et al.*, 1990). Results are shown as mean ± s.e.mean for the number of dogs indicated. The negative logarithm of the concentration of agonist that produced a response of 50% of the maximal response obtained with that agonist (pD_2 value), was calculated by linear regression analysis (Tallarida & Murray, 1981). All data were analyzed by Student's *t* test for paired and unpaired observations. *P* values of less than 0.05 were considered to be significant.

Results

During a 5-HT (3 μM)-induced contraction and in the presence of atropine (1 μM) and guanethidine (3 μM), electrical stimulation (0.25–16 Hz, 0.5 ms) induced frequency-dependent relaxations in the canine LOS (Figures 1, 3 and 4). These relaxations were not affected by hexamethonium (100 μM) or propranolol (1 μM) and phentolamine (10 μM) (Figure 1). Iso-

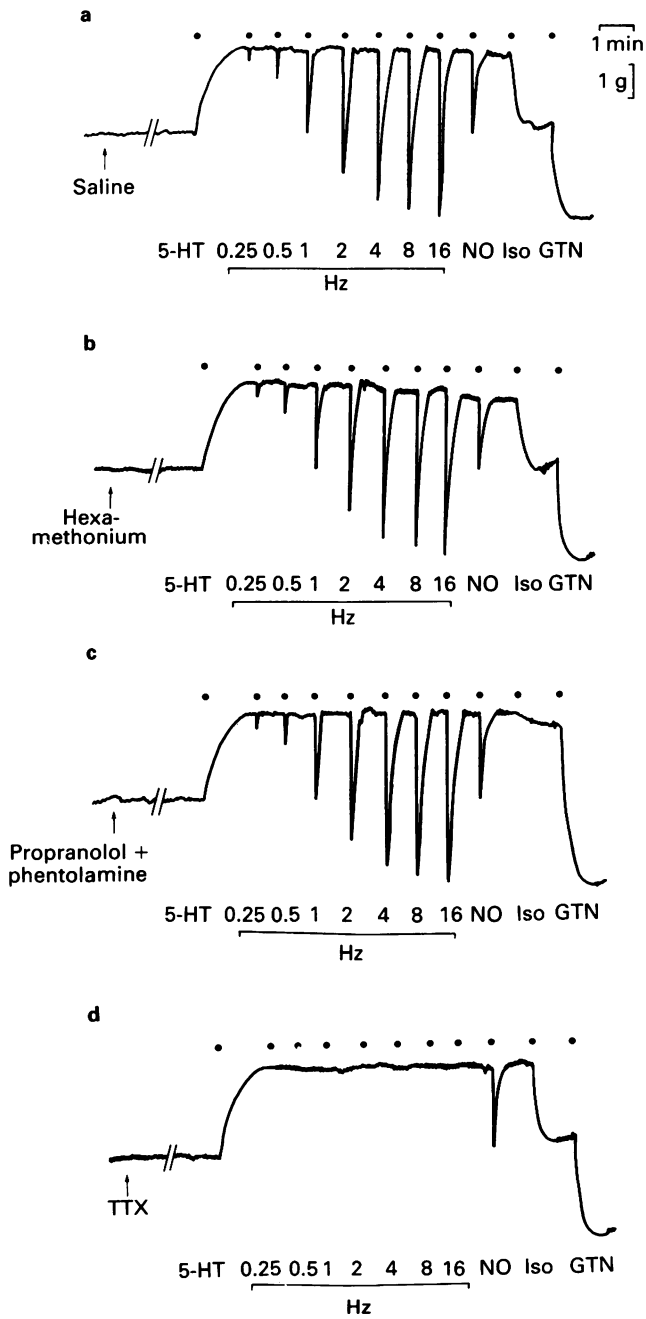

Figure 1 Isometric tension recordings showing (a) the control and the effects of (b) hexamethonium (100 μM), (c) propranolol (1 μM) plus phentolamine (10 μM), (d) tetrodotoxin (TTX, 1 μM) on the relaxations of the canine lower oesophageal sphincter induced by electrical stimulation (0.25–16 Hz, 0.5 ms), NO (3 μM), isoprenaline (Iso, 100 μM) and nitroglycerin (GTN, 10 μM). The experiments were performed during a 5-hydroxytryptamine (5-HT, 3 μM)-induced contraction and in the presence of atropine (1 μM) and guanethidine (3 μM). Similar results were obtained from five other experiments ($n = 6$). Tracing-breaks represent periods of tissue equilibration.

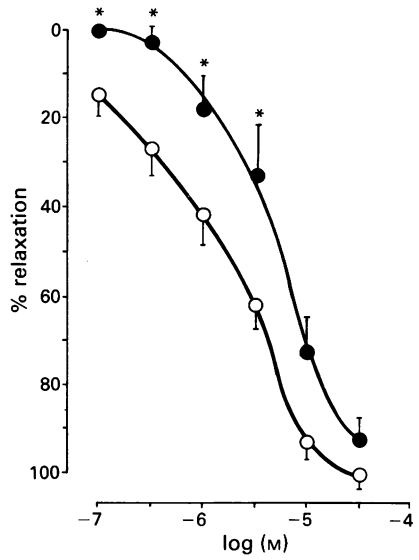

Figure 2 Effect of NO (●, 0.1–30 μM) and nitroglycerin (○, 0.1–30 μM) on canine lower oesophageal sphincter circular muscle strips. Experiments were performed during a 5-hydroxytryptamine-induced contraction and in the presence of atropine (1 μM) and guanethidine (10 μM). Results are shown as mean (s.e.mean shown by vertical bars) and are expressed as a percentage of the maximal relaxation induced by electrical field stimulation (16 Hz, 0.5 ms) at the beginning of experimentation ($n = 6$). * $P < 0.05$, significantly different, sensitivity to NO versus nitroglycerin, Student's t test for unpaired observations.

prenaline (100 μM) induced sustained relaxations (44 ± 9% of the relaxation to 16 Hz, 0.5 ms), which were not affected by hexamethonium (100 μM) but which were almost completely blocked in the presence of propranolol (1 μM) and phentolamine (10 μM) (Figure 1).

Administration of NO (0.1–30 μM, non-cumulative) and nitroglycerin (0.1–30 μM, cumulative) induced concentration-dependent relaxations (Figure 2) which were unaffected by hexamethonium (100 μM) or propranolol (1 μM) and phentolamine (10 μM) (Figures 1 and 2). The relaxations to NO mimicked those to electrical stimulation whereas the relaxations to nitroglycerin were sustained (Figure 1). The pD_2 for NO which was 5.40 ± 0.14 ($n = 5$) significantly differed from the pD_2 for nitroglycerin which was 5.87 ± 0.13 ($n = 5$).

The responses to electrical stimulation were abolished by TTX (1 μM) (Figure 1) and concentration-dependently inhibited by L-NNA (3–100 μM) and haemoglobin (10–30 μM) (Figure 3). In 6 experiments the maximal relaxation obtained by electrical stimulation (16 Hz, 0.5 ms) was inhibited from 100% to 0% by TTX (1 μM), to 20 ± 6% by L-NNA (100 μM) and to 72 ± 7% by haemoglobin (30 μM). The inhibitory effect of L-NNA was prevented by L-arginine (5 mM) but not by D-arginine (5 mM) (Figure 4). TTX and L-NNA did not affect the relaxations to NO, whereas they were abolished by haemoglobin (30 μM) (Figure 3).

Cumulative administration of VIP (1–300 nM) resulted in sustained, concentration-dependent relaxations which were slow in onset (Figure 3d). The highest concentration of VIP tested, relaxed the canine LOS muscle strips only by 52.5 ± 6.9% ($n = 4$), compared to the complete relaxation induced by electrical stimulation (16 Hz, 0.5 ms). The relaxations to VIP were not affected by L-NNA (30 μM) (Figure 4).

Discussion

The exact nature of the NANC neurotransmitter, released by inhibitory enteric neurones is still debated. Although VIP has been proposed as the most likely candidate in the canine LOS, the present results suggest NO or a NO releasing substance as the inhibitory NANC neurotransmitter.

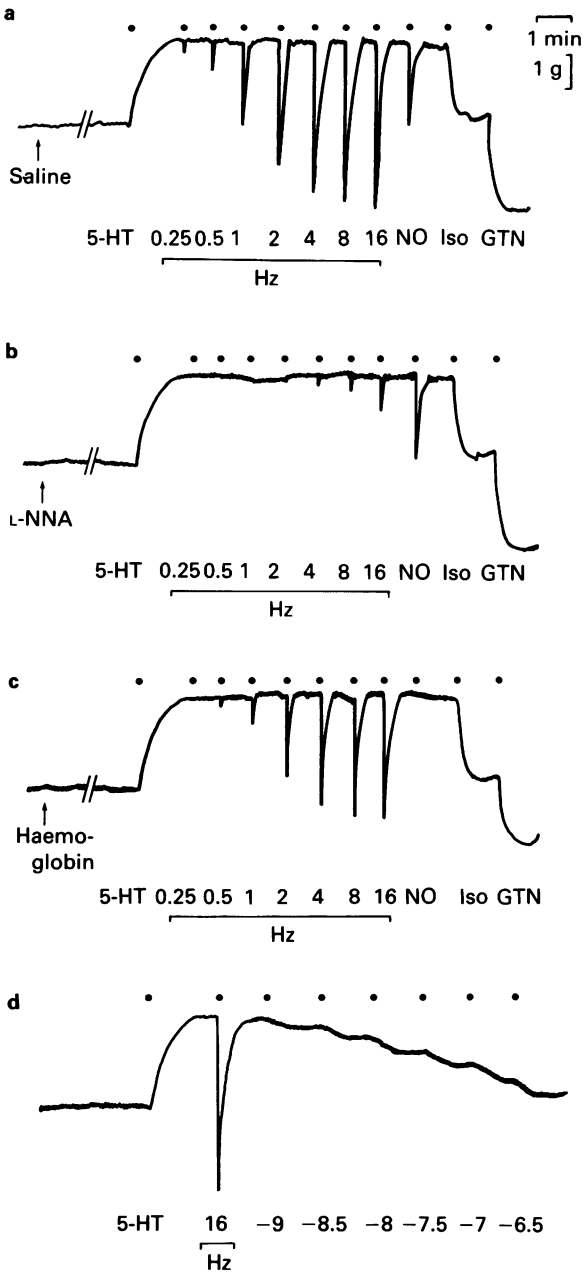

Figure 3 Isometric tension recordings of canine lower oesophageal sphincter circular muscle strips showing (a) the control and the effects of (b) N^G-nitro-L-arginine (L-NNA, 100 μM) and (c) haemoglobin (30 μM) on the relaxations induced by electrical stimulation (0.25–16 Hz, 0.5 ms), NO (3 μM), isoprenaline (Iso, 100 μM) and nitroglycerin (GTN, 10 μM). Tracing (d) shows the effect of cumulative administration of vasoactive intestinal polypeptide (VIP, 1–300 nM, log (M)). All experiments were performed during a 5-hydroxytryptamine-induced contraction and in the presence of atropine (1 μM) and guanethidine (3 μM). Similar results were obtained from additional experiments ($n = 6$, except for VIP $n = 4$). Tracing-breaks represent periods of tissue equilibration.

In the presence of adrenoceptor and cholinoceptor antagonists, electrical stimulation of circular muscle strips of the LOS caused relaxations which were abolished by tetrodotoxin, a nerve conductance blocker, suggesting that these responses resulted from NANC nerve stimulation. Since these relaxations were significantly reduced by L-NNA, an inhibitor of NO biosynthesis (Ishii *et al.*, 1990; Moore *et al.*, 1990; Mülsch & Busse, 1990), they were mediated by NO. This inhibition was stereospecific and competitive as it was prevented by L-arginine, a precursor of NO biosynthesis (Palmer *et al.*, 1988; Schmidt *et al.*, 1988) but not by its enantiomer, D-arginine. In

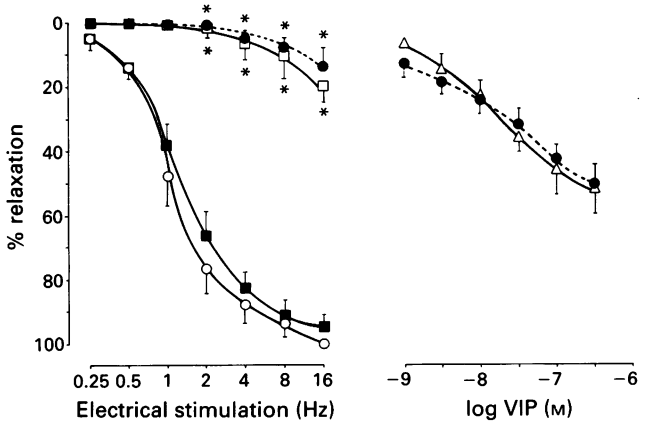

Figure 4 Effect of N^G-nitro-L-arginine (L-NNA, ●, 100 μM) on the NANC relaxations induced by electrical stimulation (○, 0.25–16 Hz, 0.5 ms) and vasoactive intestinal polypeptide (VIP, △, 1–300 nM) in the canine lower oesophageal sphincter. The effect of L-NNA on electrical stimulation was prevented by L-arginine (■, 5 mM) but not by D-arginine (□, 5 mM). L-NNA (30 μM) had no effect on the relaxations induced by VIP. Results are shown as mean (s.e.mean shown by vertical bars) ($n = 6$ for electrical stimulation and $n = 4$ for VIP). * $P < 0.05$, significantly different from control value, Student's t test for paired observations.

addition, exogenous administration of NO caused tetrodotoxin-resistant relaxations which were morphologically similar to those obtained by electrical stimulation. Since the relaxations to NO were not affected by L-NNA, the inhibitory effect of L-NNA on electrically-induced NANC relaxations was on the transmitter system rather than on the postjunctional effector cells. Compared to vascular smooth muscle, LOS tissue and other nonvascular smooth muscle (Buga *et al.*, 1989; Boeckxstaens *et al.*, 1990; 1991b,c) is relatively insensitive to NO. The pD_2 for NO in the canine LOS is 5.40 ± 0.14 ($n = 6$) whereas the pD_2 for NO in rabbit aortic rings is 6.82 ± 0.10 ($n = 10$, unpublished results). The NO solutions in these experiments were prepared identically for both tissues, indicating that the LOS is indeed less sensitive. Administration of nitroglycerin, by which NO is released intracellularly, also resulted in concentration-dependent relaxations.

Haemoglobin, which completely inactivates exogenous NO, significantly but not completely reduced the relaxations to electrical stimulation. This difference in efficacy of inhibition between the relaxation to NO and that to electrical stimulation may be explained by the large molecular size of haemoglobin so that only a small portion may have reached the neuromuscular junction.

Previously, a role in neurotransmission for NO, which accounts for the biological activity of vascular endothelium-derived relaxing factor (Palmer *et al.*, 1987), was also postulated in rat and mouse anococcygeus muscle (Gillespie *et al.*, 1989; Ramogopal & Leighton, 1989; Gillespie & Sheng, 1990; Gibson *et al.*, 1990), in rat cerebellum (Garthwaite *et al.*, 1988) and forebrain (Knowles *et al.*, 1989).

In previous studies we provided evidence for the proposal that NO or a NO releasing substance is the inhibitory NANC neurotransmitter in the canine ileocolonic junction (Boeckxstaens *et al.*, 1990; 1991b,c; Bult *et al.*, 1990) and rat gastric fundus (Boeckxstaens *et al.*, 1991a). In addition, the demonstration of the L-arginine: NO pathway in a variety of cells, suggests a more general role for this pathway in the regulation of cell function and communication (Moncada *et al.*, 1989).

Evidence has been obtained supporting the proposal that VIP is the inhibitory neurotransmitter in the LOS: VIP is released upon electrical stimulation (Biancani *et al.*, 1984) and VIP antiserum partly inhibits the relaxations to lower frequencies of electrical stimulation (Biancani *et al.*, 1984; Behar *et al.*, 1989). However, a total blockade was never achieved leading to the hypothesis that an additional neurotransmitter may be co-released by inhibitory NANC neurones (Goyal & Rattan, 1980; Behar *et al.*, 1989; Biancani *et al.*, 1989). Furthermore VIP-induced relaxations were slow in onset and sustained and were not affected by inhibitors of NO biosynthesis, in contrast to those induced by electrical stimulation. In addition, exogenous administration of even high concentrations of VIP (300 nM) failed to relax completely the canine LOS. This can be explained by the large molecular size of VIP, resulting in a limited ability to penetrate the synapse. Alternatively, it has been shown that the density of receptor binding sites for VIP in canine LOS is low (Zimmerman *et al.*, 1989), which might suggest a minor role for VIP in the inhibitory NANC mechanisms in the canine LOS. Finally, VIP-induced relaxations are mediated by a rise of intracellular cyclic AMP levels whereas electrically-induced relaxations result in a rise of intracellular cyclic GMP levels (Barnette *et al.*, 1990) providing evidence against VIP as mediator of these relaxations. Since nitrovasodilator compounds, including NO, activate guanylate cyclase and elevate cyclic GMP contents in vascular and nonvascular smooth muscle (Waldman & Murad, 1987), the combination of these results supports our hypothesis that NO is the final mediator in NANC neurotransmission in canine LOS.

In conclusion, we provide evidence that electrical stimulation of canine LOS circular muscle induced NANC relaxations which were significantly reduced by inhibitors of NO biosynthesis. Therefore, we propose NO or a NO releasing substance as the final neurotransmitter of inhibitory NANC nerves in the canine LOS.

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Mechanisms of the coronary vascular effects of platelet-activating factor in the rat perfused heart

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1 In a previous study it was demonstrated that bolus injections of platelet-activating factor (PAF) in the rat perfused heart resulted in coronary vasodilatation, vasoconstriction or the combination of both, depending on the amount of PAF that was injected. In the present study, the mechanisms of these coronary vascular effects of PAF in the rat perfused heart were investigated.

2 Pretreatment of the rat perfused heart with the PAF antagonists FR-900452 or BN-52021 did not affect the vasodilator effect of PAF but eliminated the vasoconstrictor effect of PAF. FR-900452 had no effect on the vasoconstrictor response to leukotriene C_4 (LTC_4) or LTD_4.

3 The cyclo-oxygenase inhibitor, indomethacin, did not modify the coronary vascular effects of PAF. However L-649,923 (a leukotriene antagonist) and MK-886 (a leukotriene synthesis inhibitor) eliminated both the vasodilator and vasoconstrictor effects of PAF.

4 When leukotrienes were administered by bolus injection in the rat perfused heart, LTB_4 produced vasodilatation while LTC_4 and LTD_4 produced vasoconstriction. L-649,923 blocked both the vasodilator and vasoconstrictor effects of the leukotrienes tested.

5 The results suggest that lipoxygenase products are responsible for both the vasodilator and vasoconstrictor actions of PAF in the coronary vasculature of the rat perfused heart while the cyclo-oxygenase products do not play a significant role. The ineffectiveness of PAF antagonists in blocking the vasodilatation produced by PAF is compatible with the concept that there may be multiple PAF receptors.

Keywords: Platelet-activating factor; coronary vascular effects; leukotrienes; prostaglandins; rat perfused heart

Introduction

Since the identification of the structure of platelet-activating factor (PAF) as 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, and the chemical synthesis of PAF, the effects of PAF on the coronary circulation have been examined. However, the results of these studies are diverse. PAF was shown to cause vasoconstriction in the guinea-pig perfused heart (Levi *et al.*, 1984; Piper & Stewart, 1987) and in the rat perfused heart (Piper & Stewart, 1986). In contrast, intracoronary injection of PAF to anaesthetized dogs produced only increases in coronary flow and this effect was reduced after the removal of circulating platelets (Jackson *et al.*, 1986). Other studies showed a biphasic effect, i.e. an increase in flow followed by a decrease in flow, after intracoronary injection of PAF (Feuerstein *et al.*, 1984; Mehta *et al.*, 1986; Erza *et al.*, 1987; Fiedler *et al.*, 1987). In a previous study, we demonstrated that depending on the dose, bolus injections of PAF could produce vasodilator (10 fmol–1 pmol PAF), vasoconstrictor (1 nmol PAF) or a biphasic response (10 and 100 pmol PAF) in rat perfused hearts (Man *et al.*, 1990).

The studies of Piper & Stewart (1986, 1987) clearly identified the presence of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) thromboxane B_2 and leukotriene-like bioactivity in the effluent of the perfused rat and guinea-pig hearts after PAF administration. However, only a vasoconstrictor response was observed in these studies. Hence, it is not clear what role these vasoactive compounds will have in mediating the effects of PAF, especially the vasodilator response. In the present study, the mechanism for coronary vasodilator, vasoconstrictor and biphasic responses to PAF were examined. The coronary vascular responses to PAF were determined in the absence and presence of two PAF antagonists. The cyclo-oxygenase inhibitor (indomethacin), the leukotriene antagonist (L-649, 923, Jones *et al.*, 1986) and the leukotriene synthesis inhibitor (MK-886, Gillard *et al.*, 1989; Rouzer *et al.*, 1990) were tested for their ability to inhibit the coronary vascular effects of PAF. The coronary vascular effects of leukotrienes and their ability to mimic the coronary vascular effects of PAF were also examined.

Methods

Rat heart perfusion

Following cervical dislocation, hearts from Sprague-Dawley rats (250–350 g) were rapidly excised and placed in cool Krebs-Henseleit solution (4°C) aerated with 95% O_2 : 5% CO_2. The solution had the following composition (mM): NaCl 120, NaH_2PO_4 1.18, $MgSO_4$ 1.18, KCl 4.76, $CaCl_2$ 1.25, $NaHCO_3$ 25.0 and glucose 5.5. The aorta was cannulated for coronary perfusion. The heart was allowed to beat spontaneously. The temperature of the perfusate was maintained at 37 ± 0.5°C and the coronary flow was controlled by a roller pump. The perfusion pressure was measured by a pressure transducer attached to a side arm of the aortic cannula. The perfusion pressure was recorded on a Gould chart recorder and monitored with a digital display of the perfusion pressure. The detailed methodology of the isolated heart perfusion system has been described previously (Man & Lederman, 1985).

Drug preparations and administration

Hearts were equilibrated with Krebs-Henseleit solution for 20 min. During the equilibration period, the flow rate was adjusted to obtain a control perfusion pressure of 65–75 mmHg. This perfusion pressure range was selected based on our previous experience that the vasodilator response was more prominent in this condition than at lower perfusion

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pressures (Man et al., 1990). In experiments which required a drug pretreatment, 10 min of stabilization with Krebs-Henseleit solution was followed by 10 min of pretreatment with Krebs-Henseleit solution containing a PAF antagonist, or a leukotriene antagonist, and continued throughout the administration of PAF. The cyclo-oxygenase inhibitor, indomethacin, was added to the perfusion solution at the start of the stabilization period and the effect of PAF injection was tested after 45 min of perfusion in the presence of indomethacin. All stock solutions were made fresh daily and kept at 4°C between experiments. The leukotriene antagonist, L-649,923 (sodium (*βS**, *R**)-4-(3-4(-acetyl-4-hydroxy-2-propylphenoxy)-propylthio-γ-hydroxy-β-methylbenzenebutanoate, Merck Frosst Canada Inc.) and the leukotriene synthesis inhibitor, MK-886 (3-[1-(4-chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid, Merck Frosst Canada Inc.) were dissolved in distilled water and then diluted in Krebs-Henseleit solution to a concentration of 0.1–5 μM and 1 μM respectively. BN-52021 (9H-1,7a-(epoxymethano)-1H,6aH-cyclopenta(c)furo(2,3-b)furo-(3',2':3,4)cyclopenta-(1,2-d)furan-5,9,12-(4H)-trione, 3-tert-butylhexahydro-8-methyl, Institut Henri Beaufour, France) was dissolved in dimethyl sulphoxide (DMSO) and slowly added to warm (50°C) Krebs-Henseleit solution while stirring constantly to a final concentration of 30 μM in 0.4% DMSO. FR-900452 (1-methyl-3-(1-(5-methylthiomethyl-6-oxo-3-(2-oxo-3-cyclopenten-1-ylidene)-2-piperazinyl ethyl)-2-indolinone, Fujisawa Pharmaceutical Co., Japan) was dissolved in ethanol then diluted in Krebs-Henseleit solution to a final concentration of 5 μM in 0.1% ethanol. Indomethacin (Sigma Chemical Co.) was dissolved in 0.1 M Na_2CO_3 solution and diluted in Krebs-Henseleit solution to a final concentration of 2.8 μM. Experiments were also conducted to test the effects of 10 min pretreatments with Krebs-Henseleit solutions containing 0.4% DMSO and 0.1% ethanol on the coronary vascular response to PAF. PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (prepared from bovine heart, Sigma Chemical Co.) was prepared fresh daily in saline (0.9% NaCl) containing 0.25% bovine serum albumin (Sigma Chemical Co.). Only one injection of PAF was given to each heart. Bolus injections of PAF were given in a volume of 0.1 ml and over a 1 s period, into the perfusion line 5–6 cm proximal to the aortic cannula. Changes in perfusion pressure and the time at which maximum changes occurred were recorded. Leukotriene B_4 (LTB_4), LTC_4 and LTD_4 (Merck Frosst Canada Inc.), were diluted daily in saline (0.9% NaCl) to the appropriate amounts for bolus injections of 0.1 ml.

Statistical analyses

Data were analyzed by Student's *t* test and analysis of variance (ANOVA) followed by Duncan's test where appropriate. Values are expressed as means ± standard deviations (s.d.) and $P < 0.05$ was considered statistically significant.

Results

The effects of FR-900452, BN-52021, indomethacin, L-649,923 and MK-886 on the response of rat hearts to PAF

Table 1 summarizes the effects of various additions to Krebs-Henseleit solution on some of the baseline parameters. The flow rate was adjusted in these experiments to attain a perfusion pressure between 65–75 mmHg. The flow rates in the presence of ethanol, DMSO, indomethacin, L-649,923 and MK-886 were not significantly different from the flow rate of the group perfused with normal Krebs-Henseleit only. The perfusion pressure of the group with BN-52021, although significantly higher than the group perfused with Krebs-Henseleit, was not different from the corresponding group receiving the vehicle 0.4% DMSO. However, the perfusion pressure and heart rate of the group with indomethacin was higher than the corresponding group perfused with normal Krebs-Henseleit only.

The effects of 1 and 100 pmol PAF on the perfusion pressure in hearts pretreated with FR-900452 and BN-52021 are summarized in Table 2. Bolus injections of 1 pmol PAF in hearts perfused with the vehicles 0.4% DMSO and 0.1% ethanol resulted in decreases in perfusion pressure which peaked within approximately 30 s. The effect of PAF was similar to our previous data obtained in hearts perfused with normal Krebs-Henseleit solution (Man *et al.*, 1990). FR-900452 and BN-52021 had no effect on the perfusion pressure response to 1 pmol PAF (Table 2). Bolus injections of 100 pmol PAF resulted in biphasic responses in the groups where 0.1% ethanol or 0.4% DMSO was added to Krebs-Henseleit solution. In these experiments, the initial decrease in perfusion pressure was followed by an increase in perfusion pressure which peaked at approximately 1 min after the bolus injection. Both FR-900452 (5 μM) and BN-52021 (30 μM) abolished the increase in perfusion pressure. FR-900452 and BN-52021 appeared to enhance the vasodilator component of the

Table 1 The effects of adding various compounds to Krebs-Henseleit solution on the perfusion parameters of rat hearts

Addition to normal Krebs-Henseleit	*Flow rate* (ml min^{-1} g^{-1} heart)	*Control pressure* (mmHg)	*Control heart rate* (beats min^{-1})	n
None	11.5 ± 1.7	67.7 ± 1.7	270 ± 39	9
Ethanol 0.1%	11.7 ± 3.2	67.8 ± 2.7	272 ± 46	12
DMSO 0.4%	11.9 ± 2.6	69.3 ± 2.6	289 ± 44	15
FR-900452 5.0 μM	11.9 ± 1.6	69.8 ± 2.5	252 ± 29	14
BN-52021 30 μM	12.7 ± 2.6	71.0 ± 2.6**	281 ± 36	12
Indomethacin 2.8 μM	11.0 ± 1.1	72.4 ± 3.3**	323 ± 50*	7
L-649,923 1 μM	9.8 ± 1.9	66.8 ± 1.7	260 ± 42	6
L-649,923 5 μM	12.2 ± 1.4	68.0 ± 1.8	261 ± 40	6
MK-886 1 μM	11.4 ± 0.6	70.5 ± 5.8	293 ± 18	11

For full names of substances, see Methods section.
Values represent mean ± s.d. ANOVA followed by Duncan's test was used to determine significant differences from the group perfused with normal Krebs-Henseleit solution.
* $P < 0.05$; ** $P < 0.01$.

Table 2 The effects of the PAF receptor antagonists, FR-900452 and BN-52021, on the coronary vascular response to bolus injections of 1 and 100 pmol PAF

Solution	Control pressure (mmHg)	Decrease in perfusion pressure: Maximum (mmHg)	Decrease in perfusion pressure: Time (s)	Increase in perfusion pressure: Maximum (mmHg)	Increase in perfusion pressure: Time (s)	n
(A) *Bolus injections of 1 pmol PAF*						
Ethanol 0.1%	68.3 ± 2.8	4.2 ± 5.3	25 ± 14	—	—	6
FR-900452 5.0 μM	71.0 ± 1.6	4.6 ± 1.5	14 ± 4	—	—	5
DMSO 0.4%	69.6 ± 2.8	9.5 ± 6.5	23 ± 9	—	—	8
BN-52021 30 μM	72.3 ± 2.0	4.6 ± 2.4	31 ± 9	—	—	7
(B) *Bolus injections of 100 pmol PAF*						
Ethanol 0.1%	67.3 ± 2.7	5.0 ± 3.8	10 ± 4	21.3 ± 11.1	61 ± 17	6
FR-900452 5 μM	69.1 ± 2.8	12.4 ± 8.2*	22 ± 13*	2.2 ± 3.3**	[a]	9
DMSO 0.4%	68.9 ± 2.5	5.7 ± 3.5	13 ± 6	20.0 ± 6.1	67 ± 19	7
BN-52021 30 μM	69.2 ± 2.4	14.4 ± 3.9**	23 ± 6*	0 ± 0**	[a]	5

For full names of substances, see Methods section.
Values represent mean ± s.d. Student's unpaired t test was used for statistical comparisons between vehicle and treatment groups. *$P < 0.05$; **$P < 0.01$. Time represents the time at which the maximum change occurred. [a] Since there was little or no effect of PAF injections in these groups, the time was not applicable.

response to 100 pmol PAF in these groups (approximately two times their respective vehicle control groups, $P < 0.05$).

The addition of indomethacin, 2.8 μM, to Krebs-Henseleit solution did not significantly affect the response to bolus injections of 100 pmol PAF (Table 3). The coronary vascular effects of PAF were also examined in the presence of the leukotriene antagonist, L-649,923 (0.1–5 μM). The results are summarized in Table 3. Statistically significant reduction of the decrease in perfusion pressure by PAF was observed with 1 and 5 μM L-649,923 while significant reductions of the increases in perfusion pressure were observed with 0.1–5 μM L-649,923. When the highest concentration of L-649,923 (5 μM) was used, both the initial decrease and the subsequent increase in the perfusion pressure were markedly reduced. MK-886 (1 μM) also significantly attenuated the changes in perfusion pressure produced by PAF (Table 3).

The effects of leukotrienes C_4, D_4 and B_4 on the perfusion pressure of rat hearts

The results of bolus injections of LTC_4 and LTD_4 are summarized in Table 4. Only rapid increases in perfusion pressure (within 15 s) were observed. Statistical analysis showed that the increase in perfusion pressure following LTC_4 was dose-dependent ($P < 0.001$). The increase in perfusion pressure following bolus injections of LTD_4 was also dose-dependent ($P < 0.01$). The results of bolus injections of LTB_4 are summarized in Table 4. The decrease in perfusion pressure in the presence of LTB_4 was dose-dependent ($P < 0.05$).

The effects of bolus injections of LTB_4, LTC_4 and LTD_4 were studied in hearts pretreated with 5 μM L-649,923 and compared with the effects on the perfusion pressure in the

Table 3 The effects of indomethacin, L-649,923 and MK-886 on the coronary vascular response to bolus injections of 100 pmol PAF

Solution	Control pressure (mmHg)	Decrease in perfusion pressure: Maximum (mmHg)	Decrease in perfusion pressure: Time (s)	Increase in perfusion pressure: Maximum (mmHg)	Increase in perfusion pressure: Time (s)	n
Normal Krebs-Henseleit	67.7 ± 1.7	8.9 ± 6.5	10 ± 5	27.1 ± 13.8	71 ± 22	9
Indomethacin 2.8 μM	72.4 ± 3.3	5.3 ± 3.0	11 ± 3	32.9 ± 10.6	49 ± 8	7
L-649,923 0.1 μM	68.3 ± 2.1	7.0 ± 2.2	11 ± 3	12.5 ± 7.2**	88 ± 8	6
L-649,923 0.5 μM	67.8 ± 1.7	8.7 ± 1.9	11 ± 3	6.3 ± 4.4**	99 ± 12	6
L-649,923 1.0 μM	66.8 ± 1.7	4.2 ± 3.1*	13 ± 9	2.0 ± 2.7**	[a]	6
L-649,923 5.0 μM	68.0 ± 1.8	1.8 ± 1.0**	13 ± 12	1.0 ± 1.7**	[a]	6
MK-886 1 μM	70.5 ± 5.8	3.5 ± 3.0*	10 ± 7	2.7 ± 1.4**	50 ± 22	11

For full names of substances, see Methods section.
Values represent mean ± s.d. ANOVA followed by Duncan's test was used to determine which groups were significantly different from the group perfused with normal Krebs-Henseleit. * $P < 0.05$; ** $P < 0.01$. Time represents the time at which the maximum change occurred. [a] Since there was little or no effect of PAF injections in these groups, the time was not applicable.

Table 4 The changes in the perfusion pressure of rat hearts following bolus injections of leukotriene C_4 (LTC_4), LTD_4 and LTB_4

	Control pressure (mmHg)	Changes in perfusion pressure: Maximum (mmHg)	Time (s)	n
LTC_4				
1 pmol	68.3 ± 3.8	+6.4 ± 3.5**	18 ± 9	8
10 pmol	72.0 ± 2.1	+23.8 ± 10.2**	14 ± 6	6
50 pmol	69.7 ± 1.5	+58.2 ± 23.0**	14 ± 1	6
100 pmol	66.5 ± 1.3	+70.0 ± 9.1**	11 ± 1	4
LTD_4				
1 pmol	70.7 ± 5.1	+7.3 ± 4.2	23 ± 11	3
100 pmol	68.8 ± 2.2	+39.8 ± 10.4**	15 ± 10	4
LTB_4				
1 pmol	69.7 ± 3.4	−0.7 ± 1.2	—	6
10 pmol	71.2 ± 3.3	−4.5 ± 6.4	—	6
100 pmol	69.4 ± 3.5	−12.4 ± 10.6*	11 ± 4[a]	10

Values represent mean ± s.d. The signs indicate increase or decrease in perfusion pressure. ANOVA was used for statistical analysis, and indicated that the changes in perfusion pressure induced by LTC_4, LTD_4 and LTB_4 were all dose-dependent. $P < 0.05$ and ** $P < 0.01$ when compared with the pre-injection pressure. Time represents the time at which the maximum change occurred. [a] $n = 8$, 2 hearts did not have a vasodilatation response to LTB_4.

absence of L-649,923 as shown in Table 4. Pretreatment with of L-649,923 (5 μM) significantly attenuated the decrease in perfusion pressure following bolus injections of 100 pmol LTB_4 (12.4 ± 10.6 vs 1.4 ± 1.0 mmHg, $n = 10$ and 9 in the absence and presence of L-649,923 respectively, $P < 0.01$). The increases in perfusion pressure following bolus injections of 100 pmol LTC_4 and 100 pmol LTD_4 were also attenuated in hearts pretreated with 5 μM L-649,923 (70.0 ± 9.1 vs. 3.2 ± 1.8 mmHg, $n = 4$ and 5 in the absence and presence of L-649,923 respectively for LTC_4, $P < 0.01$, and 39.8 ± 10.4 vs 5.5 ± 4.4 mmHg, $n = 4$ and 4 in the absence and presence of L-649,923 respectively for LTD_4, $P < 0.01$).

The effects of bolus injections of LTB_4, LTC_4 and LTD_4 were also studied in hearts pretreated with 5 μM FR-900452. Pretreatment with the PAF antagonists did not affect the vasoconstrictor response of the heart to 100 pmol LTC_4 (52.4 ± 5.6, and 56.5 ± 8.4 mmHg, $n = 5$ and 6 in the absence and presence of FR-900452 respectively) and 100 pmol LTD_4 (55.2 ± 5.7 and 56.5 ± 13.9 mmHg, $n = 5$ and 6 respectively). However, pretreatment with FR-900452 significantly attenuated the vasodilator response of the heart to LTB_4 (8.3 ± 2.5 and 2.4 ± 1.6 mmHg, $n = 6$ and 7 in the absence and presence of FR-900452 respectively, $P > 0.001$).

Discussion

A bolus injection of 100 pmol of PAF was used in most experimental protocols in this study since this amount consistently produced a biphasic response, i.e. an initial vasodilatation followed by vasoconstriction. This enabled us to examine the effects of various pharmacological interventions on both the coronary vasodilatation and vasoconstriction-induced by PAF in the same heart. As in previous studies on the perfused heart, a constant flow rate was used in the present study and changes in perfusion pressure were used as a reflection of the presence of coronary vasodilatation or vasoconstriction. Similar coronary vascular responses to a bolus injection of PAF under constant pressure perfusion were observed when the changes in coronary perfusion rate was measured by an electromagnetic flow probe (unpublished observations).

The effects of two different PAF antagonists, FR-900452 (5 μM) and BN-52021 (30 μM) were examined on the coronary vascular response to PAF in the rat perfused heart. These antagonists were able to block the increase in perfusion pressure following bolus injections of 100 pmol PAF, but not the decrease in perfusion pressure. The same concentrations of the PAF antagonists also had no effect on the decreases in perfusion pressure as a result of the injection of 1 pmol PAF. Thus, the coronary vasodilator and vasoconstrictor effects of PAF appear to be initiated by different mechanisms at the membrane level. The concentrations of FR-900452 and BN-52021 have been demonstrated to antagonize the platelet aggregating effect of PAF (Okamoto *et al.*, 1986; Nunez *et al.*, 1986). Higher concentrations of FR-900452 and BN-52021 were not tested due to their limited solubility in aqueous solution, the need to use solvents as vehicles and the limited availability of larger quantities of these compounds.

Piper & Stewart (1986, 1987) showed that indomethacin attenuated the increase in perfusion pressure in rat and guinea-pig hearts following bolus injections of PAF. Also, indomethacin blocked the coronary constriction produced by intracoronary injection of PAF in the pig heart (Feuerstein *et al.*, 1984). These data suggest that the effects of PAF may be mediated by cyclo-oxygenase products. However, under our experimental conditions, pretreating the rat hearts with indomethacin had no significant effect on the vasodilator and vasoconstrictor responses following bolus injections of PAF. Our data therefore suggest that cyclo-oxygenase products are not important in the mediation of the coronary vascular response of PAF and are contrary to previous results. This can be due to the differences in the perfusion pressure in our study from those of Piper & Stewart (1986, 1987). The higher starting perfusion pressure used in the present study may be more susceptible to demonstrating the vasodilator response (Man *et al.*, 1990) than the lower perfusion pressure used previously. Species difference (rat heart vs. pig heart) may also account for the difference between our results and those of Feuerstein *et al.* (1984).

The biphasic coronary vascular response to bolus injection of PAF in the rat perfused heart was blocked by the leukotriene antagonist (L-649,923) and leukotriene synthesis inhibitor (MK-886). This suggested that the PAF effects were mediated by leukotrienes. In previous studies, the leukotriene antagonist FPL-55712, was shown to attenuate the vasoconstrictor response following injections of PAF (Feuerstein *et al.*, 1984; Piper & Stewart, 1986; Fiedler *et al.*, 1987). However, the vasodilator effect, when observed, was not altered by FPL-55712 (Feuerstein *et al.*, 1984; Fiedler *et al.*, 1987). It is not known whether the differences in the effectiveness of L-649, 923 and FPL-55712 in inhibiting the vasodilator response is due to their selectivity for different leukotrienes. Bolus injections of LTC_4 and LTD_4 resulted in increases in perfusion pressure, which were blocked when the hearts were pretreated with 5 μM L-649,923. In the present study, bolus injections of LTB_4 caused decreases in perfusion pressure which were also blocked when the hearts were pretreated with L-649,923. Therefore, bolus injections of leukotrienes are capable of mimicking the individual components of the biphasic response to PAF.

In previous studies, the increase in perfusion pressure following a bolus injection of PAF in the rat heart could not be repeated by a second injection (Piper & Stewart, 1986; 1987; Man *et al.*, 1990). In this study, we were able to observe increases in perfusion pressure following repeated bolus injections of LTC_4 or LTD_4 (results not shown). This indicates that the endogenous leukotrienes may be exhausted following the first injection of PAF. However the blood vessels are still capable of responding to exogenously administered leukotrienes. This provides further indirect evidence to support the hypothesis that the PAF effects are mediated by the release of vasoactive compounds such as leukotrienes and the depletion of these compounds limits the response to subsequent exposure to PAF.

Specific binding of radiolabelled PAF was first demonstrated in platelets (Valone *et al.*, 1982; Hwang *et al.*, 1983)

and subsequently demonstrated in a number of tissues (see review of Braquet *et al.*, 1987). The binding of PAF showed the presence of high affinity sites, stereospecificity and saturation kinetics. These data suggested the presence of a specific PAF receptor on the cell membrane and the binding of PAF could be blocked by PAF antagonists (Braquet *et al.*, 1987; Saunders & Handley, 1987). The presence of multiple PAF receptors was suggested by the study of Hwang (1988). While commonly used PAF antagonists blocked receptor binding in human platelets and polymorphonuclear leukocytes with similar potency, ONO-6240 showed 6–10 times higher potency in human platelets than in leukocytes. Additional evidence for this concept was provided by the observation that the ionic requirement for PAF receptor binding was also different between the platelets and leukocytes (Hwang, 1988).

The results of the present study can be explained on the basis of the presence of two different PAF receptors in the rat heart. The activation of the first type of PAF receptor leads to the formation of LTB_4 resulting in the observed coronary vasodilatation while the activation of the second type of PAF receptor leads to the formation of LTC_4 and/or LTD_4 resulting in coronary vasoconstriction. The PAF antagonists that we used were able to block the receptors involved in the vasoconstrictor response only. In contrast, a high concentration of the leukotriene antagonist (L-649-923, 5 μM) and the leukotriene synthesis inhibitor (MK-886, 1 μM) were able to abolish the coronary vasodilator and vasoconstrictor actions of PAF.

The different time course of the vasodilator and vasoconstrictor effects of PAF (peak effect 20–30 s and 60–90 s respectively) also suggests the existence of two PAF receptor sites which when activated will mediate the vasodilator and vasoconstrictor responses at different rates. The use of specific LTB_4, LTC_4 and LTD_4 antagonists would enable us to examine this hypothesis further. Although the leukotriene antagonist L-649,923 showed specificity for LTD_4 in binding assay (Jones *et al.*, 1986), the selectivity of L-649,923 *in vivo* may be less (Ford-Hutchinson, personal communication). This would account for our results that 5 μM L-649,923 abolished both the vasodilator and vasoconstrictor actions of PAF. However, selectivity of L-649,923 can be detected when lower concentrations of L-649,923 (0.1–0.5 μM) were used. Substantial reduction of the coronary vasoconstrictor effect of PAF was observed while no significant effect on the coronary vasodilator action of PAF was detected (Table 3). Another consideration is that L-649,923 may have a non-specific effect on PAF receptors and therefore can inhibit the coronary vascular effect of PAF. However, L-649,923 (10 μM) does not affect PAF receptor binding assay in platelets (Ford-Hutchinson, personal communication). In addition, inhibition of leukotriene synthesis by MK-886 produced the same results as with the leukotriene antagonist. These data further reinforce the role of leukotriene in both the vasodilator and vasoconstrictor effects of PAF. One unresolved question in this study is that although FR-900452 did not affect the vasodilator effect of PAF, the effect of LTB_4 was significantly reduced in the presence of FR-900452. Although we have no explanation for this finding, the weight of the evidence with both a leukotriene antagonist and synthesis inhibitor suggest that the vasodilator effect of PAF could be mediated via release of a leukotriene which may be LTB_4.

Release of leukotriene-like bioactivity by PAF had been reported in the effluent of rat perfused heart. This included LTB_4, LTC_4 and LTD_4 (Piper & Stewart, 1986). However, the source for the production of these leukotrienes by PAF has not been fully identified. Based on the relatively short time course for the occurrence of the coronary vascular response to PAF (20–60 s) and the detection of leukotrienes, the endothelium or the smooth muscle cells are likely sources for PAF-induced production of these vasoactive lipoxygenase products. Perivascular mast cells have also been proposed as the source for the production of leukotrienes (Piper & Stewart, 1986). Since the PAF-induced coronary vasodilatation and vasoconstriction have different time courses, it is possible that LTB_4, and LTC_4 and LTD_4 are produced from different sources. The involvement of multiple cell types in the initiation of the complex coronary vascular effects of PAF is compatible with the different sensitivity of the vasodilator response to PAF (10 fmol–1 pmol) as compared to the vasoconstrictor response (10 pmol or more) in our previous study (Man *et al.*, 1990). However, the identification of the multiple PAF receptors in the rat heart and the possible involvement of different cell types in the generation of the vasoactive leukotrienes responsible for the vasodilator and vasoconstrictor effects of PAF remain to be elucidated.

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Adenosine-induced dilatation of the rabbit hepatic arterial bed is mediated by A_2-purinoceptors

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1 This study was carried out in order to identify the receptor responsible for adenosine-induced dilatation of the hepatic arterial vascular bed.

2 Livers of 10 New Zealand White rabbits were perfused *in vitro* with Krebs-Bülbring buffer via the hepatic artery and the portal vein at constant flows of 26 and 77 ml min^{-1} 100 g^{-1} liver respectively. The tone of the preparation was raised by the presence of noradrenaline in the perfusate (concentration: 10^{-5} M).

3 Dose-response curves for adenosine and its analogues 5'-N-ethyl-carboxamido-adenosine (NECA), the 2-substituted NECA analogue CGS 21680C, and **R**- and S-N^6-phenyl-isopropyl-adenosine (**R**- and S-PIA) were obtained after their injection into the hepatic arterial supply.

4 The order of vasodilator potency of these agents was: NECA > CGS 21680C > adenosine > **R**-PIA > S-PIA. Their potency, expressed relative to that of adenosine, was in the approximate ratio 10:3:1:0.3:0.1, consistent with that resulting from activation of P_1-purinoceptors of the A_2 sub-type (which mediate vasodilatation due to adenosine).

5 The P_1-purinoceptor antagonist 8-phenyltheophylline (10^{-5} M) caused significant attenuation of the vasodilatation to adenosine and analogues.

6 It is concluded that adenosine-induced dilatation of the hepatic arterial vascular bed is mediated by P_1-purinoceptors of the A_2 sub-type.

Keywords: Hepatic artery; portal vein; adenosine; purinoceptors

Introduction

The vasodilator response of the hepatic artery (HA) to portal vein (PV) flow interruption is mediated, at least in part, by adenosine (Lautt *et al.*, 1985; Lautt & Legare, 1985; Mathie & Alexander, 1990). Adenosine therefore plays an important role in the regulation of HA blood flow, though the actual mechanism by which it exerts physiological control of the circulation remains speculative. The release of adenosine may be continuous, so that during PV occlusion it may accumulate and result in HA dilatation (Lautt, 1985; 1988), or its release may be regulated by tissue hypoxia or oxygen supply-to-demand imbalance (Berne *et al.*, 1983; Bardenheuer & Schrader, 1986).

On the basis of studies in other tissues, it may be surmised that adenosine, whatever its mechanism of release, exerts its dilator action in the liver via a purine receptor located within the HA microvasculature (Burnstock & Kennedy, 1986), but little or no information is available in the literature to confirm or refute this supposition. Purine receptors in the cardiovascular system comprise two distinct populations (Burnstock & Kennedy, 1986; Williams, 1987): P_1-purinoceptors, which mediate responses to adenosine and adenosine monophosphate (AMP), and P_2-purinoceptors, which mediate responses to adenosine diphosphate (ADP) and adenosine triphosphate (ATP). P_1-purinoceptors of the A_2 sub-type (which mediate vasodilatation due to adenosine) have been identified in the smooth muscle of the aorta (Collis & Brown, 1983), and of the coronary (Kusachi *et al.*, 1983; 1986; Mustafa & Askar, 1985; Hamilton *et al.*, 1987) and cerebral circulations (Edvinsson & Fredholm, 1983), but this has not been investigated in the HA circulation. The present study was carried out to identify the purine receptor sub-type in the HA vascular bed which may be responsible for adenosine induced dilatation.

We have adopted a pharmacological technique for characterizing the hepatic P_1-purinoceptor population by establishing the rank order of vasodilator potency of adenosine and its analogues 5'-N-ethyl-carboxamido-adenosine (NECA) and the **R**- and S- stereoisomers of PIA (N^6-phenyl-isopropyl-adenosine) (Bruns *et al.*, 1986; Daly *et al.*, 1986; Oei *et al.*, 1988). (In keeping with current usage, we have employed the Cahn-Ingold-Prelog convention of describing stereoisomers by **R**- and **S**- prefixes instead of L- and D- respectively, while retaining the acronym PIA for the substance designated in IUPAC nomenclature as N^6-[1-methyl-2-phenylethyl]-adenosine (Bruns *et al.*, 1986).) At A_2 receptors, it is well established that NECA is 1–2 orders of magnitude more potent than **R**-PIA, and that **R**-PIA is only 2–5 times more potent than S-PIA (Collis & Brown, 1983; Kusachi *et al.*, 1983; Collis, 1985; 1989). In addition, we have investigated the vasodilator potency of the recently developed, high-affinity A_2 agonist CGS 21680C (a 2-substituted analogue of NECA: Balwierczak *et al.*, 1989; Hutchison *et al.*, 1989) in comparison to adenosine and NECA. The receptors were further characterized by use of the P_1-purinoceptor antagonist, 8-phenyltheophylline.

A novel, *in vitro*, dual-perfused, rabbit liver model has been adopted for the investigation. The HA and PV are perfused simultaneously with Krebs-Bülbring buffer at constant, physiological flow rates (Alexander *et al.*, 1991), an approach that has enabled us to investigate in detail the responses of the HA bed to pharmacological stimulation in the presence of an unchanging, normal PV flow rate.

Methods

Operative procedures

Experiments were carried out on a total of 10 New Zealand White rabbits of either sex, weighing 2.5–3.8 kg (mean 2.9 kg). The operative technique has been described elsewhere (Alexander *et al.*, 1991), but will be outlined in brief here. The rabbits were initially sedated with fentanyl-fluanisone i.p. ('Hypnorm', 0.25 ml kg^{-1}), and then anaesthetized with a

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mixture of 1 part Hypnorm (0.3 ml kg^{-1}) and 1 part midazolam ('Hypnovel', 1.5 mg kg^{-1}) in 2 parts water i.p. (total: 1.20 ml kg^{-1}) (Flecknell, 1987). A marginal ear vein was cannulated for subsequent i.v., administration of the Hypnorm/midazolam/water mixture (0.25–0.5 ml kg^{-1} h^{-1}).

The abdomen was opened through a mid-line incision, and the common bile duct cannulated. The gastroduodenal artery was cannulated (Portex 3FG), and the catheter advanced to the junction of the common and proper hepatic arteries; the common hepatic artery was then ligated and divided, and 4–5 ml saline infused into the catheter to prevent blood coagulation in the intrahepatic HA vasculature. After administration of heparin i.v. (100 units kg^{-1}), the PV was cannulated and 40–50 ml saline infused into the catheter to prevent accumulation of blood in the intrahepatic PV system. The liver was then rapidly, but carefully, excised from the animal, weighed and placed in an organ bath.

Liver perfusion

The liver was perfused at constant flow rates via the HA and the PV from a common reservoir of oxygenated (95% O_2/5% CO_2) Krebs-Bülbring buffer solution of the following composition (mM): NaCl 133, KCl 4.7, NaH_2PO_4 1.35, $MgSO_4$ 0.61, glucose 7.8 and $CaCl_2$ 2.52 at 37°C. All livers appeared evenly perfused, and this was confirmed at the end of each experiment by the local injection of Patent Blue into both the HA and the PV. Mean flow rates for all experiments were 26 ml min^{-1} 100 g^{-1} (HA) and 77 ml min^{-1} 100 g^{-1} (PV). Perfusion pressures were measured with Gould P23 pressure transducers on side-arms of the perfusion circuit, and recordings made on a Grass 79D polygraph. Bile was collected for the duration of perfusion. No evidence of oedema was observed in any liver.

After an equilibration period of 5–10 min, the tone of the preparation was raised by adding to the perfusate noradrenaline to a final concentration of 10^{-5} M.

Drug administration

Adenosine (hemisulphate), NECA, **R**-PIA, S-PIA, acetylcholine chloride, noradrenaline bitartrate and 8-phenyltheophylline (8-PT) were obtained from Sigma. CGS 21680C was obtained from CIBA-Geigy Corporation, Summit, New Jersey, U.S.A. Adenosine, NECA, CGS 21680C and acetylcholine were dissolved in distilled water; **R**- and S-PIA were dissolved in a 1:1 mixture of dimethyl formamide (DMF) and methanol; noradrenaline was made up as a 10 mM stock solution in 0.1 mM ascorbic acid (to prevent oxidation); 8-PT was made up in a mixture of methanol and 1 M NaOH (80%:20%).

Adenosine and its analogues, in the dose range 10^{-10}–10^{-6} mol, were injected in turn as 0.1 ml boluses into the HA, via a rubber septum in the circuit. They were administered in a regimen so that injections of a given concentration of all compounds were made over a minimum period of time. Injections of the vehicle (water or DMF/methanol) were given at the end of each experiment in order to account for any injection artefact or haemodynamic response; water caused no change in pressure other than the injection artefact, while DMF/methanol, at a concentration equivalent to the highest dose of **R**- and S-PIA only, caused a small decrease in HA pressure which was subtracted from the relevant PIA responses.

The above protocol was employed in 2 separate groups of rabbits: in Group I (6 rabbits) the effects of adenosine, NECA, **R**-PIA, and S-PIA were compared; in Group II (4 rabbits) the effects of adenosine, NECA and CGS 21680C were compared.

The P_1-purinoceptor antagonist 8-PT was then added to the perfusate to a final concentration of 10^{-5} M and after 10–15 min, HA injections of adenosine (Group I) or NECA, CGS 21680C and adenosine (Group II) were repeated over the same concentration range as before.

In order to confirm retained vasodilator competence of the HA bed following 8-PT administration, 10^{-9} mol and 10^{-8} mol acetylcholine were injected into the HA in 5 of the 10 livers, both in the presence and in the absence of 8-PT in the perfusate.

Statistics and presentation of data

Responses were recorded as changes in perfusion pressure (mmHg). Student's paired and unpaired t tests were used, as appropriate, to test the significance of differences between responses, $P < 0.05$ being taken as significant. All results are quoted as mean ± s.e.mean.

The vasodilator potency of each agent was defined by the PD_2, the negative logarithm of the number of mol of drug required to elicit a half-maximal response.

Results

Perfusion indices

Group I (NECA, adenosine, **R**-PIA, S-PIA). Basal perfusion pressures in the HA and PV were 102 ± 11 mmHg and 12 ± 1 mmHg respectively. Pressures in the HA and PV increased to 148 ± 8 mmHg and 15 ± 2 mmHg respectively following the addition of noradrenaline to the perfusate. The total volume of bile collected was 12.7 ± 2.8 ml over the 145 ± 8 min perfusion period, approximating to an hourly mean output of 5.3 ml.

Group II (NECA, CGS 21680C, adenosine). Basal perfusion pressures in the HA and PV were 75 ± 22 mmHg and 8 ± 2 mmHg respectively. Pressures in the HA and PV increased to 148 ± 19 mmHg and 14 ± 3 mmHg respectively following the addition of noradrenaline to the perfusate. The total volume of bile collected was 9.8 ± 1.7 ml over the 155 ± 5 min perfusion period, equivalent to an hourly mean output of 3.8 ml.

Dose-response to adenosine and analogues

Group I (NECA, adenosine, R-PIA, S-PIA). Bolus injections of NECA, adenosine, **R**- and S-PIA produced dose-dependent vasodilator responses in the HA (Figure 1). Dose-response curves for the 4 agonists are illustrated in Figure 2a. The maximum response produced by NECA and adenosine was similar, but the PD_2 for each differed significantly (8.6 ± 0.2 and 7.6 ± 0.2 respectively; $P = 0.004$). The PD_2s for **R**- and S-PIA were estimated as 7.0 and 6.5 respectively; more accurate calculations could not be made because maximum responses were not achieved due to the limited solubility of these agents. The rank order of vasodilator potency was therefore: NECA > adenosine > **R**-PIA > S-PIA; their potency, expressed relative to the PD_2 of adenosine, was in the approximate ratio 10.0:1.0:0.25:0.08.

Group II (NECA, CGS 21680C, adenosine). Dose-response curves for NECA, CGS 21680C and adenosine are illustrated in Figure 3a; each drug produced a similar maximum response, though this was about 5 mmHg less than that in Group I. The PD_2 for each drug differed (8.5 ± 0.1, 8.1 ± 0.2 and 7.6 ± 0.1 for NECA, CGS 21680C and adenosine respectively). There was a statistically significant difference between the PD_2s of NECA and adenosine ($P = 0.002$) and of CGS 21680C and adenosine ($P = 0.05$), but not between those of NECA and CGS 21680C ($P = 0.08$). The rank order of agonist potency was thus: NECA > CGS 21680C > adenosine; their potency, expressed relative to the PD_2 of adenosine, was in the ratio 7.9 : 3.2 : 1.0.

Effect of 8-phenyltheophylline

Group I (NECA, adenosine, **R**-PIA, S-PIA). 8-PT attenuated responses to adenosine, as indicated by a shift to the right in

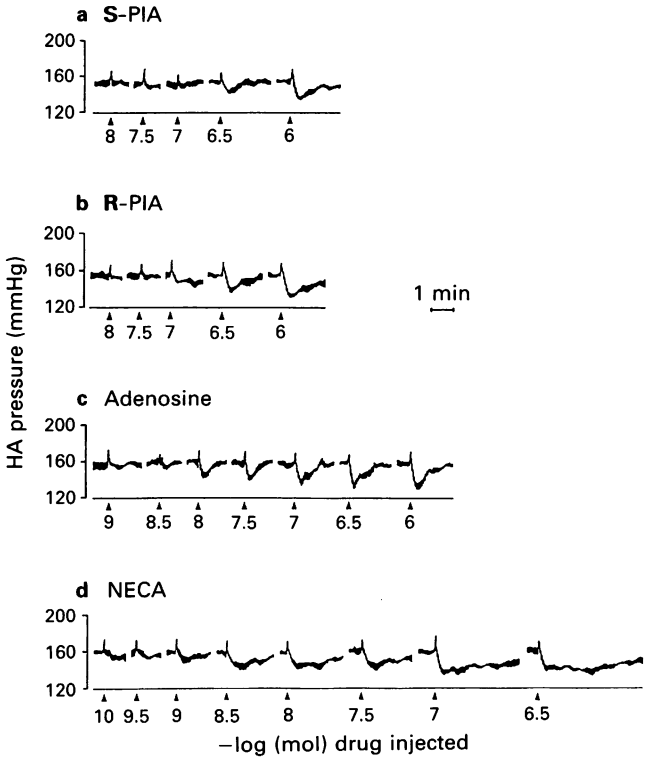

Figure 1 Vasodilator responses to (a) S-N^6-phenyl-isopropyl-adenosine (S-PIA), (b) R-PIA, (c) adenosine and (d) 5'-N-ethyl-carboxamido-adenosine (NECA) in the hepatic arterial (HA) vascular bed of an isolated, dual-perfused rabbit liver (0.1 ml of each agent at the doses indicated), showing the relative potency of the four agonists. The spike prior to response is an injection artefact.

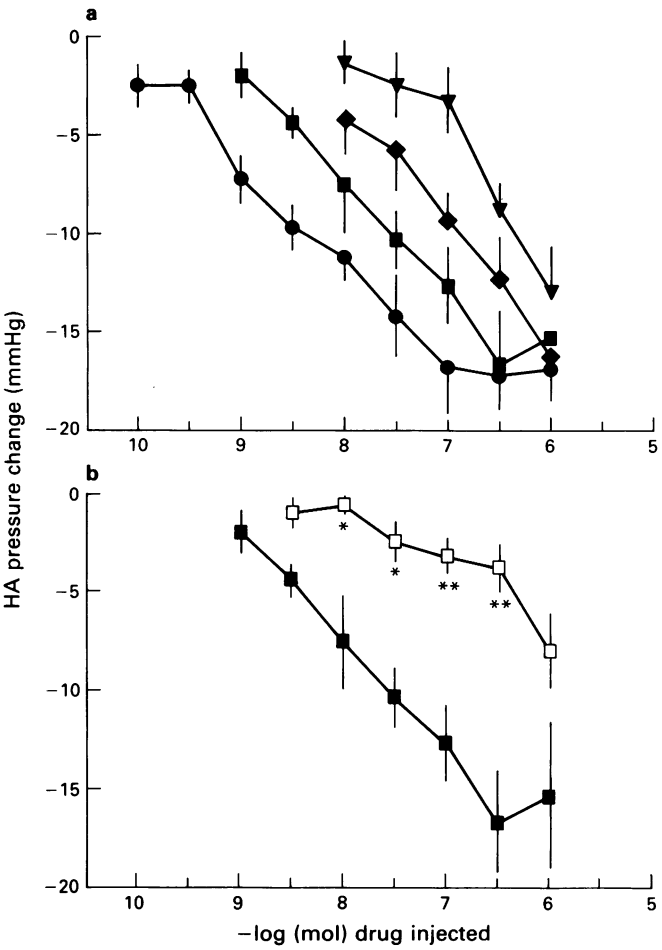

Figure 2 (a) Hepatic arterial (HA) vasodilator response to increasing doses of 5'-N-ethyl-carboxamido-adenosine (NECA) (●), adenosine (■), R-N^6-phenyl-isopropyl-adenosine (R-PIA) (◆) and S-PIA (▼) in the isolated, dual-perfused rabbit liver (Group I). (b) Hepatic arterial (HA) vasodilator response to increasing doses of adenosine before (■) and during (□) administration of 10^{-5} M 8-phenyltheophylline (8-PT) (Group I). Statistically significant differences between responses before and during 8-PT administration: * $P < 0.05$; ** $P < 0.01$.

its dose-response curve (Figure 2b). The PD_2 was 6.7 ± 0.2, which was significantly different from the normal value of 7.6 ± 0.2 ($P = 0.04$). The difference between responses to adenosine before and after the addition of 8-PT was statistically significant at the 4 doses from 10^{-8}–3×10^{-7} mol. The effect of 8-PT on responses to NECA, R- and S-PIA was not studied.

Group II (NECA, CGS 21680C, adenosine). 8-PT resulted in a substantial shift to the right of the dose-response curve of each of the three agents (Figure 3). The vasodilatation produced by each compound was significantly reduced at all doses used except the highest (10^{-6} mol; see Figure 3b).

Response to acetylcholine

Acetylcholine (10^{-9} and 10^{-8} mol) decreased HA pressure by 12.8 ± 1.2 and 17.7 ± 3.7 mmHg respectively before the addition of 8-PT to the perfusate. After the addition of 8-PT to the perfusate, the corresponding pressure decreases were almost identical (12.2 ± 1.3 and 17.6 ± 2.7 mmHg).

Discussion

The order of potency we have demonstrated for adenosine and its analogues to produce vasodilatation of the HA vascular bed is characteristic of P_1-purinoceptors of the A_2 sub-type (Collis, 1985; Burnstock & Kennedy, 1986; Williams, 1987). Moreover, the potency ratio of the 4 agents employed in Group I experiments is within the range expected from previously published studies on the A_2 receptor (Collis & Brown, 1983; Collis, 1985; 1989); for example, we have shown NECA to be 40 times more potent than R-PIA in dilating the HA, while R-PIA had only three times the potency of S-PIA. In addition, 8-PT resulted in a significant inhibition of the dilator response to adenosine, providing evidence for the existence of a population of P_1-purinoceptors in the HA vascular bed. We therefore conclude that A_2 receptors mediate adenosine-induced dilatation of the HA bed in the rabbit liver.

This conclusion was reinforced by the results from Group II, which showed a similar relative potency between adenosine and NECA as found in Group I and an inhibition by 8-PT of all three agonists used. In addition, the high-affinity A_2 agonist CGS 21680C stimulated HA vasodilatation with a potency three times greater than adenosine and one half (though not significantly different from) that of NECA. CGS 21680C is 140 fold more selective for A_2 receptors than for A_1 receptors, and has been reported to be equipotent with NECA in vasodilating the coronary artery (Hutchison *et al.*, 1989). The lesser maximal responses to adenosine and NECA in Group II compared to those in Group I cannot be explained by differences in perfusion characteristics or any other known factor; however, the maximal responses obtained from each group were internally consistent, and therefore the existence of a difference between the groups does not invalidate any of the conclusions drawn. Our use of acetylcholine before and after application of 8-PT demonstrated the retention of non-purinergic relaxation of HA smooth muscle in both groups.

We are not aware of any previous paper demonstrating the existence of A_2 purinoceptors in the HA vascular bed. However, Schütz *et al.* (1982) found evidence for A_2 receptors

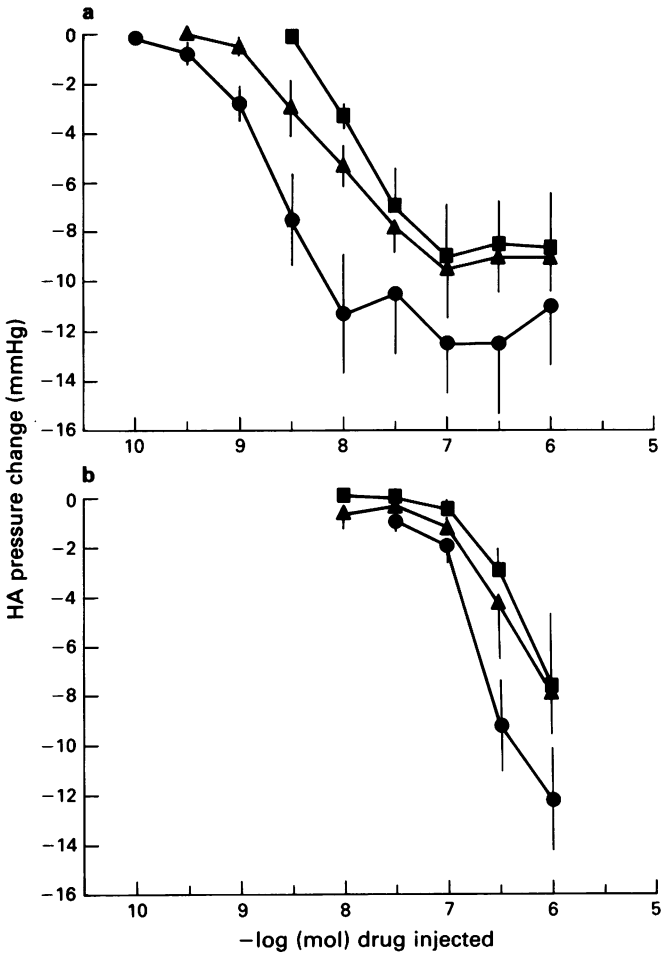

Figure 3 (a) Hepatic arterial (HA) vasodilator response to increasing doses of 5'-N-ethyl-carboxamido-adenosine (NECA) (●), CGS 21680C (▲) and adenosine (■) in the isolated, dual-perfused rabbit liver (Group II). (b) Hepatic arterial (HA) vasodilator response to increasing doses of NECA (●), CGS 21680C (▲) and adenosine (■) during administration of 10^{-5} M 8-phenyltheophylline (8-PT) (Group II). Statistically significant differences ($P < 0.05$) were observed between responses before and during 8-PT administration (cf. Figure 3a) at the following log doses of all three agonists: −8, −7.5, −7 and −6.5 mol.

on rat liver membranes, and Buxton *et al.* (1987) showed that the PV of the perfused rat liver contains A_2 receptors, which, interestingly, they found to mediate vasoconstriction due to adenosine. A_2 receptors have been demonstrated in several other sites in the body (Burnstock & Kennedy, 1986); in the cardiovascular system their presence has been demonstrated in the smooth muscle of the coronary and cerebral circulations as well as in the aorta (Kusachi *et al.*, 1983; Edvinsson & Fredholm, 1983; Collis & Brown, 1983). Further work from our own laboratory using the present experimental model has established the existence of P_2-purinoceptor sub-types in the HA (Ralevic *et al.*, 1991), while Brizzolara & Burnstock (1991) have found both P_1 and P_2 receptors to be present in the common hepatic artery of the rabbit.

It is important to acknowledge that agonist potency alone cannot provide definitive evidence for the differentiation of A_1 and A_2 receptor populations in any vascular bed (Collis, 1985; Paton, 1988). Potency of an agonist is determined by its affinity for the receptor (its ability to bind to the receptor site) and by the efficacy with which it evokes the response (its ability to activate the receptor) as well as by tissue variables such as the number of receptors and the efficiency of coupling of the stimulus to the response (Collis, 1985; 1989). In addition, systems which remove and degrade adenosine may influence the potency of the analogues used to classify receptor subtypes (Collis, 1985). However, the advantage of using agents such as NECA and PIA is that these are relatively resistant to uptake and degradation, and although this resistance was not proven in the current study the considerably longer duration of the response to NECA than to adenosine supports this idea (see Figure 1). NECA was substantially more potent at eliciting vasodilatation than R- or S-PIA, consistent with an action at the A_2 receptor. Furthermore, the absence of stereoselectivity displayed for PIA, and the results with the selective A_2 agonist CGS 21680C are also strongly indicative of the presence of A_2 receptors in the rabbit HA vasculature. Recently, several selective A_2 antagonists have been developed (Bruns & Coughenour, 1987; Ghai *et al.*, 1987; Bruns *et al.*, 1988), which should prove valuable in the identification of A_2-purinoceptors as a complement to studies using agonist potency orders.

Adenosine can cause vasodilatation indirectly by inhibiting the release of noradrenaline from adrenergic nerve terminals, through activation of pre-junctional P_1-purinoceptors (Burnstock & Kennedy, 1986). Although these are normally of the A_1 sub-type, in the rat PV they appear to be of the A_2 sub-type (Kennedy & Burnstock, 1984). The vasodilatation evoked by adenosine in the current *in vitro* preparation is unlikely to have taken place by such a pre-junctional mechanism, because of the lack of sympathetic tone. *In vivo*, however, pre-junctional P_1-purinoceptor regulation of HA blood flow is more likely to take place; in addition, ecto-enzymatic breakdown of ATP co-released with noradrenaline at the nerve terminal (Brizzolara & Burnstock, 1990) can provide a local source of adenosine for such a mechanism.

Recent studies have suggested an important role for adenosine in the physiological control of HA blood flow (Lautt, 1985; 1988), notably in the vasodilator response of the HA to PV flow interruption (the HA 'buffer response') (Lautt *et al.*, 1985; Lautt & Legare, 1985; Mathie & Alexander, 1990). Our current evidence supporting the existence of A_2-purinoceptors in the HA bed indicates a specific mechanism by which adenosine-induced vasodilatation of the HA may take place, and reinforces the probable importance of purinergic vasoactive mechanisms in the hepatic circulation.

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Characterization of P_{2X}- and P_{2Y}-purinoceptors in the rabbit hepatic arterial vasculature

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1 Responses to adenosine 5'-triphosphate (ATP) and its agonists were studied in the isolated liver of the rabbit dually perfused through the hepatic artery and the portal vein.

2 In the hepatic arterial vascular bed at basal tone, ATP and its agonists elicited vasoconstrictor responses with the rank order of potency α,β-methylene ATP > 2-methylthio ATP > ATP, consistent with their action at the P_{2X}-purinoceptor.

3 When tone was raised with noradrenaline (10^{-5} M), vasodilator responses were produced with ATP and 2-methylthio ATP; α,β-methylene ATP produced only further constriction. The rank order of vasodilator potency was 2-methylthio ATP > ATP $\gg$ α,β-methylene ATP, consistent with their action at the P_{2Y}-purinoceptor.

4 Methylene blue (10^{-5} M) antagonized vasodilator responses to acetylcholine and ATP, but not those to adenosine or sodium nitroprusside. Addition of 8-phenyltheophylline (10^{-5} M) antagonized responses to adenosine but not those to sodium nitroprusside. Responses to ATP remaining after antagonism with methylene blue were not further antagonized by 8-phenyltheophylline.

5 These results present evidence for discrete P_{2X}- and P_{2Y}-purinoceptors in the rabbit hepatic arterial bed which mediate vasoconstrictor and vasodilator responses respectively.

6 Vasodilatation produced by ATP was entirely due to direct action at the P_{2Y}-purinoceptor, and not at a P_1-purinoceptor following breakdown to adenosine. The antagonism of these responses by methylene blue is consistent with the view that vasodilatation by ATP takes place largely via endothelial P_{2Y}-purinoceptors that lead to release of endothelium-derived relaxing factor. However, we cannot exclude the possibility that P_{2Y}-purinoceptors located on the vascular smooth muscle play a contributory role in ATP-induced vasodilatation.

Keywords: Hepatic artery; portal vein; adenosine 5'-triphosphate (ATP); purinoceptors

Introduction

The liver obtains its blood supply from both the hepatic artery (HA) and the hepatic portal vein (PV), but it is only the former which contributes significantly to the regulation of hepatic blood flow. Nevertheless, there is an intimate relationship between the two systems, as shown by the ability of the HA to produce reciprocal compensatory flow changes in response to changes in PV flow (termed the 'buffer response') (Lautt, 1981; Richardson, 1982; Lautt & Greenway, 1987). While adenosine has been strongly implicated as a mediator of the buffer response (Lautt *et al.*, 1985; Lautt & Legare, 1985), there is evidence that this is not the only mediator (Mathie & Alexander, 1990), and it is possible that adenosine 5'-triphosphate (ATP) has an important role. A recent study has also implicated ATP in extrinsic regulation of the liver, as it was shown that ATP acts as a cotransmitter with noradrenaline in sympathetic nerves of the rabbit isolated hepatic artery (Brizzolara & Burnstock, 1990).

Consistent with the putative role of adenosine and ATP as mediators of hepatic blood flow regulation, these purines have been shown to have potent pharmacological effects on the rabbit isolated hepatic artery (Brizzolara & Burnstock, 1990), in isolated perfused livers of the rat (Lee & Filkins, 1988) and rabbit (Mathie *et al.*, 1991a), and *in vivo* in the livers of the cat (Lautt *et al.*, 1985) and dog (Mathie & Alexander, 1990). Adenosine and ATP elicit their effects on the vasculature by activation of P_1- and P_2-purinoceptors respectively. While the vasodilator effects of adenosine on the rabbit HA vascular bed have recently been identified as being mediated by the A_2 subtype of P_1-purinoceptor (Mathie *et al.*, 1991a), no such studies have been performed to characterize the HA P_2-purinoceptor.

According to the classification proposed by Burnstock & Kennedy (1985) P_2-purinoceptors may be subdivided into types P_{2X} and P_{2Y} based principally on the rank order of potency of ATP and its structural analogues. At the P_{2X}-purinoceptor agonist potency is α,β-methylene ATP (α,β-meATP), β,τ-methylene ATP (β,τ-meATP) > ATP = 2-methylthio ATP (2me.S.ATP), whilst at the P_{2Y}-purinoceptor the agonist potency order is 2me.S.ATP > ATP > α,β-meATP, β,τ-meATP. In general, P_{2X}-purinoceptors mediate vasoconstriction and are located on the vascular smooth muscle; P_{2Y}-purinoceptors mediate vasodilatation and are present on endothelial cells (Kennedy *et al.*, 1985; Houston *et al.*, 1987; Ralevic & Burnstock, 1988) causing relaxation through the production of endothelium-derived relaxing factor (EDRF) (Furchgott & Zawadski, 1980), although in some vessels they are also located in the medial muscle coat (Kennedy & Burnstock, 1985; Mathieson & Burnstock, 1985).

The present study was carried out to characterize the P_2-purinoceptor subtypes present in the rabbit HA vascular bed. A dual-perfused liver preparation was used (Alexander *et al.*, 1991); this model, which has been successfully used to characterize HA adenosine receptors (Mathie *et al.*, 1991a), enabled arterial responses to be elicited in the presence of normal PV flow. In the pathway of purine catabolism ATP is broken down to adenosine by degradative enzymes. To determine to what extent adenosine, formed from ATP, was contributing to the effects of ATP, and to investigate the mechanism of action of the vasodilator response, the adenosine receptor antagonist 8-phenyltheophylline (8-PT) and

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methylene blue, an inhibitor of guanylate cyclase and of endothelium-derived relaxing factor (EDRF)-mediated responses, were used.

Methods

Six male New Zealand White rabbits weighing 2.3–3.3 kg (mean 2.7 kg) were used in the study. The operative procedures have been described in detail previously (Alexander *et al.*, 1991). Briefly, rabbits were initially sedated with fentanyl/fluanisone ('Hypnorm' 0.25 ml kg^{-1}, i.p.) and then anaesthetized with a mixture of 1 part Hypnorm (0.3 ml kg^{-1}) and 1 part midazolam ('Hypnovel', 1.5 mg kg^{-1}) in 2 parts water (total 1.20 ml kg^{-1}, i.p.) (Flecknell, 1987). A marginal ear vein was cannulated for subsequent i.v. administration of the Hypnorm/Hypnovel mixture (0.25–0.5 ml kg^{-1} h^{-1}).

A mid-line abdominal incision was made and the common bile duct exposed and cannulated. The gastroduodenal artery was then cannulated (Portex 3FG), and the catheter advanced to the junction of the common and proper hepatic arteries. The common HA was then ligated and severed, and 4–5 ml saline infused into the catheter to prevent blood coagulation. Following administration of heparin (100 units kg^{-1}, i.v.) the PV was cannulated and 40–50 ml saline infused into the catheter to prevent accumulation of blood in the intrahepatic PV system. The liver was immediately excised, weighed and placed into an organ bath in the perfusion system.

Liver perfusion

The liver was perfused with Krebs-Bülbring solution containing (mM): NaCl 133, KCl 4.7, NaH_2PO_4 1.35, $NaHCO_3$ 16.3, $MgSO_4$ 0.61, glucose 7.8 and $CaCl_2$ 2.52, oxygenated (95% O_2/5% CO_2) and maintained at 37°C. Perfusion was via the HA and PV at mean flows of 24 ml min^{-1} 100 g^{-1} and 76 ml min^{-1} 100 g^{-1} respectively. Perfusion pressure was measured with Gould P23 pressure transducers on side arms of the perfusion cannulae, and recordings made on a Grass model 79D polygraph. Bile was collected throughout the experiment.

Drugs

After a 10 min equilibration period, drugs were administered as 100 μl bolus injections into the HA. Vasoconstrictor responses were examined in the preparation at basal tone. Vasodilator responses were examined in the raised tone preparation, with tone raised by the addition of 10^{-5} M NA to the perfusate. In the basal tone preparation drugs were applied in the order ATP, 2me-S.ATP, α,β-meATP to avoid desensitization by the potent P_{2X} agonist α,β-meATP. In the raised tone preparation responses to drugs were obtained in the order: ATP, 2me-S.ATP, acetylcholine (ACh), sodium nitroprusside (SNP), adenosine. The effects of α,β-meATP on the raised-tone preparation were examined in three livers. Responses to ATP, ACh, SNP and adenosine were repeated in the raised tone preparation 10–15 min after the addition of methylene blue to the perfusate to a concentration of 10^{-5} M. The maximisation of the antagonistic effect of methylene blue over this time was confirmed with consecutive applications of a fixed dose of ACh. Further dose-response curves for ATP, SNP and adenosine were obtained 10–15 min following addition of 10^{-5} M 8-PT to the perfusate (still in the presence of methylene blue).

Adenosine 5'-triphosphate (sodium salt), α,β-methylene ATP (lithium salt), adenosine (hemisulphate), acetylcholine chloride, noradrenaline bitartrate, methylene blue, 8-phenyltheophylline and sodium nitroprusside were obtained from Sigma. 2-Methylthio ATP (sodium salt) was obtained from Research Biochemicals Inc. (U.S.A.). All drugs were made up in distilled water except for NA which was made up as a 10 mM stock solution in 0.1 mM ascorbic acid (to prevent oxidation), and 8-phenyltheophylline which was made up in a mixture of 80% methanol: 20% 1 M NaOH.

Statistical analysis

Vasoconstrictor and vasodilator responses were recorded as changes in perfusion pressure (mmHg). All results are quoted as mean (± s.e.mean). Statistical significance was evaluated by the Student's t test ($P < 0.05$ was taken to be significant).

Results

The basal perfusion pressure of the preparation remained constant for the duration of the experiment: perfusion pressures following the addition of NA were 122.5 ± 9.1 mmHg for the HA and 16.3 ± 2.4 mmHg for the PV ($n = 6$). Total bile volume collected was 7.6 ± 3.3 ml over 150 ± 4 min of perfusion.

The effect of ATP and its analogues on the hepatic arterial bed at basal perfusion pressure

Bolus injections of ATP, 2me.S.ATP and α,β-meATP produced dose-dependent, vasoconstrictor responses (Figure 1). The order of potency of these agents in eliciting constriction was α,β-meATP > 2me.S.ATP > ATP (Figure 2a). pD_2 values were 8.50 ± 0.11, 7.68 ± 0.21 and 6.89 ± 0.13 respectively ($n = 6$). Despite the recovery period allowed between doses (dose cycle 4 min) α,β-meATP showed a consistent tendency to desensitize at doses subsequent to application of a bolus injection of 3×10^{-8} mol (see Figure 1c).

The effect of ATP and its analogues on the preconstricted hepatic arterial bed of the rabbit

ATP and 2me.S.ATP produced vasodilator responses in the HA bed in which tone had been raised by continuous per-

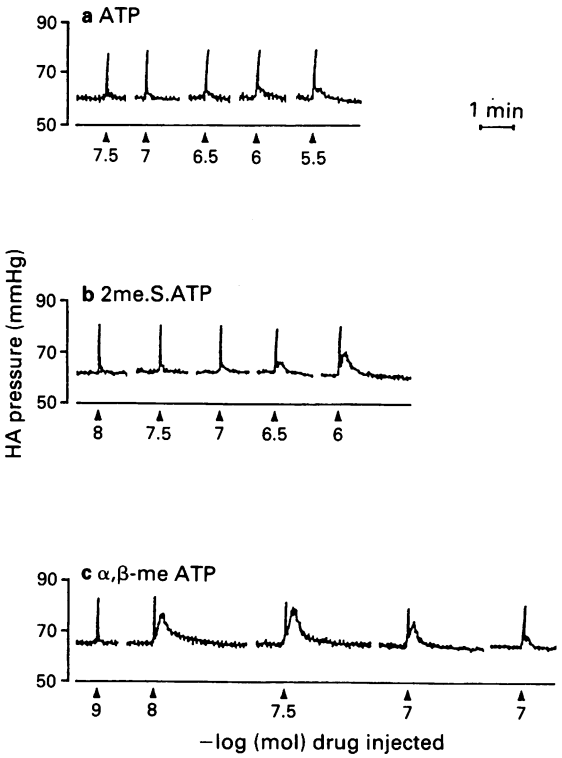

Figure 1 Vasoconstrictor responses of the isolated, dual-perfused liver preparation of the rabbit (at basal tone) to increasing doses (0.1 ml injections) of: (a) adenosine 5'-triphosphate (ATP), (b) 2-methylthio ATP (2me.S.ATP) and (c) α,β-methylene ATP (α,β-meATP). The spike prior to the response is an injection artefact.

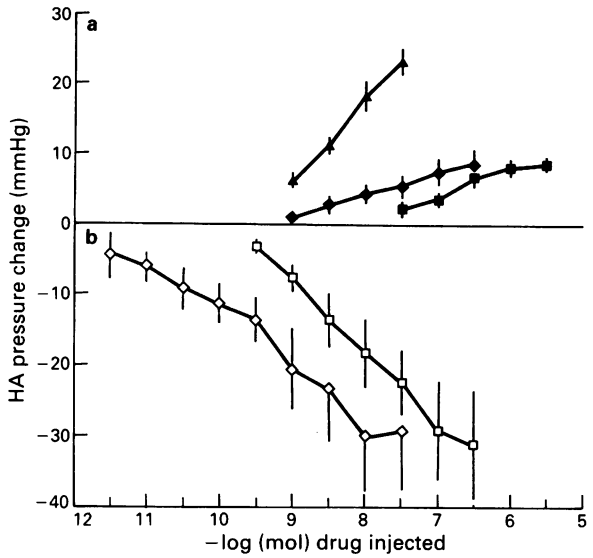

Figure 2 Dose-response curves showing the effects of adenosine 5'-triphosphate (ATP) and its analogues on the rabbit hepatic arterial bed at (a) basal tone and (b) with tone raised by the addition of noradrenaline (10^{-5} M) to the perfusate. (a) Vasoconstrictor effects of ATP (■), 2-methylthio ATP (2me.S.ATP, ◆) and α,β-methylene ATP (α,β-meATP, ▲). (b) Vasodilator effects of ATP (□) and 2me.S.ATP (◇). α,β-me ATP elicits only further contraction in the raised-tone preparation.

fusion with 10^{-5} M NA (Figures 2b and 3). 2me.S.ATP was 10 times more potent in eliciting vasodilatation than ATP (pD_2 values 9.36 ± 0.28 and 8.31 ± 0.08 respectively, $n = 6$).

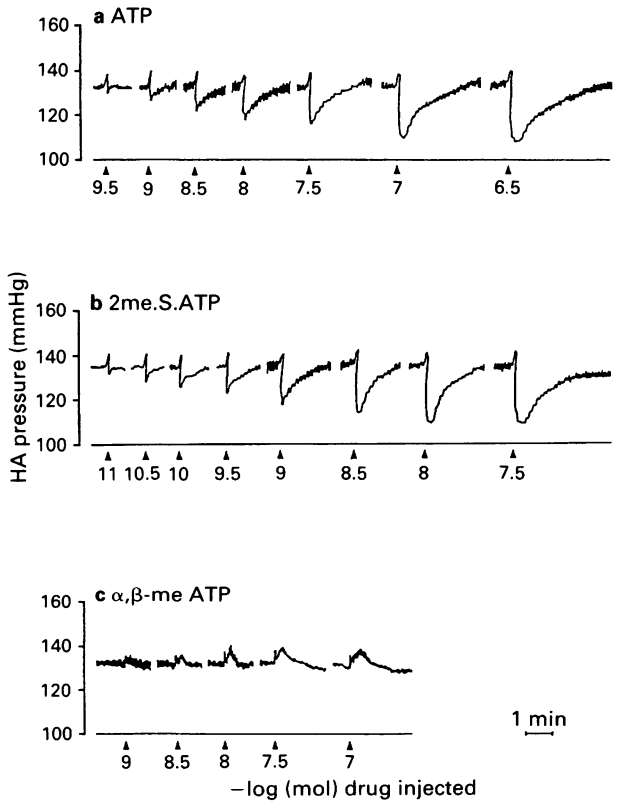

Figure 3 Responses of the rabbit hepatic arterial bed to doses (0.1 ml injections) of adenosine 5'-triphosphate (ATP) and its analogues after the tone of the preparation had been raised with noradrenaline (10^{-5} M). Vasodilator responses to (a) ATP, (b) 2-methylthio ATP (2me-S.ATP); vasoconstrictor responses to (c) α,β-methylene ATP (α, β-meATP).

Maximum dilator responses were not significantly different. α,β-meATP produced only further contraction (Figure 3c).

The effect of adenosine, acetylcholine and sodium nitroprusside on the raised tone hepatic arterial bed

Adenosine (Figure 4), ACh (Figure 5) and SNP (results not illustrated) elicited dose-dependent relaxations in the raised-tone preparation. pD_2 values for adenosine and ACh were 7.19 ± 0.17 and 9.40 ± 0.21 respectively ($n = 6$). A pD_2 value for SNP could not be determined as maximum relaxation was not attained. Comparison of the pD_2 values showed that ATP was about 13 times more potent than adenosine in eliciting vasodilatation.

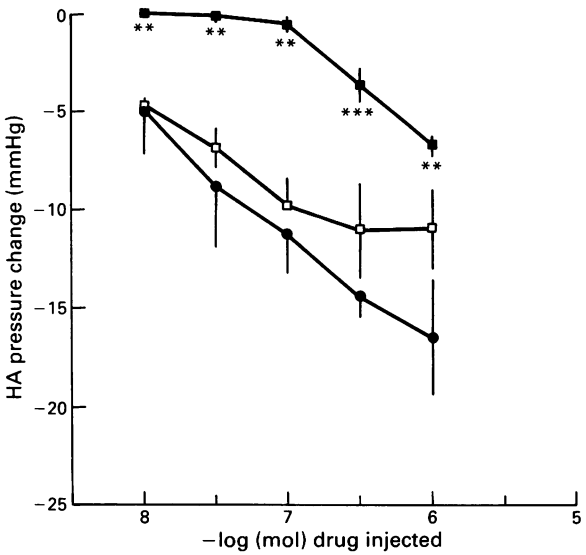

Figure 4 Dose-response curves showing vasodilator responses of the rabbit hepatic arterial bed to adenosine (●), and the lack of effect on these responses by methylene blue (meB, 10^{-5} M) (□). Responses to adenosine were antagonized by 8-phenyltheophylline (8-PT) in the same preparation (meB, 8-PT; ■). Statistical significance is denoted by: ** $P < 0.01$; *** $P < 0.001$.

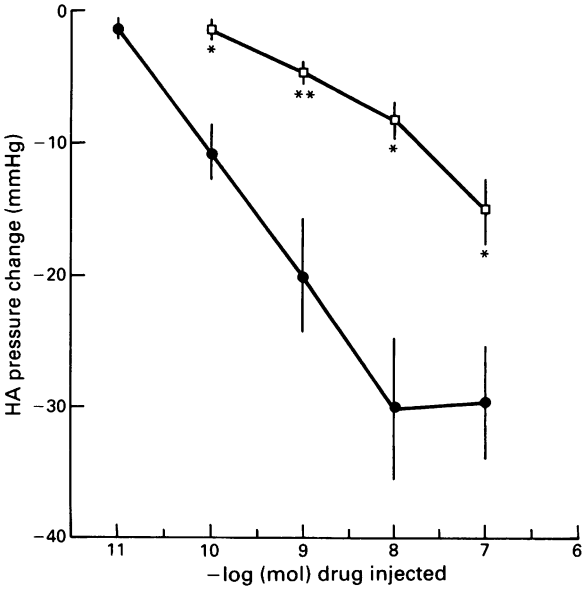

Figure 5 Dose-response curves showing vasodilator responses of the rabbit hepatic arterial bed to acetylcholine (■), and the antagonism of these responses by methylene blue (□). Statistical significance is denoted by * $P < 0.05$; ** $P < 0.01$.

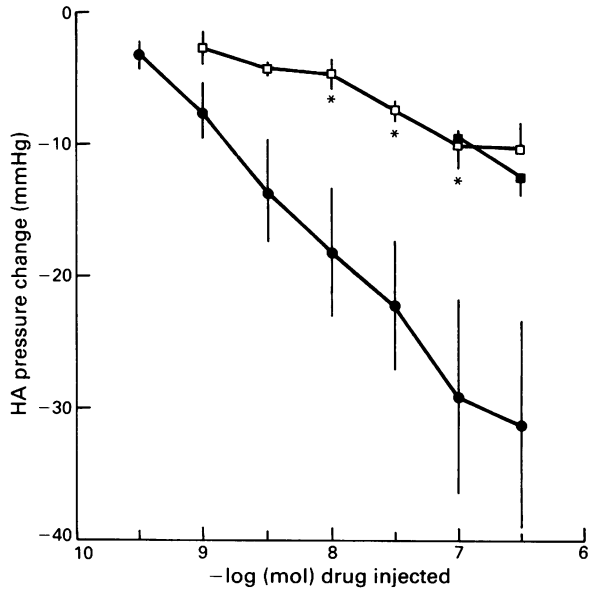

Figure 6 Dose-response curves showing vasodilator responses of the rabbit hepatic arterial bed to adenosine 5'-triphosphate (ATP) (●), and the antagonistic effect of methylene blue (□). These responses were not further antagonized by the addition of 8-phenyltheophylline (10^{-5} M) to the perfusate (■). Statistical significance is denoted by * $P < 0.05$.

The effect of methylene blue

There was no significant change in tone of the preparation following the addition of methylene blue to the perfusate (final concentration; 10^{-5} M). Methylene blue greatly antagonized responses to ACh (Figure 5), resulting in a shift to the right of the dose-response curve. Responses to ATP were also greatly reduced; the dose-response curve was shifted to the right and there was a decrease in the maximum response, indicative of non-competitive inhibition (pD_2 value 8.02 ± 0.18, $n = 6$) (Figure 6). There was no significant effect of methylene blue on responses to adenosine (Figure 4) or to SNP.

The effect of 8-phenyltheophylline

There was no significant change in the tone of the preparation in the presence of 10^{-5} M 8-PT. 8-PT greatly reduced vasodilator responses to adenosine (Figure 4). This was significant at all concentrations of adenosine tested ($P < 0.01$). It had no effect on responses to ATP remaining in the presence of methylene blue (Figure 6). Responses to SNP were also unaffected.

Discussion

The results presented in this study demonstrate the presence of two discrete P_2-purinoceptor subtypes in the HA bed of the rabbit. From the rank order of potency of ATP and its analogues, vasoconstrictor responses were shown to be mediated by the P_{2X}-purinoceptor, while vasodilatation was elicited via the P_{2Y}-purinoceptor. The mechanism of action of the P_{2Y}-mediated response was investigated by use of methylene blue, and found to be more consistent with a receptor located on endothelial cells than on the vascular smooth muscle. A direct action of ATP at P_{2Y}-purinoceptors was distinguished from the possibility of an indirect effect at P_1 receptors (following ectoenzymatic breakdown to adenosine) by the use of 8-PT.

Although portal venous blood flow accounts for about two-thirds of total liver flow it is almost exclusively within the HA vasculature that intrahepatic regulation of blood flow takes place (see Richardson, 1982; Lautt & Greenway, 1987). The ability of the HA bed to regulate liver blood flow is uniquely demonstrated by the 'buffer response' (Lautt, 1981), the ability of the HA bed to produce compensatory changes in resistance in response to changes in PV flow or pressure (Richardson, 1982; Mathie & Blumgart, 1983; Lautt *et al.*, 1985; Lautt & Greenway, 1986). There is much evidence to support the involvement of adenosine as an important mediator of the buffer response following local build up to vasoactive concentrations (Lautt & Legare, 1985; Lautt, 1988; Mathie & Alexander, 1990) and the recent demonstration of vasodilator A_2 receptors in the hepatic vasculature (Mathie *et al.*, 1991a) would also tend to support this. However, it increasingly appears that adenosine is not the sole mediator of HA dilatation (Mathie & Alexander, 1990). It has been implied that ATP, which also has potent effects on the cardiovascular system (see Burnstock & Kennedy, 1986), may have a role in the control of HA resistance (Mathie & Alexander, 1990).

In the present study, in the HA vascular bed at basal tone, ATP and its analogues elicited dose-dependent vasoconstrictor responses. The rank order of potency of these agents was α,β-meATP > 2me.S.ATP > ATP which is in accordance with their action at the P_{2X}-purinoceptor, according to the original classification by Burnstock & Kennedy (1985). In the raised tone preparation the potency order was 2me.S.ATP > ATP, which is consistent with their action at the P_{2Y}-purinoceptor; α,β-meATP produced only further contraction. In many vessels α,β-meATP has been used as a selective desensitizing agent to characterize the P_{2X}-purinoceptor further; however, this proved impractical in our preparation since the relatively high flow rates would have required large amounts of this drug to be used. Even so, there was a tendency for autodesensitization to occur following repeated application of α,β-meATP.

A number of substances, including ACh and ATP elicit vascular relaxation following activation of specific receptors on the endothelial cell surface with subsequent formation of EDRF (Furchgott & Zawadski, 1980; Peach *et al.*, 1985), now believed to be nitric oxide (Palmer *et al.*, 1987). EDRF (and other nitrovasodilators) elicit relaxation by stimulating guanylate cyclase in vascular smooth muscle cells. Hence, a final step in the chain of events leading to relaxation following activation of endothelial P_{2Y}-purinoceptors is the formation of guanosine 3':5'-cyclic monophosphate (cyclic GMP). This is in contrast to the relaxant effects of adenosine which, in general, acts on A_2 receptors on the smooth muscle to stimulate production of adenosine 3':5'-cyclic monophosphate (Burnstock, 1978; Burnstock & Kennedy, 1986). Endothelial prostacyclin production may contribute to ATP-induced vasodilatation in some vascular beds (Gordon, 1986); hence in the absence of cyclo-oxygenase inhibitors this cannot be discounted as contributing to the mechanism of ATP-induced vasodilatation in our preparation.

Methylene blue produced a significant antagonism of relaxant responses to the endothelium-dependent vasodilator ACh, and to ATP, but did not affect those to adenosine. While methylene blue is frequently regarded as exerting its effects chiefly through a mechanism involving antagonism of guanylate cyclase (Martin *et al.*, 1985), responses to the endothelium-independent nitrovasodilator, SNP were not antagonized by methylene blue. A similar lack of inhibition of SNP (Watanabe *et al.*, 1988) and of the nitrovasodilator glyceryl trinitrate (Martin *et al.*, 1985), but no lack of inhibition of ACh or EDRF (Wolin *et al.*, 1990), by methylene blue has been observed, and the possibility that EDRF is directly affected by methylene blue has been discussed (Martin *et al.*, 1985; Watanabe *et al.*, 1988; Wolin *et al.*, 1990). In all of these cases methylene blue appears to antagonize EDRF-mediated responses in preference to those mediated by agents acting directly on the vascular smooth muscle. The results of the present study are consistent with a vasodilator action of ATP at least partly mediated by EDRF (suggestive of endothelial P_{2Y}-purinoceptors), while that of adenosine is independent of

both endothelium and cyclic GMP. Results obtained by us using the L-arginine to nitric oxide pathway inhibitors, N-monomethyl-L-arginine (Rees *et al.*, 1989) and N-nitro-L-arginine methyl ester (Moore *et al.*, 1990), which are the subject of a separate paper (Mathie *et al.*, 1991b), would tend to support this. However, since methylene blue does not completely abolish P_{2Y}-mediated vasodilatation, we cannot exclude the possibility that in some part of the HA bed, P_{2Y} receptors are located on the vascular smooth muscle; this has been shown to be the case in the rabbit isolated hepatic artery (Brizzolara & Burnstock, 1991). In addition, P_{2Y}-purinoceptors have been identified on rabbit and guinea-pig hepatocytes (Keppens *et al.*, 1990).

Vasodilator responses to ATP remaining in the presence of methylene blue were not further antagonized by the adenosine antagonist 8-PT while responses to adenosine were greatly reduced, thus eliminating the possibility that adenosine was partially responsible for the effects of ATP. The integrity of the preparation over the course of the experiment was not impaired, as indicated by maintained responses to SNP. Since ATP can produce vasoconstriction and vasodilatation at P_{2x}- and P_{2y}-purinoceptors respectively, its net effect will be the resultant of these responses. Pharmacological manipulation of tone is used to favour predominantly vasoconstrictor (low tone) or vasodilator (high tone) responses. Since this reduces to a minimum the antagonistic effect of the opposing responses it is unlikely that these will significantly affect agonist potency order.

The present study which identifies P_2 purinoceptor subtypes in the HA vasculature, and the fact that ATP was shown to be about 13 times more potent than adenosine in eliciting vasodilatation, supports earlier suggestions of a role for ATP in the regulation of HA vascular tone (Brizzolara & Burnstock, 1990; Mathie & Alexander, 1990). Its role is essentially twofold since, in addition to causing vasoconstriction via smooth muscle P_{2X}-purinoceptors following release as a cotransmitter from sympathetic nerves (Brizzolara & Burnstock, 1990), it can elicit relaxation via endothelial/smooth muscle P_{2Y}-purinoceptors, predominantly following local release. There is evidence that the source of the locally released ATP may be platelets, or the endothelial cells themselves (Pearson & Gordon, 1985; Burnstock, 1987). It is possible that the HA buffer response employs the same mechanism as that advanced for hypoxic relaxation where it has been proposed that the relaxation is the resultant of a relaxation initiated by ATP and maintained by adenosine (Burnstock, 1987; Mathie & Alexander, 1990).

In conclusion, this study has shown the presence of the ATP receptor subtypes P_{2X} and P_{2Y} in the rabbit HA bed, which mediate vasoconstriction and vasodilatation respectively. The relaxant effects of ATP were mediated solely through the P_{2Y}-purinoceptor and not following breakdown to adenosine. It is likely that in the rabbit HA bed, ATP produces relaxation largely via activation of an endothelial P_{2Y}-purinoceptor with subsequent production of EDRF. Characterization of subtypes of the ATP receptor in the liver, and the demonstration that their activation produces potent effects on the vasculature, has important implications for the role of ATP in the regulation of hepatic blood flow.

The support of The British Heart Foundation is gratefully acknowledged.

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Termination of digitalis-induced ventricular tachycardias by clonidine involves central α_2-adrenoceptors in cats

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1 Effects of intravenous (i.v.) and intravertebral arterial (i.a.) administration of the α_2-adrenoceptor agonist, clonidine (Clon) and its antagonist, yohimbine (Yoh, 0.5 mg kg^{-1}, i.v.; 0.05 mg kg^{-1}, i.a.), on ventricular tachycardia (VT) induced by intravenous acetylstrophanthidin (AS) were studied in cats anaesthetized with intraperitoneal chloralose.

2 AS dose-dependently produced cardiac arrhythmias including complete atrioventricular conduction block (118 ± 14 μg kg^{-1}, i.v.), junctional tachycardia (128 ± 20 μg kg^{-1}, i.v.), multiform ventricular premature beats (157 ± 21 μg kg^{-1}, i.v.) and sustained VT (220 ± 23 μg kg^{-1}, i.v.).

3 Doses of Clon (i.v.) required for termination of VT following i.v. Yoh (62.9 ± 5.2 μg kg^{-1}) or i.a. Yoh (88.5 ± 16.3 μg kg^{-1}) were higher than those for termination of VT without Yoh administration (28.3 ± 6.2 μg kg^{-1}). Doses of Clon (i.a.) required for termination of VT without or with i.a. Yoh administration were 5.8 ± 1.0 or 14.8 ± 3.7 μg kg^{-1}, respectively, and they were significantly different.

4 These experiments demonstrate that either i.v. or i.a. Yoh antagonizes the antiarrhythmic effect of Clon on AS-induced VT. Since small doses of Clon administered i.a. act predominantly on the central nervous system, we suggest that its antiarrhythmic effect is likely to be on central α_2-adrenoceptors in the central nervous system.

Keywords: Arrhythmias; α_2-adrenoceptor agonist; α_2-adrenoceptor antagonist; digitalis

Introduction

The central nervous system is considered to be involved in the arrhythmogenic effects of digitalis. Pretreatment of animals with reserpine, β-adrenoceptor blocking agents, α_1-adrenoceptor antagonists, α_2-adrenoceptor agonists, anticholinoceptor agents, ganglionic blocking agents and dopamine receptor agonists have been shown to increase the dose of digitalis required to produce cardiac arrhythmias (Lather *et al.*, 1978; Helke *et al.*, 1979).

Clonidine, an α_2-adrenoceptor agonist, has been demonstrated to suppress the cardiac arrhythmias induced by digitalis in cats (Gillis & Quest, 1972). The reduction of this suppression after spinal transection suggests that clonidine may produce an antiarrhythmic effect through a central action. Whether the α_2-receptor in the central nervous system is specifically involved was the subject of the present experiment.

Methods

Experimental preparation

Cats of either sex weighing 1.7–3.8 kg were anaesthetized with chloralose (40 mg kg^{-1}) and urethane (400 mg kg^{-1}), intraperitoneally. All cats were cannulated with endotracheal tubes and artificially ventilated with room air. The end expiratory CO_2 concentration was maintained at 3.5–4.5%. The femoral artery and vein were catheterized for blood pressure monitoring and drug administration, respectively. In some experiments, polyethylene tubes (Intramedic) were inserted into the vertebral artery for intravertebral arterial injection of drugs (Hou *et al.*, 1988). The blood pressure was monitored with a Gould P 231 D transducer connected with an amplifier. The lead II electrocardiogram (ECG) was monitored by a Gould universal preamplifier and displayed continuously on a Tektronix 922 oscilloscope. Blood pressure and ECG were recorded continuously with a Gould ES-1000 recorder.

Induction of ventricular tachycardia

Acetylstrophanthidin (AS, Sigma Chemical Co.) was dissolved in distilled water to give a concentration of 2.5 μg ml^{-1} and administered as a bolus intravenous injection (i.v.) at a dose of 20 μg kg^{-1}, followed by subsequent infusion of 2.5 μg kg^{-1} min^{-1} until a sustained ventricular tachycardia (VT) had persisted for 5 min. Thereafter, the VT was maintained for at least 25 min. Sustained VT was considered present if a wide QRS tachycardia with atrioventricular dissociation persisted for more than 1 min.

Drug intervention

When the VT had persisted for 10 min after discontinuation of AS infusion, clonidine hydrochloride (Clon, Yung Shin Pharm, Taiwan) was administered as a bolus i.v. or i.a. injection at a dose of 16 μg kg^{-1} or 1.6 μg kg^{-1}, respectively. Additional doses of Clon (16 μg kg^{-1} for i.v.; 1.6 μg kg^{-1} for i.a.) were administered if the VT could not be converted to sinus rhythm or a junctional rhythm within 1 min after administration of Clon. Preliminary tests in five cats showed that VT was converted to sinus rhythm by 8 μg kg^{-1} in one animal but was not affected by a dose of 4 μg kg^{-1}.

Usually, the effectiveness of Clon was demonstrated within 1 min after Clon administration. Yohimbine hydrochloride (Yoh, Sigma Chemical Co.) was administered as a bolus i.v. (0.5 mg kg^{-1}) or an i.a. (0.05 mg kg^{-1}) injection at 5 min after

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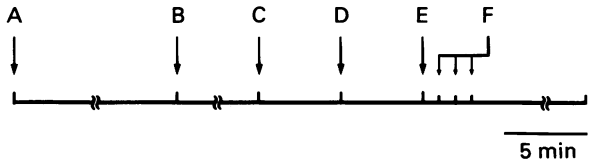

Figure 1 Protocol for experimental procedures. (A) Intravenous bolus injection of acetylstrophanthidin (AS) followed by subsequent i.v. infusion of AS. (B) VT occurrence. (C) Intravenous AS infusion discontinued if VT had persisted for 5 min. (D) Yohimbine (Yoh) administered i.v. or i.a. at 5 min after discontinuation of i.v. AS infusion. (E) Clonidine (Clon) administered i.v. or i.a. at 10 min after discontinuation of i.v. AS infusion. (F) Additional Clon injection(s) was(were) administered if no effect noted.

discontinuation of the AS infusion. This allowed Yoh to interact with the antiarrhythmic effect of Clon which was given at 10 min after the AS discontinuation.

Experimental protocol

To assess the effects of the α_2-adrenoceptor agonist and antagonist on AS-induced VT, six experimental groups were used. The experimental protocol is shown in Figure 1.

Group (I). Five cats (controls). VT was induced with i.v. AS as described previously. The duration of VT was observed.

Group (II). Ten cats. The induced VT was treated with i.v. Clon.

Group (III). Four cats. The induced VT was treated with i.a. Clon.

Group (IV). Six cats. Yoh i.v. was given at 5 min after discontinuing AS infusion, and was followed by i.v. Clon 5 min later.

Group (V). Five cats. Yoh i.a. was given at 5 min after discontinuing AS infusion and was followed by i.v. Clon 5 min later.

Group (VI). Five cats. VT was induced with i.v. AS. Yoh i.a. was given at 5 min after discontinuing AS infusion and was followed by i.a. Clon 5 min later.

Data analysis

All values are expressed as means ±s.e. Differences among multiple groups of data were examined with analysis of variance (ANOVA). Student's t test corrected for multiple comparisons was used to analyze differences in means when appropriate. A probability of less than 0.05 between two experimental groups was considered as statistically significant.

Results

Effects of acetylstrophanthidin on blood pressure and cardiac rhythm

Five cats were used in Group I. Figure 2 shows typical tracings from a cat in this group. The basal mean blood pressure (MBP) of the cat was 137 ± 6 mmHg. A gradual increase in the dose of i.v. AS produced a progressive elevation in MBP. The maximal elevation of MBP (170 + 7 mmHg, $P < 0.001$) was seen just before the occurrence of atrioventricular block (AV block). Further addition of AS produced no change or a slight decline in MBP until the onset of VT (Figure 2).

After AS was administered, the heart rate decreased slightly at the very beginning. Additional doses of AS produced AV block which was soon followed by a junctional tachycardia.

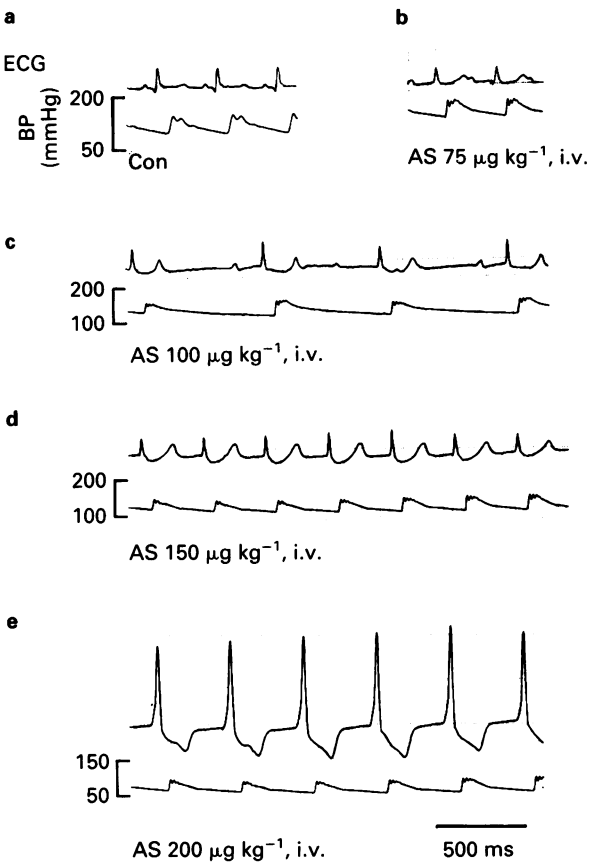

Figure 2 Typical recordings from a cat in Group I showing the effects of intravenous (i.v.) acetylstrophanthidin (AS) on the electrocardiogram (ECG) and blood pressure (BP). (a) Control (Con) sinus rhythm with cycle length (CL) 340 ms, PR interval at 70 ms, systolic blood pressure (SBP) 150 mmHg and diastolic blood pressure (DBP) 90 mmHg. (b) First degree atrioventricular (AV) block (PR interval 140 ms) with elevated SBP (180 mmHg) and DBP (130 mmHg) when AS dose increased (accumulated) to 75 $\mu g\,kg^{-1}$. (c) Complete AV block occurred when AS dose increased to 100 $\mu g\,kg^{-1}$. Note the reduction in BP and pulse pressure (SBP 160 mmHg, DBP 120 mmHg). (d) Junctional tachycardia (CL 350 ms) occurred when AS dose increased to 150 $\mu g\,kg^{-1}$. SBP (150 mmHg) and DBP (120 mmHg) appeared similar to (c). (e) Ventricular tachycardia (VT) with a CL of 410 ms occurred when AS dose reached 200 $\mu g\,kg^{-1}$. Note both SBP and DBP markedly decreased to 95 and 65 mmHg, respectively.

The dose required to produce AV block and junctional tachycardia was 118 ± 14 mg kg^{-1} and 128 ± 20 mg kg^{-1}, respectively. Further administration of AS produced multiform ventricular premature contractions (VPCs, 157 ± 21 $\mu g\,kg^{-1}$), and this was finally followed by VT (220 ± 23 $\mu g\,kg^{-1}$). The duration of sustained VT after discontinuing AS was from 25 to 48 min (37 ± 4 min, $n = 5$).

Effects of i.v. clonidine on acetylstrophanthidin-induced ventricular tachycardia

Ten cats were used in this experiment (Group II). As shown in Figure 3a, first degree AV block and ST sagging appeared when the cumulative AS dose was about 100 $\mu g\,kg^{-1}$. When AS doses were increased to 175 $\mu g\,kg^{-1}$ (Figure 3b) and 225 $\mu g\,kg^{-1}$ (Figure 3c), frequent VPCs with AV Wenckebach block and sustained VT appeared. Figure 3d shows the effect of i.v. bolus Clon 16 $\mu g\,kg^{-1}$ on the sustained VT. The cycle length of VT increased immediately and sinus rhythm was restored within 1 min after Clon. As shown in Figure 3e, regular sinus rhythm with first degree AV block (PR interval 90 ms) and lowered SBP, DBP (100 vs. 60 mmHg) were observed

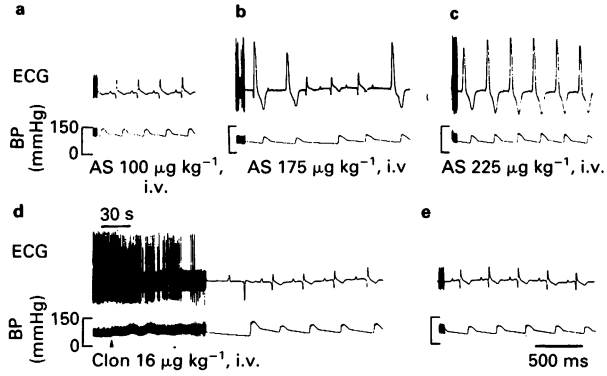

Figure 3 Recordings show the effect of intravenous clonidine (Clon) on acetylstrophanthidin (AS)-induced ventricular tachycardia (VT) in a cat in Group II. Sustained VT appeared when AS dose increased to 225 $\mu g\,kg^{-1}$ (c), cycle length of VT increased immediately and sinus rhythm restored within 1 min after Clon (d).

15 min after Clon. In this group, the average dose of i.v. Clon for termination of AS-induced VT was $28.3 \pm 6.2\,\mu g\,kg^{-1}$ (range 16 to 48 $\mu g\,kg^{-1}$). In these 10 experiments, restoration of sinus rhythm was seen in 7, and junctional rhythm in the other 3 cats, immediately after VT was terminated. In those with junctional rhythm, sinus rhythm was able to be restored within 15 min. The antiarrhythmic effect of Clon on AS-induced VT was observed for 15 min. There was no recurrence of VT.

Effects of i.a. clonidine on acetylstrophanthidin-induced ventricular tachycardia

Four cats were used in this experiment. As shown in Figure 4a, a sustained VT was achieved when the AS dose was up to 250 $\mu g\,kg^{-1}$. Injection of Clon 1.6 $\mu g\,kg^{-1}$, i.v., terminated the VT. Fifteen seconds after Clon, the sustained VT was terminated and was replaced by sinus rhythm with AV block (Figure 4b). In this experiment, sinus rhythm with 1:1 AV conduction was restored 10 min later and VT did not recur thereafter within a 15-min observation period. In this group, the average dose of i.a. Clon for terminating the AS-induced VT was $5.8 \pm 1.0\,\mu g\,kg^{-1}$. This dose was much less than that in Group II (5.8 ± 1.0 vs. $28.3 \pm 6.2\,\mu g\,kg^{-1}$, $P < 0.01$).

Role of the α_2-adrenoceptor on the antiarrhythmic effect of clonidine

To assess the role of specific central α_2-receptors in the antiarrhythmic effect of Clon, Yoh was administered via i.v. or i.a. injection to interact with the antiarrhythmic effect of Clon. As shown in Figure 5a, a sustained VT was established when the AS dose was increased up to 200 $\mu g\,kg^{-1}$. In the presence of

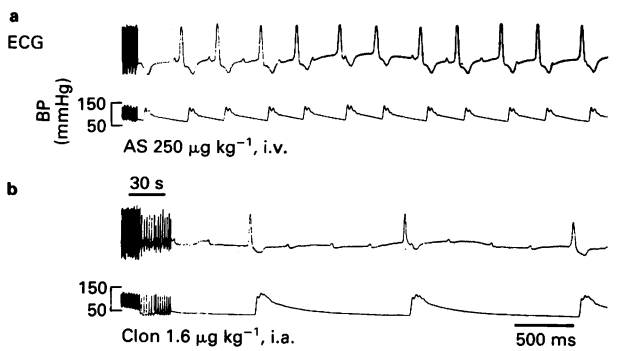

Figure 4 Recordings show the effect of intravertebral arterial (i.a.) clonidine (Clon) 1.6 $\mu g\,kg^{-1}$ on acetylstrophanthidin (AS)-induced ventricular tachycardia (VT) in a cat in Group III. Sustained VT terminated and replaced by sinus rhythm with AV block (b).

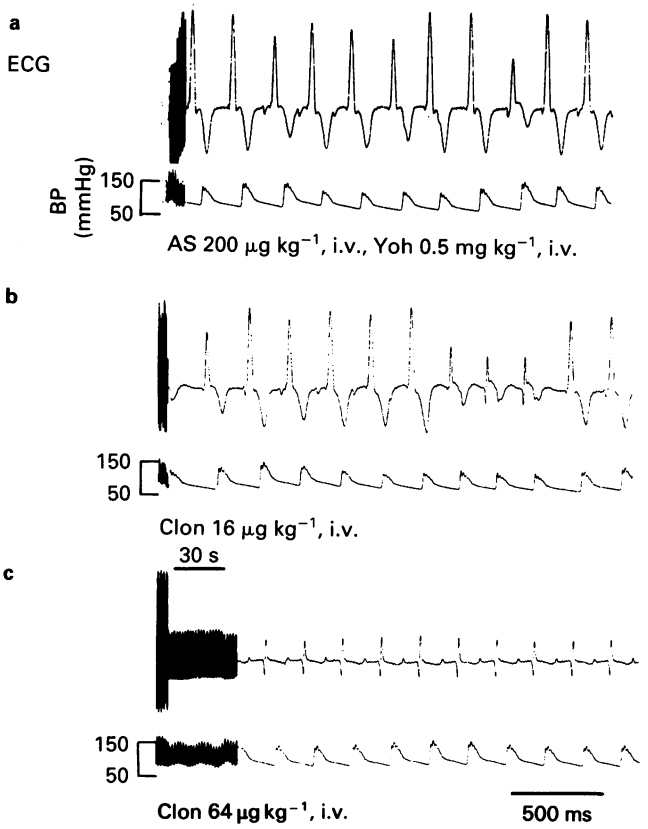

Figure 5 Recordings show the effect of i.v. Clonidine (Clon) on ventricular tachycardia (VT) induced with acetylstrophanthidin (AS) 200 $\mu g\,kg^{-1}$ i.v. Yohimbine (Yoh 0.5 $mg\,kg^{-1}$, i.v.) administered at 5 min after discontinuation of AS in a cat from Group IV (a). Sustained VT converted to sinus rhythm when Clon dose increased to 64 $\mu g\,kg^{-1}$ (c).

Yoh (0.5 $mg\,kg^{-1}$, i.v.), bolus i.v. Clon 16 $mg\,kg^{-1}$ could not terminate VT, but resulted in occasional junctional beats (Figure 5b). The sustained VT was converted to sinus rhythm when the dose of Clon was increased to 64 $\mu g\,kg^{-1}$ (Figure 5(c)). Ventricular arrhythmias never occurred thereafter within the 15 min observation period. In this group, after i.a. Yoh, two cats with VT degenerated rapidly into ventricular fibrillation leading to cardiac arrest within 5 min, and the other four cats had acceleration of VT. The average dose of Clon required to terminate the sustained VT was $62.9 \pm 5.2\,\mu g\,kg^{-1}$ and was higher than that in Group II (62.9 ± 5.2 vs. $28.3 \pm 6.2\,\mu g\,kg^{-1}$, $P < 0.01$).

In experimental Groups V and VI, Yoh (0.05 $mg\,kg^{-1}$, i.a.) was administered at 5 min after discontinuing AS infusion and Clon was given via i.v. (Group V) or i.a. (Group VI) injection to terminate VT. Acceleration of VT with rapid degeneration into ventricular fibrillation leading to cardiac arrest within 5 min after i.a. Yoh were seen in two of ten cats (one each in group V or VI). The other eight cats had acceleration of VT after i.a. Yoh. Figure 6 shows typical tracings from a cat in Group VI. The average dose of Clon for termination of VT in Group VI was significantly lower than that in Group V (14.8 ± 3.7 vs. $88.5 \pm 16.3\,\mu g\,kg^{-1}$, $P < 0.01$). The average dose of Clon for termination of VT in Group V was significantly higher than that in Group II ($P < 0.01$). The average Clon dose for termination of VT in Group VI was significantly higher than that in Group III ($P < 0.01$).

Effects of clonidine on blood pressure

Immediately after VT was converted by administration of Clon to sinus rhythm or junctional rhythm, the blood pressure rose slightly but then declined gradually during the observation period. In Groups II and III, Clon administration

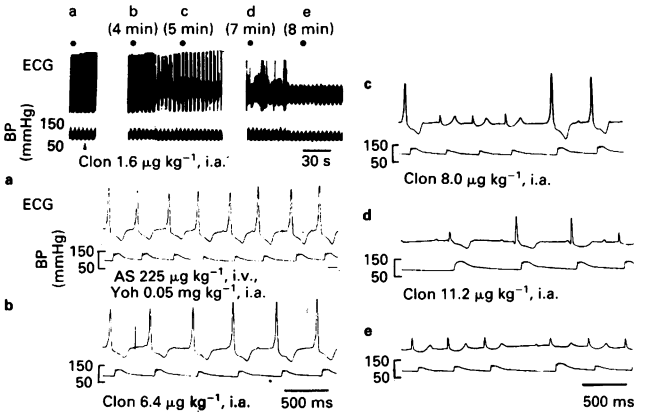

Figure 6 Recordings show the effect of i.a. clonidine (Clon) on ventricular tachycardia (VT) induced with acetylstrophanthidin (AS) 225 $\mu g\,kg^{-1}$, i.v. Yohimbine (Yoh 0.05 $mg\,kg^{-1}$, i.a.) administered at 5 min after discontinuation of AS in a cat from Group VI. Left upper panel recorded at slow speed. The letters a–e above the slow-speed recordings represent the times at which the corresponding fast-speed electrocardiogram and blood pressure in panels a–e were recorded. (a) VT with average tachycardia cycle length 350 ms induced with i.v. AS 225 $\mu g\,kg^{-1}$ and i.a. Yoh 0.05 $mg\,kg^{-1}$ administered at 5 min after discontinuation of AS. The first dose of i.a. Clon 1.6 $\mu g\,kg^{-1}$ administered at arrow, did not terminate VT. (b) Average VT cycle length increased to 470 ms after Clon dose increased to 6.4 $\mu g\,kg^{-1}$ i.a. (c) Short-run ventricular tachycardia with junctional beats seen after Clon dose increased to 8.0 $\mu g\,kg^{-1}$ i.a. (d) Occasional ventricular premature beats with junctional beats observed after the Clon dose increased to 11.2 $\mu g\,kg^{-1}$ i.a. (e) Recordings show sinus rhythm with secondary degree atrioventricular conduction block at 8 min after the first dose of Clon i.a.

resulted in significant decreases (50 ± 6 and 46 ± 4 mmHg, respectively) of MBP from the control values ($P < 0.01$). In Groups IV, V, and VI, although Yoh increased the antiarrhythmic dose of Clon, it did not affect blood pressure or prevent the Clon induced hypotension (MBP decrease 56 ± 10, 52 ± 6, and 48 ± 7 mmHg, respectively).

Discussion

Major findings

These experiments showed that Clon (i.a.) in a dose as small as one fifth to one sixth of an i.v. Clon dose could effectively terminate AS-induced VT. This antiarrhythmic effect was substantially antagonized by Yoh, an α_2-antagonist. These findings confirm the notion of Gillis & Quest (1972), that Clon produces antiarrhythmic effects through a central action. Our results further indicate that this effect involves specifically α_2-receptors in the central nervous system.

Electrophysiological effects of digitalis on the heart

Digitalis not only modifies the transmembrane action potential, but induces alterations in the slope of phase 4 depolarization of cells of the specialized conducting system. Digitalis intoxication causes increased intracellular sodium and calcium levels. Calcium overload mediating oscillatory release of calcium from the intracellular store is the primary event underlying the afterpotential which generates transient inward currents. Canine Purkinje fibres perfused with AS exhibited transient inward current (TI) which contributed to its arrhythmogenic activity (Kass & Lederer, 1978; Kass & Tsien, 1978).

Role of sympathetic neural activity in digitalis-induced ventricular arrhythmia

The relationship between the sympathetic nervous system and digitalis-induced ventricular arrhythmia is well established. Digitalis intoxication causes non-uniform discharges in cardiac sympathetic nerves which might result in non-uniformity of the electrical properties of myocardial cells (Kim *et al.*, 1984). Gillis & Quest (1972) found that a toxic dose of digitalis induced an increase in the sympathetic nerve activity in the preganglionic cardiac nerve, indicating a centrally mediated effect. Exclusion of the sympathetic nervous system by cardiac denervation or spinal cord transection significantly increased the dose of digitalis needed to produce ventricular arrhythmias (Raines *et al.*, 1967). A toxic dose of deslanoside increased the concentration of a noradrenaline metabolite, and midcollicular decerebration increased the deslanoside dose required to produce ventricular arrhythmias (Helke *et al.*, 1979). Similarly, the dose of ouabain needed to produce ventricular arrhythmias in cats increased two weeks after sympathectomy (Lather *et al.*, 1978). Lechat & Schmitt (1982) reported that the dose of digitalis glycoside required to produce ventricular arrhythmias was reduced by Yoh, an α_2-adrenoceptor antagonist. Yoh enhanced sympathetic outflow to the heart might contribute to the aggravation of digitalis-induced VT. On the other hand, inhibition of the release of neurotransmitters may be the contributory factor for the antiarrhythmic activity of Clon on AS-induced VT.

Site of effect of clonidine on digitalis-induced ventricular tachycardia

Several studies strongly indicate that the central nervous system is the primary site of antiarrhythmic action of Clon. Gillis & Quest (1972) provided several pieces of evidence to indicate that the antiarrhythmic effect of Clon was through the central adrenergic system. Firstly, Clon simultaneously converted VT and depressed sympathetic nerve discharge. A dose of Clon that did not depress nerve firing did not restore normal sinus rhythm and *vice versa*. Only when sympathetic nerve firing was depressed did Clon restore cardiac rhythm to normal. Secondly, Clon lost its antiarrhythmic effect in cats when their sympathetic nervous systems were interrupted by section of the spinal cord. If the antiarrhythmic effect of Clon was through a direct effect on myocardial cells, spinal section should not alter its antiarrhythmic effect. Lechat & Schmitt (1982) reported that α_2-adrenoceptor stimulation with Clon reduced the arrhythmogenic effects of ouabain, whereas no protection was found with ST 91, a Clon-related compound which does not cross the blood-brain barrier. All the above findings indicate that the ventricular arrhythmias induced by digitalis and its related compounds is due in part to stimulation of the central nervous system leading to an increase in sympathetic nervous activity and that the antiarrhythmic effect of Clon might be mediated through central α_2-adrenoceptors. These notions were confirmed in the present experiments. The dose of Clon via i.a. administration ($5.8 \pm 1.0\,\mu g\,kg^{-1}$) which could suppress the AS-induced VT was only about one-fifth or one-sixth of the dose required by the i.v. route ($28.3 \pm 6.2\,\mu g\,kg^{-1}$). This dose could also inhibit VT (referred to experiments in Groups II and III). As a small i.a. dose, if administered via the i.v. route, was not effective in suppressing the VT, it is suggested that Clon is not acting peripherally but rather has a primarily central action. Furthermore, following interaction with Yoh (either 0.5 $mg\,kg^{-1}$, i.v. in Group IV; or 0.05 $mg\,kg^{-1}$, i.a., in Groups V and VI), the dose of Clon required (i.v. or i.a.) to suppress VT was greatly increased. These findings indicate that the antiarrhythmic effect of Clon may specifically involve the α_2-adrenoceptor in the central nervous system.

However, Clon is not completely lacking in direct effects on the heart. Rosen & Weiss (1984) found in an *in vitro* experiment, that Clon 1×10^{-6} and 1×10^{-5} M significantly reduced V_{max} and prolonged the action potential duration of driven canine Purkinje fibres. Since these actions were not blocked by Yoh or by prazosin, a direct membrane effect not involving the adrenoceptor was suggested. However, the present study not only provides no evidence to indicate the importance of direct effects on the heart but rather suggests

that such direct cardiac effects may not be essential. Thus, while a small dose of Clon administered i.v. did not suppress the VT, the same dose given i.a. converted it to sinus rhythm.

Clinical implication

Digitalis has been commonly used for many years to treat heart failure and cardiac arrhythmias, such as atrial fibrillation with rapid ventricular response and paroxysmal supraventricular tachycardia. However, ventricular arrhythmias frequently result from digitalis intoxication caused by high myocardial digitalis concentration or central digitalis effects. Current therapy for these ventricular arrhythmias consists primarily of digitalis withdrawal along with the use of conventional antiarrhythmic agents, such as lignocaine and phenytoin. The action of these drugs is thought to be a direct suppression of the enhanced myocardial automaticity. In the present study, the antiarrhythmic effect of Clon on AS-induced VT was probably mediated through central α_2-adrenoceptors. Our findings suggest an additional avenue for treatment of digitalis-induced VT. However, use of Clon in human subjects with cardiovascular disease has been reported to produce sinus bradycardia, sinus arrest and hypotensive events. Much attention must be given to optimizing dose schedules and minimizing potential side effects before clinical use in human subjects could be recommended. However, the most effective therapy for digitalis-induced arrhythmias is a preparation of digitalis specific antibody fragments (e.g. Digibind or Digitalis antidote BM). On the other hand, drugs possessing central α_2-adrenoceptor antagonistic effect, such as Yoh, should be used carefully to avoid potentiating the occurrence of digitalis-induced VT.

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Lack of effect of zaprinast on methacholine-induced contraction and inositol 1,4,5-trisphosphate accumulation in bovine tracheal smooth muscle

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1 The effects of zaprinast (M&B 22948), a selective guanosine 3′:5′-cyclic monophosphate (cyclic GMP) phosphodiesterase inhibitor, and sodium nitroprusside on cyclic GMP content, phosphoinositide hydrolysis and airway smooth muscle tone were examined in flurbiprofen pretreated bovine tracheal smooth muscle (BTSM).

2 Anion-exchange chromatography of the soluble fraction of BTSM homogenates resolved three peaks of Ca^{2+}/calmodulin-independent phosphodiesterase (PDE) activity that corresponded to type Ia (cyclic GMP-specific, zaprinast-inhibitable), type II (cyclic GMP-stimulated) and type IV (Ro 20 1724-inhibitable) PDE isoenzymes. Zaprinast caused a selective inhibition of the type Ia PDE isoenzyme (IC_{50} 0.94 μM) with respect to the type II and IV (IC_{50}s 93 μM and 197 μM respectively) isoenzymes.

3 Pretreatment of BTSM strips with zaprinast (10 μM) for 20 min affected neither the initial rate of force development, nor the resultant magnitude of contraction induced by methacholine (10 μM). In addition, zaprinast (10 μM; 20 min) did not affect the cumulative concentration-response relationship induced by methacholine. In contrast, sodium nitroprusside (300 μM) either alone, or in combination with zaprinast (10 μM), significantly attenuated tone induced by low, but not high concentrations of methacholine. This resulted in a non-parallel, rightwards shift of the methacholine concentration-response curves (nitroprusside: 4.0 fold; nitroprusside/zaprinast: 4.8 fold at the EC_{50} values), without a reduction in the maximum tone generated.

4 In BTSM slices, zaprinast (10 or 100 μM) did not influence basal or methacholine (10 μM)-stimulated cyclic GMP accumulation or inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) mass accumulation over a 60 s incubation period, although it did significantly increase cyclic GMP content over longer (30 min) stimulation periods.

5 In [^{3}H]-inositol prelabelled BTSM slices, stimulated in the presence of 5 mM LiCl, methacholine (10 μM) caused a marked increase in total [^{3}H]-inositol phosphate accumulation. This effect was not inhibited by zaprinast (10 μM), sodium nitroprusside (300 μM), or a combination of these drugs despite these agents markedly increasing tissue cyclic GMP content.

6 These findings demonstrate that despite zaprinast being a potent and selective inhibitor of the type Ia PDE isoenzyme in a cell-free system, this drug only increases cyclic GMP content in BTSM following prolonged agonist-stimulation. This may explain its lack of inhibitory effect on methacholine-induced tone. The inability of drugs which increase tissue cyclic GMP content and exhibit anti-spasmogenic activity to inhibit methacholine-stimulated $Ins(1,4,5)P_3$ formation suggests that, unlike vascular smooth muscle, cyclic GMP-dependent mechanisms do not regulate receptor-mediated phosphoinositide hydrolysis in BTSM.

Keywords: Zaprinast (M&B 22948); inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$); cyclic GMP; bovine airway smooth muscle; sodium nitroprusside; cyclic GMP-dependent phosphodiesterase

Introduction

Despite increasing knowledge regarding the major substrates for guanosine 3′:5′-cyclic monophosphate (cyclic GMP)-dependent protein kinase in eucaryotic cells (see Casnellie & Greengard, 1974; Parks *et al.*, 1987), the exact molecular mechanism(s) underlying cyclic GMP-induced airway smooth muscle (ASM) relaxation remains uncertain. In several vascular smooth muscle preparations cyclic GMP (Hirata *et al.*, 1990), glyceryl trinitrate (Ahlner *et al.*, 1988), sodium nitroprusside and atriopeptin II (Rapoport, 1986) and α-human atrial natriuretic peptide (Kajikura & Kuriyama, 1990) have all been shown to attenuate agonist-stimulated phosphoinositide metabolism possibly by inhibiting activation of a guanine nucleotide binding protein, G_p, and uncoupling activated G_p from phosphoinositidase C (Hirata *et al.*, 1990). Since agonist-stimulated hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$) by phosphoinositidase C to form inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and 1,2-diacyglycerol is thought to play a central role in excitation-contraction coupling in smooth muscle (see Abdel-Latif, 1986; Hall & Chilvers, 1989) the inhibition of phosphoinositide metabolism by cyclic GMP has been proposed as an important mechanism underlying its dilator action in vascular smooth muscle (Rapoport, 1986; Hirata *et al.*, 1990).

In contrast to the well-documented effects of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) elevating agents on phosphoinositide responses in ASM (Hall & Hill, 1988; Madison & Brown, 1988; Hall *et al.*, 1989; 1990), little is known about the influence of cyclic GMP in this tissue. In a recent study in guinea-pig tracheal rings, Langlands *et al.* (1989) examined the effects of zaprinast (M&B 22948), a Ca^{2+}/calmodulin-independent, cyclic GMP-specific phosphodiesterase (PDE; type Ia, see Torphy & Cielinski, 1990) inhibitor, on

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cyclic GMP content, Ins(1,4,5)P_3 mass and methacholine (MCh)- and histamine-induced changes in tone. Since zaprinast increased basal and agonist-stimulated cyclic GMP levels and abolished Ins(1,4,5)P_3 generation without influencing the rate or magnitude of tension development, the authors concluded that agonist-induced Ins(1,4,5)P_3 formation was not involved in the contractile response induced by either of these agonists. As these data are complicated by the heterogeneous nature of the tissue studied, conflict with the findings of Hall et al. (1990) who demonstrated no effect of zaprinast on inositol phosphate responses in bovine tracheal smooth muscle (BTSM), and challenge much of the available data that support a pivotal role for Ins(1,4,5)P_3 in initiating ASM contraction (Hashimoto et al., 1985; Chilvers et al., 1989a,b; 1990a; Hall & Chilvers, 1989; Coburn & Baron, 1990) we have re-investigated the potential interaction between cyclic GMP, Ins(1,4,5)P_3 formation and tone in BTSM, an essentially homogeneous ASM preparation (Katsuki & Murad, 1977). A preliminary account of some of these data has been presented to the British Pharmacological Society (Chilvers et al., 1990c).

Methods

Tissue preparation

Tracheae from 12–18 month old cows were obtained from a local abattoir and transported to the laboratory in oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit (KH) buffer (composition in mM: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.7, $CaCl_2$ 1.3) at 4°C. The cervical trachealis muscle was dissected free of epithelium and surrounding connective tissue and either maintained in oxygenated ice-cold KH buffer for tension, [^{3}H]-InsP and cyclic GMP measurements or snap frozen in liquid nitrogen and stored at −80°C for subsequent phosphodiesterase studies.

Tension measurements

The effects of zaprinast, sodium nitroprusside and the combination of these drugs on methacholine-induced tension development in BTSM were studied as described by Langlands et al. (1989). Essentially BTSM strips measuring approximately 2 × 2 × 10 mm were mounted under an initial tension of 20 mN in 5 ml tissue baths containing oxygenated KH buffer at 37°C. Following a 60–120 min equilibration period, during which time the tension was periodically readjusted to 20 mN and the buffer frequently changed, tissues were pretreated for 20 min with either vehicle (20 μl 0.1 M NaOH), 10 μM zaprinast, 300 μM sodium nitroprusside or the latter two drugs in combination. Cumulative concentration-response curves to methacholine were then constructed according to Van Rossum (1963) from which estimates of agonist potency ($pD_2 = -\log_{10} EC_{50}$) were determined. To account for intrinsic changes in sensitivity (pD_2) of BTSM strips to methacholine between tissues obtained from different animals (see Table 1), the potential inhibitory effects of zaprinast, sodium nitroprusside and zaprinast-plus-sodium nitroprusside on the methacholine concentration-response relationship were performed using a paired experimental design where control (no drug pretreatment) and experimental strips obtained from the same animal were studied in parallel.

In a separate series of experiments designed to assess the effects of zaprinast on the kinetics ($t_{1/2}$) and magnitude (T_{max}) of methacholine-induced tone, BTSM strips were preincubated with 10 μM zaprinast or vehicle prior to challenge with 10 μM methacholine (approx. EC_{90} for tension). Changes in tension were measured isometrically with Grass FT03.c force-displacement transducers and displayed on a Grass 7D curvilinear ink-writing polygraph.

Flurbiprofen (1 μM) was present in the KH buffer throughout the tension, InsP and cyclic GMP experiments to prevent the spontaneous and drug-stimulated release of cyclooxygenase products from the BTSM and to prevent sodium nitroprusside from increasing the tissue cyclic AMP content (Torphy et al., 1985).

Separation and assay of cyclic nucleotide-dependent phosphodiesterase isoenzymic activities

For the separation of PDE isoenzymes, approx. 5 g of BTSM were homogenized (Polytron 2 × 10 s bursts at setting 8) in 10 vol. of ice-cold buffer A (composition in mM: bis-Tris 20 (pH 6.5), sodium acetate 50, dithiothreitol (DTT) 2, EDTA 1, ethylene glycol 30% (v/v) supplemented with benzamidine 2, phenylmethylsulphonylfluoride 0.05, bacitracin (100 μg ml^{-1}) and soybean trypsin inhibitor (10 μg ml^{-1})). To prevent DTT-induced activation of thiol-dependent proteases which may reduce PDE-yield, 60 μM leupeptin was also added to buffer A prior to homogenization (see Degerman et al., 1987). The homogenate was centrifuged at 45000 *g* at 4°C for 30 min to provide soluble (supernatant) and particulate (pellet) fractions. The pellet was resuspended in 2 vol. buffer A containing 300 mM KCl to remove any electrostatically bound PDE and re-centrifuged as above. The combined supernatants were filtered through gauze, diluted in buffer B (composition in mM: bis-Tris 20 (pH 6.5), EDTA 1, ethylene glycol 30% (v/v)) to reduce conductivity to <4 mS at 4°C and applied at a flow rate of 1 ml min^{-1} to a Q-sepharose column (Bio-Rex 1.5 × 1.8 cm) pre-equilibrated in buffer A. The column was then washed with buffer A until the absorbance at 280 nm returned to baseline and then eluted with a linear sodium acetate gradient (50–900 mM, flow rate 0.5 ml min^{-1}, total volume 400 ml). Forty × 10 ml fractions were collected and aliquots assayed for cyclic nucleotide PDE activity as described below. Peak fractions were subsequently pooled and could be stored at −18°C for at least 2 months without loss of Ca^{2+}/calmodulin-independent PDE activity. Cyclic nucleotide-dependent PDE activity was measured by a modification (Schwartz & Passonneau, 1974) of the method described by Thompson & Appleman (1971). The assay is based upon the phosphodiesteric cleavage of [^{3}H]-cyclic AMP or [^{3}H]-cyclic GMP to [^{3}H]-5′-AMP or [^{3}H]-5′-GMP respectively which are dephosphorylated to their corresponding labelled nucleosides and separated from the charged nucleotides by Dowex anion exchange resin. Assays were performed in triplicate at 37°C by adding 30 μl of the relevant column fraction to 270 μl of reaction medium containing (final concentration in mM): triethanolamine 40 (pH 8.0), EGTA 2, DTT 2, $Mg(CH_3COO)_2$ 10, BSA (500 μg ml^{-1}), cyclic AMP (1 μM for type IV PDE isoenzyme) or cyclic GMP (1 μM for type Ia and type II PDE isoenzymes) supplemented with 250000–350000 d.p.m. [^{3}H]-cyclic GMP or [^{3}H]-cyclic AMP, 0.25 u alkaline phosphatase and approximately 5000 d.p.m. [^{14}C]-adenosine to estimate recovery. Reactions were terminated after 10 min by the addition of 1 ml of Dowex AG1-X8 (acetate form, 200–400 mesh, propan-2-ol, water, 1:2:1 (w/v/v)). Samples were thoroughly mixed and after 10 min centrifuged at 12000 *g* for 2 min. The radioactivity in 650 μl aliquots of the resulting supernatant was determined by liquid scintillation counting. One unit of enzyme activity was defined as the amount of PDE which catalysed the formation of 1 pmol 5′-AMP/5′-GMP min^{-1} mg^{-1} protein at 37°C after correction for the recovery (routinely 70–85%) of [^{14}C]-adenosine.

Measurement of cyclic GMP

BTSM slices (300 μm × 300 μm) were prepared as previously described (Chilvers et al., 1989a) and preincubated for 60 min in 100 ml oxygenated (95% O_2:5% CO_2) KH buffer at 37°C. Two ml of gravity packed slices were then transferred to 50 ml Erlenmeyer flasks containing 10 ml oxygenated KH buffer and incubated for a further 60 min at 37°C. After this second incubation period, 50 μl aliquots of gravity-packed BTSM slices were transferred to flat bottom vials containing 230 μl KH

buffer. Zaprinast (final concentration 10 μM) or vehicle (0.3 mM NaOH) were added in a 10 μl volume 20 min before stimulation with methacholine (10 μM). Reactions were terminated at appropriate times with 300 μl ice-cold 1 M trichloroacetic acid and extracts neutralized according to Downes *et al.* (1986). Aliquots (250 μl) of the neutralized tissue extracts were diluted 2 fold in 100 mM sodium acetate (pH 6.2) and acetylated by the consecutive addition of triethylamine (10 μl) and acetic anhydride (5 μl). Cyclic GMP levels were then measured by radioimmunoassay as described by Brooker *et al.* (1979) and Lappin *et al.* (1984). In brief, 100 μl of acetylated sample were added to 25 μl guanosine-3', 5'-cyclic monophosphate, 2-0-succinyl 3-[^{125}I]-iodotyrosine methyl ester in 0.1% BSA (approx. 2000 d.p.m.) and 100 μl anti-cyclic GMP antibody in 0.1% BSA. After vortex mixing, samples were incubated overnight at 4°C and free and antibody-bound cyclic GMP separated by charcoal precipitation in 100 mM phosphate buffer (pH 7.4). The detection limit and sensitivity of this assay were approximately 2 fmol and 15 fmol cyclic GMP respectively.

Measurement of Ins(1,4,5)P_3 and [^{3}H]-InsPs

Ins(1,4,5)P_3 mass was measured in the same neutralized trichloroacetic acid tissue extracts used for the above cyclic GMP estimations with a radioreceptor assay exactly as described previously (Challiss *et al.*, 1988). The protein content of each tissue pellet was determined according to Lowry *et al.* (1951).

The accumulation of total [^{3}H]-InsPs in the presence of 5 mM LiCl was used as an index of agonist-stimulated phosphoinositide hydrolysis (Berridge *et al.*, 1982). BTSM slices were prepared and pre-incubated in KH buffer for 60 min as detailed above and labelled with 0.1 μM *myo*-[^{3}H]-inositol for 60 min in the absence of agonist, exactly as described previously (Chilvers *et al.*, 1989a). Tissue slices were dispensed, pretreated with zaprinast (10 μM), sodium nitroprusside, (300 μM), vehicle or zaprinast in combination with sodium nitroprusside for 20 min in the presence of LiCl (5 mM) and stimulated with methacholine (10 μM). Trichloroacetic acid extracts were prepared as outlined above and total [^{3}H]-InsPs separated by Dowex AG1-X8 anion-exchange resin (100–200 mesh, Cl^- form; 0.5 cm × 1.75 cm columns) (Chilvers *et al.*, 1989a). Unlabelled samples were run in parallel to assess changes in tissue cyclic GMP content.

Materials

Acetyl-β-methylcholine, alkaline phosphatase (P-4252), bacitracin, benzamidine, bovine serum albumin (grade III), cyclic AMP (sodium salt), cyclic GMP (sodium salt), dithiothreitol, EDTA, EGTA, fluorbiprofen, leupeptin, phenylmethylsulphonylfluoride, sodium nitroprusside and soybean trypsin inhibitor were purchased from Sigma. Zaprinast (M&B 22948) was obtained from Rhone-Poulenc Rorer Inc., Dagenham, Essex. Anti-cyclic GMP antibody, cyclic GMP 2'-O-succinyl 3-[^{125}I]-iodotyrosine methyl ester (1980 Ci mmol^{-1}), [2,8-^{3}H]-cyclic AMP (36.1 Ci mmol^{-1}), [8-^{3}H]-cyclicGMP (23.7 Ci mmol^{-1}), [8-^{14}C]-adenosine (55 Ci mmol^{-1}) and D-inositol 1,4,5-trisphosphate were from Amersham International Dowex AG1-X8 (200–400 mesh, acetate form and 100–200 mesh, chloride form) were from BioRad. *myo*-[2-^{3}H]-inositol (17 Ci mmol^{-1}) and D-[^{3}H]-inositol 1,4,5-trisphosphate (17–20 Ci mmol^{-1}) were purchased or received as gifts from NEN (DuPont). All other reagents were of analytical grade obtained from commercial sources.

Data analysis

Significant differences between means were assessed by unpaired *t* test for non-contractile data and non-parametric analysis (Mann-Whitney U-test) for contractile studies. The null hypothesis was rejected when $P < 0.05$; IC_{50} and slope factor values were determined by computer-assisted iterative curve fitting (ALLFIT).

Results

Effect of zaprinast on cyclic nucleotide phosphodiesterase activity

Three peaks of Ca^{2+}/calmodulin-independent, cyclic-nucleotide hydrolytic activity were obtained following anion exchange chromatography of a crude BTSM supernatant eluting at 14, 41 and 57 mS (measured at 27°C). Using the classification of Torphy & Cielinski (1990), and the substrate specificity, kinetics of cyclic AMP/cyclic GMP hydrolysis and sensitivity of the PDE activity of each peak to a range of selective inhibitors (rolipram, Ro 20 1724, SK 94120, imazodan and zaprinast), these peaks corresponded to type Ia, II and IV PDE isoenzymes respectively (M.A. Giembycz & P.J. Barnes, unpublished results). The specific activity of these peaks, measured in the absence of Ca^{2+}/calmodulin and with 1 μM cyclic GMP (type Ia and II PDE) and 1 μM cyclic AMP (type IV PDE) as substrate and allowing correction for [^{14}C]-adenosine recovery was 4.3, 3.8 and 7.8 pmol 5'-GMP/5'-AMP formed min^{-1} mg^{-1} protein for types Ia, II and IV respectively.

Zaprinast caused a concentration-dependent inhibition of all three PDE activities (Figure 1), but exhibited marked selectivity (>100 fold) as an inhibitor of the Ca^{2+}/calmodulin-independent, cyclic GMP-specific (type Ia) isoenzyme (IC_{50} 941 ± 111 nM) over both the type II isoenzyme (IC_{50} 93 ± 10 μM) and the type IV isoenzyme (IC_{50} 197 ± 28 μM). The concentration of zaprinast chosen for the subsequent studies (10 μM) gave an 84.2 ± 0.6% inhibition of type Ia PDE hydrolytic activity and 5.9 ± 2.3 and 13.7 ± 3.8% inhibition of types II and IV PDE activity respectively and hence, in the cell-free assay system employed, this concentration of zaprinast was the most discriminating to permit selective inhibition of type Ia (Ca^{2+}/calmodulin-independent, cyclic GMP specific) PDE activity.

Effect of zaprinast and sodium nitroprusside on methacholine-induced tone

Pretreatment of BTSM strips with zaprinast (10 μM) for 20 min in the presence of flurbiprofen (1 μM) failed to attenuate the

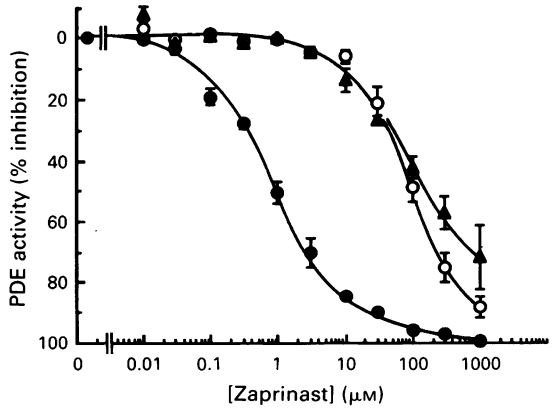

Figure 1 Effect of zaprinast on type Ia, II and IV phosphodiesterase (PDE) activities in the soluble fraction of BTSM homogenates. PDE isoenzymes were separated from 45 000 *g* soluble fractions of BTSM homogenates by Q-sepharose anion exchange chromatography and cyclic nucleotide-dependent PDE activity measured in the presence of various concentrations of zaprinast. Cyclic AMP (1 μM) was used as substrate for type IV (▲), and cyclic GMP (1 μM) for types Ia (●) and II (○) PDE isoenzymes respectively. Values are presented as mean for 8 determinations, each performed in triplicate, from 4 separate tissue preparations; vertical bars shows s.e.mean.

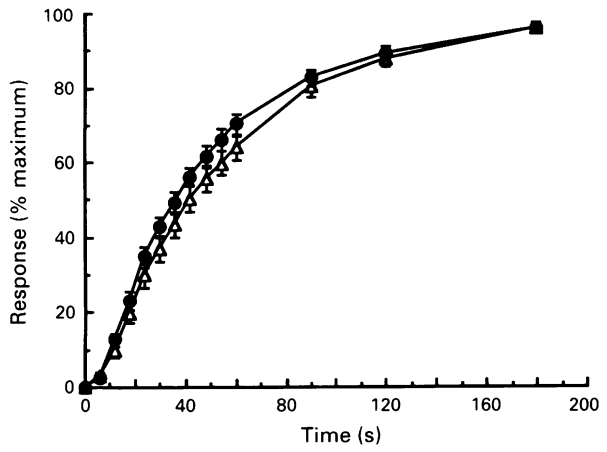

Figure 2 Effect of zaprinast on kinetics of methacholine-induced tone in BTSM strips. Strips were incubated in the absence (△) or presence (●) of zaprinast (10 μM) for 20 min, prior to addition of methacholine (10 μM; approx. EC_{90} for tension development). Data are presented as mean for 10 tissue samples in 4 separate experiments; s.e.mean shown by vertical bars.

initial rate or magnitude of tone induced by 10 μM methacholine (approx. EC_{90}). There was no significant difference in the lag-time between addition of agonist and the onset of contraction in control (4.2 ± 0.7 s) or zaprinast pretreated tissues (3.8 ± 0.3 s) and no difference ($P > 0.05$) in the time to achieve half maximal ($t_{1/2}$) contraction (control: $t_{1/2} = 39.6 \pm 3.9$ s; zaprinast pretreatment: $t_{1/2} = 37.3 \pm 2.4$ s) or in the maximum tension developed (control: 204.8 ± 12.6 mN; zaprinast pretreatment: 243.5 ± 18.9 mN; Figure 2). In addition, the cumulative concentration-response relationship induced by methacholine was also unaffected by pretreatment of tissue with zaprinast (Figure 3a). In contrast, pretreatment of BTSM for 20 min with sodium nitroprusside (300 μM) alone or in combination with zaprinast (10 μM) significantly attenuated tone induced by low, but not high concentrations of methacholine (Figures 3b, 3c; Table 1). Thus, the methacholine concentration-response curve was shifted to the right in a non-parallel manner by both nitroprusside (4.0 fold at EC_{50}) and by nitroprusside and zaprinast in combination (4.8 fold at EC_{50}) without a reduction in the maximum response attained (Table 1).

Effect of zaprinast on methacholine-stimulated Ins(1,4,5)P_3 and cyclic GMP accumulation

Methacholine (10 μM) caused a rapid, transient increase in Ins(1,4,5)P_3 mass in BTSM slices similar to that observed with carbachol (Chilvers *et al.*, 1989b) with a maximal response (175% over basal) observed at 5 s (Table 2). The increase in Ins(1,4,5)P_3 caused 5 s after methacholine addition was about 70% of that caused by a maximally-effective concentration of this agonist (100 μM, see Table 2). Pretreatment of slices with zaprinast (10 μM) for 20 min prior to stimulation with methacholine did not influence the time course of methacholine-induced Ins(1,4,5)P_3 accumulation (Table 2).

Table 2 Effect of zaprinast on methacholine-stimulated accumulation of inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) in BTSM slices

		Ins(1,4,5)P_3 (pmol mg^{-1} protein)	
Time (s)	*+/− MCh*	*Control*	*+Zaprinast*
5	−	10.0 ± 0.5	9.8 ± 0.7
	+	17.1 ± 0.6	17.4 ± 0.9
10	+	14.7 ± 0.7	14.9 ± 0.8
15	+	11.9 ± 0.9	12.5 ± 0.8
30	+	10.2 ± 0.8	10.2 ± 0.8
60	−	10.4 ± 0.4	10.7 ± 0.4
	+	8.6 ± 0.7	8.1 ± 0.8

BTSM slices (300 μm × 300 μm) were pre-incubated with vehicle or zaprinast (10 μM) for 20 min prior to addition of vehicle or methacholine (MCh, 10 μM). Incubations were terminated at the indicated time-points by addition of trichloroacetic acid and samples neutralised and assayed for Ins(1,4,5)P_3 concentrations. Addition of 100 μM methacholine for 5 s increased Ins(1,4,5)P_3 accumulation to 21.4 ± 0.8 pmol mg^{-1} protein. Increasing the concentration of zaprinast (100 μM) had no effect on the maximal methacholine (10 μM) stimulation of Ins(1,4,5)P_3 accumulation (5 s: 17.4 ± 1.0 pmol mg^{-1} protein). Data represent mean ± s.e.mean, for 3 separate experiments, each performed in triplicate.

Methacholine (10 μM) also caused a time-dependent increase in the cyclic GMP content (Figure 4) with levels increasing from control values of 105 ± 21 fmol mg^{-1} protein under resting conditions, to 453 ± 29 fmol mg^{-1} protein by 30 s post-methacholine challenge. Zaprinast pretreatment did not significantly affect either basal or methacholine-stimulated (0–60 s) cyclic GMP accumulation (Figure 4) although significant increases in cyclic GMP were observed in the presence of zaprinast with a longer (30 min) methacholine-stimulation period (Figure 5a). In two further experiments pretreatment of BTSM slices with zaprinast (100 μM) for 20 min likewise failed to attenuate methacholine-stimulated increases in Ins(1,4,5)P_3 or cyclic GMP at 5 s (data not shown).

Effect of zaprinast and sodium nitroprusside on [^{3}H]-InsP and cyclic GMP accumulation

Since zaprinast (10 or 100 μM) did not influence basal or short-term (60 s) methacholine-stimulated cyclic GMP levels, but was able to enhance the accumulation of cyclic GMP over longer periods of methacholine stimulation (Figure 5a), the effect of zaprinast (10 μM) on methacholine-stimulated phosphoinositide metabolism was examined 30 min after methacholine challenge. Since only a transient increase in

Table 1 Effect of zaprinast and sodium nitroprusside on methacholine-induced contraction: cumulative concentration-response curves to methacholine were constructed using BTSM strips, pretreated for 20 min with vehicle, zaprinast (10 μM), sodium nitroprusside (300 μM) or a combination of the latter two agents as indicated

	Methacholine concentration-response curve				
		Fold rightwards shift at			T_{max}
	pD_2	EC_{20}	EC_{50}	EC_{80}	(mN)
Control	6.48 ± 0.10				352 ± 32
+ zaprinast	6.42 ± 0.09	1.0	1.2	1.2	323 ± 31
Control	6.89 ± 0.21				324 ± 21
+ sodium nitroprusside	6.29 ± 0.07*	5.1	4.0	2.3	328 ± 16
Control	6.59 ± 0.07				376 ± 34
+ zaprinast + sodium nitroprusside	5.91 ± 0.13**	5.2	4.8	3.4	331 ± 31

Data are presented as means ± s.e.mean, derived from 8 tissue samples in 4 separate experiments. Statistical significance is indicated as *$P < 0.05$; **$P < 0.01$.

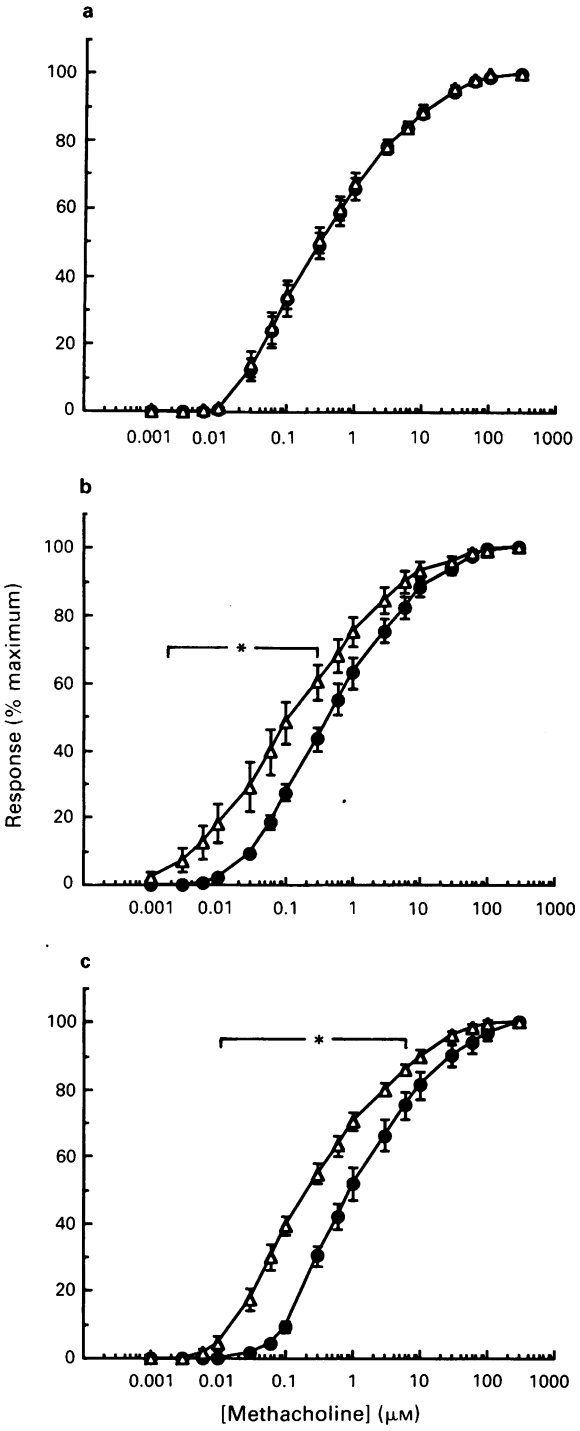

Figure 3 Effect of zaprinast and sodium nitroprusside on methacholine-induced tone in BTSM strips. Cumulative concentration-response curves to methacholine were constructed in BTSM strips pretreated for 20 min in the absence (△) or presence (●) of (a) zaprinast (10 μM); (b) sodium nitroprusside (300 μM); or (c) a combination of zaprinast (10 μM) and sodium nitroprusside (300 μM). pD_2 and T_{max} values and the degree of rightward shift observed are presented in Table 1. Values are presented as mean of 8 observations in 4 separate experiments; s.e.mean shown by vertical bars. Statistical significance is indicated as $*P < 0.05$.

Ins(1,4,5)P_3 accumulation is observed following muscarinic receptor-stimulation in this tissue (Table 2), despite continued $PtdInsP_2$ hydrolysis (Chilvers *et al.*, 1990b), total [^{3}H]-InsP accumulation in the presence of Li^+ was used as an index of receptor-mediated phosphoinositide hydrolysis. In addition, to investigate whether the elevation of cyclic GMP levels by other means (i.e. through direct stimulation of soluble guanylyl cyclase) was able to influence phosphoinositide

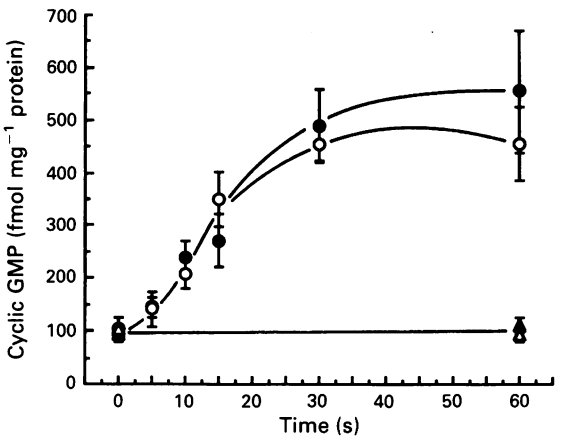

Figure 4 Effect of zaprinast on methacholine-stimulated accumulation of cyclic GMP in BTSM slices. Slices (300 μm × 300 μm) were pre-incubated with vehicle (○, △) or zaprinast (10 μM) (●, ▲) for 20 min prior to addition of methacholine (10 μM) (○, ●) or KH buffer (△, ▲). Incubations were terminated at the indicated time-points by addition of trichloroacetic acid and samples neutralised and assayed for cyclic GMP concentration. Data represent mean for 3 separate experiments, each performed in triplicate; s.e.mean shown by vertical bars.

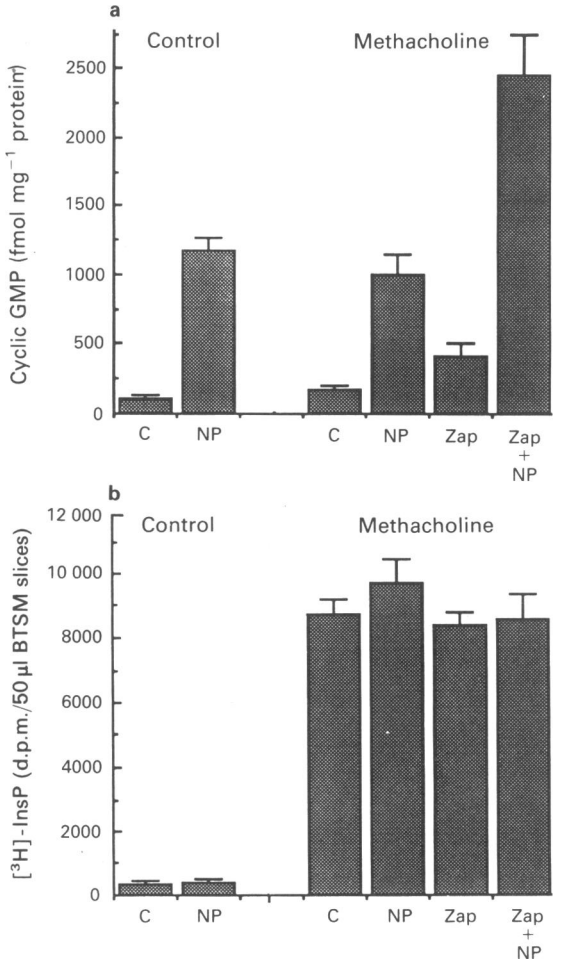

Figure 5 Effect of zaprinast and sodium nitroprusside on 30 min methacholine-stimulated cyclic GMP and [^{3}H]-inositol phosphate (InsP) accumulations. Unlabelled (a) or [^{3}H]-inositol pre-labelled (b) BTSM slices were pre-incubated with vehicle (c), zaprinast (Zap) (10 μM), sodium nitroprusside (NP) (300 μM) or a combination of these agents as indicated for 20 min prior to stimulation with methacholine (10 μM) or KH buffer for 30 min. Cyclic GMP (a) and [^{3}H]-InsP (b) accumulations were determined in neutralised trichloroacetic acid extracts as described in the Methods section. Data are presented as means for 3 separate experiments performed in triplicate; vertical bars shown s.e.mean.

hydrolysis, the effects of sodium nitroprusside alone at a concentration (300 μM) known to inhibit methacholine-induced tone, or in combination with zaprinast, on cyclic GMP and [^{3}H]-InsP accumulation were examined.

Figure 5a shows that incubation of BTSM slices for 30 min with sodium nitroprusside caused a marked increase in basal and methacholine-stimulated cyclic GMP levels. When added in combination, sodium nitroprusside and zaprinast acted synergistically producing an 8 fold increase in the cyclic GMP content. Despite these marked increases in cyclic GMP observed with zaprinast, sodium nitroprusside or a combination of these agents in methacholine-stimulated tissue, no effect on methacholine-stimulated [^{3}H]-InsP formation was observed (Figure 5b).

Discussion

These data indicate that in the soluble fraction of BTSM homogenates, zaprinast appears to be an effective inhibitor of the type Ia (Ca^{2+}/calmodulin-independent, cyclic GMP-specific) PDE isoenzyme with an approximate 100 and 200 fold selectivity over the type II and IV PDE isoenzymes, respectively. The potency of zaprinast in inhibiting Type Ia PDE activity in BTSM (IC_{50} 0.94 μM) is very similar to that previously reported in canine trachealis (Silver *et al.*, 1988) and bovine photoreceptors (Gillespie & Beavo, 1989). The concentration of zaprinast (10 μM) used for the studies employing tissue slices was chosen not only to allow a direct comparison with the study by Langlands *et al.* (1989) but also because this concentration caused near maximal inhibition of type Ia PDE without major effects on either the type II or IV isoenzymes in BTSM.

The complete lack of effect of zaprinast on basal and methacholine-stimulated Ins(1,4,5)P_3 accumulation and on cyclic GMP levels over the first 60 s following stimulation with methacholine contrasts markedly with the findings of Langlands *et al.* (1989) in guinea-pig tracheal rings where an identical experiment protocol resulted in a significant elevation in tissue cyclic GMP and a complete inhibition of methacholine- and histamine-stimulated increase in Ins(1,4,5)P_3. Our additional experiments demonstrating no effect of higher concentrations of zaprinast (100 μM) on methacholine-stimulated Ins(1,4,5)P_3 accumulation, or any effect of zaprinast on phosphoinositide hydrolysis over longer stimulation periods, where significant increases in tissue cyclic GMP levels were observed, and the lack of effect of sodium nitroprusside which induced large increases in tissue cyclic GMP levels on [^{3}H]-InsP responses all argue against a significant role of cyclic GMP-dependent mechanisms in regulating muscarinic-cholinoceptor-mediated phosphoinositide hydrolysis in BTSM. This conclusion is supported by the observations by Hall *et al.* (1989; 1990) that pretreatment of [^{3}H]-inositol-labelled BTSM slices with zaprinast (100 μM) for 20 min failed to influence either carbachol (1 μM)- or histamine (1 mM)-stimulated accumulation of [^{3}H]-InsPs, despite marked attenuation of both responses by a range of cyclic AMP elevating agents. These findings in BTSM are also consistent with the recent demonstration in rat glioma C_6Bu_1 cells (Kim *et al.*, 1989) that dibutyryl cyclic AMP, but not 8-bromo-cyclic GMP, causes the phosphorylation of PLC-γ, a mechanism which is thought to be involved in the regulation of phosphoinositidase C by cyclic AMP-dependent protein kinase.

As it has been demonstrated that cyclic GMP is an important regulator of phosphoinositide metabolism in other bovine tissues, notably mesenteric arterial smooth muscle (Ahlner *et al.*, 1988), it is unlikely that a species effect alone explains the differences that exist between our own study and that of Langlands *et al.* (1989). It is probable that since ASM is a relatively minor cellular component in intact guinea-pig tracheal rings, in contrast to BTSM preparations which are >95% smooth muscle (Katsuki & Murad, 1977), that some of the biochemical responses observed in the former tissue reflect changes in non-airway smooth muscle cells. However, since it is clear that major differences do exist in the ability of cyclic GMP-elevating agents and non-hydrolysable cyclic GMP analogues to influence phosphoinositide metabolism in smooth muscle in different tissues, a specific species difference cannot be fully excluded. In addition, our study does not exclude the possibility that the apparent differences that exist between vascular and airway smooth muscle with regard to the sensitivity of agonist-stimulated inositol phosphate response to cyclic GMP elevating agents are dependent on the type of receptor activated, since studies in vascular tissue have examined the modulation of α_1-adrenoceptor responses, while investigations in airway tissue have used agonists that interact with histamine H_1-receptors and M_3-muscarinic-cholinoceptors.

In contrast, therefore, to vascular smooth muscle where cyclic GMP-mediated inhibition of phosphoinositide metabolism may well be an important mechanism mediating relaxation (Rapoport *et al.*, 1983; Ahler *et al.*, 1988; Hirata *et al.*, 1990); an alternative mechanism(s) appears to be responsible for cyclic GMP-induced relaxation in BTSM (Ishii & Murad, 1989) as judged by the dissociation between the effect of sodium nitroprusside on methacholine-induced tone and on phosphoinositide metabolism. Although BTSM is known to contain relatively large amounts of cyclic GMP-dependent protein kinase (Torphy *et al.*, 1982) and have a high cyclic GMP to cyclic AMP-dependent protein kinase ratio (Fiscus *et al.*, 1984; Felbel *et al.*, 1988) the mechanisms underlying the ability of 8-bromo-cyclic GMP, and cyclic GMP-dependent protein kinase, to inhibit carbachol-induced intracellular Ca^{2+} mobilisation in dissociated BTSM cells (Felbel *et al.*, 1988) are largely unknown. In BTSM, activation of cyclic GMP-dependent protein kinase is known to phosphorylate several sarcolemmal-associated proteins (Hogaboom *et al.*, 1982), two of which correspond to GS_1 and GS_2 subtrates identified in guinea-pig vas deferens, uterus and ileum (Casnellie & Greengard, 1974). In addition, cyclic GMP-dependent protein kinase promotes the monophosphorylation of myosin light chain kinase (Nishikawa *et al.*, 1984), but unlike the diphosphorylation caused by cyclic AMP-dependent protein kinase has no effect on the Ca^{2+}/calmodulin dependence of myosin light chain phosphorylation and consequently little functional effect. Activation of cyclic GMP-dependent protein kinase has been shown to stimulate Ca^{2+} extrusion (Kobayashi *et al.*, 1985) and Ca^{2+} uptake into the sarcoplasmic reticulum (probably via the phosphorylation of phospholamban; Raemaekers *et al.*, 1988) in aortic smooth muscle cells and bovine pulmonary artery respectively. The agonist-stimulated increases in cyclic GMP which result from Ca^{2+} activation of the soluble guanylyl cyclase or through protein kinase C-induced phosphorylation of guanylyl cyclase (Zwiller *et al.*, 1985) may therefore play an important role in the feedback control of Ca^{2+} movements in ASM although evidence that this occurs via modulating agonist-stimulated phosphoinositide metabolism in bovine ASM is lacking.

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Effects of brevetoxin-B on motor nerve terminals of mouse skeletal muscle

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1 The effects of brevetoxin-B, a red tide toxin, on motor nerve terminal activity were assessed on mouse triangularis sterni nerve-muscle preparations. The perineural waveforms were recorded with extracellular electrodes placed in the perineural sheaths of motor nerves.

2 At 0.11 μM, brevetoxin-B increased the components of waveforms associated with sodium and potassium currents while it decreased the calcium activated potassium current and the slow calcium current of the nerve terminal. The fast calcium current and slow potassium current were not affected.

3 At 1.11 μM, brevetoxin-B decreased all of the components of waveforms associated with sodium, potassium and calcium currents.

4 It is concluded that brevetoxin-B affects sodium, potassium as well as calcium currents in the nerve terminal. The effects may contribute to its pharmacological actions on synaptic transmission.

Keywords: Brevetoxin; nerve terminal current; red tide toxin; neuromuscular transmission

Introduction

The dinoflagellate *Ptychodiscus brevis* (formerly *Gymnodinium breve*) is responsible for numerous fish kills during its blooms ('red tides') along the coast of the Gulf of Mexico and for human toxicity due to ingestion of contaminated shellfish (Steidinger, 1979). In nerve and muscle preparations, Shinnick-Gallagher (1980) reported that the crude fraction of *Gymnodinium breve* toxin depolarized the resting membrane potential of rat diaphragm. Tetrodotoxin antagonized the toxin depolarized membrane. Wu *et al.* (1985) reported that T-17, a fraction of purified toxin isolated from *Ptychodiscus brevis* caused an increase in the frequency of miniature endplate potentials in rat and frog neuromuscular junctions. Similar results were found in rat hemidiaphragm (Gallagher & Shinnick-Gallagher, 1985). T-17 also depolarized the squid giant axons causing the sodium channels to open at the normal resting potential (Wu *et al.*, 1985). Based on the electrophysiological studies on vertebrate synaptic transmission and on squid giant axon, it was suggested that the brevetoxin-enhanced transmitter release was caused by the effect of brevetoxins on the sodium channel in the motor nerve terminal. However, there is no direct evidence proving brevetoxin acts on the sodium channel in the motor nerve terminal.

Brevetoxin-B can be isolated in crystallized form (Lin *et al.*, 1981; Baden *et al.*, 1981; Chou & Shimizu, 1982) and the method of perineural waveform recordings allowed the characterization of channels in the nerve terminals (McArdle *et al.*, 1981; Penner & Dreyer, 1986). The effects of various toxins on motor nerve terminal currents have been well studied (Anderson *et al.*, 1988; Anderson & Harvey, 1988a, b). The aim of this study was to elucidate the possible modes of action of brevetoxin-B on the nerve terminal currents.

Methods

Experiments were carried out *in vitro* on the isolated triangularis sterni nerve-muscle preparation (McArdle *et al.*, 1981) of adult I.C.R. mice (*Mus musculus* from Institute of Cancer Research, U.S.A.) of either sex weighing between 17–25 g. Tissues were immersed in a physiological solution containing (mM): NaCl 115, KCl 5.0, $CaCl_2$ 2.5, $MgSO_4$ 1.0, $NaHCO_3$ 25, Na_2HPO_4 1.0 and glucose 11. The bath was maintained at room temperature (22–25°C) and continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2, maintaining the physiological solution at pH 7.3. (Penner & Dreyer, 1986). For recording the perineural waveform, preparations were visualized at a $\times$400 magnification by a Zeiss microscope equipped with Normarksi interference contrast optics (Dreyer *et al.*, 1979). The preparation was continuously perfused (3–6 ml min^{-1}) with modified Krebs solutions of the compositions described above.

Signals following nerve stimulation through a suction electrode were recorded inside the endothelial tube of nerve bundles (containing 2–4 nerve fibres) with glass microelectrodes filled with 0.5 M NaCl (resistance 4–10 megaohm). The reference electrode was a silver/silver chloride wire in the recording chamber. The potential difference between the recording electrode and the reference electrode in the bath was measured by a high impedance unity gain amplifier (Axoclamp-2), displayed on a dual beam storage oscilloscope and simultaneously stored on FM tape. Wave-forms were evoked by stimulating the motor nerve via a suction electrode every 2–30 s with supramaximal pulses of 0.05 ms duration. To avoid the contribution of postsynaptic responses, the preparation was treated with (+)-tubocurarine (30 μM). Investigations on the 'Ca currents' were made in the presence of tetraethylammonium chloride (TEA, 1 mM) and 3,4-diaminopyridine (3,4-DAP, 100–500 μM) as indicated in the results.

As the shape of the waveform recorded was very dependent on the electrode position, waveforms were monitored continuously from the same site before and throughout application of drugs.

Materials

Tetraethylammonium, 3,4-diaminopyridine, (+)-tubocurarine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Brevetoxin-B was purified by Hong-Nong Chou according to the method of Chou & Shimizu (1982) and it was dissolved in absolute ethanol as a stock solution.

Results

Effect on the perineural waveforms

The effect of brevetoxin-B on the nerve terminal currents was tested in nerve-muscle preparations immobilized by pretreatment with (+)-tubocurarine (30 μM). Figure 1a shows a nerve

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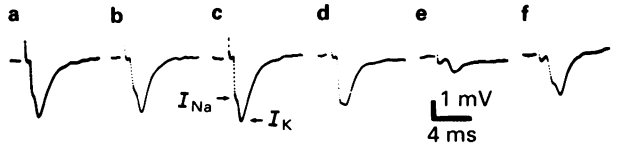

Figure 1 The effects of brevetoxin-B on nerve terminal current of mouse motor nerve terminal. The preparation was incubated in normal physiological solution containing 2.5 mM $CaCl_2$ and (+)-tubocurarine (30 μM). The nerve was stimulated at 0.5 Hz. (a) An example of a perineural waveform; (b), (c), (d) and (e) were perineural waveforms 10 min after brevetoxin-B (0.055, 0.11, 0.55, 1.11 μM) application, respectively. (f) Perineural waveform 30 min after (e) and washing. Note that sodium current (I_{Na}) and potassium current (I_K) showed a biphastic perineural waveform.

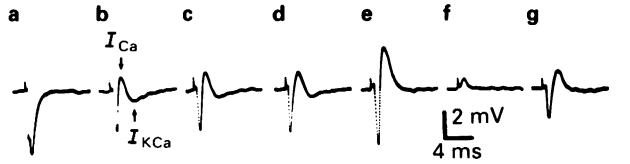

Figure 2 The effects of brevetoxin-B on calcium-dependent potassium current (I_{KCa}) of mouse motor nerve terminal. The perineural waveforms were recorded in the same area of nerve terminal. The nerve was stimulated at 0.03 Hz. At (a) the preparation was incubated in normal physiological solution containing 30 μM (+)-tubocurarine and 2.5 mM calcium; (b) perineural waveform recorded 20 min after addition of 30 μM (+)-tubocurarine ((+)-Tc) and 500 μM 3,4-diaminopyridine (3,4-DAP). At (c), (d), (e) and (f) brevetoxin-B, (0.011, 0.055, 0.11, 1.11 μM) were added respectively for 10 min. At (g) brevetoxin-B was washed with physiological solution containing 500 μM 3,4-DAP and (+)-Tc (30 μM) for 30 min from (f). Note that brevetoxin-B decreased the calcium-dependent potassium current when it increased the sodium and calcium (I_{Ca}) currents in the nerve terminal as shown in (e).

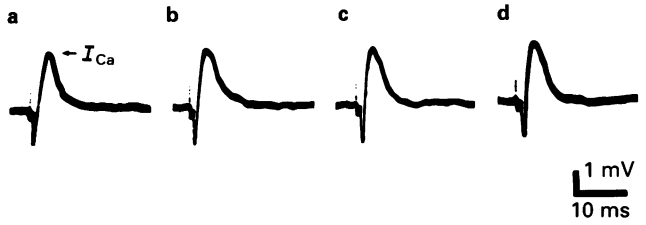

Figure 3 The effects of brevetoxin-B on the slow potassium current of motor nerve terminal. Calcium current (I_{Ca}) was displayed after suppression of the fast potassium current and calcium-dependent potassium current by tetraethylammonium (TEA, 30 mM). The perineural waveforms were recorded in the same area of motor nerve terminal. (+)-Tubocurarine (30 μM) and TEA (30 mM) were present throughout the experiment from (a) to (d). The nerve was stimulated at 0.03 Hz. (a) Control signal; at (b), (c) and (d), brevetoxin-B (0.011, 0.055, 0.11 μM) were added respectively for 10 min. Note that brevetoxin-B did not affect the calcium current, indicating that brevetoxin-B did not alter the slow potassium current of the nerve terminal.

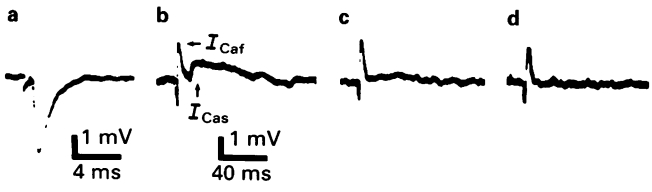

Figure 4 The effects of brevetoxin-B on presynaptic calcium currents in mouse motor nerve terminals. The nerve was stimulated at 0.3 Hz. (a) Control signal; from (b) to (d), the preparation was incubated in a solution containing tetraethylammonium (1 mM) and 3,4-diaminopyridine (100 μM). At (c) and (d) brevetoxin-B (0.11, 0.55 μM), respectively was further added to the preparation for 10 min. Note that brevetoxin-B did not alter the fast calcium current (I_{Caf}) of the motor nerve terminal, while it decreased the slow calcium current (I_{Cas}).

signal following supramaximal nerve stimulation. It consisted of a predominant biphasic negativity which was often preceeded by a small positivity. This waveform closely resembled focally recorded signals obtained at the transition between myelinated axon and non-myelinated terminal where evidence has been presented that the first negativity is due to Na^+ influx (I_{Na}) and the second negative phase corresponds to a passive current generated by the K^+ efflux (I_K) in the nerve terminals (Brigant & Mallart, 1982; Penner & Dryer, 1986; Anderson & Harvey, 1988a, b).

Brevetoxin-B affected the perineural waveform in a concentration-dependent manner. The concentration-effect relationships of brevetoxin-B on the perineural waveforms are shown in Figure 1. At 0.11 μM, brevetoxin-B increased both the sodium and potassium currents in the nerve terminal (Figure 1c). At a higher concentration (1.11 μM), brevetoxin-B decreased both sodium and potassium currents in the nerve terminal (Figure 1e). The effect of brevetoxin-B on the perineural waveform was reversible. After 30 min of washing, the perineural waveform was recovered. Similar results were obtained in five experiments.

Effect of brevetoxin-B on the presynaptic calcium-activated potassium current

For investigations of calcium-activated potassium currents (I_{KCa}) in the nerve terminal, 3,4-DAP (500 μM) was added to the bath solution. This concentration ensured maximal responses and consistently revealed the typical triphasic signals shown in Figure 2b. The signal component of the calcium-activated potassium current was decreased after addition of brevetoxin-B (0.11 μM) as showing in Figure 2e. Note that the sodium and outward current (calcium current, I_{Ca}) in the nerve terminal were increased after brevetoxin-B (0.11 μM)-treatment (Figure 2e). However, the sodium and calcium currents in the nerve terminal were decreased if a higher concentration of brevetoxin-B (1.11 μM) was applied (Figure 2f).

After repetitive washing for 30 min, the sodium and calcium currents in the nerve terminal recovered while the I_{KCa} blocked by brevetoxin-B had not yet recovered (Figure 2g). Similar results were found in 4 experiments.

Effect on slow tetraethylammonium-resistant potassium current

A dose of TEA as high as 30 mM failed to elicit full calcium plateau responses. Prolonged calcium responses could only be obtained by subsequent addition of 3,4-DAP (200 μM). This was taken as evidence for the presence of a TEA-resistant K^+-current in mammalian motor nerve terminals (Penner & Dreyer, 1986). We studied the sensitivity of this current to brevetoxin-B by looking for the ability of the compound to promote full calcium plateau in 30 mM TEA-treated preparations. As shown in Figure 3, the calcium plateau could not be elicited after addition of brevetoxin-B (0.11 μM). If a higher concentration of brevetoxin-B (1.11 μM) was applied, the calcium current as well as the sodium current was decreased. The results indicate that brevetoxin-B has no effect on the slow TEA-resistant potassium current. Similar results were found in 4 experiments.

Effect of brevetoxin-B on the presynaptic calcium current

TEA and 3,4-DAP are potassium channel blockers. A combination of both TEA and 3,4-DAP gave rise to a large positive deflection of the presynaptic current, which was blocked by Cd^{2+}, indicating its underlying cause to be a calcium current (Penner & Dreyer, 1986). There were two different presynaptic calcium currents in mouse motor nerve terminal. The fast positive signal component (fast calcium current, I_{Caf}) was attributed to the voltage-dependent calcium channel, responsible for the initiation of transmitter release. The slow

positive signal component (slow calcium current, I_{Cas}) also depended on extracellular concentration although its physiological role remained unknown (Penner & Dreyer, 1986).

The effects of brevetoxin-B on the nerve terminal calcium currents are shown in Figure 4. Brevetoxin-B, at 0.11–0.55 μM, decreased the slow component of the calcium channel in motor nerve terminal. The fast component of the calcium channel was not affected at this concentration. However, at 1.11 μM, brevetoxin-B decreased both fast and slow components of calcium currents in the nerve terminal. The sodium current in the nerve terminal was also decreased. Similar results were found in 5 experiments.

Discussion

In the present experiments, we found that brevetoxin-B (0.11 μM), not only increased the sodium and potassium currents of motor nerve terminal, it also decreased the I_{KCa} of the nerve terminal. At this concentration, brevetoxin-B had no effect on the potassium current of the terminal currents.

The I_{KCa} of the nerve terminal current may be important in regulating nerve terminal excitability (either by altering the frequency of depolarizations, or shortening the duration of depolarization), preventing excessive accumulation of internal Ca^{2+} and thereby inhibiting transmitter release. Therefore, the decreasing of I_{KCa} may contribute in some part to the effect of brevetoxin-B on the transmitter releasing process. It is interesting to note that charybdotoxin, a blocker of calcium activated potassium channel, also produced a moderate increase in the evoked release of acetylcholine after 3,4-DAP pretreatment (Anderson *et al.*, 1988).

Brevetoxin-B (0.11 μM) did not alter the fast calcium current of the nerve terminal (Figure 4), while it increased the outward current in a 3,4-DAP (500 μM)-pretreated preparation (Figure 2e). The reason for the different effects of brevetoxin-B on those currents remained unknown. However, brevetoxin-B also increased the sodium current and calcium-activated potassium current. Any change in the operation of the neuronal sodium current will alter the driving force for the nerve terminal currents.

In the mouse isolated diaphragm preparation, brevetoxin-B initially induced spontaneous muscle twitching, it also increased the frequency of miniature endplate potentials and depolarized the resting membrane potential of the mouse diaphragm (Tsai *et al.*, 1991). Similar results were also found in rat isolated diaphragm and frog cutaneous pectoris muscle (Wu *et al.*, 1985; Gallagher & Shinnick-Gallagher, 1980; 1985). Brevetoxins caused a concentration-dependent depolarization of the crayfish and squid giant axons membranes. Based on the results from rodent skeletal muscle and squid giant axon, it was suggested that the primary action of the toxin on the membrane is to cause the sodium channels to open at the normal resting potential (Wu *et al.*, 1985). However, from the studies on the nerve terminal currents, it appeared that brevetoxin-B affected not only the sodium current, it also acted on the calcium-activated potassium current and slow calcium current in the nerve terminal. It appeared that some of the effects of brevetoxin-B on the signals recorded may be due to alterations in the magnitude of the nerve terminal depolarization.

At 0.11 μM, brevetoxin-B had no effect on the fast calcium current and slow potassium current of the nerve terminal. However, those currents were decreased after treatment with a higher concentration (1.11 μM) of brevetoxin-B. The effect may be due to brevetoxin-B decreasing the sodium current. The sodium current which provided the driving force for nerve terminal currents was decreased after treatment with a high concentration of brevetoxin-B.

It is concluded that brevetoxin-B acts on the sodium, slow calcium and calcium-activated potassium currents of the motor nerve terminal. The effects may contribute to its pharmacological actions on synaptic transmission.

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Endothelins: vasoconstrictor effects and localization in canine cerebral arteries

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1 The vascular effects of endothelin and localization of endothelin-like immunoreactivity were characterized in isolated cerebral arteries of dogs.

2 Endothelin-like immunoreactivity was detected in a few populations of endothelial cells of dog basilar artery.

3 Endothelin-1, endothelin-2 and endothelin-3 contracted isolated ring preparations of cerebral arteries in a dose-dependent manner independently of the presence of endothelium. The ED_{50} values (and 95% confidence intervals) for the contraction were 411 pM (242–697 pM) and 478 pM (295–776 pM) for endothelin-1 and endothelin-2, respectively. Endothelin-3 induced vascular contraction at a higher concentration (ED_{50} = 26.5 nM, 95% confidence interval = 15.7–45.7 nM).

4 The increases in tone induced by endothelin-1 and endothelin-2 did not return to the resting level after repeated washings, while a rinse with Krebs solution reversed the vasoconstrictor response to endothelin-3. The endothelins did not cause any vasodilator response in arteries precontracted with uridine 5'-triphosphate even in the presence of intact endothelial cells.

5 $NiCl_2$ (1 mM) attenuated the contractions induced by endothelin-3 (10–300 nM) and those to relatively low doses (1 nM) but not higher doses (10–100 nM) of endothelin-1 and endothelin-2. The contractions in response to endothelin-1, endothelin-2 and endothelin-3 were greatly attenuated in Ca^{2+}-free solutions although high concentrations of endothelin-1 and endothelin-2 still evoked contractions.

6 These results suggest that the vasoconstriction induced by endothelin-3 and lower doses of endothelin-1 and endothelin-2, largely depends on the influx of Ca^{2+} ions. The apparent insensitivity to Ni^{2+} shows that additional distinct mechanisms also operate in the vasoconstrictor responses to high concentrations of endothelin-1 and endothelin-2.

7 The presence of endothelin-like immunoreactivity in endothelial cells suggests that endothelin is a potential endogenous spasmogen.

Keywords: Dog cerebral artery; endothelin; immunohistochemistry; nicardipine; nickel; vasoconstriction

Introduction

Cultured vascular endothelial cells produce endothelin, a vasoconstrictor peptide with 21 amino acid residues (Yanagisawa *et al.*, 1988). Analysis of genomic DNA has shown the presence of three members of an endothelin family, endothelin-1, endothelin-2 and endothelin-3, in mammals including man (Inoue *et al.*, 1989a; Yanagisawa & Masaki, 1989). Endothelin-3 is a less potent constrictor of porcine coronary arteries *in vitro*, while endothelin-2 is equally or more potent than endothelin-1 (Inoue *et al.*, 1989a). Although quantitative differences in the vasoconstrictor effects of these endothelins have been shown in porcine coronary arteries, it is not known whether or not there is a qualitative difference in the vasoconstrictor mechanism of these peptides.

Endothelin-1 causes a direct and sustained vasoconstriction of cerebral arteries from cats and dogs *in vitro* and *in vivo* (Saito *et al.*, 1989b; Mima *et al.*, 1989; Asano *et al.*, 1989). Although endothelin-1 constricts various vascular tissues, there seems to be a considerable difference in the pharmacological effects of endothelin-1 among the tissues. For example, the vasoconstrictor responses induced by endothelin-1 are reportedly sensitive to nicardipine, a Ca^{2+}-channel blocker, in porcine coronary and cat cerebral arteries but not in the aortae of rats and rabbits (Kasuya *et al.*, 1989a; Saito *et al.*, 1989b; Sakata *et al.*, 1989; Ohlstein *et al.*, 1989). Endothelin-1 also evokes an endothelium-dependent vasodilator response in rat aorta and mesenteric arteries (Sakata *et al.*, 1989; De Nucci *et al.*, 1988) but not in cat cerebral arteries.

Recently the presence of endothelin-1 in both plasma and cerebrospinal fluid was demonstrated in patients with subarachnoid haemorrhage (Masaoka *et al.*, 1989; Fujimori *et al.*, 1990). In the present study, we have characterized the cerebrovascular effects of the endothelin family and investigated the localization of endothelin by an immunohistochemical method in dog cerebral arteries.

Methods

Mongrel dogs of either sex (11–19 kg) were anaesthetized with sodium pentobarbitone (Nembutal; Abbot, North Chicago, U.S.A.; 60 mg kg^{-1}, i.v.) and exsanguinated. The brains were removed, then the middle cerebral and basilar arteries were dissected out and placed in a Krebs solution. The data obtained from these arteries were pooled since there is no difference in the responses of these arteries to agonists (Toda, 1990). The composition of the Krebs solution was (mM): NaCl 113, KCl 4.8, $CaCl_2$ 2.2, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 5.5. A high K^+ (100 mM) Krebs solution was prepared by replacing NaCl with equimolar amounts of KCl. A Ca^{2+}-free Krebs solution was prepared by omitting $CaCl_2$ from the Krebs solution. An 0.1 M stock solution of ethylene glycol *bis*(β-aminoethyl ether)N,N,N',N',-tetra-acetic acid (EGTA) was prepared with distilled water and the pH of this solution was adjusted to 7.4; in some experiments, EGTA was

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added to the Ca^{2+}-free Krebs solution to give a concentration of 200 μM.

A stainless rod and a short piece of tungsten wire were carefully passed through the lumen of ring segments (4 mm long) of the arteries which were then mounted in tissue baths containing 30 ml Krebs solution. The tungsten wire was anchored to a plastic gate and the steel rod was connected to a transducer (Nihon Kohden TB 612T) for isometric recordings of changes in the contractile force. The organ baths were siliconized before the experiments to prevent nonspecific adhesion of the peptides. The Krebs solutions were maintained at 37°C and bubbled with a gas mixture of 95% O_2 and 5% CO_2 throughout the experiment. The resting tension of the tissue was adjusted to 1.0 g. At the beginning of the experiment, the vasoconstrictor response to 100 mM KCl was obtained in each tissue so that the vasoconstrictor responses to the endothelins could be expressed as a % of K-induced contractions. In some tissues uridine 5'-trisphosphate (UTP; 30–100 μM) was applied to gain an active tone of 0.5 to 1.0 g.

Full dose-response curves were constructed by cumulative addition of the endothelin peptides. In some preparations the endothelium was removed by rubbing the intimal surface with a scored needle (Saito *et al.*, 1989a). The functional absence of endothelium was confirmed by the lack of an endothelium-dependent vasodilator response to substance P. From the ED_{50} values of the preparations the geometric mean and the 95% confidence interval were obtained (Fleming *et al.*, 1972).

For immunohistochemical experiments, basilar arteries were fixed with formaldehyde and picric acid, dehydrated with ethanol, cleared with xylene, rehydrated and then frozen (Saito *et al.*, 1989a). Transverse sections (10 μm) of the arteries were obtained by cutting with a cryostat. Monoclonal antibodies (KM 565; Goto *et al.*, 1990) were applied (1 : 2000) and then the avidin-biotin peroxidase complex (ABC) method (Hsu *et al.*, 1981) was used to demonstrate the localization of endothelin-like immunoreactivity. The immunoreaction was observed under a microscope (Axioskop; Carl Zeiss; Oberkochen, Germany). In the same sections, the endothelium was identified as a monolayer of cells covering the inner surface of the internal elastic lamina of the artery (Lüsher, 1988) by use of a differential interference contrast. The monoclonal antibodies used in the present study cross-react with endothelin-1, endothelin-2, endothelin-3 and porcine big endothelin-1. Since the antibodies might have cross-reacted with unknown substances, the immunoreactive material was termed "endothelin-like" in the present study.

The drugs used were endothelin-1, endothelin-2 and endothelin-3 (Peptide Institute; Osaka, Japan), EGTA (Dojin; Kumamoto, Japan), nicardipine (Sigma; St Louis, U.S.A.), nickel chloride (Wako; Osaka, Japan), substance P (Peptide Institute) and UTP (Sigma). The endothelins were prepared in a phosphate buffered saline (pH 7.4) containing 0.05% bovine serum albumin. The statistical analysis was carried out by Dunnett's test using a computer package (YUKUMUS Statistic Library; Yukumus; Kawasaki, Japan).

Results

Endothelin-1 and endothelin-2 caused slowly developing and dose-dependent vasoconstrictor responses at doses from 20 pM–20 nM. Repeated washings of the tissue with fresh Krebs solution did not reduce the tone induced in the arteries by these endothelins (Figure 1). In the presence of tone induced by UTP, additional vasoconstrictor responses occurred in response to relatively high concentrations of endothelins. In these precontracted tissues, substance P induced a vasodilator response (Figure 2).

Endothelin-3 also caused a vasoconstrictor response. In contrast to the responses caused by endothelin-1 and endothelin-2, the endothelin-3-induced vasoconstriction was not sustained after rinsing the tissue with fresh Krebs solution (Figure 1). Endothelin-3 did not cause a vasodilator response in the presence of UTP-induced tone (Figure 2).

Figure 3 shows the cumulative dose-response curves in endothelium-intact and endothelium-free preparations; the ED_{50} values and the maximum responses induced by the endothelins are shown in Table 1. The vasoconstrictor effects did not differ between endothelium-intact and endothelium-free preparations. There was no difference in the ED_{50} values between endothelin-1 and endothelin-2. The ED_{50} value for endothelin-3 was about 60 times greater than those for endothelin-1 and endothelin-2. Endothelin-1 and endothelin-3 induced similar maximum responses, but endothelin-2 evoked a significantly larger maximum response than those given by endothelin-1 and endothelin-3.

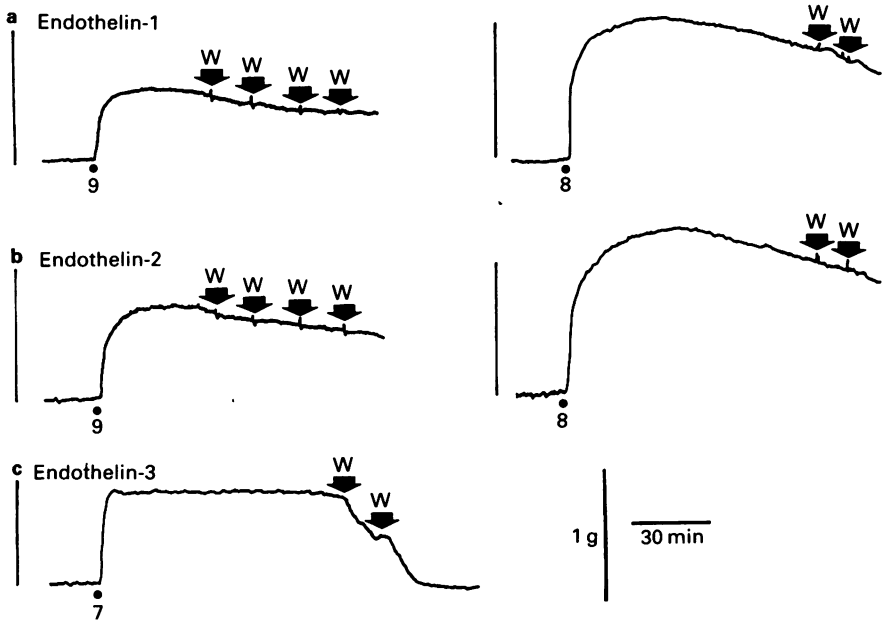

Figure 1 Typical vasoconstrictor responses to endothelin-1 (a), endothelin-2 (b) and endothelin-3 (c). Washing with fresh Krebs solution (W) did not reduce the increased tone by endothelin-1 or endothelin-2. The increased tone induced by endothelin-3 returned to a resting level by repeated washing with Krebs solution. The numbers in the figure indicate the negative logarithm of the peptide concentration. Each trace was obtained from separate preparations.

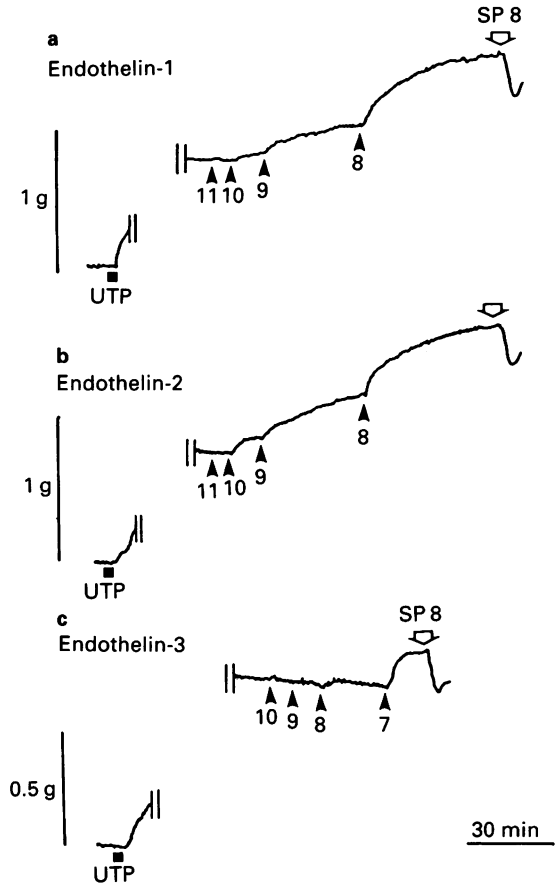

Figure 2 The effect of endothelins on precontracted dog cerebral arteries. Endothelin-1 (a), endothelin-2 (b) and endothelin-3 (c) did not cause any vasodilator responses in tissues precontracted with uridine 5'-trisphosphate (UTP). In all these tissues substance P (SP) caused a vasodilator response. The numbers in the figure indicate the negative logarithm of the concentration of the peptides.

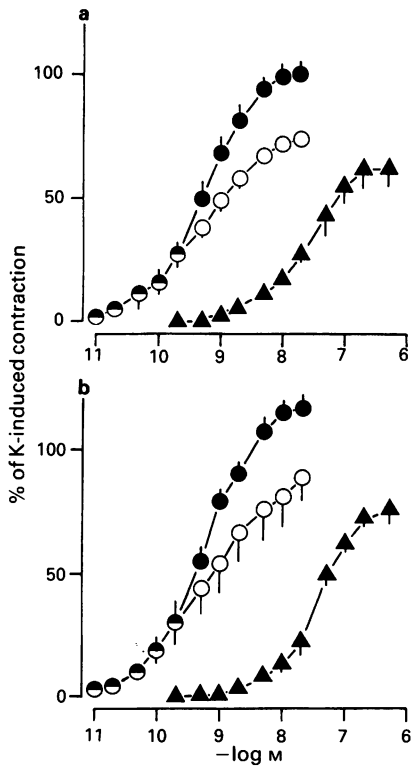

Figure 3 Dose-response curves to the cumulative addition of endothelin-1 (○), endothelin-2 (●) and endothelin-3 (▲) in endothelium-intact (a) and endothelium-free (b) preparations. The vasoconstrictor response was expressed as a percentage of the contraction induced by 100 mM KCl. Each point and bar represents the mean and s.e.mean. At some points the bars are within the symbol. In the endothelium-intact preparations the number of tissues (*n*) and number of animals (N) were 9 and 6, respectively, for all the peptides. In endothelium-denuded preparations, (*n*, N) were (7, 4), (8, 5) and (5, 4) for endothelin-1, endothelin-2 and endothelin-3, respectively.

Addition of 1 mM $NiCl_2$ caused a slight vasodilatation and then a slight vasoconstriction in some tissues (Figure 4). $NiCl_2$ suppressed the vasoconstrictions induced by 1 nM but not by 10 or 100 nM of either endothelin-1 or endothelin-2 (Figure 4 and Figure 5). The vasoconstrictor response to endothelin-3 (10–300 nM) was profoundly attenuated by 1 mM $NiCl_2$. Nicardipine (10 nM) also caused a vasodilator response in some arterial preparations. The vasoconstrictor responses induced by the three endothelins were similarly reduced by nicardipine (Figure 5).

Removal of Ca^{2+} ions also caused a vasodilatation from

Table 1 ED_{50} values and the maximum responses for endothelins in dog isolated cerebral arteries

	ED_{50} (nM) (geometric mean and 95% confidence interval)	*Maximum response* (% of K-induced contraction) mean ± s.e.mean	*n*, N
Endothelium-intact			
Endothelin-1	0.411 (0.242–0.697)	74.4 ± 2.8	9, 6
Endothelin-2	0.478 (0.295–0.776)	95.5 ± 4.2[c]	9, 6
Endothelin-3	26.5[ab] (15.7–45.7)	62.6 ± 8.7[d]	9, 6
Endothelium-removed			
Endothelin-1	0.741 (0.468–1.17)	88.5 ± 9.5	7, 4
Endothelin-2	0.513 (0.331–0.812)	117.7 ± 4.4[c]	8, 5
Endothelin-3	35.8 (22.9–56.2)	75.6 ± 5.7[d]	5, 4

n, N: number of tissues and number of aminals.
[a] Significant difference between the ED_{50} for endothelin-1 and endothelin-3 ($P < 0.01$).
[b] Significant difference between the ED_{50} for endothelin-2 and endothelin-3 ($P < 0.01$).
[c] Significant difference between the maximum response to endothelin-1 and endothelin-2 ($P < 0.05$).
[d] Significant difference between the maximum response to endothelin-2 and endothelin-3 ($P < 0.01$).
There were no significant differences ($P > 0.05$) between the ED_{50}s for endothelin-1 and endothelin-2 and the maximum responses to endothelin-1 and endothelin-3.

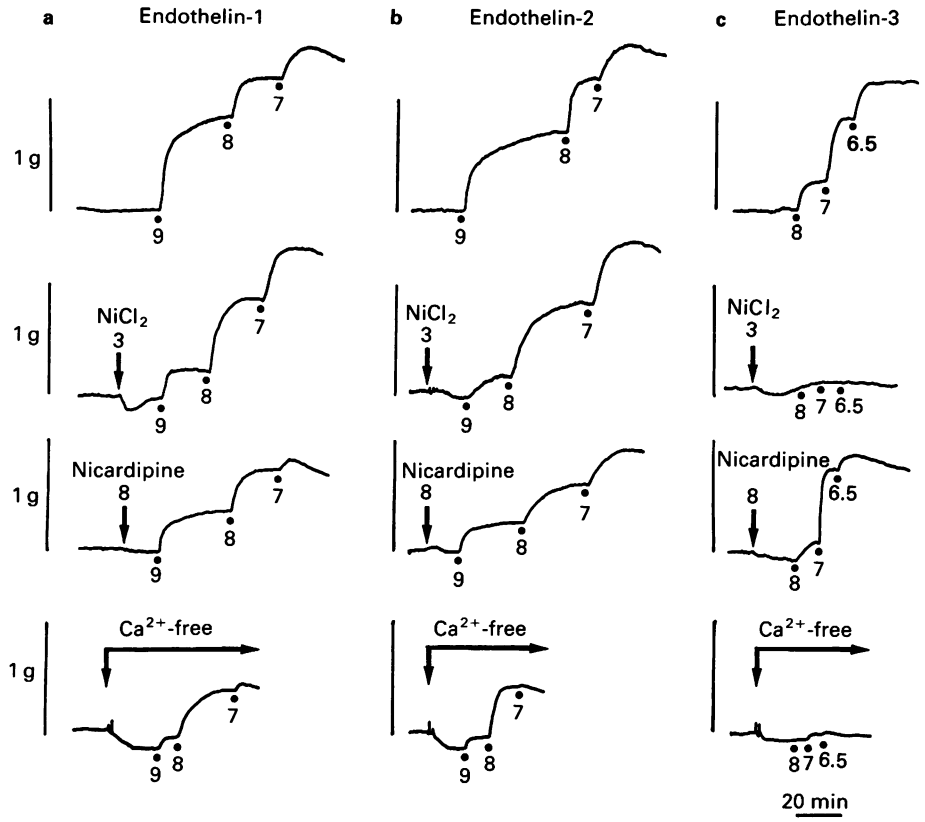

Figure 4 Representative tracings of the effects of 1 mM $NiCl_2$, 10 nM nicardipine and Ca^{2+}-removal (no EGTA) on the vasoconstrictor effects of endothelin peptides. Three doses (1, 10 and 100 nM for both endothelin-1 and endothelin-2, and 10, 100 and 300 nM for endothelin-3) were examined. The numbers in the figure indicate the negative logarithm of the concentration of the peptide.

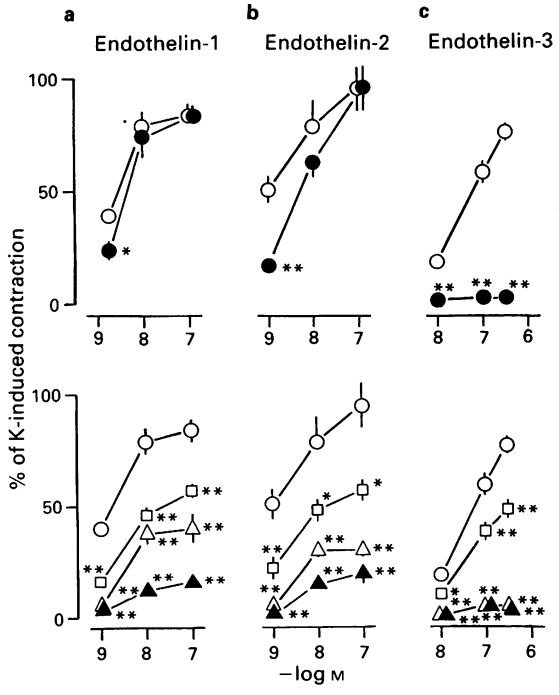

Figure 5 Effects of various treatments on the vasoconstrictor responses induced by endothelin-1, endothelin-2 and endothelin-3. Each point and bar represents the mean and the s.e.mean. Number of tissues (*n*) and number of animals (N) for control (○), 1 mM $NiCl_2$ (●), 10 nM nicardipine (□), Ca^{2+}-removal without EGTA (△) and Ca^{2+}-removal with 0.2 mM EGTA (▲) were as follows (*n*, N): endothelin-1 (7, 4), (7, 4), (14, 8), (6, 4), (8, 5); endothelin-2 (7, 4), (8, 5), (6, 3), (6, 4), (6, 3) and endothelin-3 (9, 6), (7, 4), (8, 5), (6, 4), (6, 3). *Significantly different from the control ($P < 0.05$). **Significantly different from control ($P < 0.01$).

the resting state (Figure 4). In Ca^{2+}-free solutions, the contraction to endothelin-3 was abolished whereas the endothelin-1- and endothelin-2-induced contractions were reduced, but not abolished. Addition of EGTA (0.2 mM) to Ca^{2+}-free solutions further suppressed the endothelin-1- and endothelin-2-induced vasoconstrictions (Figure 5).

In 8 out of 9 tissues (from 6 animals), endothelin-like immunoreactivity was found to be present in endothelial cells of the basilar arteries (Figure 6). Even in these positive tissues only a few cells in each section exhibited an endothelin-like immunoreactivity. The immunoreaction was not observed when the antibodies were preincubated with 10 μM endothelin-1 overnight.

Discussion

Endothelin-1 causes potent and long-lasting vasoconstriction of cerebral arteries of cats and dogs *in vitro* (Saito *et al.*, 1989b; Asano *et al.*, 1989). The present study showed that not only endothelin-1 but also endothelin-2 and endothelin-3 were potent vasoconstrictors of dog cerebral arteries. Judging from the ED_{50} values, endothelin-2 was as potent as endothelin-1; however, endothelin-3 was 60 times less potent than endothelin-1. Although the maximum contractile responses induced by endothelin-1 and endothelin-3 were not significantly different, endothelin-2 caused a larger maximum response than endothelin-1. The orders of the potency of, and the maximum responses to, the endothelins were similar to those observed in the porcine coronary artery (Inoue *et al.*, 1989a). The vasoconstrictions induced by endothelin-1 and endothelin-2 were not easily reversed by removing the peptide from the bathing solution. The long-lasting effect of endothelin-1 may be due to its low dissociation rate from the binding sites (Hirata *et al.*, 1988). In contrast, the elevated tone of the artery induced by endothelin-3 returned to the

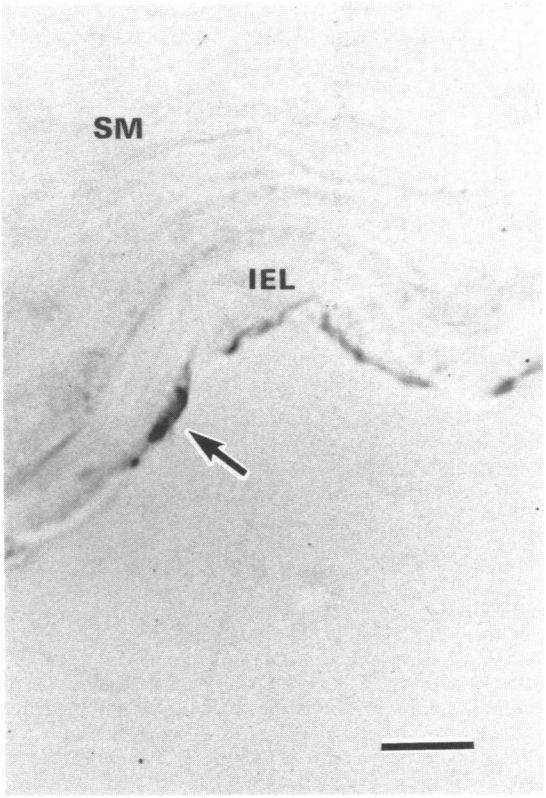

Figure 6 Endothelin-like immunoreactivity (arrow) in endothelial cells of the basilar artery of the dog. Bar equals 10 μm. IEL: internal elastic lamina; SM: smooth muscle.

resting level upon washing with a fresh solution. Since endothelin-3 is less hydrophobic than endothelin-1 and endothelin-2, endothelin-3 may dissociate from its receptor more easily than endothelin-1 and endothelin-2.

Although endothelins evoke endothelium-dependent vasodilatation in some peripheral vascular tissues (De Nucci *et al.*, 1988; Sakata *et al.*, 1989; Warner *et al.*, 1989; Douglas & Hiley, 1990), a vasodilator response was not evoked by the endothelins in dog cerebral arteries. Acetylcholine, arachidonic acid and angiotensins induce vasoconstriction by releasing a vasoconstrictor substance from endothelial cells in dog cerebral arteries (Shirahase *et al.*, 1987; Manabe *et al.*, 1989). However, the vasoconstrictor actions of the endothelin were independent of the presence of endothelial cells in the present study. Thus, in dog cerebral arteries all these endothelins cause strong vasoconstriction by acting directly on the smooth muscle rather than indirectly by acting on endothelial cells. Further, it is suggested that endothelins do not release endothelium-derived relaxing factor in cerebral arteries.

Endothelin-1 seems to cause vasoconstriction by both increasing the influx of Ca^{2+} and also releasing Ca^{2+} from intracellular store sites in coronary arteries of pigs (Kasuya *et al.*, 1989a,b). Although the vasoconstrictor responses induced by the endothelins were profoundly attenuated, small but consistent vasoconstrictor responses were evoked by endothelin-1 and endothelin-2 in Ca^{2+}-free solutions in dog cerebral arteries. This suggests that the endothelins cause vasoconstrictor responses by an extracellular Ca^{2+}-dependent mechanism and that endothelin-1 and endothelin-2 may act also by a mechanism which is independent of extracellular Ca^{2+}. Since the removal of Ca^{2+} from the medium, expecially in the presence of EGTA, may reduce the vasoconstriction induced by not only Ca^{2+}-influx but also mobilization of intracellular Ca^{2+} (Ohlstein *et al.*, 1989), the effects of the blockade of Ca^{2+}-influx by Ni^{2+} and of nicardipine, an organic Ca^{2+}-channel blocker, were further examined in the present study.

The vasoconstrictor responses induced by endothelin-3 and those caused by low (1 nM), but not those by high (10–100 nM), doses of endothelin-1 and endothelin-2 were sensitive to Ni^{2+}. The pharmacological effects of Ni^{2+} on membrane Ca^{2+}-channels are not known in dog cerebral arteries. However, Ni^{2+} suppresses the Ca^{2+}-influx through channels which are distinct from L-type Ca^{2+}-channels in the smooth muscle of the aorta (Lawson & Cavero, 1989). Accordingly, it appears that the vasoconstrictor responses induced by either endothelin-3 or low doses of endothelin-1 and endothelin-2 are mediated by the influx of Ca^{2+} into the smooth muscle cells through the Ni^{2+}-sensitive pathway.

Organic Ca^{2+}-channel blockers inhibit the endothelin-1-induced vasoconstrictor responses of porcine coronary and cat cerebral arteries but not of aorta or mesenteric arteries of rats and rabbits (Kasuya *et al.*, 1989a; Saito *et al.*, 1989b; Ohlstein *et al.*, 1989; D'Orleans-Juste *et al.*, 1989). In the present study, nicardipine attenuated the vasoconstrictor responses to all three endothelins to a similar extent. It has been shown that endothelin-1 causes its vasoconstrictor effects by the activation of L-type Ca^{2+}-channels in porcine coronary arteries (Yanagisawa *et al.*, 1988; Goto *et al.*, 1989). However, Ca^{2+}-channel blockers inhibit the vasoconstrictor responses induced by various vasoconstrictors in dog cerebral arteries by not only suppressing the influx of Ca^{2+} through Ca^{2+}-channels but also by depleting Ca^{2+} from intracellular stores (Asano *et al.*, 1987; Yamamoto *et al.*, 1983; Fujiwara *et al.*, 1982). In either case, the results suggest that Ca^{2+}-channel blockers may be useful in the relief of any cerebral vasoconstriction induced by endogenous endothelins.

The vasoconstrictor responses induced by high doses of endothelin-1 and endothelin-2 were entirely resistant to Ni^{2+}. Thus it may be suggested that the mode of Ca^{2+}-influx induced by high doses of endothelin-1 and endothelin-2 is different from that by low doses in dog cerebral arteries. However, endothelin-1, at doses above 1 nM induces contraction of vascular smooth muscles by mobilization of Ca^{2+} from intracellular stores both in porcine coronary arteries (Kasuya *et al.*, 1989b) and in the aortae of rats and rabbits (August *et al.*, 1988; Sakata *et al.*, 1989; Huang *et al.*, 1990; Ohlstein *et al.*, 1989). Therefore, it is likely that in dog cerebral arteries, endothelin-1 and endothelin-2 at high doses evoke vasoconstriction by an additional extracellular Ca^{2+}-independent mechanism.

The attenuation by Ni^{2+} of the vasoconstriction induced by endothelin-3, but not that caused by high doses of endothelin-1 or endothelin-2, indicates that the vasoconstrictor effect of endothelin-3 was not only quantitatively but also qualitatively distinct from those of endothelin-1 and endothelin-2. Recently, the presence of multiple binding sites for the endothelins has been shown in chicken cardiac membranes; a binding site for endothelin-1 and endothelin-2, and the other for endothelin-3 (Watanabe *et al.*, 1989). It is conceivable that these receptor subtypes couple to different intracellular messenger systems or channels. Therefore, high doses of endothelin-1 and endothelin-2 may activate distinct receptor subtypes from that for endothelin-3, although the vascular endothelin-binding sites are yet to be characterized.

The present study revealed the presence of endothelin-like immunoreactivity in a few endothelial cells of the cerebral arteries of dogs. Similar endothelin-like immunoreactivity is also present in neurones of the porcine spinal cord (Yoshizawa *et al.*, 1989). Unlike neuronal cells, secretory granules are not present in endothelial cells. Thus it has been supposed that endothelial cells release endothelin synthesized *de novo* (Yanagisawa & Masaki, 1989). Nevertheless, the demonstration of endothelin-like immunoreactivity suggests that some cells do retain a certain amount of an endothelin-like substance.

The antibodies used in the present study cross-reacted with endothelin-1, endothelin-2 and endothelin-3. Cultured endothelial cells from human umbilical vein produce mRNA for preproendothelin-1 but not preproendothelin-2 or

preproendothelin-3 (Inoue *et al.*, 1989b). Furthermore, cerebral microvessels of pigs also produce mRNA for preproendothelin-1 (Yoshimoto *et al.*, 1990). Although this observed endothelin-like substance is likely to be endothelin-1, it cannot be determined exactly which form of endothelin is present in endothelial cells of cerebral arteries. As shown in the present study, endothelin-1, as well as endothelin-2 and endothelin-3, causes a sustained vasoconstriction of cerebral arteries. Accordingly, endogenous endothelin produced in endothelial cells, even if it is endothelin-2 or endothelin-3, may affect the tone of the underlying vascular smooth muscles.

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Rapid decrease in neuropeptide Y gene expression in rat adrenal gland induced by the α_2-adrenoceptor agonist, clonidine

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1 The mechanism of regulation of the neuropeptide Y (NPY) gene by pharmacological treatment with the α_2-adrenenoceptor agonist, clonidine, was investigated by quantitative Northern blot analysis of the effects of this drug on the NPY mRNA levels in rat adrenal gland and medulla oblongata/pons.

2 In the adrenal gland, clonidine-treatment ($50\,\mu g\,kg^{-1}$, s.c., once daily) resulted in decrease in the amount of NPY mRNA to $44 \pm 10\%$ of the control level in 24 h and then its increase to $162 \pm 16\%$ of the control level after 5 days. Concomitant changes in putative NPY pre-mRNA species (7.0 and 3.3 kb) were observed, probably due to changes at the level of NPY gene transcription.

3 The short-term (24 h) effect of clonidine was blocked by yohimbine ($5\,mg\,kg^{-1}$, i.p., once daily). Yohimbine alone tended to increase the NPY mRNA level after 24 h.

4 The recovery/increase in the NPY mRNA level in the adrenal gland after 5 days treatment with clonidine was similar to its increase after treatment with reserpine ($0.5\,mg\,kg^{-1}$, i.p., once daily).

5 NPY gene expression in the medulla oblongata/pons was not changed by short- or long-term treatment with clonidine.

6 These results suggest that clonidine suppresses NPY gene expression in the adrenal gland, probably at the level of transcription, by activation of the α_2-adrenoceptor.

Keywords: Neuropeptide Y; gene expression; clonidine; α_2-adrenoceptor; rat adrenal; rat medulla oblongata/pons

Introduction

Neuropeptide Y (NPY) is a 36-amino acid peptidergic cotransmitter/neuromodulator of central and peripheral catecholaminergic neurones (especially perivascular sympathetic neurones), including the adrenal gland derived from sympathetic neurones (Tatemoto, 1982; Everitt *et al.*, 1984; de Quidt & Emson, 1986). The recent finding that NPY coexists with catecholamines and is co-released with the latter on nerve stimulation suggests its importance in sympathetic neurotransmission (Lundberg *et al.*, 1982; Håkanson *et al.*, 1986; Higuchi *et al.*, 1988a). In addition to its role in sympathetic neurotransmission and/or neuromodulation, NPY in the circulation seems to be involved in regulation of the peripheral and cardiac arterial blood pressure and cardiac functions (Allen *et al.*, 1983; Gray & Morley, 1986; Edvinsson *et al.*, 1987). Central administration of NPY results in marked hypotension, whereas its peripheral systemic administration induces prolonged hypertension (Fuxe *et al.*, 1983; Dahlöf *et al.*, 1985).

High concentrations of NPY-immunoreactivity (NPY-I) have been found in chromaffin cells and nerve fibres in the adrenal gland (de Quidt & Emson, 1986; Schalling *et al.*, 1988b; Higuchi *et al.*, 1990). NPY-I is stored in chromaffin granules (Majane *et al.*, 1985) and is co-released with catecholamines from the adrenals into the circulation (Allen *et al.*, 1984). NPY functions as an endogenous inhibitor of catecholamine release from the adrenals (Higuchi *et al.*, 1988a) and a potent vasoconstrictor in the circulation (Dahlöf *et al.*, 1985; Edvinsson *et al.*, 1987). Judging from these effects, NPY synthesized in the adrenal gland seems to participate in regulation of the systemic blood pressure and release of adrenal catecholamines into the circulation.

On the other hand, in the medulla oblongata/pons many NPY-immunoreactive neurones are located in ventrolateral C1 and dorsal vagal C2 adrenergic cell groups as well as in A1 and A4 noradrenergic cell groups (Everitt *et al.*, 1984; Gray & Morley, 1986). NPY cell groups in the ventrolateral medulla seem to be important in regulation of the vasomotor centre (Ward-Routledge & Marsden, 1988; Tseng *et al.*, 1988; McAuley *et al.*, 1989; Sun & Guyenet, 1989).

There is increasing evidence that the steady-state level of NPY is regulated by physiological factors such as aging, innervation, stress and pharmacological treatments that influence sympathetic functions or blood pressure (Higuchi & Yang, 1986; Lundberg *et al.*, 1987; Higuchi *et al.*, 1988b; Higuchi, 1989). During aging, NPY gene expression increases specifically in the adrenal gland and medulla oblongata/pons (Higuchi *et al.*, 1991).

The antihypertensive drug, reserpine, changes the steady-state level of NPY and also prepro-NPY gene (NPY gene) expression in peripheral sympathetic organs (Schalling *et al.*, 1988a,b,c; Higuchi *et al.*, 1990). Thus one effect of antihypertensive drugs may be to modify the level of vasoconstrictive NPY peptide, by changing its rate of biosynthesis and/or turnover.

The imidazoline derivative clonidine, an α_2-adrenoceptor agonist, lowers the blood pressure by central inhibition of sympathetic nerve activity (Haeusler, 1974). Moreover, short-term treatment with clonidine increases the steady-state level of NPY in peripheral organs, such as the heart and adrenal gland, in contrast to reserpine, which has a suppressive effect (Nagata *et al.*, 1986; Franco-Cereceda *et al.*, 1987; Lundberg *et al.*, 1987). One way in which clonidine elevates the NPY peptide level seems to be by inhibiting NPY release by activating presynaptic α_2-adrenoceptors (Dahlöf *et al.*, 1986). However, like reserpine, it might also affect NPY gene expression in neuronal cells.

To investigate whether clonidine modulates gene expression of the vasoconstrictive NPY peptide, we examined its effects on the level of NPY mRNA in the adrenal gland and medulla oblongata/pons, in relation to vascular control. For this purpose we carried out quantitative Northern blot analyses with a cloned rat NPY cDNA (Higuchi *et al.*, 1988c), as a probe.

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Methods

Drug treatment

Male Sprague-Dawley (SD) rats were used. Clonidine hydrochloride ($50\,\mu g\,kg^{-1}$, s.c.), yohimbine hydrochloride ($5\,mg\,kg^{-1}$, i.p.) and reserpine ($0.5\,mg\,kg^{-1}$, i.p.) in saline were injected into 8–week-old rats (body weight 250–280 g) once daily for 1 or 5 days. Control rats received the same volume (0.1 ml) of saline only. Rats were killed by decapitation 24 h after the last injection.

Preparation of cellular RNA

Brains were dissected as described by Glowinski & Iversen (1966). Pairs of adrenal glands and medulla oblongata/pons, obtained immediately after decapitation, were homogenized in at least 5 volumes of 4 M guanidinium thiocyanate solution. Total cellular RNA in the extracts was purified by centrifugation through 5.7 M CsCl, and quantitated by their absorbance at 260 nm (Higuchi *et al.*, 1988c). The RNA yields were not affected by drug treatment and mean values were as follows: $89 \pm 6\,\mu g$ per pair of adrenal glands ($n = 30$); $108 \pm 12\,\mu g$ per the medulla oblongata/pons ($n = 31$).

Quantitation of neuropeptide Y mRNA

The level of NPY mRNA was determined by Northern blot analysis and comparison of autoradiographic signals with those of standard RNA samples run at the same time, as described previously (Higuchi *et al.*, 1988c). For standardization, at least 5 different amounts (2.5–100 pg) of pBL-NPY1 transcripts (NPY sense RNA) and a standard rat striatum RNA preparation were run simultaneously with or without carrier rat liver RNA. The autoradiographic signals were quantitated by densitometric scanning and integration of peak areas corresponding to mature NPY mRNA (approximately 800 bases). Northern blot rather than dot blot analysis was used because the latter method is usually not sufficiently sensitive for quantitation of NPY mRNA (Higuchi *et al.*, 1988c; 1990; Sabol & Higuchi, 1990).

Statistical methods

Statistical significance was determined by Student's *t* test.

Materials

Clonidine hydrochloride (Sigma), yohimbine hydrochloride (Nakarai Tesque) and reserpine (Daiichi Seiyaku Co) were used.

Results

Figure 1 shows results of Northern blot analysis on change in NPY mRNA from the adrenal gland after clonidine treatment. The rat NPY cDNA probe hybridized with at least three mRNA species of different sizes. Besides mature NPY mRNA (800 bases), larger RNA species (3300 and 7000 bases) were detected in samples from the adrenal gland and brain (Figures 1 and 3). These larger RNA species (7.0 and 3.3 kb) are consistent in size with the unspliced and incompletely spliced transcripts predicted from the structure of the rat NPY gene, suggesting the presence of putative NPY pre-mRNA species (Larhammar *et al.*, 1987; Sabol & Higuchi, 1990; Higuchi *et al.*, 1990).

The amounts of NPY mRNA and the two putative NPY pre-mRNA species (0.8, 3.3 and 7.0 kb, respectively) in the adrenal gland decreased to an equal extent after treatment with the α_2-adrenoceptor agonist, clonidine, for 24 h (Figures 1, 2, Tables 1 and 2). Their decreases induced by clonidine were blocked in the presence of the α_2-adrenoceptor antagonist yohimbine (Figure 1, Tables 1 and 2). Yohimbine alone tended to increase the levels of the NPY mRNA and NPY pre-mRNA species (0.8, 3.3, and 7.0 kb, respectively), but not significantly (Figures 1 and 2, Tables 1 and 2). As these changes in the level of mature NPY mRNA were associated with changes of a similar extent in the level of the putative NPY pre-mRNA species (3.3 and 7.0 kb), the changes in NPY gene expression probably occurred at the level of gene transcription. The absolute amounts of NPY mRNA in the adrenal glands after short-term treatments with clonidine and/or yohimbine were quantitated by the comparison with signals of the synthesized pBL-NPY1 transcripts (NPY sense RNA) (Table 1). The results suggested that NPY gene expression in the adrenal gland is decreased by activation of the

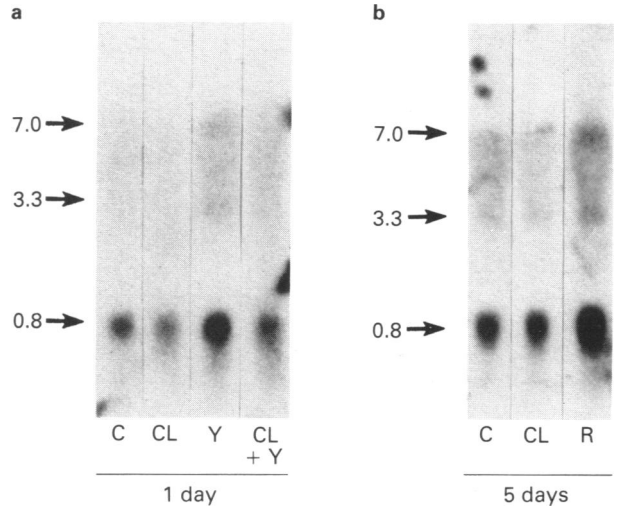

Figure 1 Northern blot analysis of neuropeptide Y (NPY) mRNA from rat adrenal gland after drug treatment. Male rats (8 weeks old) were treated with clonidine (CL, $50\,\mu g\,kg^{-1}$, s.c.), yohimbine (Y, $5\,mg\,kg^{-1}$, i.p.), reserpine (R, $0.5\,mg\,kg^{-1}$, i.p.), and/or vehicle (C) once daily for 1 day or 5 days as indicated. Then tissues were promptly excised and lysed in 5–10 volumes of 4 M guanidinium thiocyanate solution. Total cellular RNAs were prepared, purified, and quantitated as described in the Methods. Total cellular RNAs ($25\,\mu g$/sample) were separated by electrophoresis in formaldehyde agarose gel, blotted and hybridized with the 511-bp EcoRI insert of the pBL-NPY1 plasmid. Numbers at the left indicate lengths of hybridized RNAs (0.8, 3.3 and 7.0 kb). The density of NPY mRNA band of control samples are a little different in (a) and (b) due to different exposure time.

Table 1 Effect of clonidine treatment on the level of neuropeptide Y (NPY) mRNA in rat adrenal gland

Treatment		*NPY mRNA* ($pg\,\mu g^{-1}$ tcRNA)
Control	(1 day)	1.70 ± 0.25 (6)
Clonidine	(1 day)	0.75 ± 0.17 *(6)
Yohimbine	(1 day)	2.20 ± 0.24 (3)
Clonidine + yohimbine	(1 day)	1.76 ± 0.07 (3)
Control	(5 days)	1.54 ± 0.14 (3)
Clonidine	(5 days)	2.50 ± 0.24 †(3)
Reserpine	(5 days)	3.17 ± 0.30 **(3)

Male rats (8 weeks old) were treated with clonidine ($50\,\mu g\,kg^{-1}$, s.c.), yohimbine ($5\,mg\,kg^{-1}$, i.p.) and reserpine ($0.5\,mg\,kg^{-1}$, i.p.) once daily for 1 day or 5 days, as indicated. The adrenal glands were immediately excised and their NPY mRNA abundances were quantitated as described in the Methods. tcRNA is total cellular RNA. Values are means ± s.e.mean for 3 or 6 independent experiments (as shown in parentheses). Each experiment was done with an extract of tissue from two or three rats. Significantly different from the control value: $*P < 0.01$; $**P < 0.05$.
† Significantly different from the value after clonidine treatment for 1 day: $P < 0.01$.

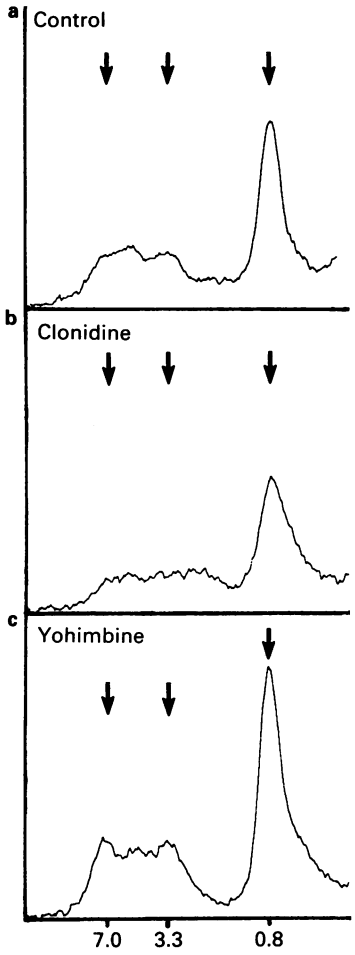

Figure 2 Densitogram of drug-induced changes in neuropeptide Y (NPY) mRNA in rat adrenal gland. Autoradiographic signals of NPY mRNA from the adrenal gland after drug treatment for 1 day were quantitated by scanning densitometry. Exposure times of autoradiograms were chosen so that peak areas were within the range of linear variation with the amount of RNA applied. Numbers below the traces indicate the lengths of hybridized RNAs (0.8, 3.3 and 7.0 kb).

α_2-adrenoceptor with clonidine, probably at the level of NPY gene transcription.

After the initial decrease to 44 ± 10% of the control level, the amount of NPY mRNA in rat adrenal gland increased significantly to 162 ± 16% of the control level after 5 days treatment with clonidine (Table 1). As shown in Figure 1 and Table 2, this increase in the amount of NPY mRNA was associated with increases in the level of the putative NPY pre-mRNA species (3.3 and 7.0 kb) in the adrenal gland, suggesting increase in the level of NPY gene transcription. Similar increase in NPY gene expression in the adrenal gland was observed after 5 days treatment with reserpine, although the hypotensive mechanisms of clonidine and reserpine are different (Figure 1, Tables 1 and 2).

In contrast, the level of NPY mRNA in the medulla oblongata/pons was not changed significantly by treatment with clonidine for 1 or 5 days (Figure 3 and Table 3), suggesting that α_2-adrenoceptors are not involved in regulation of NPY gene expression in the medulla oblongata/pons.

Discussion

The antihypertensive drug reserpine is known to deplete sympathetic neurones of monoamines by interfering with Mg^{2+}-dependent vesicular storage mechanisms for amines. Recently short-term treatment with reserpine was found to decrease the level of NPY-I without changing that of NPY mRNA in peripheral sympathetic organs including the adrenal gland (Lundberg *et al.*, 1986; Nagata *et al.*, 1987; Higuchi *et al.*, 1990). This effect was due to accelerated release of NPY from the nerve terminals and the chromaffin cells by increase in

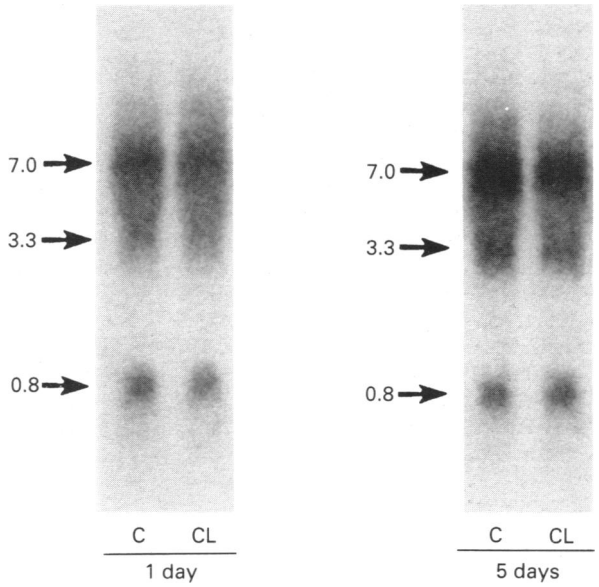

Figure 3 Northern blot analysis of neuropeptide Y (NPY) mRNA from rat medulla oblongata/pons after drug treatment. Male rats (8 weeks old) were treated with drugs or vehicle once daily for 1 or 5 days as for FIgure 1. The RNAs from the medulla oblongata/pons were prepared, quantitated and separated by electrophoresis in formaldehyde agarose gel as described in the Methods. Treatment: C; vehicle, CL; clonidine.

Table 2 Effect of clonidine treatment on the putative neuropeptide Y (NPY) pre-mRNA levels in rat adrenal gland

		NPY pre-mRNA level (% of control)	
Treatment		3.3 kb-species	7.0 kb-species
Control	(1 day)	100 ± 11	100 ± 10
Clonidine	(1 day)	44 ± 12*	41 ± 9*
Yohimbine	(1 day)	110 ± 11	143 ± 25
Clonidine + yohimbine	(1 day)	110 ± 15	95 ± 2
Control	(5 days)	100 ± 14	100 ± 4
Clonidine	(5 days)	118 ± 5	104 ± 5
Reserpine	(5 days)	190 ± 21*	200 ± 32*

Male rats (8 weeks old) were treated with clonidine, yohimbine, and reserpine as described for Table 1. Total cellular RNA (tcRNA) was prepared from the adrenal glands and subjected to Northern blot analysis, as described in the Methods. The relative quantities of the putative NPY pre-mRNA species (3.3 and 7.0 kb) were determined by comparison of the densities of autoradiographic bands of samples (25 μg tcRNA) and are shown as percentages of the control values (means ± s.e.mean) from three independent experiments. Each experiment was done with two independent extracts of tissue from two or three rats. Significantly different from the control value: $*P < 0.05$.

Table 3 Absence of change in the neuropeptide Y (NPY) mRNA level in rat medulla oblongata/pons on clonidine treatment

Treatment	*NPY mRNA* (pg μg^{-1} tcRNA)
Control (1 day)	2.63 ± 0.52
Clonidine (1 day)	2.66 ± 0.18
Control (5 days)	3.04 ± 0.70
Clonidine (5 days)	2.89 ± 0.64

Total cellular RNA (tcRNA) from the medulla oblongata/pons of the same rats as for Figure 1 were subjected to quantitative Northern blot analysis and levels of NPY mRNA were measured as described in the Methods. Values are means ± s.e.mean for three independent experiments. Each experiment was done with an extract of tissue from two rats.

sympathetic nerve activity in response to hypotension (Lundberg *et al.*, 1986; Higuchi *et al.*, 1990). Long-term treatment with reserpine results in gradual increase in NPY gene expression in chromaffin cells in the adrenal gland or ganglion cells in sympathetic ganglia, due to trans-synaptic activation as the result of increased activity of the preganglionic sympathetic nerves (Higuchi *et al.*, 1990; Schalling *et al.*, 1988c). This induction of NPY gene expression resulted in increased biosynthesis of NPY peptides (Higuchi *et al.*, 1990). Thus, reserpine clearly induces both release (/turnover) of NPY peptides and gene expression and biosynthesis of NPY.

In contrast, short-term treatment with clonidine increases the tissue content of NPY-I in peripheral tissues (Nagata *et al.*, 1986; Franco-Cereceda *et al.*, 1987). One reason for this effect is its inhibition of presynaptic release of NPY from sympathetic nerve terminals by activation of the α_2-adrenoceptor (Dahlöf *et al.*, 1986). Interestingly, short-term treatment (24 h) with clonidine at 50 $\mu g\,kg^{-1}$ decreased NPY gene expression, probably at the level of gene transcription, by activating the α_2-adrenoceptor in the adrenal gland *in vivo* (Figure 1, Tables 1 and 2). Suppression of NPY gene expression can result in decreased production of NPY peptides and so its inhibition of NPY gene expression may also decrease the amount of usable/releasable NPY peptides from the adrenal gland. If so, this inhibition of NPY gene expression in the adrenal gland probably participates in the hyptensive action of clonidine.

Clonidine (50 $\mu g\,kg^{-1}$) changed the levels of NPY mRNA and putative NPY pre-mRNA species in the adrenal gland, but not in the medulla oblongata/pons. This finding suggested that this α_2-adrenoceptor agonist regulates NPY gene expression preferentially in peripheral sympathetic neurones, including those in the adrenal gland, but does not produce hypotension by interfering with NPY biosynthesis in the medulla oblongata/pons.

One possible cause of the clonidine-induced suppression of NPY gene expression in the adrenal gland may be decrease in splanchnic nerve activity, because clonidine causes central inhibition of sympathetic nerve activity (Svensson, 1987; Pernow *et al.*, 1988) and consequently decreases trans-synaptic activation of chromaffin cells, which is one mechanism inducing NPY gene expression (Higuchi *et al.*, 1990). However, this possibility is unlikely, because elimination of trans-synaptic activation by denervation did not change NPY gene expression in the adrenal gland under ordinary conditions (Higuchi *et al.*, 1990). Therefore, the inhibition of NPY gene expression in the adrenal gland by clonidine seems to depend on its direct activation of the α_2-adrenoceptor on the chromaffin cells.

The normal counterpart of the PC12 rat phaeochromocytoma cell line is the chromaffin cells of the rat adrenal medulla. In PC12 cells, elevation of the intracellular adenosine 3′:5′-cyclic monophosphate (cyclic AMP) level, alone or in combination with other second messengers and/or hormones, results in rapid activation of NPY gene transcription with consequent increase in NPY mRNA abundance (Higuchi *et al.*, 1988c; Sabol & Higuchi, 1990). Therefore, decrease in the intracellular cyclic AMP content via the α_2-adrenoceptor on the chromaffin cells induced by clonidine may result in decrease in NPY gene expression at the level of transcription.

The suppression of NPY gene expression in the adrenal gland by clonidine is rapid and transient (Figure 1, Tables 1 and 2). The subsequent increase in NPY gene expression may result from desensitization of the α_2-adrenoceptor. However, the mean NPY mRNA level in the rat adrenal gland after 5 days was more than the control level (162 ± 16%). This overshoot was probably due to increase in splanchnic nerve activity in response to the hypotensive state, as in the case with reserpine (Figure 1; Higuchi *et al.*, 1990). Thus, on long-term treatment with clonidine, NPY gene expression in the adrenal gland may recover quickly due to trans-synaptic activation of chromaffin cells as an adaptation to hypotension.

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Characterization of bombesin receptors in peripheral contractile organs

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1 Guinea-pig and rat urinary bladders, rat stomach and the guinea-pig gall bladder, four isolated organs that show high sensitivity to bombesin, were used to characterize bombesin receptors in peripheral organs.

2 The order of potency of agonists was determined with several naturally occurring peptides of the bombesin series, namely bombesin (BBS), litorin (Lit), neuromedin B (NMB), the gastrin-releasing peptide (GRP 18–27), neuromedin C (NMC) and with some bombesin fragments. It was found that bombesin, neuromedin C, litorin and two bombesin fragments, BBS (6–14) and AcBBS (6–14) had similar activities in the four preparations, while neuromedin B and [Phe^6]-neuromedin C were more active on the rat urinary bladder than on the other tissues.

3 The order of potency of agonists determined in the rat urinary bladder was as follows: BBS = NMB > Lit > NMC > [Phe^6]NMC = GRP and it was found to be different from that observed in the other preparations: BBS > GRP = Lit $\geqslant$ NMC $\gg$ NMB > [Phe^6]NMC, suggesting the existence of two different bombesin receptors, BBS_1 and BBS_2.

4 This interpretation was convalidated by the finding that bombesin antagonists, namely Ac.GRP(20–26)OCH_3 and Ac.GRP(20–26)OC_2H_5 reduced or blocked the effects of bombesin-related peptides on BBS_2 receptor systems while being completely inactive on the rat urinary bladder (BBS_1 system).

Keywords: Bombesin; receptor subtypes; antagonist; smooth muscles

Introduction

Bombesin is a tetradecapeptide isolated from amphibian skin (Anastasi *et al.*, 1971), and shown to have similarities to litorin (Anastasi *et al.*, 1975) and other mammalian peptides, the gastrin releasing peptide (GRP) (McDonald *et al.*, 1979), neuromedin B (NMB) (Minamino *et al.*, 1983) and neuromedin C (NMC), (Minamino *et al.*, 1984).

Bombesin and its homologues exert a variety of biological effects both on the central nervous system and in peripheral organs; they modulate thermoregulation (Brown *et al.*, 1977), activate gastric, pancreatic or intestinal secretions (Bertaccini *et al.*, 1973; Girard *et al.*, 1984; Otsuki *et al.*, 1987 and see also Jensen *et al.*, 1988a) and motility (Erspamer *et al.*, 1970; Erspamer & Melchiorri, 1973; Girard *et al.*, 1984; Severi *et al.*, 1988).

Erspamer *et al.* (1970) first observed that bombesin has a stimulant effect on isolated smooth muscles. More recently, Falconieri-Erspamer *et al.* (1988) have shown that litorin and phyllolitorin (two bombesin homologues) are potent stimulants of the rat urinary bladder but are rather weak on other smooth muscle preparations, which however show high sensitivity to bombesin. They concluded that multiple bombesin receptor types may be present in peripheral organs. Studies with antagonists, both the substance P (SP)-analogues (Jensen *et al.*, 1984; Regoli *et al.*, 1988) and the pseudopeptide analogues of bombesin (Jensen *et al.*, 1988a,b) have provided further indication that BBS and related peptides may act on two different receptor types.

Recent developments in the field of bombesin antagonists have centred on the GRP (20–26) sequence. Heimbrook *et al.* (1989) have shown that GRP (20–26)OCH_3 and GRP (20–26) OC_2H_5 competitively block the binding of GRP to Swiss 3T3 mouse fibroblasts.

In this study, we have attempted to characterize bombesin receptors by using a few bombesin homologues and fragments as well as some antagonists bearing the GRP (20–26) sequence. These peptides were tested on four smooth muscle preparations, the rat and guinea-pig urinary bladders (RUB; GPUB), the rat stomach (RS) and the guinea-pig gall bladder (GPGB).

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Methods

The experiments were carried out on tissues taken from rats (albino, Sprague Dawley, 200–300 g) and guinea-pigs (albino, 200–300 g) killed by stunning and exsanguination through sectioning of the carotid arteries. The organs were rapidly taken out and plunged in oxygenated Tyrode solution of the following composition (in mM): NaCl 137.0, KCl 2.7, $CaCl_2.6H_2O$ 1.8, $MgCl_2.6H_2O$ 1.05, Na_2HPO_4 0.4, $NaHCO_3$ 11.9 and dextrose 5.6. Strips of the four organs were prepared according to the procedure described by Vane (1957) for the rat stomach. The strips were then suspended in 10 ml organs baths containing Tyrode solution oxygenated with 95% O_2 and 5% CO_2 and kept at 32°C. A tension of 1 g was initially applied to the strips of GPUB, RUB and RS and 0.5 g to those of GPGB. Tissues were allowed to equilibrate for 60 to 80 min during which time they were washed and the tension readjusted at 15 min intervals. Changes of tension produced by bombesin and other substances were recorded isometrically with Grass force transducers (FTO3C) on Grass polygraphs (Model 7D).

Experimental protocols

Concentration-response curves for bombesin and several fragments and homologues were obtained by applying consecutively increasing concentrations of each peptide up to 10^{-5} M. The maximal response was measurable for the majority of the peptides at this concentration. Large variations in the maximal responses to the various peptides were observed in some of the tissues (for instance the rat stomach) and the maximal response of each preparation to BBS was taken as 100%, knowing that α^E in this condition is purely an empirical parameter useful to compare maximal effects of agonists. Peptide affinities were calculated from a minimum of six concentration-response curves and are expressed in terms of pD_2, the negative logarithm of the concentration of peptides that produces 50% of the maximum effect of each compound.

This method of calculation inevitably leads to underestimating the activities of the potent (α^E higher than 1.0) and to overestimating those of the weak (α^E lower than 1.0) peptides. Relative activities are expressed as a percentage of that of bombesin.

Apparent affinities of antagonists (pA_2, the negative logarithm of the concentration of antagonist that reduces the effect of a double dose of agonist to that of single dose; Schild, 1947) were determined by measuring biological responses to BBS in the absence and in the presence of the antagonist, the latter being added 7–8 min before measuring the effect of bombesin. Higher concentrations of some antagonists were also tested to estimate pA_{10} values and evaluate the competitivity of the antagonists from the pA_2–pA_{10} difference, according to Schild (1949). Schild plots were calculated according to Schild (1949) for the two antagonists.

In other experiments, peptidase inhibitors, namely captopril (1×10^{-6} M), thiorphan (2.3×10^{-6} M) and bestatin (10^{-6} M) were tested on the rat stomach, the rat urinary bladder and the guinea-pig urinary bladder, according to Rouissi et al. (1990a,b). In short, the effects of average high concentrations of each peptide were measured in the absence and presence of the peptidase inhibitor. These compounds were applied 20 to 30 min before testing the peptide in order to obtain a stable inhibition of the intramural enzymes.

Peptides and other drugs

Bombesin, litorin, neuromedin C, neuromedin B were purchased from Bachem; human GRP (1–27) was a gift from Dr S. St-Pierre, INRS, Montreal; BBS (6–14), Ac-BBS (6–14), Ac-GRP (20–26) OCH_3, Ac-GRP (20–26) OC_2H_5, Ac-GRP (20–26), Ac-[D-Phe^{12}]-BBS (6–14), [Phe^6]-neuromedin C, bradykinin and angiotensin II were prepared in our laboratory by Dr G. Drapeau by the solid-phase method (Drapeau & Regoli, 1988). The primary structures of the bombesin-related peptides are shown in Table 1. Peptides prepared in our laboratory were purified by high pressure liquid chromatography and their structures were assessed by fast atomic bombardment (FAB) mass spectrometry.

Atropine, methysergide, indomethacin, acetylcholine and histamine were purchased from Sigma, EDTA from Fisher and 3-amino-1-[*m*-(trifluoromethyl)-phenyl]2 pyrazoline (BW 755C) was a gift from Dr S. Moncada of Burroughs-Welcome. Thiorphan was purchased from Institut Armand Frappier Biochem International Inc., bestatin from Sigma and captopril was a gift from Dr J.G. Joly of Squibb Canada.

Concentrated solutions (1 mg ml^{-1}) of all peptides were made in distilled water and kept at −20°C. Daily solutions were made in 0.9% saline and discarded at the end of the experiment. Solutions of indomethacin were made in Trizma base.

Activities of agonists are presented in terms of pD_2 and those of antagonists in terms of pA_2 or pA_{10}. Data obtained in the absence and in the presence of antagonists or peptidase inhibitors have been compared, by Student's *t* test for paired samples. Probability values lower than 0.05 were considered to be significant.

Results

Effects of bombesin and related peptides on isolated organs

The four isolated organs used in the present study responded to BBS and related peptides with concentration-dependent contractions which developed rapidly; for instance, in the GPUB (Figure 1) and the GPGB (not shown). These contractions were reversible in a few minutes after washing out the peptides. In the other preparations, the RUB (Figure 1) and the RS (not shown), the contractile responses consisted of an initial rapid and late slow phase and took 5 to 8 min to reach the maximum: they were reversible, but more slowly than those of the tissues from the guinea-pig (Figure 1).

Concentration-response curves were measured with all the bombesin-related peptides agonists in the four tissues by applying consecutively, increasing concentrations of each peptide from threshold to 10^{-5} M.

Examples of such curves are shown in Figure 2 for bombesin and neuromedin B. The two peptides showed similar activities in the rat urinary bladder, but bombesin was much more active than neuromedin B on the other three tissues, particularly the GPGB. The other peptides gave concentration-response curves that occupied an intermediate position between bombesin and neuromedin B, and showed parallelism with that of BBS. This is shown in Figure 2 for the GPUB. From such curves, the activities of the various peptides were compared with that of BBS as mentioned under Methods.

Order of potency of bombesin homologues and fragments

Relative affinities (expressed as a percentage of that of BBS) of some homologues and fragments of bombesin, including those already tested by Regoli et al. (1988) are presented in Table 2. In all preparations, bombesin and its fragment Ac-BBS (6-14) were the most potent stimulants. The other fragment, BBS (6–14) was also very active on the RUB and the GPUB, but less active than bombesin on the others. Neuromedin B was as active as BBS on the RUB, while showing little activity on the other three preparations (see also Figure 2). The affinities of bombesin homologues in the GPUB and the GPGB were very similar and the rank order of potency of the investigated peptides was as follows: BBS > GRP = Lit ≥ NMC ≫ NMB > [Phe^6]-NMC.

The rat stomach showed some differences compared to the GPUB and GPGB. Firstly, NMC was much less active than BBS; however, NMB was a full agonist with almost 10% of

Table 1 Primary structure of bombesin-related peptides

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Bombesin (BBS)	pGlu	Gln	Arg	Leu	Gly	Asn	Gln	Trp	Ala	Val	Gly	His	Leu	Met	NH_2
BBS (6–14)						—	—	—	—	—	—	—	—	—	—
Ac-BBS (6–14)					Ac	—	—	—	—	—	—	—	—	—	—
Ac-[D-Phe^{12}]BBS (6-14)					Ac	—	—	—	—	—	—	DPhe	—	—	—
Neuromedin C (NMC)					—	—	His	—	—	—	—	—	—	—	—
[Phe^6]-NMC					—	—	His	—	—	Phe	—	—	—	—	—
Neuromedin B (NMB)					—	—	Leu	—	—	Thr	—	—	Phe	—	—
Litorin						pGlu	—	—	—	—	—	—	Phe	—	—
		15	16	17	18	19	20	21	22	23	24	25	26	27	
GRP (human)		R-Tyr	Pro	Arg	—	—	His	—	—	—	—	—	—	—	NH_2
Ac-GRP (20–26)						Ac	His	—	—	—	—	—	—	NH_2	
Ac-GRP (20–26) OCH_3						Ac	His	—	—	—	—	—	—	OCH_3	
Ac-GRP (20–26) OC_2H_5						Ac	His	—	—	—	—	—	—	OC_2H_5	

R = Val-Pro-Leu-Pro-Ala-Gly-Gly-Gly-Thr-Val-Leu-Thr-Lys-Met.

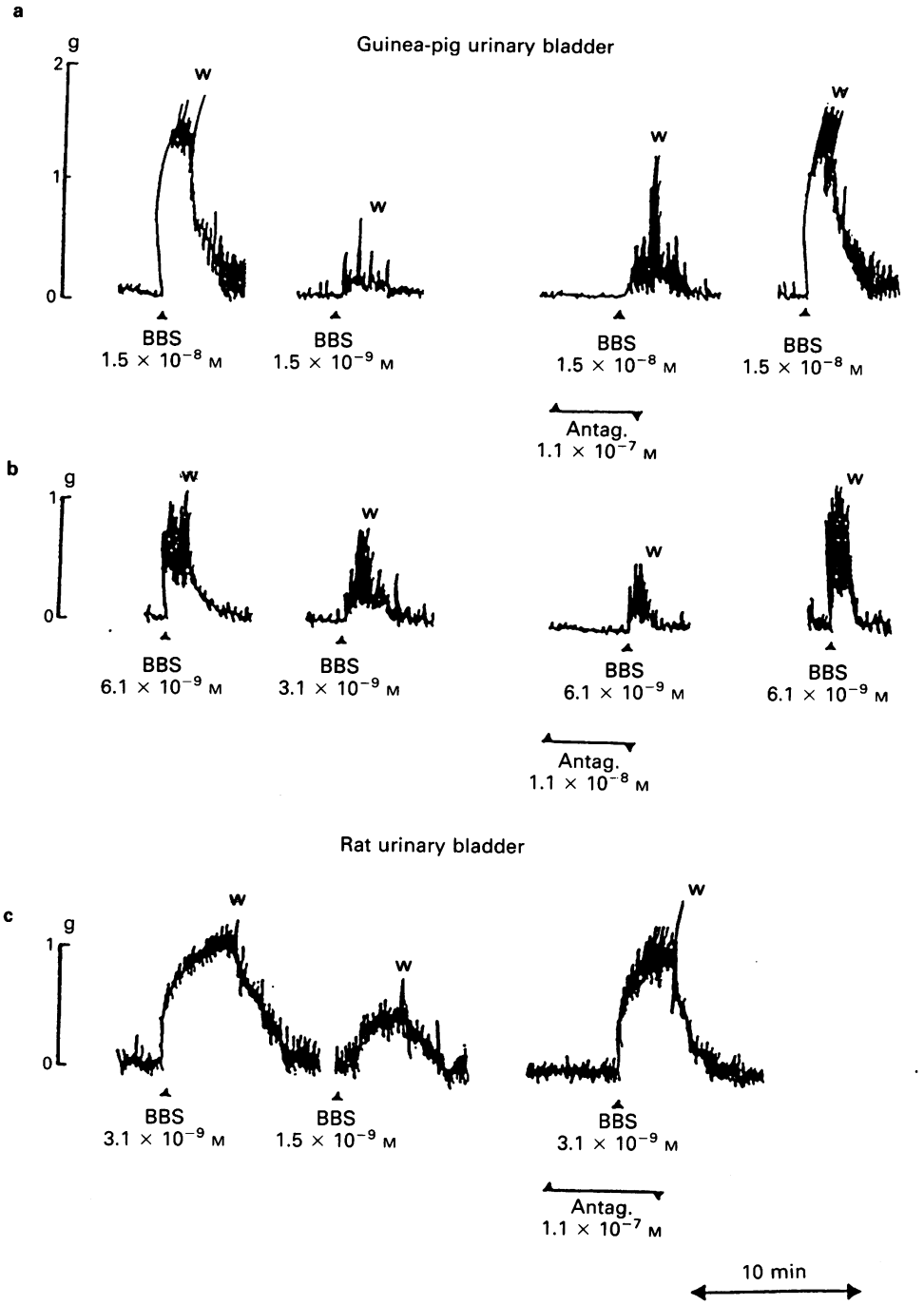

Figure 1 Myotropic effects of bombesin (BBS) obtained on the guinea-pig urinary bladder (a and b) and the rat urinary bladder (c). In (a) and (b), the first two tracings represent the contractile effect of BBS when the peptide is kept in contact with the tissue for 4 min. The third tracing represents the effects of BBS obtained in the presence of the antagonist AcGRP(20–26)OCH_3 applied 7 min beforehand. The fourth tracing shows the effect of BBS, measured 30 min later and indicates that the effect of the antagonist is reversible. Tracings shown in (c) were obtained on the rat urinary bladder with BBS in the absence (first two tracings) and presence of the antagonist (third tracing). At the point indicated by W, the bath fluid was replaced with fresh Tyrode solution. Abscissa scale: time in min; ordinate scale: changes of tension in g.

the activity of BBS: it was therefore more active than in the GPUB and especially in the GPGB. The order of potency of these peptides on the RS was: NMC > BBS > GRP = Lit > NMB > [Phe^6]-NMC.

In the RUB, two major differences were observed, the high affinity of neuromedin B which was equivalent to bombesin and a weak potency of GRP. In this preparation, the order of potency of BBS-related peptides was BBS = NMB > Lit > NMC > [Phe^6]-NMC = GRP.

This order of potency of agonists was different from those obtained in the other preparations, especially the GPUB and GPGB. The RS appeared to occupy an intermediate position, because of the high activity of NMC and a relatively high residual activity of NMB. Moreover, the two fragments of BBS showed exceptionally high maximal effects in this tissue (Table 2).

Affinities and competitivity of antagonists

Three analogues of the gastrin releasing peptide and an analogue of Ac-BBS (6–14) were evaluated as antagonists in the four preparations. As shown in Table 3, AC-[D-Phe^{12}]-BBS (6–14) and Ac-GRP (20–26) were found to be inactive both as

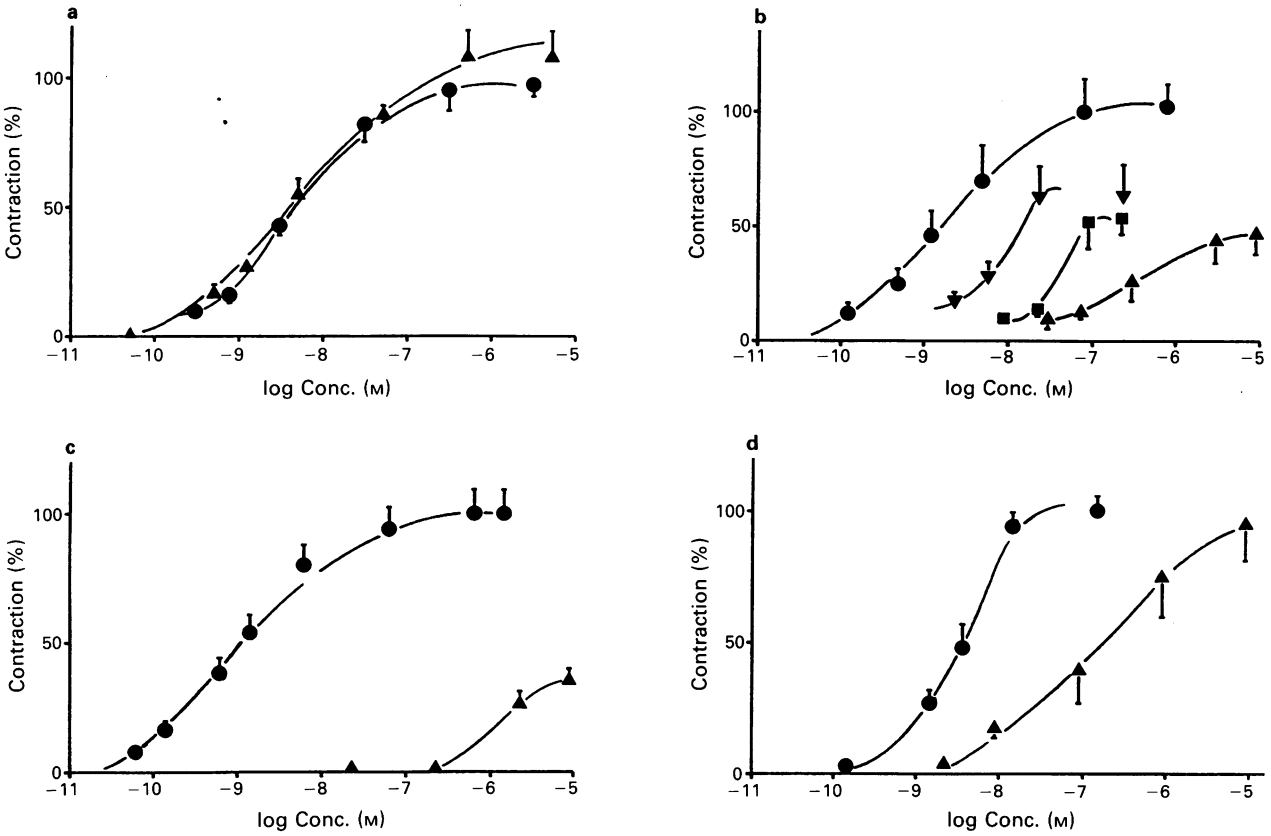

Figure 2 Concentration-response curves of bombesin (●) and neuromedin B (▲) obtained in four isolated organs, and neuromedin C (■) and GRP (▼) in the guinea-pig urinary bladder. Abscissa scale: −log of the concentration of agonist; ordinate scale: biological responses (contraction) as a percentage of that of bombesin. Points are mean and vertical bars are the s.e. of at least 6 determinations obtained in different tissues.

stimulants and as antagonists: conversely, Ac-GRP (20–26) OCH_3 and Ac-GRP (20–26) OC_2H_5 exerted a potent inhibitory effect against bombesin on the RS, the GPGB and the GPUB, showing pA_2 values of almost 8.0; the two compounds were however completely inactive on the RUB. pA_{10} was measured for the two compounds on the RS and the GPUB in order to establish the type of antagonism from the difference pA_2–pA_{10}. As shown in Table 3, the differences were near to 1.0 for the GPUB, suggesting that both antagonists exerted a competitive type of inhibition. This was confirmed by the Schild plot (Figure 3) which was linear and showed a slope of 0.981. The pA_2–pA_{10}, evaluated in the RS, were 1.10 and 0.92 respectively for Ac-GRP (20–26) OCH_3 and Ac-GRP (20–26) OC_2H_5.

When tested against substance P, bradykinin and angiotensin II on the GPUB, Ac-GRP (20–26) OCH_3 and Ac-GRP (20–26) OC_2H_5 were found to be inactive: the antagonists appeared therefore to be specific for bombesin receptors.

The tracings of Figure 1 show that one of the peptides (Ac-GRP (20–26) OCH_3) had no direct effect at the concentration of 1.1×10^{-8} M or higher (1.1×10^{-7} M): the antagonist did not exert any antagonistic effect on the RUB at a concentration of 1.1×10^{-7} M and higher (data not shown). These findings and other similar obtained in the other two preparations (RS and GPGB) suggested the existence of at least two different receptors for bombesin and related peptides in peripheral organs.

Table 2 Apparent affinities (pD_2), relative affinities (RA) and maximum responses (α^E, expressed in fraction or multiple of that of bombesin) of bombesin-related peptides in four isolated organs

	RUB			*GPUB*			*GPGB*			*RS*		
	pD_2	RA	α^E	pD_2	RA	α^E	pD_2	RA	α^E	pD_2	RA	α^E
Bombesin (BBS)*	8.33	100	1.0	8.83	100	1.0	8.92	100	1.0	8.43	100	1.0
Litorin*	8.20	74	0.8	8.36	34	1.3	8.53	41	0.9	8.26	68	1.0
Neuromedin C (NMC)*	7.80	30	0.7	7.91	12	0.6	7.93	10	0.9	8.62	155	1.2
[Phe^6]-NMC	7.24	8	0.6	6.24	0.3	0.5	6.12	0.2	1.1	6.72	2	1.0
Neuromedin B (NMB)*	8.29	91	1.1	6.71	0.8	0.5		P.Ag.		7.26	7	1.0
GRP (human)*	7.13	6	0.8	8.20	23	0.6	8.68	58	0.8	8.26	68	1.0
BBS (6–14)	8.52	155	1.0	8.92	123	1.0	8.53	41	0.9	8.16	54	2.0
Ac-BBS (6–14)	8.48	141	1.2	8.83	100	1.3	9.20	191	1.0	8.68	178	1.7

RUB: rat urinary bladder; GPUB: guinea-pig urinary bladder; GPGB: guinea-pig gall bladder; RS: rat stomach. GRP: gastrin releasing peptide; P.Ag.: partial agonist.

pD_2: −log concentration of agonist that produces 50% of the maximal effect. RA: relative affinities expressed as a percentage of that of BBS = bombesin = 100.

* Data from Regoli *et al.*, 1988.

Each value was obtained from at least 10 determinations.

Table 3 Antagonistic effects (pA_2 and pA_{10}) of bombesin antagonists against bombesin on 3 of the 4 preparations

	Preparations[a]							
	RUB	*GPGB*	*RS*			*GPUB*		
Antagonists	pA_2	pA_2	pA_2	pA_{10}	pA_2–pA_{10}	pA_2	pA_{10}	pA_2–pA_{10}
AcGRP (20–26) OCH_3	Inact.	8.07	7.94	6.84	1.10	7.95	6.96	0.99
AcGRP (20–26) OC_2H_5	Inact.	8.07	7.55	0.92	0.92	7.95	6.94	1.01
Ac-[D-Phe^{12}]-BB (6–14)	Inact.	Inact.	Inact.			Inact.		
Ac-GRP (20–26)	Inact.	ND	Inact.			Inact.		

[a] Abbreviations as in Table 2.
Inact.: inactive; ND: not determined. All data were calculated from at least 6 determinations.
pA_{10}: −log concentration of antagonist that reduces the effect of a dose of agonist 10 times higher than that of a single dose.

Specificity of the effects of bombesin in four preparations

Several antagonists or inhibitors of other naturally occurring agents (e.g. acetylcholine, angiotensin II, bradykinin, etc.) were used to determine the specificity of the responses of the RUB, RS, GPGB and GPUB to bombesin. Thus, atropine (1.5×10^{-7} M), methysergide (7.1×10^{-6} M), the kinin B_2 receptor antagonists D-Arg[Hyp^3, D-Phe^7, Leu^8]BK (Regoli *et al.*, 1990) (1.8×10^{-7} M), [Leu^8] angiotensin II (8.4×10^{-8} M), as well as the inhibitors of the arachidonic acid cascade, namely indomethacin (7.0×10^{-6} M) and BW-755C (1.1×10^{-5} M) were found to be inactive against bombesin (3.9×10^{-9} M), while blocking the effects of acetylcholine (1.5×10^{-5} M), 5-hydroxytryptamine (6.4×10^{-6} M), bradykinin (8.1×10^{-9} M) and angiotensin II (9.1×10^{-8} M) respectively. Similar results were obtained with the same antagonists, applied at the same concentrations and tested against bombesin (3.9×10^{-9} M) on the RUB. Again, the antagonists were found to be active in blocking the effects of the corresponding agonists, applied at the same concentrations as in the RS. As already mentioned, the responses of the RS, the GPUB and the GPGB to bombesin were blocked by two bombesin antagonists, while that of the RUB was not affected.

Peptidase inhibitors

Captopril (4.6×10^{-6} M), thiorphan (1.0×10^{-6} M) and bestatin (8.1×10^{-6} M) were tested to evaluate the possible interference by the angiotensin converting enzyme or other peptidases and by the bestatin-sensitive aminopeptidases on the biological response of the RUB, GPUB and RS to bombesin and its fragments. None of these inhibitors were found to be active in reducing or increasing the effects of bombesin, BBS (6–14) or Ac-BBS (6–14) in any of the preparations. These findings suggest that the three tissues do not contain active peptidases that are able to hydrolyse bombesin-related peptides.

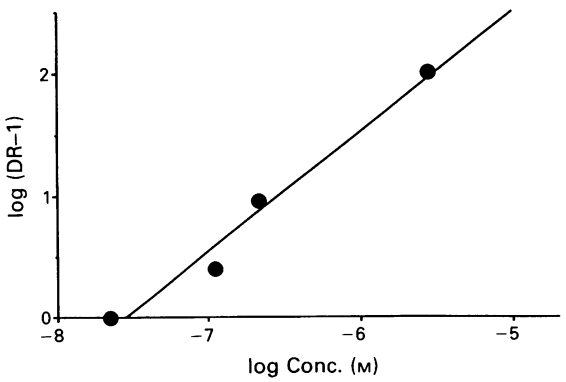

Figure 3 Schild plot of AcGRP (20–26) OC_2H_5 against bombesin on the guinea-pig urinary bladder. Abscissa scale: log molar concentration of the antagonist; ordinate scale: log (DR − 1) of the agonist. Slope = 0.98.

Discussion

The present study was directed to identifying and characterizing bombesin receptors in peripheral organs. Four isolated tissues were chosen from several pharmacological preparations (Broccardo *et al.*, 1975) that have been shown to be sensitive to bombesin and related peptides. The major reason for the choice of the RUB, GPUB, GPGB and RS was their high sensitivity to bombesin, described and documented by Girard *et al.* (1984) for the RS, and by Mizrahi *et al.* (1985) for the RUB and GPUB and confirmed recently by Falconieri-Erspamer *et al.* (1988) who have also studied the GPGB (Regoli *et al.*, 1988). Indeed, the four preparations showed pD_2 values for bombesin between 8.3 and 8.9 (RUB 8.33, GPUB 8.83, GPGB 8.92, RS 8.43).

Bombesin and related peptides evoked rapid contractions of the GPUB (Figure 1) that were not maintained at a stable plateau and faded even if the peptide was kept in contact with the tissue. Similar effects of bombesin were observed in the GPGB. In these tissues, concentration-response curves can only be measured by consecutive applications of the peptides in increasing concentrations. When enough time (20–40 min) is left between two doses, full sensitivity is maintained and desensitization can be avoided. Contractile effects of bombesin or related peptides in the rat urinary bladder (Figure 1) and the rat stomach developed more slowly and persisted at stable plateaux such that cumulative concentration-response curves could be measured in these preparations. In the present study however, the curves were measured by the same experimental protocol in all tissues to facilitate comparison of data between the various peptides and between the four preparations.

The contractile responses of the four preparations to bombesin appear to be the results of the direct effects of the peptide on the various smooth muscles. In fact, indirect effects by other endogenous agents such as acetylcholine, 5-hydroxytryptamine, prostaglandins have been excluded by the use of specific antagonists or inhibitors that reduce or block the effect or the release of the respective stimulants without influencing the responses to bombesin and related peptides. The possible interference by bombesin with receptors for neurokinins, kinins and angiotensin was excluded by the use of specific antagonists for each peptide. The present results confirm a previous specificity study by Girard *et al.* (1984) on the RS and by Mizrahi *et al.* (1985) on the RUB and the GPUB.

Bombesin and related peptides appear to be fairly well protected from the degradation by proteases sensitive to captopril, thiorphan or bestatin. These findings suggest that bombesin-like peptides may not be inactivated by the converting enzyme, or enkephalinase, or the aminopeptidase sensitive to bestatin, in contrast to other peptides, for instance substance P, the myotropic activity of which in the rat urinary bladder was found to be potentiated by captopril (Rouissi *et al.*, 1990b). In a few experiments, performed on the RUB and

GPUB, it was also found that bombesin and related peptides had the same effects in the absence and in the presence of phosphoramidon, suggesting that endopeptidase 24-11 was not active in these tissues. It is concluded that metabolic degradation by endo and exopeptidases did not interfere with the myotropic effects of bombesin and related peptides in the RUB, GPUB, GPGB and RS.

Characterization of bombesin receptors in the four isolated organs was attempted by measuring the order of potency of agonists and the affinity of antagonists, according to Schild (1973). Several naturally occurring bombesin-related peptides were used, in particular bombesin itself, neuromedin C, neuromedin B, litorin and the gastrin-releasing peptide (the human bombesin). We also measured the effect of some bombesin fragments. It was found that the order of potency of agonists was different between the RUB and the other three tissues, since, neuromedin B was much more active on the RUB than on the other preparations. The opposite was true for GRP which is more active on the GPUB, GPGB and RS than on the RUB (Table 2). On the other hand, BBS, bombesin fragments as well as neuromedin C and litorin were found to be nonselective as they showed fairly high affinities in all four preparations (Table 2). Therefore, on the basis of the order of potency of agonists we suggest that there are two different receptors for bombesin, one of which is present in the three preparations (RS, GPUB, GPGB) and the other in the RUB. In molecular terms, it appeared that the replacement of Val in position 10 of bombesin, by Phe (as in [Phe^6] neuromedin C) or by Thr as in neuromedin B favoured the RUB receptor and decreased affinity on the other three preparations.

The hypothesis that bombesin acts on two different receptors was confirmed by the use of two antagonists recently described by Heimbrook *et al.* (1989). The two compounds were found to be very active (they showed pA_2 higher than 7.5) on three preparations, (GPUB, GPGB and RS), while being inactive on the RUB. This represents the best evidence that bombesin acts on two different receptors which were named BB_1 and BB_2 by Regoli *et al.* (1988). These names should be changed to BBS_1 and BBS_2 to be consistent with the peptide nomenclature utilized by Falconieri-Erspamer *et al.* (1988) and adopted in the present paper.

Different orders of potency of agonists and different affinities of antagonists have also been reported for other tests, for instance the rat pancreas secretion which is almost insensitive to neuromedin B (Jensen *et al.*, 1984; 1988a; Otsuki *et al.*, 1987). In binding assays, Von Schrenck *et al.* (1989) have shown the existence of two bombesin binding sites, one of which is to be found in the rat oesophagus and shows high affinity for bombesin and neuromedin B, while the other (in the rat pancreas) shows high affinity for bombesin and very little for neuromedin B (Jensen *et al.*, 1988). Furthermore, Severi *et al.* (1990) have obtained good evidence that the rat gastric smooth muscle cells contain two different receptor sites. All together, the present results and recent findings in other laboratories suggest that bombesin and related peptides exert their contractile or secretory effects by activating at least two different receptor types.

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Comparison of the effects of the K^+-channel openers cromakalim and minoxidil sulphate on vascular smooth muscle

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1 The actions of the potassium channel openers, cromakalim and minoxidil sulphate, were compared in a range of isolated blood vessel preparations.

2 Cromakalim and minoxidil sulphate inhibited spontaneous mechanical activity of the guinea-pig portal vein and relaxed the noradrenaline precontracted rat aorta with similar potency. In contrast, minoxidil sulphate was less potent than cromakalim in inhibiting spontaneous activity in the rat portal vein and was essentially inactive in the noradrenaline precontracted rat mesenteric artery and rabbit aorta.

3 Minoxidil sulphate did not antagonize the effects of cromakalim in the rabbit aorta indicating it was not acting as a partial 'agonist'.

4 Charybdotoxin, noxiustoxin and rubidium failed to discriminate between cromakalim and minoxidil sulphate indicating that the apparently selective effects of minoxidil sulphate were not mediated by either Ca^{2+}-activated potassium channels, delayed rectifiers or rubidium impermeable potassium channels.

5 Glibenclamide antagonized the effects of cromakalim in an apparently competitive manner whereas the effects of minoxidil sulphate were antagonized in a non-competitive manner. The involvement of subtypes of ATP-sensitive potassium channels is discussed.

Keywords: Cromakalim; minoxidil sulphate; blood vessels; K^+-channels

Introduction

Cromakalim and minoxidil sulphate are thought to belong to a class of vasodilators collectively known as potassium channel openers. Thus, cromakalim has been shown to relax a variety of vascular smooth muscle preparations and these effects are generally found to be associated with increases in the $^{86}Rb^+$ or $^{42}K^+$ efflux rate coefficient and membrane hyperpolarization to a value close to the calculated potassium equilibrium potential (Hamilton *et al.*, 1986). Studies with minoxidil sulphate however, are far less numerous. Meisheri *et al.* (1988) reported that minoxidil sulphate relaxed the rabbit isolated mesenteric artery by a mechanism that was associated with stimulation of $^{42}K^+$ efflux. The ability of minoxidil sulphate to increase $^{42}K^+$ efflux and hyperpolarize the vascular smooth muscle cell membrane has subsequently been demonstrated in rat portal vein and rat aorta (Newgreen *et al.*, 1990) and in rabbit portal vein (Leblanc *et al.*, 1989), confirming a role for potassium permeability in the mechanism of action of this compound. However, despite the obvious similarities in the mechanisms of these compounds, several subtle differences have emerged. For example, although the hypoglycaemic sulphonylurea glibenclamide has been found to inhibit the effects of cromakalim in an apparently competitive manner (Buckingham *et al.*, 1989; Winquist *et al.*, 1989), glibenclamide antagonizes the effects of minoxidil sulphate in a non-competitive manner (Winquist *et al.*, 1989; Newgreen *et al.*, 1990). Furthermore, minoxidil sulphate, unlike cromakalim, failed to increase $^{86}Rb^+$ efflux from rat aorta (Newgreen *et al.*, 1990).

In order to investigate further similarities and differences in the mechanism of action of cromakalim and minoxidil sulphate the present study compares the actions of these compounds in a range of isolated vascular preparations. In addition, the interaction of cromakalim and minoxidil sulphate with a range of potassium channel blockers was investigated in an attempt to elucidate the nature of the potassium channels opened by these vasodilators.

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Methods

Tissue bath experiments

Longitudinal sections of hepatic portal vein from male Dunkin Hartley guinea-pigs (200–350 g), longitudinal sections of portal vein and rings of thoracic aorta and superior mesenteric artery from female Alderley Park rats (200–300 g) or rings of thoracic aorta from male New Zealand White rabbits (2.5–3 kg) were suspended in 20 ml organ baths containing Krebs buffer of the following composition (mM): NaCl 120, $NaHCO_3$ 25, D-glucose 11.2, KH_2PO_4 1.2, $MgSO_4 7H_2O$ 1.2, KCl 4.7, $CaCl_2$ 2.5, ethylenediamine-tetra-acetic acid disodium salt (EDTA) 0.026, maintained at 37°C and bubbled with 95% O_2/5% CO_2. No attempt was made to remove the endothelium in any of the preparations studied. Isometric tension was recorded under a resting tension of 1 g. Tissues were allowed to equilibrate for a period of 1 h during which the Krebs buffer solution was changed every 20 min. Following the equilibration period, rings of aorta and mesenteric artery were challenged with a sub-maximal concentration of noradrenaline every 30 min until responses were consistent. At this point the spasmogen was allowed to remain in contact with the tissue and, once a stable plateau had developed (approximately 20 min), cumulative concentration-response curves were constructed to either cromakalim or minoxidil sulphate. Results were expressed as percentage relaxation of the response to the spasmogen. Additionally, cumulative concentration-response curves were constructed to cromakalim or minoxidil sulphate in guinea-pig or rat portal vein. A 30 min contact time was allowed for each concentration. The mean amplitude of the spontaneous changes in tension was calculated over the final 10 min of each contact period and expressed as a percentage inhibition of the mean control amplitude. Studies were also undertaken with potassium channel blockers. Cumulative concentration-response curves were constructed following 30 min incubation with potassium channel blocker or vehicle. Results are expressed as percentage inhibition of the mean amplitude of spontaneous activity

following addition of blocker or vehicle. In experiments involving replacement of potassium with rubidium, cumulative concentration-response curves were constructed using strips of rat portal vein following a 1 h equilibration in a modified Krebs solution (KH_2PO_4 replaced with an equimolar concentration of NaH_2PO_4, 2 mM KCl and 2 mM RbCl).

Schild analysis

Results showing evidence of competitive antagonism (parallel shifts without a reduction in maxima) were subjected to Schild analysis. Concentration ratios were calculated at the IC_{50} point from the mean control IC_{50} value determined in separate tissues in the presence of vehicle. Providing the slope of the Schild regression did not differ significantly from unity, pA_2 values were calculated from a least squares regression with slope corrected to unity.

Materials

Noradrenaline, glibenclamide and rubidium chloride were all obtained from Sigma. Cromakalim, minoxidil sulphate, noxiustoxin (NTX) and charybdotoxin (ChTX) were obtained from Chemistry Department II, ICI Pharmaceuticals. Cromakalim (1 mM), noxiustoxin (0.1 mM) and charybdotoxin (0.1 mM) were all dissolved in distilled water. Minoxidil sulphate (1 mM) was dissolved in 40% PEG200. Higher concentrations were obtained from a 10 mM stock dissolved in dimethylsulphoxide (DMSO). Noradrenaline (1 mM) was dissolved in water with an equal weight of ascorbic acid to prevent oxidation. All stocks were made up fresh every day.

Results

Guinea-pig portal vein

In control experiments cromakalim inhibited spontaneous mechanical activity in the guinea-pig portal vein with a mean pD_2 ($\pm$s.e.mean) of 7.4 $\pm$ 0.32 ($n = 4$, Figure 1a). Minoxidil sulphate also inhibited spontaneous mechanical activity with a mean pD_2 ($\pm$s.e.mean) of 7.5 $\pm$ 0.13 ($n = 4$, Figure 1b). These pD_2 values were not significantly different ($P > 0.05$, Student's t test).

Glibenclamide produced a marked, concentration-related rightward shift in the cromakalim concentration-response curve without altering the maximum inhibition of spontaneous activity (Figure 1a). Mean pD_2 values were 7.05 $\pm$ 0.14, 6.88 $\pm$ 0.1, 6.14 $\pm$ 0.12 and 5.16 $\pm$ 0.05 in the presence of vehicle, 0.1, 1 and 10 μM glibenclamide respectively. Schild analysis of concentration-ratios revealed a linear relationship with a slope of 1.07, which was not significantly different from unity and a pA_2 value of 6.8 $\pm$ 0.1. At the maximum concentration tested, glibenclamide was found to produce only a small increase in spontaneous activity (<20%).

Glibenclamide also produced a concentration-related rightward shift in the minoxidil sulphate concentration-response curve (Figure 1b). Mean minoxidil sulphate pD_2 values were 7.73 $\pm$ 0.25, 7.45 $\pm$ 0.35, 6.99 $\pm$ 0.39 and 6.1 $\pm$ 0.33 in the presence of vehicle, 0.1, 0.3, and 1 μM glibenclamide respectively. In the presence of 10 μM glibenclamide, minoxidil sulphate failed to inhibit spontaneous activity by 50% at concentrations up to and including 10 μM. Thus, unlike the antagonism of cromakalim the antagonism of minoxidil sulphate by glibenclamide was associated with a reduction in maximum inhibition of spontaneous activity. The maximum inhibition of spontaneous activity by minoxidil sulphate was 100 $\pm$ 0%, 100 $\pm$ 0%, 73 $\pm$ 12%, 59 $\pm$ 12% and 24 $\pm$ 8% in the presence of vehicle, 0.1, 0.3, 1 and 10 μM glibenclamide respectively.

ChTX (0.1 μM) enhanced the spontaneous mechanical activity of the guinea-pig portal vein by 37 $\pm$ 8% ($n = 8$). However, ChTX (0.1 μM) failed to antagonize the effects of cromakalim.

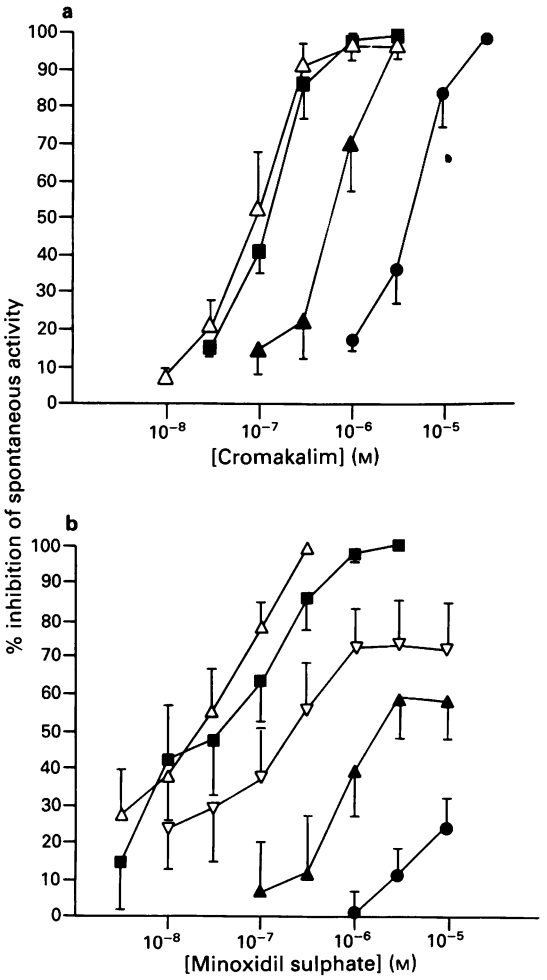

Figure 1 Effect of (a) cromakalim and (b) minoxidil sulphate on the spontaneous mechanical activity of the guinea-pig portal vein in the presence of vehicle (△) and glibenclamide, 0.1 μM (■), 0.3 μM (▽) 1 μM (▲) and 10 μM (●). Symbols represent the mean of 6 experiments. Bars represent the s.e.mean.

Mean cromakalim pD_2 values were 7.13 $\pm$ 0.31 and 7.53 $\pm$ 0.13 ($P > 0.05$) in the presence of vehicle and ChTX respectively. Similarly, ChTX (0.1 μM) failed to antagonize minoxidil sulphate induced inhibition of spontaneous activity. Thus, mean minoxidil sulphate pD_2 values were 7.30 $\pm$ 0.12 and 7.15 $\pm$ 0.12 ($P > 0.05$) in the presence of vehicle and ChTX respectively.

NTX (1 μM) enhanced the spontaneous mechanical activity of the guinea-pig portal vein by 29 $\pm$ 17% ($n = 8$). NTX (1 μM) was also without effect on the concentration-effect curves for cromakalim and minoxidil sulphate. Thus, mean cromakalim pD_2 values were 7.16 $\pm$ 0.11 and 7.57 $\pm$ 0.15 ($P > 0.05$) in the presence of vehicle and NTX respectively and mean minoxidil sulphate pD_2 values were 7.19 $\pm$ 0.17 and 7.28 $\pm$ 0.01 ($P > 0.05$) in the presence of vehicle and NTX respectively.

Rat portal vein

Cromakalim completely inhibited the spontaneous mechanical activity of the rat portal vein with a mean pD_2 value of 6.9 $\pm$ 0.08 ($n = 8$; Figure 2a). Minoxidil sulphate also inhibited the spontaneous activity of the rat portal vein. However, minoxidil sulphate was both less potent (mean $pD_2 = 6.2 \pm 0.12$) and less efficacious (mean max. inhibition = 70 $\pm$ 4%) than cromakalim ($P < 0.05$, Student's t test, Figure 2b). Following incubation in modified Krebs solution (Rb Krebs) the concentration-response curve to cromakalim was shifted significantly ($P < 0.05$) to the right by a factor of 8.3 $\pm$ 3.5 (Figure 2a). Cromakalim mean pD_2 values were 6.9 $\pm$ 0.08

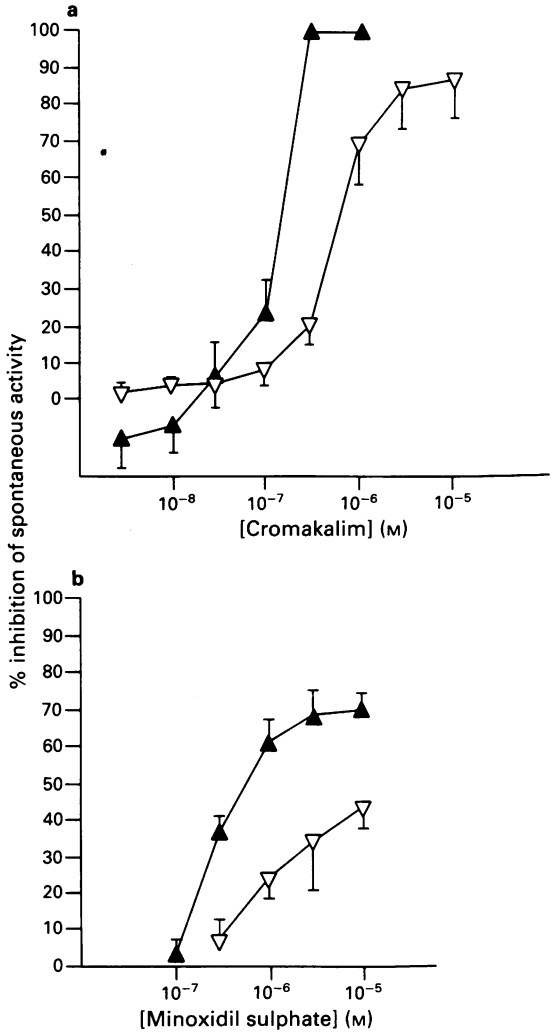

Figure 2 The effect of (a) cromakalim and (b) minoxidil sulphate on the spontaneous activity of the rat portal vein. Tissues were incubated in normal (▲) or rubidium (▽) Krebs solution. Symbols represent the mean of 8 experiments. Bars represent s.e.mean.

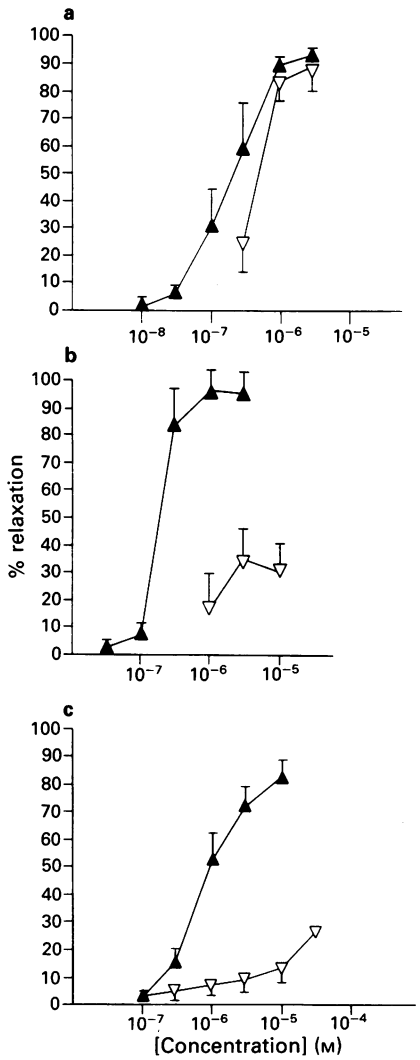

Figure 3 Comparison of the effects of cromakalim (▲) and minoxidil sulphate (▽) on (a) the noradrenaline (0.03 μM) precontracted rat thoracic aorta, (b) the noradrenaline (0.3 μM) precontracted rat mesenteric artery and (c) the noradrenaline (1 μM) precontracted rabbit thoracic aorta. Symbols represent the mean of 4 experiments. Bars represent the s.e.mean.

and 6.15 ± 0.13 in normal and Rb Krebs respectively. This shift was associated with a reduction in the maximum inhibition of spontaneous activity achieved by cromakalim (100 ± 0% and 87 ± 9% in normal and Rb Krebs respectively). Similar effects were observed with minoxidil sulphate. Thus following incubation with Rb Krebs the concentration-response curve to minoxidil sulphate was shifted to the right and the maximum inhibition reduced to the extent that an IC_{50} was achieved in only two out of four experiments (Figure 2b). Mean maximum % inhibition was 70 ± 4% and 44 ± 6% in normal and Rb Krebs respectively. Comparison of concentration-response curves at the IC_{30} level revealed a mean concentration ratio (from mean IC_{30} in normal Krebs) of 14.2 ± 8.0 (mean −log IC_{30} values = 6.6 ± 0.06 and 5.6 ± 0.2 in normal and Rb Krebs respectively; $P < 0.05$). Concentration-ratios for cromakalim and minoxidil sulphate were not significantly different ($P > 0.05$).

Rat thoracic aorta

Noradrenaline (0.03 μM) contracted rat thoracic aorta by 95 ± 1% of the noradrenaline maximum response (data not shown). Cromakalim and minoxidil sulphate relaxed the noradrenaline (0.03 μM) precontracted rat aorta with mean (± s.e.mean) pD_2 values of 6.7 ± 0.21 ($n = 4$) and 6.29 ± 0.07 ($n = 4$) respectively. These pD_2 values were not significantly different ($P > 0.05$; Figure 3a).

Rat mesenteric artery

Noradrenaline (0.3 μM) contracted the rat mesenteric artery by 97 ± 3% of the noradrenaline maximum response (data not shown). Cromakalim relaxed the rat mesenteric artery precontracted with noradrenaline (0.3 μM) with a mean pD_2 of 6.71 ± 0.09 ($n = 4$, Figure 3b). Minoxidil sulphate however, failed to relax the noradrenaline precontracted rat mesenteric artery by 50% at concentrations up to 10 μM (Figure 3b). The mean maximum % relaxation produced by minoxidil sulphate was 36 ± 11%.

Rabbit thoracic aorta

Noradrenaline (1 μM) contracted the rabbit thoracic aorta by 83 ± 1.3% of the noradrenaline maximum response (data not shown). Cromakalim relaxed the noradrenaline (1 μM) precontracted rabbit aorta with a mean pD_2 of 5.97 ± 0.15 ($n = 4$, Figure 3c). As found in the rat mesenteric artery, (Figure 3b), minoxidil sulphate failed to relax the rabbit aorta by 50% at concentrations up to 30 μM. The mean maximum % relaxation produced by minoxidil sulphate was 27 ± 1.0%. In additional experiments the relaxant effect of a single concentration of minoxidil sulphate (10 μM) was assessed. Minoxidil sulphate (10 μM) relaxed the NA (1 μM) precontracted rabbit aorta by

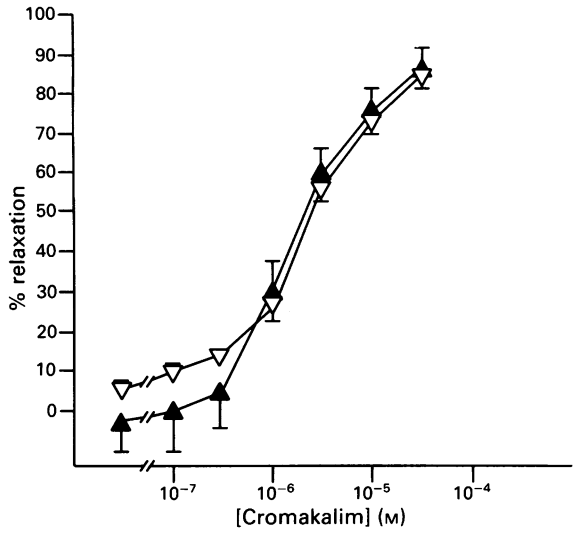

Figure 4 The effect of cromakalim on the noradrenaline (1 μM) precontracted rabbit thoracic aorta in the presence of vehicle (▲) or minoxidil sulphate (▽). Symbols represent the mean of 4 experiments. Bars represent the s.e.mean.

16.1 ± 3.1% when allowed to remain in contact with the tissues for a period of 60 min. Minoxidil sulphate was tested as an antagonist of the effects of cromakalim (Figure 4). Minoxidil sulphate (10 μM, 30 min incubation) failed to inhibit cromakalim-induced relaxation in the rabbit aorta (mean ± cromakalim pD_2 values were 5.68 ± 0.12 and 5.61 ± 0.03 in the presence of vehicle and minoxidil sulphate respectively).

Discussion

The present study compares the activity of cromakalim and minoxidil sulphate in a range of vascular preparations. Minoxidil sulphate was equipotent with cromakalim at inhibiting the spontaneous mechanical activity of the guinea-pig portal vein and at relaxing the noradrenaline precontracted rat aorta, was less potent and was less efficacious than cromakalim at inhibiting spontaneous activity in the rat portal vein and was essentially inactive against noradrenaline-induced tone in the rat superior mesenteric artery and the rabbit thoracic aorta. Thus, from the present study, it appears that striking differences exist in the profile of activity of the potassium channel openers cromakalim and minoxidil sulphate. Interestingly literature reports support this conclusion. For example, Winquist *et al.* (1989) and Newgreen *et al.* (1990) have reported that both cromakalim and minoxidil sulphate potently inhibited spontaneous activity in the rat portal vein and relaxed potassium-induced tone in the rat aorta. Piper & Hollingsworth (1989) however, have reported that minoxidil sulphate is essentially inactive compared to cromakalim in the rat uterus.

There are several possible explanations for the reported inactivity of minoxidil sulphate. Firstly, minoxidil sulphate is an unstable substance which readily breaks down to its parent compound minoxidil at room temperature. Minoxidil itself is a poor dilator of vascular smooth muscle (Towart, 1982; Kauffman *et al.*, 1986). However, such an explanation can be discounted since the sample used in the present study was stored at −20°C, the activity confirmed at regular intervals on the guinea-pig portal vein and the content of the sample verified by thin layer chromatography (results not shown).

Secondly, Newgreen *et al.* (1990) reported that the effects of minoxidil sulphate were somewhat slower than those of cromakalim in the rat portal vein. Thus, cromakalim was found to induce a maximum hyperpolarization over a period of 6 min whereas minoxidil sulphate produced a maximum hyperpolarization over a period of 12 min. Such a slowed or delayed response is unlikely to explain the findings of the present study since minoxidil sulphate failed to relax the precontracted rabbit aorta when allowed to remain in contact with the tissue for a period of 1 h.

A decrease in the number of spare receptors typically reduces the potency of full agonists whilst reducing both the potency and the maximum effect of partial agonists. Comparison of the concentration-response curves for cromakalim and minoxidil sulphate in the preparations used in the present study reveals such a trend raising the possibility that minoxidil sulphate may possess partial 'agonist' activity. Thus, minoxidil sulphate was maximally active in the guinea-pig portal vein, the tissue in which cromakalim was most potent, showed intermediate activity with a reduction in its maximum achievable effect in the rat portal vein and was only weakly active/inactive in the rat mesenteric artery and rabbit aorta, tissues in which cromakalim was least potent. However, minoxidil sulphate failed to antagonize the effect of cromakalim in tissues in which it is incapable itself of inducing a response suggesting that partial agonism is not an adequate explanation for the findings of this study.

It has been reported that although cromakalim and minoxidil sulphate are both capable of increasing $^{42}K^+$ efflux, only cromakalim was capable of increasing $^{86}Rb^+$ efflux from rat aorta (Newgreen *et al.*, 1990). Similar results have been reported in the rat portal vein (Newgreen *et al.*, 1990). On the basis of these results it is possible to speculate that minoxidil sulphate opens a different potassium channel from that modulated by cromakalim and that this channel is relatively impermeable to $^{86}Rb^+$. Previous electrophysiological studies have demonstrated the ability of cromakalim to open calcium-activated potassium channels (Kusano *et al.*, 1987; Trieschmann *et al.*, 1988; Gelband *et al.*, 1989) and the delayed rectifier (Beech & Bolton, 1989) in vascular smooth muscle. In addition, minoxidil sulphate has been reported to increase a potassium current which was sensitive to removal of extracellular calcium in coronary smooth muscle (Wilde & Lee, 1988). The possibility that cromakalim and minoxidil sulphate act via different potassium channels was therefore investigated in the present study by comparing the interactions of these compounds with charybdotoxin, a potent inhibitor of large conductance, calcium-activated potassium channels in a variety of tissues including vascular smooth muscle (Talvenheimo *et al.*, 1988) and noxiustoxin, a blocker of the delayed rectifier (Carbone *et al.*, 1982). The findings that neither charybdotoxin or noxiustoxin differentiate between the actions of cromakalim and minoxidil sulphate suggests that neither agent functionally interacts with large conductance calcium-activated potassium channels or delayed rectifiers and rules these channels out as mediators of the apparently selective effects of minoxidil sulphate. The lack of effect of these peptide channel blockers is unlikely to be due to their breakdown in the tissue bath since charybdotoxin induced an increase in the amplitude of the spontaneous activity of the guinea-pig portal vein which was sustained for several hours (data not shown). In addition, the effect of replacing potassium with rubidium was investigated. Rubidium has previously been shown to inhibit the actions of cromakalim in tracheal smooth muscle and in bladder smooth muscle and would be expected to block effects mediated via rubidium impermeable channels to a greater extent than those mediated via rubidium permeable channels. The finding that rubidium failed to block the effects of minoxidil sulphate to a greater extent than those of cromakalim in the rat portal vein is somewhat surprising and suggests that the channels mediating the effects of both cromakalim and minoxidil sulphate show a similar permeability to rubidium. The reason for the apparent discrepancy between the present functional studies and previously reported efflux studies is unclear (Foster *et al.*, 1989; Morris & Taylor, 1989).

The only other agent to antagonize effectively the actions of cromakalim and minoxidil sulphate was glibenclamide, which

antagonized the effects of both cromakalim and minoxidil sulphate over a similar concentration-range. However, whereas the antagonism of cromakalim by glibenclamide was apparently competitive, the antagonism of minoxidil sulphate by glibenclamide appeared non-competitive, findings consistent with previous reports (Buckingham et al., 1989; Winquist et al., 1989; Newgreen et al., 1990). If glibenclamide were to interact with more than one potassium channel with similar affinity then such observations could still be explained in terms of cromakalim and minoxidil sulphate interacting with different potassium channels. The channels activated by cromakalim and minoxidil sulphate may therefore represent subtypes of glibenclamide-sensitive potassium channels. In the absence of any compelling evidence to support an interaction between glibenclamide and any class of potassium channel other than the ATP-sensitive potassium channel, it remains a strong possibility that such sub-types may represent sub-types of ATP-sensitive channels. Sub-types of ATP-sensitive potassium channels have previously been suggested by Quast & Cook (1989) to account for differences in the properties of ATP-sensitive potassium channels in pancreatic, cardiac and neuronal tissues and arterial smooth muscle. Furthermore, ATP-sensitive potassium channels from different sites in the vasculature appear to show different properties. For example, ATP-sensitive potassium channels in cells dissociated from rat and rabbit mesenteric artery are reported to have a single channel conductance of 135 pS (Standen et al., 1989) whereas cells from rat portal vein are reported to express ATP-sensitive potassium channels with a single channel conductance of 10 pS (Kajioka et al., 1990).

Another possible explanation for the observed results is that cromakalim and minoxidil sulphate may modulate the same channel by different mechanisms. Indirect opening of potassium channels by minoxidil sulphate could explain the non-competitivity of the interaction between this agent and glibenclamide.

It has recently been reported that [^{35}S]-minoxidil sulphate labelled a 116 kD protein in rabbit mesenteric artery (Meisheri et al., 1990). Whether this site forms part of a particular subtype of ATP sensitive potassium channel or part of a cascade of events initiated by minoxidil sulphate which lead to potassium channel activation is unclear and is worthy of further study.

In summary, the results of the present study indicate that whereas cromakalim uniformly relaxes a variety of different blood vessel preparations, the actions of minoxidil sulphate appear more selective. The reasons for this are unclear. Minoxidil sulphate may interact with a different potassium channel from cromakalim although this channel is not charybdotoxin- or noxiustoxin-sensitive. The involvement of sub-types of the ATP sensitive potassium channel in the actions of cromakalim and minoxidil sulphate remains an attractive hypothesis and studies are planned to follow up this possibility.

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Cardioprotective and endothelial protective effects of $[Ala\text{-}IL8]_{77}$ in a rabbit model of myocardial ischaemia and reperfusion

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1 We studied the effects of a form of interleukin-8 (i.e., $[Ala\text{-}IL8]_{77}$) on endothelial dysfunction and myocardial injury in rabbits. Pentobarbitone-anaesthetized rabbits were subjected to 1.5 h occlusion of the marginal coronary artery and 3.5 h reperfusion. $[Ala\text{-}IL8]_{77}$ (50 μg or its vehicle) was given i.v. as a bolus 10 min prior to reperfusion. $[Ala\text{-}IL8]_{77}$ was also studied in isolated perfused hearts of rabbits.

2 Myocardial ischaemia plus reperfusion in untreated rabbits produced severe endothelial dysfunction and myocardial injury, including marked myocardial necrosis, elevated cardiac myeloperoxidase (MPO) activity in ischaemic cardiac tissue, and loss of response of marginal coronary rings to the endothelium-dependent vasodilators, acetylchloline (ACh) and A23187.

3 Administration of $[Ala\text{-}IL8]_{77}$ 10 min prior to reperfusion resulted in significant protective effects in post-ischaemic reperfusion. Compared with untreated rabbits, $[Ala\text{-}IL8]_{77}$ caused a reduced necrotic zone ($P < 0.01$), lower MPO activity in the necrotic zone ($P < 0.05$), and significantly preserved vasorelaxant responses of marginal coronary artery rings to endothelium-dependent vasodilators, ACh ($P < 0.001$) and A23187 ($P < 0.001$).

4 These results indicate that myocardial ischaemia and reperfusion result in a severe endothelial dysfunction and myocardial injury which involved the interaction of neutrophils and endothelial cells. However, $[Ala\text{-}IL8]_{77}$ did not appear to exert a direct endothelial protective effect in the absence of neutrophils in rabbit isolated perfused hearts.

5 Inhibition of neutrophil accumulation in the myocardium, perhaps by prevention of endothelial dysfunction resulting from $[Ala\text{-}IL8]_{77}$, leads to significant protective effects in ischaemia and reperfusion in rabbits.

Keywords: Endothelial cell interleukin-8; granulocytes; endothelium-derived relaxing factor; reperfusion injury; myeloperoxidase activity; neutrophil adherence

Introduction

Prolonged myocardial ischaemia without reperfusion inevitably results in myocardial cell death. Although early reperfusion is a desired therapeutic goal, reperfusion itself may contribute to additional myocardial cell injury (Braunwald & Kloner, 1985). Recent evidence strongly implicates neutrophils in the genesis of reperfusion injury (Engler *et al.*, 1986; Lucchesi *et al.*, 1989; Forman *et al.*, 1990). Neutrophils promote cellular damage by releasing superoxide radicals, proteolytic enzymes, and cytotoxic cytokines. Experimental strategies which have involved prevention of activation (Bednar *et al.*, 1985; Simpson *et al.*, 1987; Simpson *et al.*, 1988a) or depletion (Romson *et al.*, 1983) of neutrophils have been shown to reduce myocardial reperfusion injury. A monoclonal antibody directed against one of the adhesive glycoproteins (i.e., CD-11b/CD-18) on the neutrophil cell surface has also been shown to reduce infarct size significantly in dogs (Simpson *et al.*, 1988b). Therefore, substances which inhibit neutrophil adherence would appear to have particular utility in preventing reperfusion injury.

Recently , a variety of interleukin-8 (i.e., $[Ala\text{-}IL8]_{77}$, a 77 amino acid peptide identical to the 72 amino acid sequence of IL-8, with a pentapeptide extension at the NH_2-terminus; Gimbrone *et al.*, 1989) which is produced by endothelial cells, has been shown, at nanomolar concentrations, to be a potent inhibitor of neutrophil adhesion to cytokine-activated endothelial monolayers and to protect these monolayers from neutrophil-mediated damage (Gimbrone *et al.*, 1989). It has been proposed that $[Ala\text{-}IL8]_{77}$ may attenuate inflammatory events at the interface between the blood and the vessel wall (Gimbrone *et al.*, 1989). However, the effects of $[Ala\text{-}IL8]_{77}$ on endothelial dysfunction and myocardial injury produced by myocardial ischaemia and reperfusion have not been studied. Since endothelial dysfunction has recently been found to be an important early event leading to myocardial cell injury (Tsao *et al.*, 1990; Lefer *et al.*, 1990), this interrelationship may be of fundamental pathophysiological significance.

The purposes of the present investigation were to examine the effects of $[Ala\text{-}IL8]_{77}$ in myocardial ischaemia plus reperfusion to determine its activity in (a) prevention of neutrophil accumulation in the myocardium, (b) preservation of endothelial function as assessed by release of endothelium-derived relaxing factor (EDRF), and (c) subsequent limitation of myocyte necrosis following myocardial ischaemia and reperfusion in the rabbit.

Methods

Experimental procedure

Twenty adult male New Zealand white rabbits (2.2 to 3.1 kg) were anaesthetized with sodium pentobarbitone (30 mg kg^{-1} body weight), intravenously. An intratracheal tube was inserted through a midline incision and all rabbits were given intermittent positive-pressure ventilation via a Harvard small animal respirator (Harvard Apparatus, Inc., S. Natick, MA, U.S.A.). A polyethylene catheter was inserted into the external jugular vein, and the right femoral artery was cannulated and connected to a Statham P23C pressure transducer (Spectromed, Inc., Critical Care Division, Oxnard, CA, U.S.A.) for the measurement of arterial blood pressure. A midline

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thoratocomy was performed, the pericardium opened and the heart was exposed. A 3-0 silk ligature was carefully placed around the major marginal branch of the left circumflex coronary artery located on the dorsal surface of the heart, 10–12 mm from its origin. Heart rates and ST segment changes were obtained from standard lead II of the scalar electrocardiogram every 20 min. Arterial blood pressure and the electrocardiogram were continuously recorded on a Grass Model 7 oscillographic recorder (Grass Instrument Co., Quincy, MA, U.S.A.). The pressure-rate index, calculated as the product of the mean arterial blood pressure and heart rate divided by 1000, was employed as an approximation of myocardial oxygen demand.

Experimental protocol

After a 30 min period of stabilization following thoracotomy, myocardial ischaemia (MI) was initiated by complete ligation of the marginal coronary artery. This was designated as time 0. After 1.5 h of ischaemia, the ligature was untied and the ischaemic myocardium was reperfused for 3.5 h. Recombinant *E. coli* $[Ala\text{-}IL8]_{77}$ (J. Baker, Genentech, Inc., South San Francisco, CA, U.S.A., greater than 98% pure) was given as an intravenous bolus (50 μg) starting 10 min prior to reperfusion (6 rabbits). An additional series of 7 rabbits were given an equal volume of 0.9% NaCl as a vehicle. Five sham-operated control rabbits were subjected to all of the procedures except that the coronary artery ligature was not tightened. Two rabbits died due to surgical complications, one sham-operated control due to haemorrhage, and one myocardial ischaemic rabbit due to pulmonary failure prior to assignment to a group.

At the end of the 5 h experimental period, the ligature around the marginal coronary artery was retightened; 30 ml of 5% Evans blue dye was injected into the left atrium to stain the area of the myocardium perfused by the patent coronary arteries. The area-at-risk was therefore determined by negative staining. The atria, right ventricle, and major blood vessels were subsequently removed from the heart. The left ventricle was then sliced into sections 3 mm thick parallel to the atrioventricular groove. The unstained portion of myocardium (i.e., the area-at-risk) was separated from the stained portion (i.e., the area-not-at-risk). The unstained portion was again sliced into 1 mm thick sections and incubated in a 0.1% solution of nitroblue tetrazolium stain in phosphate buffer at pH 7.4 and 37°C for 15 min to detect the presence of coenzyme and dehydrogenase. The necrotic portion of the myocardium which did not stain was separated from the stained portion (i.e., the non-necrotic area-at-risk). Samples from all three portions of left ventricular cardiac tissue (i.e., non-ischaemic, ischaemic non-necrotic and ischaemic necrotic) were weighed and stored at −70°C for subsequent assay of myeloperoxidase activity.

Measurement of myeloperoxidase activity in cardiac tissue

Myeloperoxidase, an enzyme which is specific for neutrophils, was determined in cardiac tissue by the method of Bradley *et al.* (1982), as modified by Mullane *et al.* (1985). MPO activity was used as an index of neutrophil accumulation in the heart since it correlates closely with numbers of neutrophils present in the heart (Mehta *et al.*, 1989). Cardiac tissue samples were homogenized in 0.5% hexadecyltrimethyl ammonium bromide (HTAB) (Sigma Chemical Co., St. Louis, MO, U.S.A.), dissolved in 50 mM potassium phosphate buffer at pH 6 in a Polytron (PCU-2) homogenizer (Kinemutica GmbH, Luzern, Switzerland) for 15 s at 7000 r.p.m. Homogenates were centrifuged for 20 min at 12,000 *g* and 2°C. The supernatants were decanted and added to 0.167 mg ml^{-1} *o*-dianisodine dihydrochloride (Sigma Chemical Co., St. Louis, MO, U.S.A.) and 0.0005% H_2O_2 in 50 mM phosphate buffer at pH 6. The change in absorbance was measured spectrophotometrically at 460 nm. One unit of MPO activity is defined as that quantity of enzyme degrading 1 mmol of peroxide per min at 25°C.

Studies on coronary ring responses

After injection of Evans blue, hearts were excised and placed in warmed, oxygenated Krebs-Henseleit (K-H) buffer. The marginal coronary artery was carefully isolated and 10–12 mm long segments were removed both above and below the ligature. The segment above the ligature (i.e., the proximal segment) was used as a non-ischaemic, non-reperfused control, and the portion of the vessel which had undergone ischaemia and reperfusion (i.e., the distal segment) was used as an ischaemic vessel. These segments were placed in warmed K-H consisting of (in mM): NaCl 118, KCl 4.75, $CaCl_2$ 2.54, KH_2PO_4 1.19, $MgSO_4$ 1.19, $NaHCO_3$ 12.5 and glucose 10.0. Isolated coronary vessels were cut into rings of 2 to 3 mm in length. The rings were suspended in 20 ml tissue baths filled with K-H buffer warmed to 37°C and connected to Grass FT-03 force-displacement transducers (Grass Instrument Co., Quincy, MA, U.S.A.). Responses were recorded on a Grass Model 7 oscillographic recorder. Coronary rings were initially stretched to give a preload of 0.5 g of force and allowed to equilibrate for 1 h. Preloads of 1.0 g or higher caused injury to the endothelium and thus could not be used. Coronary rings were then exposed to 100 nM U-46619 (9,11-methanoepoxy PGH_2) (The Upjohn Co., Kalamazoo, MI, U.S.A.), a thromboxane-mimetic, to generate about 0.5 g of developed force. Once a stable contraction was observed, ACh was added to the bath to achieve the following final concentrations: 0.1, 1, 10 and 100 nM. After the response stabilized, the rings were washed and allowed to equilibrate to baseline once again. The procedure was repeated with A-23187 (1, 10, 100 and 1000 nM) and then to $NaNO_2$ (0.1, 1, 10 and 100 μM), final concentrations. $NaNO_2$ was dissolved in 0.1 N HCl and the solution titrated to pH 2.0. Distilled water at pH 2.0 added to the bath did not produce any vasorelaxation.

Rabbit isolated perfused hearts

Adult male rabbits were anaesthetized with pentobaritone sodium (30 mg kg^{-1}) injected intravenously, and then given heparin (1000 u kg^{-1}) intravenously. Hearts were rapidly isolated following a midsternal thoracotomy and placed in oxygenated Krebs-Henseleit (K-H) solution at pH 7.3 to 7.4. Following rinsing of all blood, hearts were transferred to a Langendorff perfusion apparatus, and perfused retrogradely through the aorta. Hearts were perfused with K-H solution at 37°C oxygenated with 95% O_2 plus 5% CO_2. Hearts were equilibrated for 5 min at a coronary perfusion pressure of 50 mmHg, and then constant flow perfusion was instituted at 50 ml min^{-1}. Coronary perfusion pressure (CPP) was then continuously recorded from a side-arm in the aortic inflow tract via a Statham P23AC pressure transducer (Spectramed, Inc., Oxnard, CA) and a Grass Model 7 oscillographic recorder (Grass Instrument Co., Quincy, MA).

After 5 min of constant flow perfusion, a bolus injection of the coronary vasoconstrictor U-46619 was given at 1 μg ml^{-1} of flow into the aortic inflow line. When CPP reached a stable plateau, the endothelium-dependent dilator acetylcholine (ACh), was injected at doses of 2, 20 and 200 ng ml^{-1} of flow. Following washout of all vasoactive agents, the process was repeated for U-46619 followed by the endothelium-independent dilator nitroglycerin at 0.5, 5 and 50 μg ml^{-1} of flow. Hearts were then shifted to a recirculation mode of perfusion with 200 ml of K-H solution. Hearts were then tested for vasodilator responses 50 min later, either after a period of control flow, or ischaemia (i.e., coronary flow reduced to 15% of control for 30 min), followed by 20 min of reperfusion. Ischaemic hearts received 2 ml of either 0.9% NaCl or 50 μg of $[Ala\text{-}IL8]_{77}$ 10 min prior to reperfusion. Myeloperoxidase analysis of rabbit hearts at the end of the experiments failed to show any detectable MPO activity.

Generation of superoxide radicals

Superoxide radicals were generated synthetically from a xanthine-xanthine oxidase system from a solution consisting of 50 mmol l^{-1} potassium phosphate (pH 7.8), 10 mmol l^{-1} EDTA, 10 μmol l^{-1} ferricytochrome C type III, 50 μmol l^{-1} xanthine, 200 μmol ml^{-1} potassium cyanide and 1 μg ml^{-1} dithionite. Xanthine oxidase (12.3 u ml^{-1}) was added to reaction mixture at 30°C and absorption measured every 15 s at 550 nm according to the method of McCord & Fridovich (1969).

Statistical analysis

All values in the text and figures are presented as means ± s.e. mean of n independent experiments. Data were analyzed by Kruskal-Wallis non-parametric analysis of variance due to inequalities of variances among the groups. Differences between specific means were tested for post-hoc analysis by the Mann-Whitney U test. A value of $P < 0.05$ was accepted as being statistically significant.

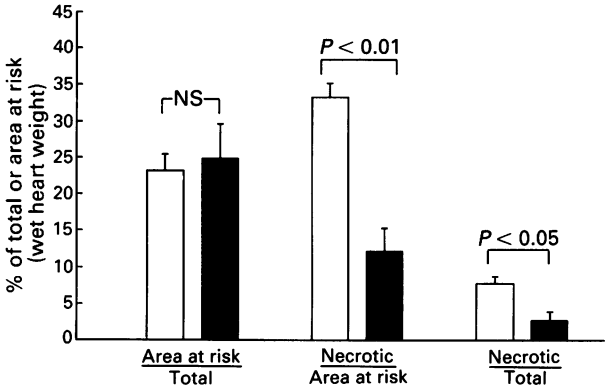

Figure 1 Area-at-risk indexed to total left ventricle (area-at-risk/total left ventricle × 100) and necrotic area indexed to area-at-risk (necrotic area/area-at-risk × 100) and to total left ventricle (necrotic area/area-at-risk × 100) in wet weight percent. Heights of columns are means and vertical bars indicate s.e.mean Treatment with [Ala-IL8]$_{77}$ (solid columns) significantly reduced the size of the necrotic area when compared to the untreated MI group (open columns).

Results

All sham MI rabbits maintained stable mean arterial blood pressures and heart rates over the course of the experimental period, so that the pressure-rate index did not change. Neither was there any change in ST segment of the electrocardiograms (Table 1). However, ST segments were significantly elevated in both of the MI groups (0.3 to 0.4 mV at 20–40 min post-occlusion) relative to the sham MI group. Moreover, there were no significant differences between the MI groups in ST segment elevation indicating that the severity of ischaemia was similar between the groups of rabbits subjected to myocardial ischaemia.

There were no significant differences between the MI groups for heart rates, mean arterial blood pressures (MABP) or the pressure-rate product, which was used as an index of myocardial oxygen demand. The initial MABP for all ischaemic rabbits combined was 100 ± 2 mmHg and the final MABP for all ischaemic rabbits combined was 89 ± 2 mmHg. Heart rate did not change over time in any of the experimental groups. Moreover, there were no statistically significant changes in the pressure-rate index (PRI) among any of the three experimental groups (Table 1). Therefore, changes in myocardial oxygen demand cannot account for any subsequent differences observed in any of the indices of severity of myocardial ischaemia.

Myocardial necrosis was assessed by staining techniques following 90 min of ischaemia and 3.5 h reperfusion in order to determine an anatomical index of the myocardial area jeopardized and that which became necrotic. The area-at-risk determined by negative staining following perfusion with Evan's blue stain, showed no significant differences between either of the MI groups (Figure 1), indicating that a similar amount of tissue was jeopardized by occlusion of the marginal coronary artery in both MI groups. In contrast, the necrotic area, which was measured by negative staining with nitroblue tetrazolium, indicated that a relatively large amount of the myocardial tissue at risk became necrotic (33%) in the untreated MI group. However, treatment with [Ala-IL8]$_{77}$ 10 min prior to reperfusion significantly reduced myocardial necrosis. This significant reduction in necrosis was observed whether the necrotic area was expressed as a percentage of the area-at-risk or as a percentage of the total left ventricle. Thus, [Ala-IL8]$_{77}$ afforded significant cardioprotection.

Figure 2 summarizes the cardiac myeloperoxidase activities for the groups of rabbits studied. Very low MPO activities were observed in the sham MI group, while significantly elevated MPO activities were observed in the area-at-risk in both MI groups. However, treatment with [Ala-IL8]$_{77}$ resulted in a significantly lower MPO activity in the necrotic area. MPO activities in the area-not-at-risk were very low for all three of the groups and there were no significant differences in MPO activities among any of them. Thus, these data suggest that [Ala-IL8]$_{77}$ can significantly limit the amount of neutrophil infiltration into the necrotic zone of the ischaemic myocardium.

In an effort to assess the extent of coronary vascular endothelial dysfunction, we isolated marginal coronary artery rings and assessed the ability of endothelium-dependent vasodilators to promote release of endothelium-derived relaxing factor (EDRF) in these preparations. U-46619 was used to contract marginal coronary artery rings, prior to testing with endothelium-dependent (e.g., ACh, A-23187) and endothelium-independent (i.e., $NaNO_2$) vasodilators. Figure 3 illustrates typical responses to ACh and $NaNO_2$ of isolated coronary artery rings taken from the ischaemic marginal artery segment below the site of occlusion. ACh added to rings from sham MI rabbits produced a full relaxation response. In marked contrast, rings isolated from the untreated MI rabbits were essentially unresponsive to addition of ACh. However, coronary artery rings isolated from rabbits treated with [Ala-IL8]$_{77}$ relaxed normally to ACh compared with the untreated MI group. Responses of these same coronary artery rings to the endothelium-independent vasodilator acidified $NaNO_2$ were

Table 1 Peak ST-segment elevation, initial and final pressure-rate index (PRI)

Group	*ST-segment* (mV)	*Initial PRI* (HR × MABP/1000)	*Final PRI* (HR × MABP/1000)
Sham MI + Vehicle (5)	0 ± 0	29.3 ± 1.6	27.1 ± 2.3
MI + vehicle (7)	0.3 ± 0.06	26.2 ± 2.0	20.9 ± 1.9
MI + [Ala-IL8]$_{77}$ (6)	0.4 ± 0.07	28.2 ± 0.9	22.0 ± 0.8

All values are means ± s.e.mean.
Numbers in parentheses are numbers of rabbits studied in each group.
HR = heart rate; MABP = mean arterial blood pressure. MI = mycardial ischaemia.

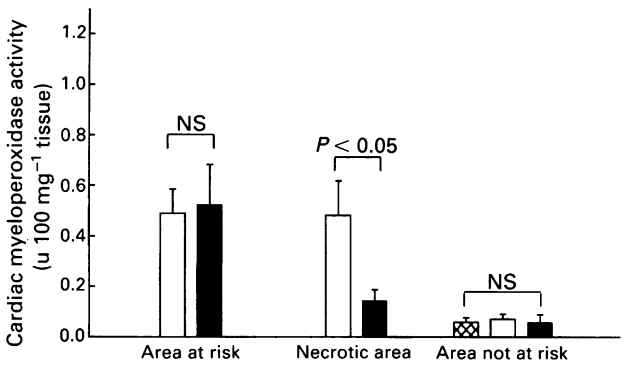

Figure 2 Myeloperoxidase (MPO) activity in the area-at-risk, necrotic area and area-not-at-risk in units $100\,mg^{-1}$ tissue. Heights of columns are means and vertical bars indicate s.e.mean. Treatment with $[Ala\text{-}IL8]_{77}$ (solid columns) significantly reduced the MPO activity in the necrotic zone when compared to the untreated MI group (open columns). The cross-hatched columns represent the Sham MI + vehicle group.

not different among the three groups studied. All groups relaxed fully in response to acidified $NaNO_2$ indicating that the coronary vascular smooth muscle was fully capable of relaxing to a vasodilator.

Figure 4 illustrates the mean responses to ACh and to $NaNO_2$ which were shown by the representative tracings in Figure 3. In addition, responses to the endothelium-dependent, non-receptor mediated vasodilator, A-23187 (calcium ionophore), are also shown. The pattern of responses to A-23187 was similar to that of ACh in all groups. Coronary rings isolated from sham MI rabbits relaxed fully, while significant impairment was observed in the untreated ischaemic MI rings. Treatment with $[Ala\text{-}IL8]_{77}$ produced virtually a complete relaxation which was significantly greater than the

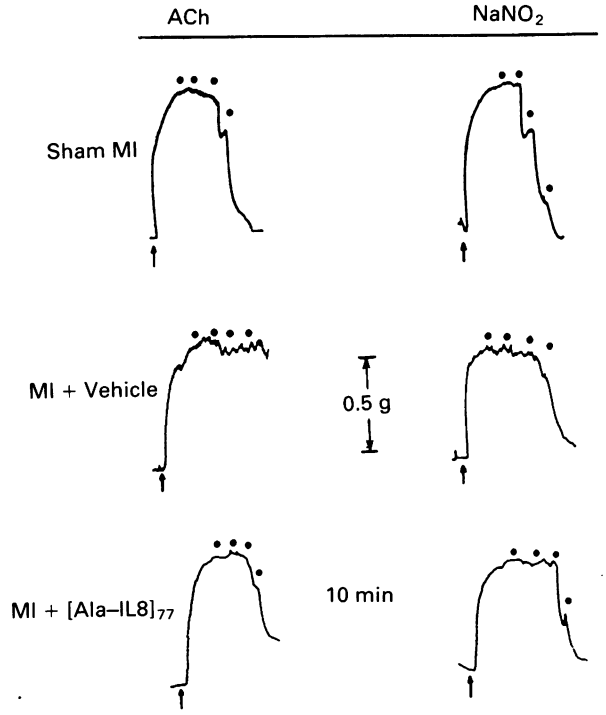

Figure 3 Representative recordings of marginal coronary artery rings of rabbit from the three groups studied in the presence of the endothelium-dependent vasodilator acetylcholine (ACh) and the non-endothelium-dependent vasodilator acidified $NaNO_2$. Rings were precontracted with U-46619, a thromboxane mimetic, as shown by the arrows. Dots above the tracings indicate addition of vasodilator substances. The endothelium-dependent relaxation was abolished by ischaemia/reperfusion in the untreated MI group, whereas treatment with $[Ala\text{-}IL8]_{77}$ significantly preserved this response.

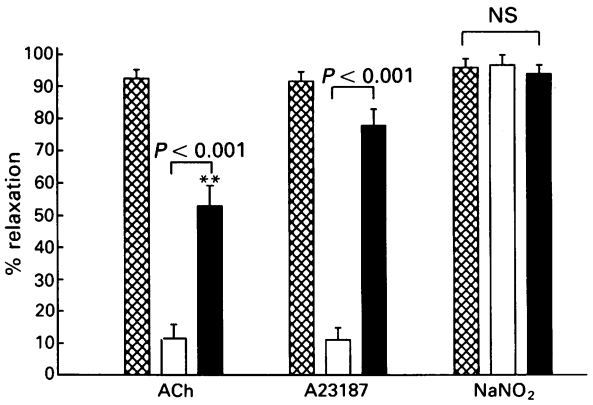

Figure 4 Group data from ischaemic coronary artery rings are summarized for the four groups studied following addition of the endothelium-dependent receptor mediated vasodilator acetylchloline (ACh), the endothelium-dependent nonreceptor-mediated vasodilator A-23187, and the endothelium-independent vasodilator acidified $NaNO_2$. Responses are expressed as percentage relaxation of a U-46619-induced contraction. Significant response decrements were observed in the untreated MI group (open columns) in response to ACh and A-23187 which were significantly preserved in the MI group treated wh $[Ala\text{-}IL8]_{77}$ (solid columns). $^{**}P < 0.001$ compared with Sham MI group (cross-hatched column).

untreated ischaemic MI rings and was not significantly different from the sham MI rings. This indicates that both receptor-mediated and non-receptor-mediated endothelium-dependent responses were fully protected by $[Ala\text{-}IL8]_{77}$ treatment. All ischaemic rings fully relaxed in the presence of $NaNO_2$, indicating that the vascular smooth muscle had not been damaged during the course of the experiment.

Figure 5 illustrates the responses of the control coronary artery rings which were taken from the proximal portion of the marginal coronary artery above the site of occlusion. There was no significant difference among the groups in the relaxation to ACh. There was a small but significant difference in response to A-23187 between the untreated MI control rings and the sham MI control rings. This difference is probably of no physiological consequence, and no other group comparisons were significant with regard to responses to A-23187. Finally, there were no significant differences in

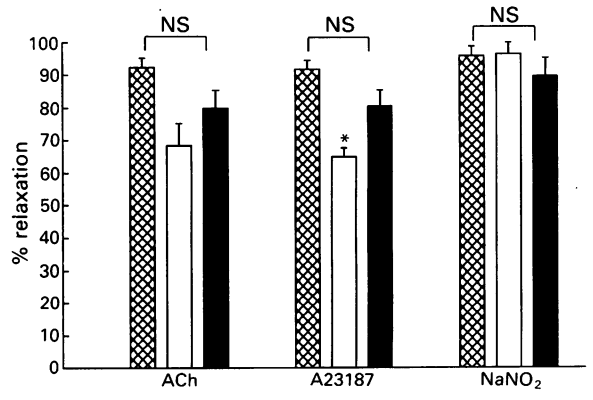

Figure 5 Group data from control coronary artery rings are summarized for the four groups studied following addition of the endothelium-dependent receptor mediated vasodilator acetylcholine (ACh), the endothelium-dependent non-receptor-mediated vasodilator A-23187, and the endothelium-independent vasodilator acidified $NaNO_2$. Responses are expressed as percentage relaxation of a U-46619-induced contraction. The only significant response decrement observed was between the sham MI group (hatched columns) and the untreated MI group (open columns) in response to A-23187. $^{*}P < 0.05$ compared with Sham MI group. The solid columns represent the MI + $[Ala\text{-}IL8]_{77}$ group.

Table 2 Effect of [Ala-IL8]$_{77}$ on vasodilator responses in rabbit isolated perfused hearts

Group	n	*% response to acetylcholine*[a]	*% response to nitroglycerin*[b]
Control hearts	5	104 ± 7	103 ± 5
MI + Vehicle	6	44 ± 8*	100 ± 7
MI + [Ala-IL8]$_{77}$ (50 μg)	6	55 ± 7*	99 ± 5

All values are means ± s.e. mean. The numbers represent the second response (i.e., 20 min post-reperfusion or sham reperfusion) calculated as a percentage of the initial control response (i.e., prior to ischaemia or sham ischaemia). Coronary vascular resistances decreased to ACh by about 65% of their U-46619 added tone.

[a] Concentration = 200 ng ml^{-1}.

[b] Concentration = 50 μg ml^{-1}.

n = number of hearts studied; MI = myocardial ischaemia.

* $P < 0.01$ from control hearts.

response to $NaNO_2$ among any groups. These data indicate that there was no significant endothelium impairment in the non-ischaemic segment of the marginal coronary artery. The endothelium dysfunction occurred only in the ischaemic segment of the marginal coronary artery of rabbits subjected to myocardial ischaemia.

Rabbit hearts perfused with Krebs-Henseleit solution without any blood plasma or blood cells responded normally to ACh and nitroglycerin initially, and also after 50 min of perfusion at control flow (Table 2). However, rabbit hearts subjected to 30 min of global ischaemia (i.e., to 15% of control flow) and 20 min of reperfusion at control flows experienced a significant impairment in vasodilatation to ACh but not to nitroglycerin, indicating an endothelial dysfunction. Moreover, addition of [Ala-IL8]$_{77}$ to the perfusate 10 min prior to reperfusion failed to restore vasodilator responsiveness to ACh, indicating that [Ala-IL8]$_{77}$ under the conditions of the buffer-perfused heart, failed to exert a direct endothelium protecting effect in the rabbit coronary vasculature. Additional studies are necessary to determine the precise interaction between neutrophils and the endothelium in the presence of all of the substances present in blood during myocardial ischaemia.

Finally, [Ala-IL8]$_{77}$ added to the synthetic xanthine-xanthine oxidase mixture at a concentration of 0.3 μg ml^{-1} did not significanly alter the absorbance at 550 nm from 0.124 ± 0.013 to 0.126 ± 0.015 whereas recombinant human superoxide dismutase (hSOD) reduced the absorbance to 0.019 ± 0.006 ($P < 0.001$ from control). Thus, [Ala-IL8]$_{77}$ clearly lacks intrinsic superoxide radical scavenging activity.

Discussion

Myocardial ischaemia produced by the occlusion of a large coronary artery results in functional alterations due to the prolonged interruption of blood flow. Although reperfusion restores flow, it initiates generation of reactive oxygen metabolites and stimulates release of pro-inflammatory mediators (McCord, 1985; Hess & Manson, 1984; Zweier, 1988). Prominent targets for post-ischaemic reperfusion injury include both endothelial cells and cardiac myocytes. Damage to the endothelium may impair control of vascular tone by inhibiting release of vasodilator substances such as endothelium-derived relaxing factor (EDRF) which can result in a loss of vasodilator reserve (Van Benthuysen *et al.*, 1987; Mehta *et al.*, 1989; Johnson *et al.*, 1990). This defect has been shown to occur within 10–20 min after reperfusion in cats and rats (Tsao *et al.*, 1990; Lefer *et al.*, 1990).

Neutrophils have been shown to accumulate in the post-ischaemic myocardium (Sommers & Jennings, 1964; Romson *et al.*, 1982; Johnson *et al.*, 1990) and can cause cellular dysfunction by a variety of mechanisms. Neutrophils release cytotoxic oxygen metabolites, enzymes (e.g., proteases and collagenases), and cytokines such as tumor necrosis factor (TNF) which has been shown to inhibit EDRF release from the vascular endothelium (Aoki *et al.*, 1989). Neutrophils also release superoxide radicals which have been shown to inactivate EDRF (Rubanyi & Vanhoutte, 1987) and may disrupt cellular membranes through lipid peroxidation reactions, thus promoting increased microvascular permeability. In addition, neutrophil aggregates may participate in microvascular plugging leading to the 'no-reflow' phenomenon (Kloner *et al.*, 1974; Engler *et al.*, 1986). Experimental strategies which have involved prevention of activation of neutrophils (Bednar *et al.*, 1985; Simpson *et al.*, 1987; Simpson *et al.*, 1988a) or neutrophil depletion (Romson *et al.*, 1983) have been shown to reduce reperfusion injury.

Adherence of neutrophils to the endothelium appears to represent a critical step in the pathogenesis of ischaemia-reperfusion injury and can lead to endothelial damage (Harlan, 1987). Cytokines such as IL-1 and TNF can act directly on endothelial cells to induce the expression of endothelial-leukocyte adhesion molecules (ELAMs) (Bevilacqua *et al.*, 1987). The expression of these adhesion molecules promotes adherence of neutrophils to the surface of the endothelium, which is a prerequisite for diapedesis of these cells into ischaemic reperfused tissues.

[Ala-IL8]$_{77}$ is a recently discovered 10-kD protein which is produced by the endothelium and acts as a soluble leukocyte adhesion inhibitor (Gimbrone *et al.*, 1989). It has been sequenced and shares considerable sequence identity over 72 amino acids (with an additional pentapeptide added) with mononuclear leukocyte-derived IL-8 [(Ser)-IL8]$_{72}$ produced by cells of the immune system. When activated by IL-1 or TNF, endothelial cells produce this leukocyte adhesion inhibitor [Ala-IL8]$_{77}$ which attenuates the adhesive interaction between leukocytes and endothelial cells (Wheller *et al.*, 1988; Strieter *et al.*, 1989; Schroder & Christophers, 1989). [Ala-IL8]$_{77}$ has been shown to be a potent inhibitor of neutrophil adhesion to cytokine-activated endothelial monolayers (Gimbrone *et al.*, 1989) and to protect these monolayers from neutrophil-mediated damage (Gimbrone *et al.*, 1989). It has been proposed that [Ala-IL8]$_{77}$ may attenuate inflammatory events at the interface between the blood and the vessel wall (Gimbrone *et al.*, 1989). Therefore, an important new strategy in prevention of reperfusion injury may be the identification of substances which protect the endothelium from damage by inactivation of neutrophil adherence mechanisms, thus inhibiting neutrophil induced endothelial dysfunction which later leads to infiltration of neutrophils into the myocardium.

There is evidence for a reduced neutrophil accumulation in ischaemic rabbit cardiac tissue evidenced by the MPO data. This anti-inflammatory effect of intravenous [Ala-IL8]$_{77}$ confirms the earlier report of this phenomenon by Hechtman *et al.* (1990). Although [Ala-IL8]$_{77}$ significantly retarded MPO activity in the necrotic zone, it did not do so in the non-necrotic area-at-risk. This may be due to two different neutrophil pools, a circulating pool migrating to the necrotic area, and another pool (e.g., marginated or rolling neutrophils) which behave differently and which adhere to the endothelium or migrate into the extravascular compartment

in the area-at-risk. Further work is necessary to clarify these relationships.

We have shown a remarkable degree of endothelial protection by $[Ala\text{-}IL8]_{77}$ delivered 10 min prior to reperfusion compared to untreated MI rabbits. In this connection, $[Ala\text{-}IL8]_{77}$ maintained EDRF effectiveness during the reperfusion phase of myocardial ischaemia and reperfusion. This endothelial protective effect may retard neutrophil adherence to endothelial cells and the progressive infiltration of neutrophils into deeper areas of the myocardium. This is supported by the finding that EDRF (i.e., nitric oxide) prevents neutrophil adherence to endothelial cells (McCall *et al.*, 1988). Thus, protection and preservation of endothelial cell function by prevention of neutrophil adherence would appear to be a major mechanism of action which could explain the protective effects of $[Ala\text{-}IL8]_{77}$ observed in this model. However, it is unlikely that a direct endothelial preservation by $[Ala\text{-}IL8]_{77}$ could explain our results since $[Ala\text{-}IL8]_{77}$ failed to prevent endothelial dysfunction in rabbit isolated perfused hearts in the absence of blood or blood cells. Moreover, $[Ala\text{-}IL8]_{77}$ did not scavenge superoxide radicals in a totally synthetic xanthine-xanthine oxidase system.

Nevertheless, several possibilities still exist with regard to the mechanism of action of $[Ala\text{-}IL8]_{77}$ in myocardial ischaemia and reperfusion. $[Ala\text{-}IL8]_{77}$ may exert (a) direct effects on neutrophils to reduce the number of these leukocytes accumulating in myocardial tissue following reperfusion; (b) direct inhibitory effects on chemotactic function of neutrophils to prevent their migration along chemotactic gradients; (c) direct inhibitory effects on the ability of neutrophils to express or upregulate adhesion molecules on their surface; (d) direct or indirect inhibitory effects on neutrophils to prevent damage to endothelial cells by inhibition of cytotoxic mediator release; and (e) other, as yet unknown effects, such as cell membrane stabilizing activity. These possibilities remain for future investigation.

In summary, we have observed that coronary artery occlusion for 1.5 h followed by 3.5 h reperfusion resulted in significant endothelial dysfunction, neutrophil accumulation in the myocardium and myocardial injury in the anaesthetized rabbit. Intravenous administration of $[Ala\text{-}IL8]_{77}$, a novel endothelial cell-derived leukocyte adhesion inhibitor, preserved endothelial function, reduced neutrophil infiltration into the necrotic myocardium and reduced myocardial necrosis, thus providing a significant degree of endothelial and myocardial protection in this model. Since the rabbit is known to have very little collateral coronary flow (Schaper, 1981) it is unlikely that $[Ala\text{-}IL8]_{77}$ could protect by opening collateral vessels. This is even more remote since $[Ala\text{-}IL8]_{77}$ failed to exert any direct coronary vasoactivity in isolated coronary rings (data not shown), and did not exert any systemic vasodilation *in vivo* (i.e., pressure-rate index (PRI) was not decreased). Despite the beneficial effects observed in our short term model of myocardial ischaemia and reperfusion in rabbits, it should be emphasized that we have no information on long term effects of infarction (e.g., 24–48 h) nor do we know whether these results would be directly applicable in clinical cases of human myocardial ischaemia where the time of onset of ischaemia or of reperfusion may not be clearly defined.

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Modulation by the epithelium of the extent of bronchial narrowing produced by substances perfused through the lumen

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1 Airway narrowing was determined *in vitro* as a measure of bronchial reactivity. A bronchial segment from pig lung was perfused with a Krebs solution and the change in flow rate to drugs and small ions perfused intraluminally was compared with that obtained by application to the serosal surface.

2 The sensitivity (EC_{50}) to acetylchloline was 30 times greater on the serosal surface than on the luminal surface. Concentrations of histamine and carbachol which had threshold responses on flow rate when perfused intraluminally virtually stopped flow on the serosal surface. Potassium depolarizing solutions (containing either KCl or K_2SO_4) and vanadate (VO_3^-) had little or no effect intraluminally but completely stopped flow through the bronchial segment when applied to the serosal surface, i.e. they closed off the airway.

3 After removal of the epithelium the sensitivity to drugs and K^+ perfused intraluminally was increased to equal that on the serosal surface.

4 No evidence for suppression of smooth muscle contraction by a putative epithelium-derived inhibitory factor (EpDIF) could be obtained: no inhibition of smooth muscle contractility was seen when the agents listed above were perfused intraluminally and their perfusion continued while they were applied to outside.

5 It was concluded that the epithelium plays a crucial role as an impermeant barrier in modulating the responsiveness of the airways smooth muscle.

Keywords: Bronchial epithlium; airway narrowing; perfused bronchial segments; luminal vs. serosal responses; epithelium-derived inhibitory factor (EpDIF); K^+ depolarizing solution; vanadate; acetylcholine

Introduction

The bronchial epithelium is abnormal in the lungs of asthmatics and epithelial damage has been implicated in the hyperresponsiveness of airways (Dunnill, 1960; Laitinen *et al.*, 1985; Holgate & Finnerty, 1988). While the epithelium provides a barrier to the access of inhaled stimulants (Holroyde, 1986; Goldie *et al.*, 1988) it has been suggested that it has a more active role in protecting the airways by elaborating an epithelium-derived inhibitory factor (EpDIF) (Vanhoutte, 1988; Goldie *et al.*, 1990). The evidence for this arises firstly from isolated strips of airway wall *in vitro*, where a leftward shift in the dose-response curve or an increase in maximum response is seen in epithelium-denuded compared with epithelium-intact preparations (Flavahan *et al.*, 1985; Barnes *et al.*, 1985; Goldie *et al.*, 1986; Stuart-Smith & Vanhoutte, 1988; Iriarte *et al.*, 1990). The magnitude of this sensitivity shift is not large, generally within the range of 2–5 fold for acetylcholine, histamine and 5-hydroxytryptamine. Secondly, by use of a co-axial assay technique, challenge of the epithelium of tracheal tubes with histamine and other stimuli causes the release of a substance(s) which relaxes rat aorta (Fernandes *et al.*, 1989) or reduces the sensitivity of tracheal strips (Hay *et al.*, 1987).

The use of strips or rings of trachea *in vitro* does not permit an unequivocal interpretation of the role of the epithlium where drugs added to the organ bath can contract the smooth muscle by diffusing via the serosal, the mucosal and the cut surfaces at the same time. Recently Pavlovic *et al.* (1989), Munakata *et al.* (1989) and Small *et al.* (1990) have measured isometric force or perfusion pressures developed by intact tracheae from guinea-pigs in response to agents applied from the serosal and the luminal side. The sensitivity was much less on the inside than on the outside and this difference disappeared when the epithelium was removed. These workers concluded that the epithelium may act both as a barrier and by the release of a putative EpDIF.

But the airway narrowing which causes an increase in resistance to air flow in bronchial hyperreactivity and in asthma occurs in the bronchi or bronchioles. Airway narrowing can be measured in intact segments of the bronchial tree either as a change in flow or in resistance to flow by use of a perfused bronchial segment preparation (Mitchell *et al.*, 1989). This technique takes into account the important influence of the lumen diameter, the thickness of the airway wall and its geometrical configuration during narrowing, the transmural force acting across the wall and the presence of a complete epithelial lining covering the luminal surface of the wall. We show here, using this preparation, that the major function of the epithelium is that of an impermeant barrier preventing acess of substances to the underlying smooth muscle. Evidence for an EpDIF released by the epithelium could not be obtained. A preliminary account of this was presented at the workshop on 'Mediators in Airway Hyperreactivity', a satellite conference of the 11th International Congress of Pharmacology.

Methods

Lungs were obtained from 25–35 kg (approximately 12 week old) Largewhite/Landrace-cross pigs. Animals were housed in the University Biosciences Animal Unit and were killed with a bolt gun and bled immediately. The lungs were removed and packed in ice for dissection of the bronchi from lower right or left lobes.

Preparation of bronchial segments

Bronchial segments of approximately 20 mm in length were dissected as previously described (Mitchell *et al.*, 1989). One bronchial segment with a relaxed distal external diameter of 3 mm was dissected from each lower lobe. The epithelium was removed, when required, by inserting a cotton applicator tip soaked in Krebs solution into the lumen and very gently dragging it several times along the entire length of the segment.

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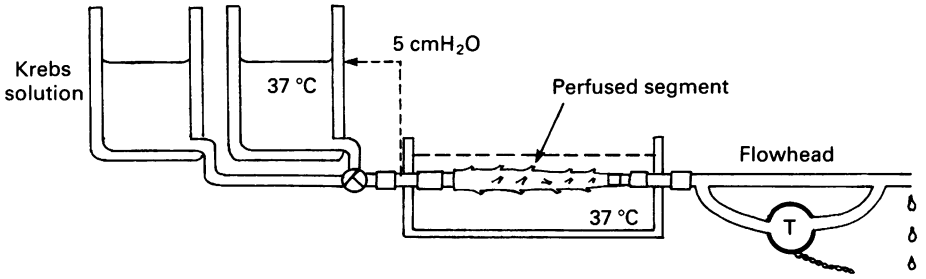

Figure 1 Apparatus used to record flow in bronchial segments. Drugs could be added to the bronchial lumen (i.e. inside) via the perfusate or to the outside via the bathing medium.

Both ends were cannulated with polythene tubing of a slightly larger internal diameter than that of the relaxed segment. Segments were then mounted in horizontal chambers containing Krebs solution (37°C, pH 7.4, gassed with 5% CO_2 in O_2) which was exchanged for fresh solution at regular intervals throughout the experiment. Krebs solution was perfused through the lumen from a reservoir set at a constant pressure head of 5 cmH_2O. The flow rate through the segment was measured with a differential pressure transducer (type MPX10DP, Motorola semiconductors, Phoenix, U.S.A.) which was set to detect the pressure-drop across a custom built flowhead set up in series with the tissue (Figure 1). The flowhead and transducer were previously checked for linearity and were calibrated over the range of flows encountered in an experiment. The resistance of the flowhead alone was less than that of the relaxed bronchial segment. Resting flow with the bronchial segment connected was 33 ml min^{-1}. Airway narrowing reduced the pressure drop across the flowhead because of the lesser flow rate through the tissue. Flow (i.e., pressure drop) was monitored on a chart recorder.

Protocols

Two experimental protocols were followed. In the first experiment involving only epithelium-intact bronchial segments, tissues were primed by adding a submaximum concentration of acetylcholine (ACh) to the Krebs solution bathing the outside of the tissue. Following washout and complete recovery, a cumulative concentration-response curve (CCRC) to perfused ACh (i.e. inside), then a CCRC to ACh added to the solution bathing the outside of the segment were recorded. The CCRC to perfused ACh was then repeated. Subsequently, the Krebs solution bathing the outside of the tissue, or the perfusate, was replaced by K^+ depolarizing solution or KCl solution or vanadate (VO_3^-) 9 mM in Krebs solution. Throughout the experiment electrical field stimulation (70 V, 1 ms, 10 Hz) was used as a control to monitor any change in tissue responsiveness with time. This same procedure was adopted with histamine or carbachol as bronchoconstrictors. At the conclusion of each experiment, segments were frozen in isopentane cooled in liquid nitrogen in preparation for histological examination.

In the second experiment, tissues were paired, one segment having an intact epithelium and the other being stripped of its epithelium. After priming the tissues to ACh (outside), two CCRCs to ACh were recorded, the first inside and the second outside. These were followed by K^+ depolarizing solution and VO_3^- applied to the inside and then the outside. Electrical field stimulation was again used to compare tissue responsiveness throughout the experiment. Segments were frozen in preparation for histological examination.

Histology

To examine the extent of removal of the epithelium, the segments frozen at −190°C were divided into proximal, mid and distal regions, embedded in CRYO-M-BED (Bright) and 15 μm cryostat sections were cut in triplicate from each region. Sections were dried and fixed in 100% methanol for 10 min, then stained with haematoxylin and Chromotrope 2R. The proportion of the internal perimeter denuded of its epithelium was estimated by examining each section under a light microscope at low magnification.

Solutions

Krebs solution had the following composition (mM): NaCl 121, KCl 5.4, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.5 and $CaCl_2$ 2.5. Depolarizing Krebs solutions contained 80 mM K_2SO_4 in place of NaCl or 121 mM KCl in place of NaCl. The vanadate solution was ammonium vanadate (NH_4VO_3) made up to give a 9 mM concentration in Krebs solution. The solutions were continuously gassed with 95%O_2/5%CO_2 mixture. The drugs used were acetylcholine chloride, carbamylcholine chloride (carbachol), histamine acid phosphate (Sigma Chemicals) and ammonium vanadate (Aldrich Chemical Company). These were prepared in distilled water or Krebs on the day of each experiment. All drug solutions were kept on ice.

Statistics

Data shown are mean ± s.e.mean with n = number of preparations. Mean EC_{50}s were calculated by finding the arithmetic means of pD_2 values (pD_2 is the negative logarithm of the EC_{50}) and then taking the antilog of the mean pD_2 values. Mean fold differences in EC_{50} were found by calculating individual fold differences and taking the geometric mean. The significance of the difference between means was compared by Student's t test with $P < 0.05$ considered significant.

Results

Segments perfused at a pressure of 5 cmH_2O maintained a steady flow for 5 to 6 h which averaged 32.9 ± 1.1 ml min^{-1} in 10 preparations. Figure 2 shows the responses to electrical field stimulation (EFS). The flow rate decreased rapidly in response to 1 ms pulses at 5 Hz, 70 V and at 20 Hz the flow was completely abolished. On cessation of the stimulus the flow was restored to normal. Reproducible responses to EFS could be elicited throughout the course of an experiment of 6 h duration. The response of the perfused bronchial segment to other stimuli such as acetylcholine (ACh), histamine, a K^+ depolarizing solution or vanadate ions (VO_3^-) depended on whether they were perfused through the lumen or added to the solution bathing the outside of the segment: the outside was much more responsive (Figure 2). With ACh the EC_{50} was shifted 29 fold, from 69.0 ± 5.1 μM on the luminal surface to 2.40 ± 0.5 μM on the serosal side ($P < 0.001$ $n = 4$ pigs, Figure 3). The difference in sensitivity was unaffected by the order of applying ACh. In Figure 3 the order of stimulus was inside/outside/inside. Reversing the order gave the same result. Flow was reduced to zero at 0.1 mM ACh on the outside and at 10 mM on the inside.

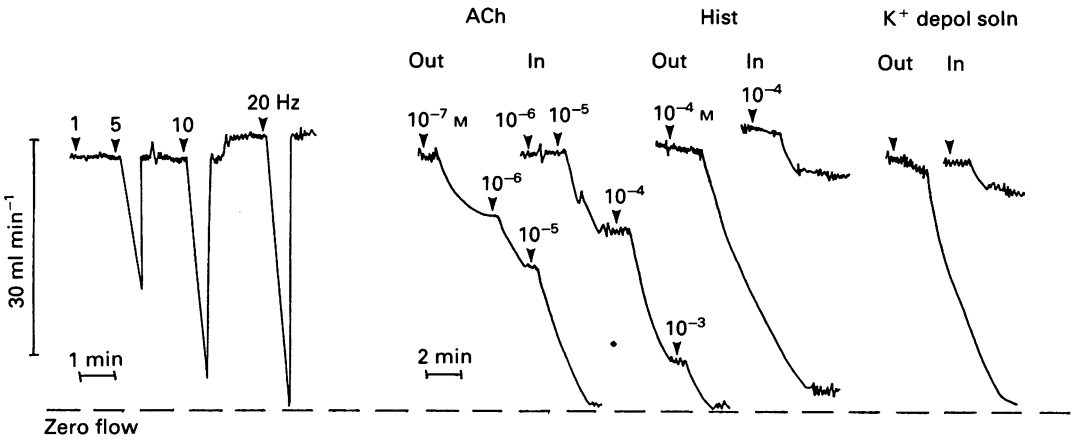

Figure 2 Airway narrowing, shown as a decrease in flow of perfusate through a bronchial segment, to electrical field stimulation (70 V, 1 ms) and to contractile agonists. Left to right: responses to electrical field stimulation, at 10–20 Hz flow approaches zero. Acetylcholine (ACh) applied to the outside of the bronchial segment, ACh perfused through the lumen (inside): histamine (Hist) outside, histamine inside, K^+ depolarizing solution (NaCl replaced with 80 mM K_2SO_4) outside, K^+ depolarizing solution inside.

This difference in responsiveness between the inside and outside was even more striking when K^+ depolarizing solutions were used (Figure 4). When the K_2SO_4 solution was perfused the flow was unchanged in 4 out of 6 tissues with a small decrease in flow (< 15% response) in the other two tissues. In contrast, on the outside it caused a rapid fall in flow with complete closure in 3 of the 6 tissues and a greater than 75% reduction in flow in the others. A similar result was obtained with a KCl depolarizing solution (Figure 4).

Vanadate causes contraction of airway smooth muscle by a direct effect on the smooth muscle (Nayler & Sparrow, 1983). When perfused through the lumen at 9 mM, it had no effect in 3 out of 3 tissues whereas on the outside of the segment this supramaximum concentration completely abolished flow in 3 of 5 segments and caused a marked reduction in flow (>85%) in the remaining two (Figure 4).

The results suggested that the epithelial layer was acting as a barrier, reducing or preventing the diffusion of these agents to the smooth muscle. To test this possibility, the effect of removing the epithelial layer from bronchial segments was next investigated with paired segments, one stripped and unstripped, from the same lung, with ACh perfused via the lumen. Figure 5 shows that the sensitivity of the stripped segment was increased 37 fold, the EC_{50} shifting from $60.3 \pm 7.1\,\mu M$ to $1.62 \pm 0.5\,\mu M$ ($P < 0.01$, $n = 6$ pigs). The sensitivity of these epithelium-denuded segments perfused via the lumen was now comparable with that seen outside (EC_{50} $1.62\,\mu M$ vs $2.40\,\mu M$, not significant). Likewise perfusion of K^+ depolarizing solutions or 9 mM VO_3^- now produced complete or near complete abolition of flow (data not shown).

The effectiveness of stripping was confirmed by histological examination. In 3 out of 6 stripped tubes, sections from the proximal, mid and distal region showed complete removal of the epithelium. In the other stripped tubes sections from two of these regions showed complete stripping with the remaining sections being >85% stripped. In the unstripped tissues only one region from one segment showed epithelial damage (≈10%), while in all others, the epithelial layer was fully intact.

If an EpDIF was elaborated by the epithelium in response to stimulants applied to the luminal surface, it would diffuse across the mucosa to suppress smooth muscle contraction. To test whether this was occurring, the above agents were perfused through the lumen and then added to the outside while the perfusion was continued. Figure 6 shows that there was no suppression of the smooth muscle responsiveness produced by

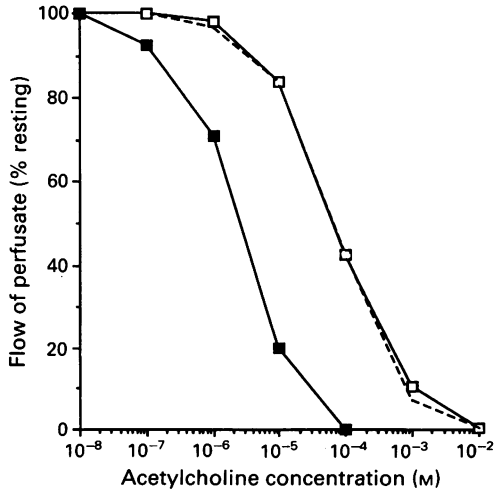

Figure 3 Narrowing of perfused bronchial segments, shown as a decrease in flow of perfusate, in response to acetylcholine (ACh) added to the perfusate (□ inside) then to the Krebs solution bathing the outside of the segment (■ outside) and then again to the inside (- - - -). Flow is expressed as a percentage of resting flow which averaged 33 ± 1.4 ml min^{-1}. Data points represent the mean responses of four segments from four pigs. Horizontal bars show the s.e. of the mean EC_{50}.

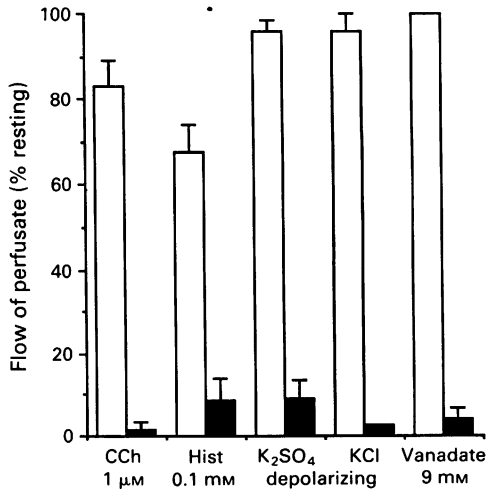

Figure 4 Narrowing of perfused bronchial segments in response to single doses of stimulants in the perfusate (open columns) and applied to the outside (filled columns). Height of columns represents mean of 3 to 6 tissues; s.e.mean shown by vertical bars. CCh, carbachol; Hist, histamine.

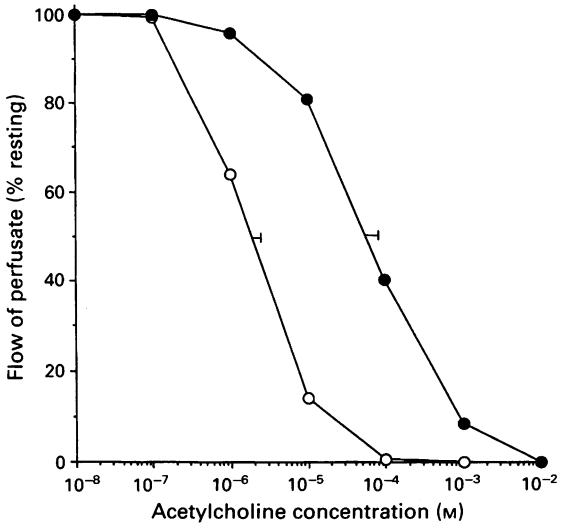

Figure 5 The effect of stripping the epithelium from bronchial segments on airway narrowing to acetylcholine. Increasing concentrations of acetylcholine were added to the perfusate in epithelium-intact (●) and epithelium-denuded (○) bronchial segments. Flow is expressed as a percentage of resting flow. Data points represent mean responses ($n = 6$ preparations). Horizontal bars show the s.e.mean of the mean EC_{50}.

perfusion of carbachol 10^{-6} M (5 segments, 4 pigs) or by histamine 10^{-4} M (3 segments, 3 pigs). Similarly with K_2SO_4 depolarizing solution the resting flow was 34 ml min^{-1}, with K^+ perfused 32 ml min^{-1}, with K^+ on the outside 6 ml min^{-1} and with K^+ in the perfusate and on the outside 5 ml min^{-1}. Using this same test procedure with submaximal concentrations of histamine, carbachol, ACh and a K^+ depolarizing solution (25% of the NaCl replaced by KCl) the flow rate in response to their application on the serosal side was not influenced by their prior perfusion intraluminally (Table 1).

Discussion

We have demonstrated here that the epithelium lining the bronchi in pigs lungs behaves as a very effective barrier. It prevents, almost completely, the actions of K^+ depolarizing solutions and vanadate from contracting the smooth muscle at concentrations which effectively close off the airway when applied to the outside. The sensitivity to acetylcholine is reduced by almost 30 fold compared with its effect on the serosal surface; other agents, carbachol and histamine, similarly show a decreased sensitivity when perfused intraluminally. The response of these agents after removal of the epithelium was the same as that on the outside. Electrical stimulation of the nerves which releases ACh from postganglionic nerve endings was unaffected by removal of the epithelium as would be predicted, since electrical field stimulation acts directly on nerve terminals thereby bypassing epithelial modulation. If the airway smooth muscle is a target for an EpDIF released from the epithelium, as has been widely suggested, then these quite disparate agents ACh and the other drugs, K^+ and VO_3^- would have to be able to act on the epithelium to synthesize or release EpDIF which would then diffuse across the submucosa to inhibit smooth muscle contraction. But we could not show that this was happening because perfusing submaximal doses of any of the above agents intraluminally for several minutes prior to adding them to the outside whilst continuing their perfusion did not show any suppression of the outside response. A reduction in response would have been predicted if a putative EpDIF had inhibited the smooth muscle. The speed of narrowing was not slowed, neither was there a delay before initiation of narrowing.

Our data obtained by measuring airway narrowing in response to agents applied to the lumen and to the serosa is best explained by the epithelium acting as a barrier to diffusion. This is the function of epithelial cells lining tubular structures and organs (Wright, 1983) and the tight junctions between the cells vary in their capacity to hinder the penetration of molecules. In the bladder and stomach the tight junctions are impermeant to small inorganic ions but in the intestinal epithelial lining they are 10,000 times more leaky (Alberts *et al.*, 1989). In view of the lack of response to K^+ and VO_3^- applied to the luminal surface of the airway the tight junctions would seem to be impermeant. The route by which drug molecules are transported is more likely to be via the epithelial cell membrane (Wright, 1983) but specific experimental evidence using airway epithelia is needed to establish this (see Cereijido *et al.*, 1988).

The use of strips of airway wall (Barnes *et al.*, 1985; Farmer *et al.*, 1986; Stuart-Smith & Vanhoutte, 1988) has considerable limitations in assessing the characteristics of the airways. Both an epithelial surface and a still greater non-luminal surface which includes damaged edges due to cutting, is exposed to the bathing fluid. This is possibly why relatively small shifts of 2–5 fold in sensitivity are seen after removing the epithelium (Goldie *et al.*, 1986; Flavahan *et al.*, 1985) compared with the 30 fold or more shifts reported in our experiments and those using intact trachea (Munakata *et al.*, 1989; Small *et al.*, 1990) where an intact tube completely isolates the

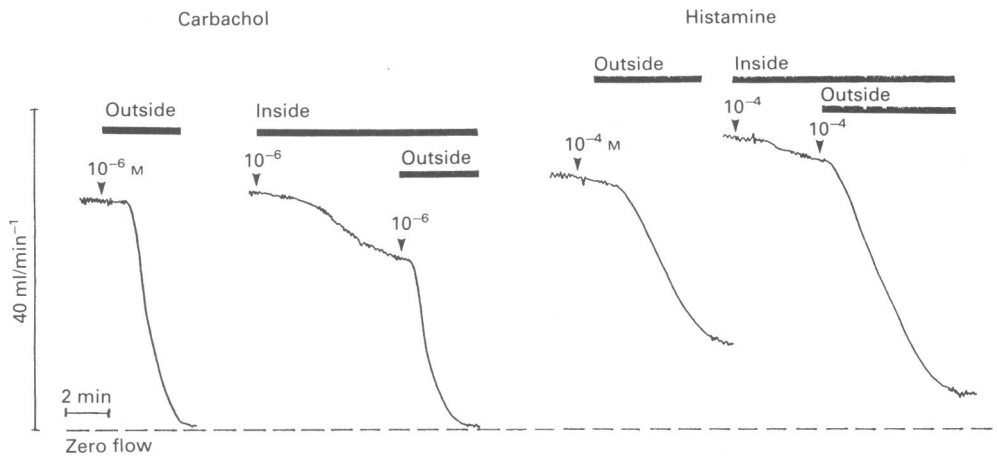

Figure 6 Typical narrowing responses of bronchial segments from different pigs elicited by (a) carbachol and (b) histamine given as a control response on the outside followed by washout and recovery, then perfused via the lumen for 5 min prior to adding the drug to the outside whilst continuing its perfusion intraluminally.

Table 1 A comparison of flow rates expressed as % of resting flow (= 100%)* when submaximal concentrations of acetylcholine (ACh), histamine, carbachol and a K^+ depolarizing solution were added to the outside of a bronchial segment, perfused via the lumen alone or via the lumen and the outside together as shown in the design used in Figure 6

	Outside (%)	*Inside* (%)	*Inside + outside* (%)
ACh 10^{-6} M	69.3 ± 1.7	97.6 ± 1.4	64.3 ± 2.3 (NS)
Carbachol 10^{-7} M	58.3 ± 2.0	96.6 ± 1.3	56.0 ± 1.8 (NS)
Histamine 10^{-5} M	75.2 ± 2.1	98.4 ± 1.6	76.2 ± 3.2 (NS)
KCl depol. soln†	54.5 ± 2.9	95.3 ± 2.9	51.0 ± 2.6 (NS)

Values are means ± s.e.mean of three segments. NS not significantly different from outside alone.
* Average resting flow rate 33 ml min^{-1}.
† 25% of the NaCl in Krebs replaced by KCl.

mucosa from the outside of the airway. But more importantly, isometric force developed by strips is not an appropriate way of assessing narrowing of the airway wall. Complete closure of the lumen occurs with approximately 46% of muscle shortening in small airways (i.d. 2.5 mm, transmural pressure 5 cmH_2O) and even in large airways (6 mm i.d.) where narrowing is restrained by the airway wall components, e.g. cartilage, maximum narrowing is obtained at about 57% of maximum muscle shortening (Mitchell & Sparrow, 1989; Sparrow & Mitchell, 1990). Thus, the upper portions of dose-response curves are not relevant to small airway narrowing at physiological transpulmonary pressures so that evidence for EpDIF based on E_{max} and EC_{50} data (Barnes *et al.*, 1985; Farmer *et al.*, 1987; Stuart-Smith & Vanhoutte, 1988) should be viewed with circumspection. The present study using the perfused bronchial segment and that recently reported by Small *et al.* (1990) using perfused trachea provides a clear demonstration of the crucial role of the epithelium as an impermeant barrier in modulating the responsiveness of airways smooth muscle.

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Evidence for prejunctional inhibitory muscarinic receptors on sympathetic nerves innervating guinea-pig trachealis muscle

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1 Relaxation responses induced by stimulation of the postganglionic sympathetic nerve trunk were studied in the isolated, fluid-filled, innervated tracheal tube preparation of the guinea-pig.

2 The thromboxane-mimetic U46619, prostaglandin $F_{2\alpha}$ and histamine each caused concentration-dependent increases in the intraluminal pressure (ILP) of the fluid-filled tracheal tube, reflecting contraction of the trachealis muscle. Sympathetic nerve stimulation in the presence of the spasmogens caused relaxations which increased with increasing ILP. Relaxant responses evoked in the presence of these three spasmogens were comparable at any given ILP.

3 Muscarinic agonists caused concentration-dependent increases in ILP, pilocarpine being more potent than acetylcholine. Sympathetic nerve-induced relaxations were reduced in the presence of pilocarpine and acetylcholine when compared to those obtained at the same ILP in the presence of U46619. This inhibitory effect of muscarinic agonists on sympathetic nerve-induced responses was concentration-dependent.

4 Exogenously applied noradrenaline opposed the contractile effect of U46619 and acetylcholine to a similar extent, indicating that a comparable degree of postjunctional functional antagonism exists between the sympathetic neurotransmitter noradrenaline and both spasmogens.

5 The selective M_2 muscarinic antagonists, gallamine and methoctramine, altered neither the postjunctional contractile action of acetylcholine nor its inhibitory effect on sympathetic nerve-induced relaxations. In addition, the inhibitory effect of acetylcholine was not modified by concentrations of pirenzepine known to block M_1 muscarinic receptors.

6 The postjunctional contractile action of acetylcholine and its inhibitory effect on sympathetic neurotransmission were antagonized by atropine, by the M_3 muscarinic antagonist hexahydrosiladiphenidol and by higher concentrations of pirenzepine.

7 These results suggest that in the guinea-pig trachea, muscarinic cholinoreceptor agonists inhibit sympathetic neurotransmission via activation of muscarinic receptors located on the sympathetic nerve endings. These inhibitory prejunctional muscarinic heteroreceptors are of the M_3 subtype.

Keywords: Inhibitory muscarinic receptors; sympathetic nerve endings; innervated guinea-pig trachea

Introduction

It is known that neural control of airway calibre is very complex. The dominant control of airway calibre is exerted via excitatory parasympathetic nerves, stimulation of which releases acetylcholine onto muscarinic receptors present on the airway smooth muscle with are of the M_3 subtype. In contrast, in some species, for example, the guinea-pig, cat and dog, sympathetic nerve stimulation evokes the opposite response, that is relaxation of airway smooth muscle via activation of β-adrenoceptors (Mann, 1971; O'Donnell & Saar, 1973).

The varicose terminals of the parasympathetic nerves are an important site for modulation of pulmonary cholinergic neurotransmission by other neurotransmitters including noradrenaline and by autacoids, such as histamine, prostaglandins and thromboxane. Acetylcholine can also inhibit its own release via activation of prejunctional muscarinic autoreceptors which are of the M_2 subtype (Maclagan, 1988). Very little is known, however, about the factors which modulate transmitter release from sympathetic noradrenergic nerves innervating airway smooth muscle.

In other tissues exogenously applied muscarinic agonists have been shown to inhibit noradrenaline release via a prejunctional mechanism. These include the heart (Loffelholz & Muscholl, 1970), gut (Manber & Gershon, 1979), and blood vessels such as the rat portal vein (Vanhoutte *et al.*, 1973) and the rabbit ear artery (Rand & Varma, 1970).

Although previous studies suggest that exogenously applied muscarinic agonists may also inhibit pulmonary sympathetic neurotransmission in the airways of the rabbit (Tong *et al.*, 1978) and dog (Russell & Bartlett, 1981) the source of the noradrenaline released in these studies was unclear. It could have been released from sympathetic nerves innervating pulmonary vascular smooth muscle rather than airway smooth muscle, as the vasculature is more densely innervated by noradrenergic nerves.

It is important to establish whether the prejunctional mechanisms which regulate noradrenaline release in other organs also exist in the airways. The aim of the present study was to investigate the effects of muscarinic agonists and other spasmogens on relaxation responses induced by selective stimulation of the postganglionic sympathetic nerves innervating the guinea-pig trachealis smooth muscle and to determine whether these effects were exerted via pre- or post-junctional actions. A preliminary account of these findings was communicated to the British Pharmacological Society (Pendry & Maclagan, 1989).

Methods

Innervated tracheal tube preparation

Guinea-pigs (200–400 g) of the Dunkin-Hartley strain (Graystoke, Hampshire) were anaesthetized with urethane ($1.5\,g\,kg^{-1}$, i.p.). The sympathetic nerve trunk on the right hand side was dissected down to the stellate ganglion, as described by Blackman & McCaig (1983). During the dissection the pulmonary nerves were kept moist with Krebs-

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Henseleit solution (composition mM: NaCl 118.4, KCl 4.7, $NaHCO_3$ 25.0, glucose 11.1, KH_2PO_4 1.16, $MgSO_4$, $7H_2O$ 1.19 and $CaCl_2$ 2.6) gassed with 95% O_2 and 5% CO_2. When the dissection was complete, the animal was killed with an anaesthetic overdose and the trachea was removed with the sympathetic nerves attached. The trachea was cannulated at both ends and mounted horizontally at its *in vivo* length in an organ bath containing gassed Krebs-Henseleit solution maintained at 37°C. The lumen of the trachea was filled with Krebs-Henseleit solution to form a fluid-filled tube which was closed at one end with a clamp and the other end was attached to a Statham (P23AC) transducer to record intraluminal pressure (ILP).

Stimulation of the sympathetic nerve trunk

The stellate ganglion was placed on bipolar platinum electrodes and stimulated with trains of rectangular pulses (40 Hz, max. voltage, 0.2 ms for 5 s at 90 s intervals using a Grass S44 stimulator) to induce relaxation of the trachea. Increases or decreases in intraluminal pressure reflected smooth muscle contraction or relaxation, respectively.

Tissues were left to equilibrate for 1 h in the presence of indomethacin (5×10^{-6} M) to remove any prostaglandin-induced tone, after which time a stable baseline was obtained. The trachea could, however, be relaxed further and the ILP induced by isoprenaline (10^{-3} M) was established at the end of each experiment. All ILP's were expressed as values in mmH_2O above this minimum. After the equilibration period, drugs which induced airway smooth muscle contraction were added cumulatively to the organ bath and sympathetic nerve-induced relaxations were measured when the ILP had reached a plateau.

Effect of muscarinic agonists

The muscarinic agonists, acetylcholine and pilocarpine, were administered cumulatively to the organ bath at regular intervals (3 min between doses for acetylcholine, and 7.5 min for pilocarpine). Sympathetic nerve-induced relaxation responses were measured in the presence of different concentrations of the agonists and compared to those at a comparable ILP when the tone had been raised with the stable thromboxane-mimetic, U46619. Tissues were washed for at least 30 min between exposure to the muscarinic agonist and subsequent exposure to U46619. This time period was sufficient to wash out the agonist and restore the intraluminal pressure to pre-drug control values.

Simultaneous addition of exogenous noradrenaline and a spasmogen in the absence of nerve stimulation

Cumulative contractile concentration-response curves to U46619 and acetylcholine were constructed in the absence and presence of noradrenaline (10^{-6} or 10^{-5} M); 3 min were allowed between concentration increments for acetylcholine and 6 or 7.5 min between U46619 concentration increments. At the end of each experiment acetylcholine (10^{-3} M) was added to the organ bath to obtain a maximum contraction. The ILP changes produced by U46619 and acetylcholine were expressed as a percentage of this maximum. The concentrations of U46619 and acetylcholine to produce a 30% maximum contraction in the absence and presence of noradrenaline were calculated (EC_{30}).

The ratio of the EC_{30} for the spasmogen in the presence and absence of noradrenaline was used as a measure of the degree of functional antagonism and called the 'concentration ratio'.

Drugs used

These included acetylcholine bromide, pilocarpine nitrate, atropine sulphate, histamine acid phosphate (BDH), gallamine triethiodide (May & Baker), hexahydrosiladiphenidol (a gift from Dr Lambrecht), methoctramine (a gift from Dr Melchiorre), pirenzepine dihydrochloride (Boots), prostaglandin $F_{2\alpha}$ trimethamine salt, urethane, indomethacin (Sigma), 11α,9α-epoxymethano-prostaglandin H_2 (U46619, Sigma/Semat), isoprenaline sulphate (Wellcome), noradrenaline acid tartrate (Winthrop), propranolol hydrochloride (ICI), hexamethonium bromide (Koch Light).

Indomethacin (1 mg ml^{-1}) was dissolved daily in buffer, (KH_2PO_4 19.76 mM and Na_2PO_4 118.34 mM; pH adjusted to 7.8 with NaOH) by warming and sonication before addition to the Krebs-Henseleit solution (final concentration 5×10^{-6} M). Stock solutions of U46619 and prostaglandin $F_{2\alpha}$ were prepared by dissolving 1 mg of the solid in 0.1 ml of ethanol, and 0.9% saline was added to give a 1 mM solution (2.75 ml for U46619 and 2 ml for prostaglandin $F_{2\alpha}$). Aliquots (100 μl) of these stock solutions were stored at −70°C and were diluted daily in 0.9% saline. All solutions of U46619 and prostaglandin $F_{2\alpha}$ were stored on ice throughout the experiment. All other drugs were dissolved and diluted in 0.9% saline. Stock solutions of isoprenaline were never kept longer than 15 min, and so no antioxidant was used. Ascorbic acid (10^{-5} M) was added to prevent oxidation of solutions of noradrenaline and histamine, which were kept throughout the experiment.

Statistical analysis of data

Sympathetic nerve-induced relaxations in the presence of different drugs were compared by unpaired Student's t tests and were deemed significantly different when $P < 0.05$.

Results

When the tissue was first set up in the organ bath, stimulation of the sympathetic nerve trunk resulted in little or no relaxation. In order to observe a relaxation, the intraluminal pressure (ILP) had to be increased with a spasmogen, such as the stable thromboxane-mimetic U46619, which induced a concentration-dependent increase in the ILP of the guinea-pig trachea. When the ILP had been raised with U46619, stimulation of the sympathetic nerves caused a rapid fall in the ILP, reflecting relaxation of the trachealis muscle (Figure 1). These sympathetic nerve-induced relaxations were abolished by propranolol (10^{-5} M), but not by hexamethonium (5×10^{-5} M), indicating that postganglionic sympathetic nerves had been stimulated to release noradrenaline onto β-adrenoceptors on airway smooth muscle. The relaxations increased with increasing ILP over the range 100–300 mmH_2O. The size of the sympathetic nerve-induced relaxation response at any specific ILP was reproducible during two consecutive cumulative U46619 concentration-response curves. Therefore, sympathetic nerve-induced relaxations in the presence of different spasmogens were always

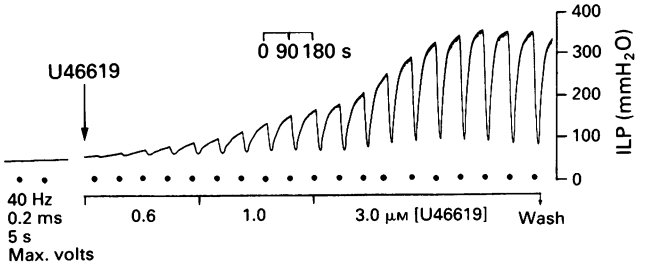

Figure 1 Experimental record showing relaxation responses of the guinea-pig trachea, recorded as rapid decreases in the intraluminal pressure (ILP), following stimulation of the postganglionic sympathetic nerve trunk (indicated by the dots; 40 Hz, max. voltage, 0.2 ms, 5 s). The size of the relaxation responses increased as the pressure was raised by cumulative addition of U46619. Indomethacin (5×10^{-6} M) was present throughout.

compared at identical ILP's in order to eliminate the effect of changes in muscle tone.

Tissues did not respond immediately to stimulation of the sympathetic nerve trunk. Approximately 4 s elapsed between the start of the stimulus train and the beginning of the relaxation response, and the duration of the relaxation was often greater than 1 min. Sympathetic nerve-induced relaxations increased with stimulation frequency, a maximum being observed at 40 Hz.

When the ILP had been raised, stimulation of the sympathetic nerve trunk via the stellate ganglion evoked a pure relaxation response in 90% of the preparations. However, in the remaining tissues an initial contraction occurred before the relaxation response (< 5% relaxation response), which was blocked with atropine (10^{-7} M) and could not be avoided by changing the position of the stimulating electrodes or by taking more care when separating the sympathetic and parasympathetic nerves.

Figure 2 shows a comparison of sympathetic nerve-induced relaxations observed in the presence of spasm induced by U46619, histamine and prostaglandin $F_{2\alpha}$. All three spasmogens induced concentration-dependent increases in the ILP of the guinea-pig trachealis muscle; the order of potency was U46619 > prostaglandin $F_{2\alpha}$ > histamine (Figure 2b). When sympathetic nerve-induced relaxations in the presence of all three spasmogens were studied at comparable ILP (0–250 mmH_2O), they were not significantly different (Figure 2). This indicates that the size of the relaxation response was directly related to the tone of the trachealis muscle.

Effect of muscarinic agonists

The muscarinic agonists, acetylcholine and pilocarpine, caused a concentration-dependent increase in the ILP of the guinea-pig trachea. The order of potency on the postjunctional muscarinic receptor on the trachealis muscle was pilocarpine > acetylcholine (Figure 3b).

Figure 3 summarizes experiments in which sympathetic nerve-induced relaxation responses in the presence of the muscarinic agonists, acetylcholine and pilocarpine, were compared to those recorded in the same tissues in the presence of U46619. At any given ILP (for example 300 mmH_2O above the minimum ILP in the presence of isoprenaline), sympathetic nerve-induced relaxations in the presence of acetylcholine or pilocarpine were smaller than those obtained when the tissue had been contracted to the same tone with U46619. This suggests that acetylcholine and pilocarpine were inhibiting sympathetic nerve-induced relaxations. From Figure 3, it appears that acetylcholine has a greater efficacy as an inhibitor of sympathetic relaxations than pilocarpine; yet acetylcholine is less potent than pilocarpine in increasing intraluminal pressure. However, responses to the muscarinic agonists were also calculated in terms of the percentage inhibition of the relaxant response to nerve stimulation as observed at an identical ILP in the presence of U46619. When responses were calculated in this way, it became evident that acetylcholine and pilocarpine were approximately equipotent and equi-efficacious in producing concentration-dependent inhibition of responses to sympathetic nerve stimuli (Figure 4).

Simultaneous addition of exogenous noradrenaline and a spasmogen

Both U46619 and acetylcholine caused a concentration-dependent contraction of the guinea-pig trachealis muscle via activation, respectively, of prostanoid receptors and muscarinic cholinoceptors situated postjunctionally on the airway smooth muscle (Figure 3b). In contrast, β-adrenoceptor agonists such as isoprenaline and the sympathetic neurotransmitter, noradrenaline, caused relaxation of the trachea. The

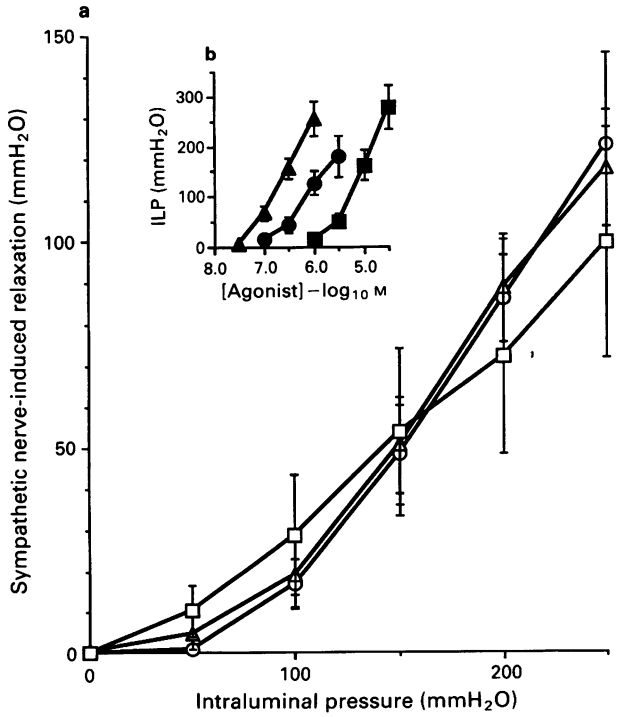

Figure 2 (a) Relaxation responses of the guinea-pig trachea induced by stimulation of the postganglionic sympathetic nerve trunk (40 Hz, max. voltage, 0.2 ms, 5 s) when the intraluminal pressure (ILP) was raised with U46619 (△), histamine (□) and prostaglandin $F_{2\alpha}$ (○). Insert (b) shows the postjunctional contractile action of U46619 (▲), histamine (■) and prostaglandin $F_{2\alpha}$ (●) increasing the ILP of the tracheal tube. At any given ILP, the sympathetic nerve-induced relaxations in the presence of U46619, histamine and prostaglandin $F_{2\alpha}$ were not significantly different (mean with s.e.mean; $n > 5$).

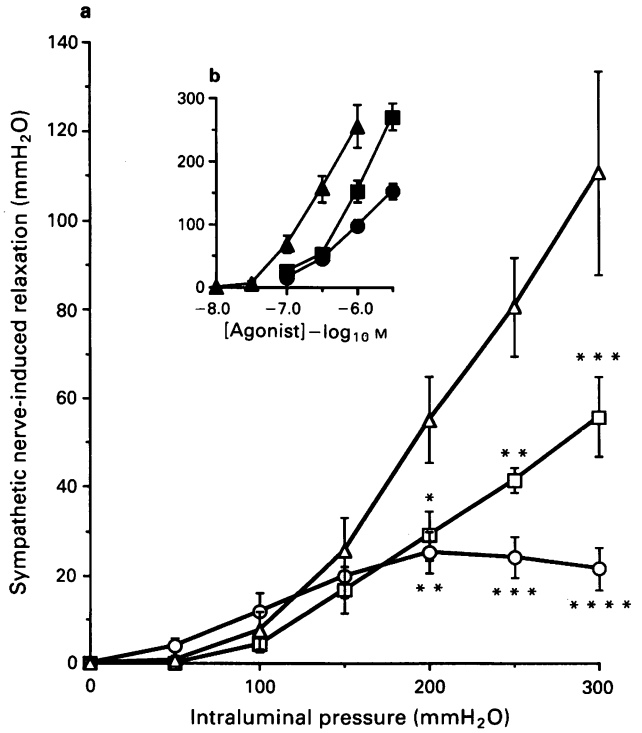

Figure 3 (a) Comparison of the relaxation responses of the guinea-pig trachea following stimulation of the postganglionic sympathetic nerve trunk (40 Hz, max. voltage, 0.2 ms, 5 s) when the intraluminal pressure was raised with U46619 (△), pilocarpine (□) and acetylcholine (○) (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$, mean with s.e.mean shown by vertical bars, $n > 5$). Insert (b) shows the increase in pressure within the tracheal tube (mmH_2O) in the same preparations caused by U46619 (▲), pilocarpine (■), and acetylcholine (●).

possibility that the observed inhibitory effect of the muscarinic agonists compared to U46619 was due to differences in the postjunctional functional antagonism existing between the spasmogens and the sympathetic neurotransmitter noradrenaline was investigated.

Table 1 shows the concentration-ratios (EC_{30} ratios) obtained when the guinea-pig trachea was contracted with either U46619 or acetylcholine in the presence of noradrenaline (10^{-6} and 10^{-5} M), compared to the spasmogen alone. At a concentration of 10^{-6} M, noradrenaline did not inhibit the postjunctional contractile action of either spasmogen to any significant amount, and as a result the calculated concentration-ratios were close to unity. Noradrenaline at a higher concentration (10^{-5} M), however, inhibited the postjunctional action of U46619 and acetylcholine to a similar degree and caused about a 20 fold shift of both U46619 and acetylcholine contractile concentration-response curves (concentration-ratio of 21.8 ± 3.7 and 22.5 ± 5.4 respectively). Thus, the degree of postjunctional functional antagonism existing between the sympathetic neurotransmitter and either spasmogen appears to be comparable.

Table 1 Comparison of the functional antagonism existing between noradrenaline and either acetylcholine or U46619, expressed as concentration ratios

Noradrenaline concentration (μM)	*Concentration ratio*	
	Acetylcholine	*U46619*
0	1	1
1	2.2 ± 0.5	1.7 ± 0.4
10	22.5 ± 5.4	21.8 ± 3.7

$$\text{Concentration ratio} = \frac{EC_{30} \text{ in the presence of noradrenaline}}{EC_{30} \text{ in the absence of noradrenaline}}$$

where the EC_{30} is the concentration of the spasmogen which induced an ILP increase of 30% of the maximum observed in the presence of acetylcholine ($\leq 10^{-3}$ M) administered at the end of each experiment (mean ± s.e.mean, $n > 5$).

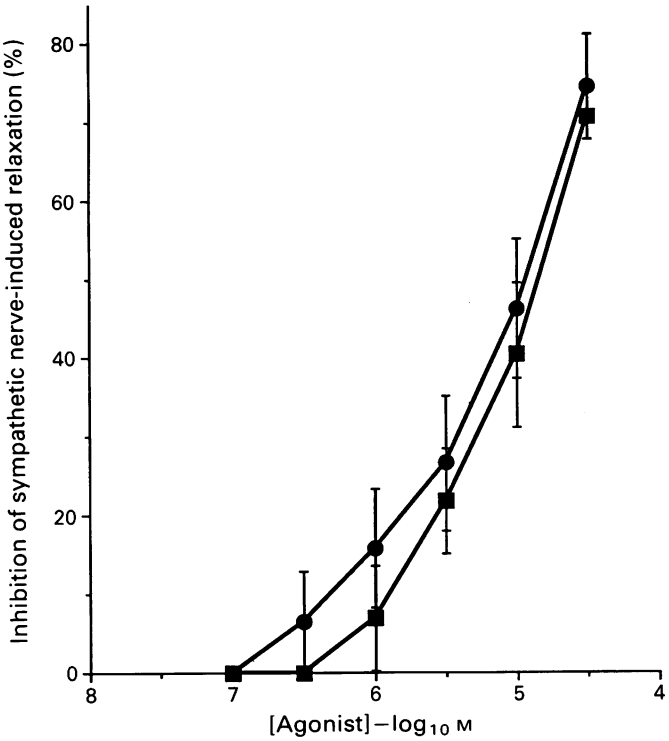

Figure 4 Relationship between the concentrations of the muscarinic agonists pilocarpine and acetylcholine and their inhibitory effect on relaxation responses of the guinea-pig trachea induced by stimulation of the postganglionic sympathetic nerve trunk (40 Hz, max. voltage, 0.2 ms, 5 s).

$$\% \text{ Inhibition} = \frac{A - B}{A} \times 100\%$$

where A is the sympathetic nerve-induced relaxation response in the presence of U46619 and B is the relaxation response at an identical intraluminal pressure (ILP) when the ILP has been raised with either pilocarpine (■) or (●) acetylcholine (mean with s.e.mean shown by vertical bars; $n > 5$).

Effect of muscarinic antagonists

The effect of the selective M_2 muscarinic receptor antagonists, gallamine ($<10^{-5}$ M) and methoctramine ($<10^{-5}$ M) was investigated on the inhibition of sympathetic nerve-induced relaxations by acetylcholine. Figure 5a and b shows the sympathetic nerve-induced relaxations measured in the presence of U46619 and acetylcholine respectively.

As previously shown, sympathetic relaxations at any given ILP were smaller when the ILP had been raised with acetylcholine than those in the presence of U46619. Figure 5c shows sympathetic nerve-induced relaxations in the presence of acetylcholine following at least 15 min preincubation with the M_2 muscarinic antagonist, methoctramine. The similarity of

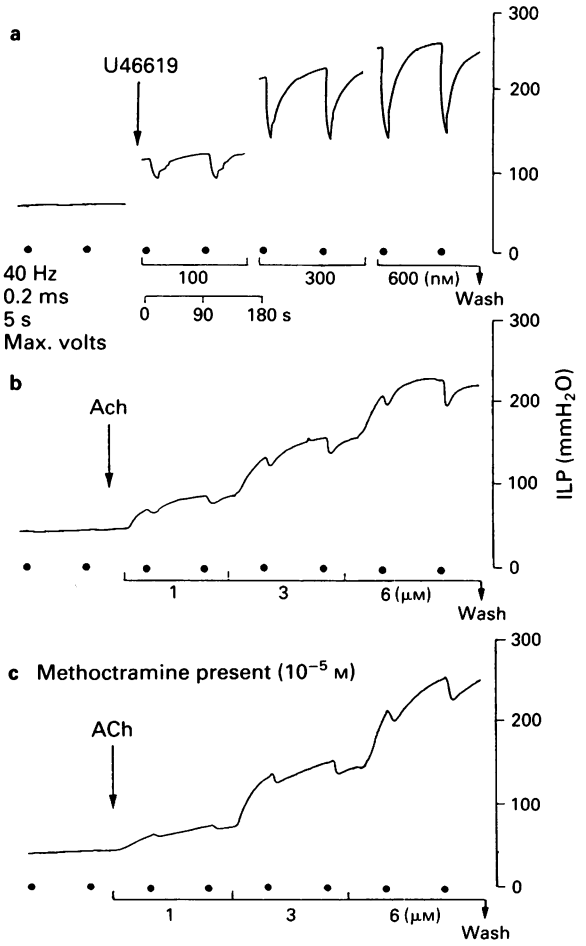

Figure 5 Experimental record showing the effect of the muscarinic M_2 antagonist, methoctramine (10^{-5} M), on the intraluminal pressure (ILP), and relaxation responses of the guinea-pig trachea induced by stimulation of the postganglionic sympathetic nerve trunk (shown by the dots; 40 Hz, max. voltage, 0.2 ms, 5 s). The ILP was raised with (a) U46619; (b) acetylcholine (ACh) and (c) acetylcholine in the presence of methoctramine (10^{-5} M). Both the postjunctional contractile action and inhibitory effect of acetylcholine on sympathetic nerve-induced relaxations were unaltered by methoctramine.

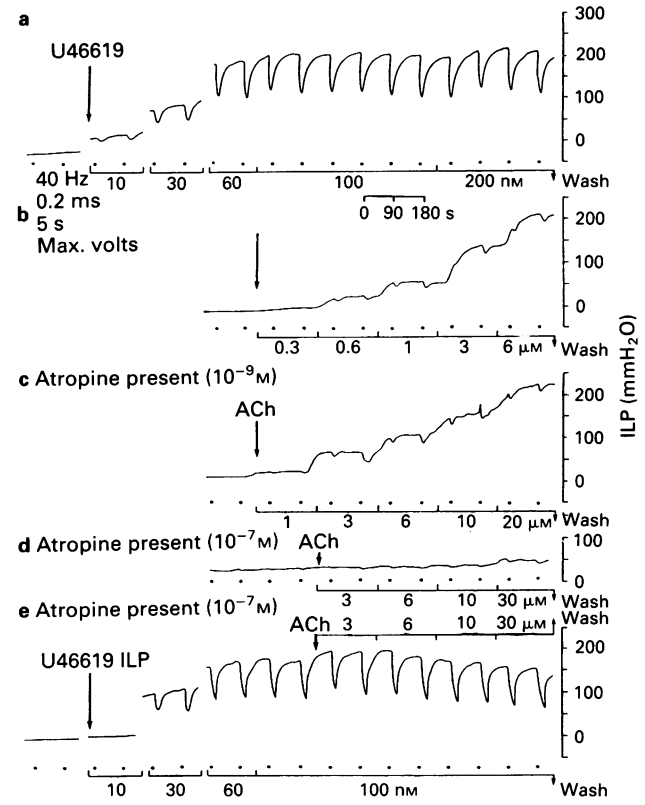

Figure 6 Experimental record showing the effect of the muscarinic antagonist, atropine, on the intraluminal pressure (ILP) and on relaxation responses of the guinea-pig trachea induced by stimulation of the postganglionic sympathetic nerve trunk (indicated by the dots; 40 Hz, max. voltage, 0.2 ms, 5 s). Sympathetic nerve-induced relaxations and ILPs are shown when the ILP was raised with (a) U46619; (b) acetylcholine (ACh); (c) acetylcholine in the presence of atropine (10^{-9} M); (d) acetylcholine in the presence of atropine (10^{-7} M); and (e) U46619 and acetylcholine in the presence of atropine (10^{-7} M). Atropine (10^{-7} M) abolished both the postjunctional contractile action of acetylcholine and its inhibitory action on sympathetic nerve-induced relaxations.

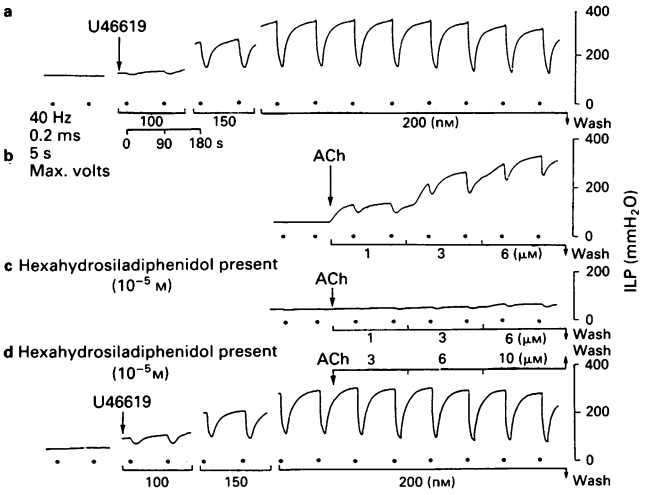

Figure 7 Experimental record showing the effect of the M_3 muscarinic receptor antagonist, hexahydrosiladiphenidol, on the intraluminal pressure (ILP) and on relaxation responses of the guinea-pig trachea induced by stimulation of postganglionic sympathetic nerve trunk (indicated by the dots; 40 Hz, max. voltage, 0.2 ms, 5 s). Sympathetic nerve-induced relaxations and ILPs are shown when the ILP was raised with (a) U46619; (b) acetylcholine (ACh); (c) acetylcholine in the presence of hexahydrosiladiphenidol (10^{-5} M); and (d) U46619 and acetylcholine in the presence of hexahydrosiladiphenidol (10^{-5} M). Hexahydrosiladiphenidol 10^{-5} M blocked both the postjunctional contractile action of acetylcholine and its inhibitory action on sympathetic nerve-induced relaxations.

the recordings shown in Figure 5b and c indicates that the presence of the M_2 antagonist altered neither the postjunctional action of acetylcholine in contracting the airway smooth muscle nor its inhibitory effect on sympathetic nerve-induced relaxations. Similar results were obtained for the M_2 muscarinic antagonist, gallamine (10^{-5} M; not shown). In addition, M_1 selective concentrations of pirenzepine ($< 10^{-6}$ M) did not antagonize the inhibitory effect of acetylcholine.

Figure 6 is a record of an experiment in which the effects of atropine on the inhibition of sympathetic nerve-induced relaxations and the contraction of guinea-pig trachealis smooth muscle by acetylcholine were investigated. Sympathetic nerve-induced relaxations were reduced in the presence of acetylcholine (Figure 6b) when compared to those at similar ILPs in the presence of U46619 (Figure 6a). Atropine at lower concentrations (10^{-9} M) altered neither the postjunctional contractile action of acetylcholine nor its inhibitory action on sympathetic nerve-induced relaxations (Figure 6c). At higher concentrations ($> 10^{-8}$ M), atropine profoundly antagonized the postjunctional contractile action of acetylcholine via blockade of M_3 muscarinic receptors on the airway smooth muscle (Figure 6d). Therefore in order to investigate the inhibitory effect of acetylcholine on sympathetic nerve-induced relaxations in the presence of concentrations of atropine which block M_3 muscarinic receptors, the trachea had to be precontracted with a spasmogen which does not act through muscarinic receptors, such as U46619. When the ILP had reached a plateau, acetylcholine was administered at concentrations which had been shown previously in the same tissue to inhibit sympathetic nerve-induced relaxations (Figure 6b). Acetylcholine no longer had an inhibitory effect on sympathetic neurotransmission in the presence of concentrations of atropine which block M_3 receptors on the airway smooth muscle.

Figure 7 shows a record of an experiment in which the spasmogenic effect of acetylcholine and its ability to inhibit sympathetic nerve-induced relaxation responses was investigated in the presence of the M_3 muscarinic receptor antagonist hexahydrosiladiphenidol. Hexahydrosiladiphenidol (10^{-5} M) antagonized the postjunctional contractile action of acetylcholine via blockade of M_3 muscarinic receptors on the airway smooth muscle (Figure 7c). The trachea, in the presence of a similar concentration of hexahydrosiladiphenidol, was then precontracted with U46619. When the ILP had achieved a plateau, acetylcholine was administered at concentrations which had previously inhibited sympathetic nerve-induced relaxations (Figure 7 a and b). These concentrations of acetylcholine no longer caused inhibition of sympathetic nerve-induced relaxations in the presence of M_3 blocking concentrations of hexahydrosiladiphenidol (Figure 7d). Similar results were obtained in the presence of nonselective concentrations of pirenzepine ($\leqslant 10^{-6}$ M). In this case the antagonist also attenuated the postjunctional contractile action of acetylcholine.

Discussion

The present experiments were designed to evaluate the effect of a variety of spasmogens on transmission in sympathetic nerves innervating the tracheal smooth muscle by investigating their effects on tracheal relaxations evoked by selective stimulation of the postganglionic sympathetic nerve trunk.

The muscarinic agonists, acetylcholine and pilocarpine, inhibited sympathetic nerve-induced relaxations in a concentration-dependent manner. The evidence suggests that this was due to activation of inhibitory prejunctional M_3 muscarinic heteroreceptors located on the sympathetic nerve endings.

A maximum relaxation was obtained when the sympathetic nerve fibres were stimulated at a frequency of 40 Hz, which is

in good agreement with Blackman & McCaig (1983) who found that the size of the depressor responses increased with frequency to a maximum at between 20 and 80 Hz. In our preparations, a slight delay occurred between stimulation of the sympathetic nerve trunk and the evoked relaxation. Although the tracheal smooth muscle contains a relatively high proportion of β-adrenoceptors, pulmonary innervation is relatively sparse. Therefore, not all airway smooth muscle cells are innervated but they are electrically coupled to each other via gap junctions (Richardson & Ferguson, 1979). In addition, transmitter release from sympathetic nerve endings is quantal and occurs intermittently (Cunnane, 1984). Thus the lapse between sympathetic stimulation and the evoked relaxation may be related to the lack of pulmonary sympathetic innervation and intermittency of transmission, which leads to delays in the build-up of noradrenaline concentration in the synapse. The time lapse may also be sufficient to allow noradrenaline to overflow from sympathetic nerves innervating pulmonary blood vessels and secretory cells.

In contrast to the preparations of Blackman & McCaig (1983), indomethacin (5×10^{-6} M) was present throughout the present experiments to remove any prostaglandin-induced tone. This was necessary because prostaglandins have been shown to alter the tone of the airway smooth muscle (Mathe & Hedqvist, 1975; Coleman & Kennedy, 1980), modulate pulmonary parasympathetic and sympathetic neurotransmission (Ito & Tajima, 1981; Walters *et al.*, 1984; Tong *et al.*, 1978) and evoke spontaneous activity of the trachealis smooth muscle (Boyle *et al.*, 1988). Thus the presence of indomethacin allowed greater control of airway smooth muscle tone, which was essential as sympathetic nerve-induced relaxations were found to increase with increasing tone.

At any given intraluminal pressure, sympathetic nerve-induced relaxation responses were comparable in the presence of three spasmogens which contract the guinea-pig trachea via different receptors namely U46619, prostaglandin $F_{2\alpha}$ and histamine. It therefore seems unlikely that drugs acting via such diverse mechanisms were modulating sympathetic neurotransmission to the same extent. In support of this, it has previously been shown *in vivo* that prostaglandin $F_{2\alpha}$ does not modulate sympathetic neurotransmission in canine lungs (Tong *et al.*, 1978).

This suggestion differs from the conclusions previously published by McCaig (1986) who reported that histamine (2×10^{-5} M) potentiated sympathetic inhibitory responses of the guinea-pig trachea at frequencies comparable to those used in the experiments described in this paper (20–80 Hz). However, in McCaig's experiments the change in ILP of the preparations due to the postjunctional contractile action of histamine was not taken into consideration. In McCaig's experiments, histamine (2×10^{-5} M) was reported to increase the ILP by $13.5 + 2.7\,cmH_2O$ ($n = 6$). It is therefore probable that the potentiation observed by McCaig was due to the increase in tone caused by histamine, as sympathetic nerve-induced relaxations would be expected to increase with tone, as shown in the experiments reported in this paper.

The results described here show that when the muscarinic agonists acetylcholine and pilocarpine were used as the spasmogens, sympathetic nerve-induced relaxations were reduced in a concentration-dependent manner compared to those obtained at the same ILP in the presence of U46619. This inhibitory effect could be due either to differences in the postjunctional contractile effect of the spasmogens opposing the relaxant action of noradrenaline on airway smooth muscle or to a prejunctional action of the muscarinic agonist inhibiting noradrenaline release via activation of inhibitory muscarinic receptors on the pulmonary sympathetic nerve terminals.

When two substances interact with different receptor systems present postjunctionally on the effector tissue to oppose each other, functional antagonism is said to occur. For example, spasmogens such as U46619 or muscarinic agonists functionally oppose the relaxant action of the sympathetic neurotransmitter, noradrenaline, on the trachealis smooth muscle. The possibility that the degree of functional antagonism existing between the sympathetic neurotransmitter noradrenaline on one hand and either U46619 or the muscarinic agonists on the other hand are different was excluded by the results, which showed that noradrenaline suppressed the postjunctional contractile actions of acetylcholine and U46619 to the same extent.

In the guinea-pig trachea, therefore, muscarinic agonists appear to inhibit noradrenaline release via inhibitory muscarinic receptors present on the sympathetic nerves innervating the trachealis smooth muscle. As the nerves were stimulated at a postganglionic location, these heteroreceptors are probably located on the final terminals of the noradrenergic nerves.

If the inhibitory effect of the muscarinic agonists is due to an action on muscarinic heteroreceptors the effect should be abolished with muscarinic antagonists. Muscarinic cholinoceptors were initially classified as M_1 or M_2 receptors, depending on the receptor binding and pharmacological profile of pirenzepine. Receptors with a high affinity for pirenzepine were designated M_1 and those of a low affinity as M_2 receptors. With the discovery of more selective antagonists, M_2 receptors were further subdivided into M_2 and M_3 receptors. M_2 receptors are predominantly found in cardiac tissue and are selectively blocked by gallamine, AFDX116, himbacine and methoctramine (Giachetti *et al.*, 1986; Melchiorre *et al.*, 1987); whereas M_3 receptors are found on smooth muscle and glands, and are selectively antagonised by 4-DAMP and hexahydrosiladiphenidol (Barlow *et al.*, 1972; Lambrecht *et al.*, 1986).

The presence of more than one subtype of muscarinic receptor has now been recognised in many tissues including the guinea-pig trachea (Eglen & Whiting, 1986; Mitchelson, 1989; Maclagan & Barnes, 1989). The muscarinic receptors present postjunctionally on guinea-pig airway smooth muscle which mediate contraction, are classified as M_3 because they are blocked by 4-DAMP and hexahydrosiladiphenidol. In contrast, neural muscarinic autoreceptors which inhibit acetylcholine release from pulmonary parasympathetic nerve endings of the cat, guinea-pig and rat are sensitive to gallamine and methoctramine and are therefore classified as M_2 (Fryer & Maclagan, 1984; 1987; Faulkner *et al.*, 1986; Watson *et al.*, 1989). Although no muscarinic receptors appear to be present in pulmonary parasympathetic ganglia, M_1 receptors have been demonstrated in the sympathetic ganglia innervating the lung in the guinea-pig (Maclagan *et al.*, 1989).

In the present study atropine, which nonselectively blocks all muscarinic receptors, antagonized the inhibitory effect of the muscarinic agonists on sympathetic nerve-induced relaxations confirming the involvement of a muscarinic receptor. In contrast, the M_2 muscarinic receptor antagonists, gallamine and methoctramine, depressed neither the postjunctional contractile action of acetylcholine nor its inhibitory effect on sympathetic relaxations. An M_2 muscarinic receptor does not, therefore, appear to be involved; and it also seems unlikely that the inhibitory action of muscarinic agonists such as acetylcholine is mediated via an M_1 muscarinic receptor, as concentrations of pirenzepine which block M_1 cholinoceptors did not alter the inhibitory effect of acetylcholine on sympathetic neurotransmission.

The inhibitory effect of acetylcholine on sympathetic nerve-induced relaxations was, however, sensitive to the M_3 muscarinic receptor antagonist, hexahydrosiladiphenidol, and higher concentrations of pirenzepine which also blocked the postjunctional contractile action of acetylcholine mediated via M_3 muscarinic receptors. Thus the prejunctional muscarinic receptors on pulmonary sympathetic nerve endings appear to be of the M_3 subtype and differ from the muscarinic autoreceptor on pulmonary parasympathetic nerve endings which are of the M_2 subtype. They also differ from the excitatory M_1 receptors found in the sympathetic ganglion in this species.

If the prejunctional inhibitory M_3 receptors on sympathetic nerves are activated by endogenous acetylcholine under *in*

vivo conditions when both branches of the pulmonary autonomic nervous system are active, a selective M_3 antagonist would be expected to decrease the bronchoconstrictor action of the parasympathetic nervous system on airway smooth muscle via blockade of postjunctional M_3 muscarinic receptors and also remove the prejunctional inhibitory effect of the dominant parasympathetic nervous on sympathetic neurotransmission, that is, have a sympathoexcitatory effect.

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The effect of *E. coli* STa enterotoxin on the absorption of weakly dissociable anti-malarial drugs from rat intestine *in vivo*

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1 The effect of *E. coli* heat stable (STa) enterotoxin on the absorption of radiolabelled anti-malarial weak bases and their appearance in peripheral blood was assessed *in vivo* by a recirculation procedure in rat intestinal loops.

2 Enterotoxin increased the jejunal disappearance of quinine ($P < 0.05$), trimethoprim ($P < 0.05$), proguanil ($P < 0.05$) and chloroquine ($P < 0.001$) and left pyrimethamine disappearance unaltered. Peripheral blood levels of trimethoprim ($P < 0.02$) and proguanil ($P < 0.05$) were higher after STa exposure.

3 In the ileum, enterotoxin increased the luminal disappearance ($P < 0.05$) and peripheral blood appearance ($P < 0.001$) of chloroquine. The luminal disappearance rate of trimethoprim was reduced ($P < 0.05$) and that of pyrimethamine unchanged.

4 The increased jejunal absorption of the anti-malarial drugs occurred despite STa causing a reduction in the amount of net fluid absorption. It seems likely that the enhanced absorption with STa exposure is related to the effect of STa on the microclimate pH. An elevation in the microclimate pH would increase the amount of undissociated weak base available for non-ionic diffusion.

5 The favourable elevation of microclimate pH by STa seemed to be outweighed by the reduced fluid absorption in the ileum. Only chloroquine still showed enhanced absorption in the ileum and this may have been because unlike the other antimalarial drugs, chloroquine has two dissociable groups likely to be affected by the mucosal surface pH changes.

Keywords: Microclimate pH; weak electrolytes; absorption, small intestine; anti-malarials; *E. coli* enterotoxin

Introduction

The well established dependence of intestinal weak electrolyte absorption on the extent of dissociation is caused by changes in the concentration of undissociated and therefore membrane permeable form. However, the correlation of absorption with intestinal luminal pH is often only approximate, particularly in the jejunum. Early studies additionally proposed the existence in the jejunum of an acid microclimate, not in equilibrium with the luminal pH (Hogben *et al.*, 1959). This acid microclimate hypothesis, which would now be regarded as a boundary layer model, explicitly recognises that the pH-dependent partition step depends on the pH immediately next to the enterocytes and not the more distant luminal pH. Conversely, it also implicitly recognises that discrepancy between absorption and the extent of ionisation calculated with reference to the luminal pH is not necessarily evidence for deviation from pH-partition. While the broad dependence of absorption on luminal pH is acknowledged, this boundary layer aspect of weak-electrolyte absorption has been overlooked in the search for explanations for apparent deviations from pH-partition behaviour.

Both *in vitro* and *in vivo* investigations with pH-electrodes and *in vitro* indicator experiments (Lucas *et al.*, 1975; Shiau *et al.*, 1985) have detected mucosal surface pH values of around pH 6.0 in the proximal jejunum. More recently, heat-stable STa enterotoxin from enterotoxigenic strains of *E. coli* and a shorter, synthetic peptide have been shown to neutralise the microclimate (McEwan *et al.*, 1988). Both native STa from strain P16 and STh(5-19) rapidly and reversibly elevate the surface pH from 6.2 to 6.9 and provide a means of evaluating the relevance of the acid boundary layer to weak electrolyte absorption. After exposure to STa, which also reduces net fluid absorption to zero, the proximal jejunum demonstrates enhanced uptake of some weak bases (McEwan & Lucas, 1990). If fluid entrainment is a significant factor for weak electrolyte absorption as proposed (Leahy *et al.*, 1989), then the enhanced uptake after STa exposure is all the more noteworthy. For this reason, the effect of STa exposure on the absorption of a selection of weakly basic anti-malarial drugs was studied in order to detect further examples of enhanced uptake. The investigations additionally included the ileum to see whether STa would have similar effects in the distal intestine. The various anti-malarial drugs were studied principally to test the microclimate model but the focus on anti-malarials arose from intermittent reports of unaffected or even enhanced absorption in the presence of small intestinal disease. This curious lack of effect or enhancement of anti-malarial weak base uptake in intestinal diseases more often characterized by malabsorption (Maier, 1948; Mattila *et al.*, 1973; Parsons *et al.*, 1975) provided additional interest because of the opportunity of providing an explanation for these clinical findings. In addition, it would be of some clinical relevance if some forms of intestinal secretory diarrhoeal disease led to enhanced anti-malarial absorption and others led to malabsorption.

Methods

Physiological procedures

The methods used in this study have been described previously (McEwan & Lucas, 1990), and are given here in an abbreviated form. Adult male Wistar rats (245–255 g) were anaesthetized (80 mg kg^{-1} body weight, i.p.) with pentobarbitone (Sagatal; May & Baker, U.K.) and body temperature was maintained at 37°C. A 15 cm length of proximal jejunum distal to the ligament of Treitz or a 15 cm length of terminal ileum proximal to the caecum with intact mesenteric vasculature was cannulated at both ends and returned to the peritoneal cavity. The left carotid artery was cannulated and connected via a 3-way tap to a syringe filled with a heparinised 0.9% saline solution, to collect peripheral blood.

The loops of intestine were perfused by the standard recirculation procedure (Schanker *et al.*, 1958). Krebs-bicarbonate buffer (Krebs & Henseleit, 1932) at 37°C, gassed with 95:5% O_2:CO_2 (v/v) additionally containing 5 mg ml^{-1} of polyethylene glycol 4000 (PEG 4000) with 1 μCi [^{3}H]-PEG 4000 as a non-absorbable marker for fluid transport was perfused at

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1 ml min^{-1}. Individual drugs were added to this solution at a concentration of 1 mmol l^{-1} with 1.0 μCi ^{14}C-labelled drug as a marker for drug absorption, with the exception of pyrimethamine which was perfused at 0.1 mmol l^{-1}.

Experimental protocol

Prior to the experiment, 15 ml of the appropriate solution was added to the reservoir and the pH was adjusted to 7.40. This solution perfused the intestine for 15 min to allow equilibration with residual fluid in the loop and adsorption of PEG 4000 onto the mucosal surface. After this equilibration period, the 'zero time' pH was measured and a 50 μl perfusate sample was withdrawn. Perfusate samples and pH measurements were then taken at 15 min intervals for 3 h. Samples were dissolved in 10 ml of Ecoscint in polythene vials prior to counting the ^{3}H and ^{14}C activity in a Packard Tri-carb 2425 liquid scintillation spectrophotometer. In addition, 100 μl blood samples were withdrawn at 'zero time' and at 30 min intervals for the duration of the perfusion. Withdrawn blood was replaced by heparinised 0.9% saline. Blood samples for liquid scintillation counting of ^{14}C activity underwent prior oxidation with peroxide (Moore, 1981). At the end of the experiment, the intestinal loop was removed for drying overnight to constant weight at 100°C. Acidification of the luminal fluid was calculated by titration of the luminal fluid back to pH 7.4 and expressed as rate of appearance of hydrogen ion per minute per 100 mg tissue dry weight.

A significant difference in the jejunal trimethoprim and pyrimethamine protocols is that the perfused volume was 25 and not 15 ml. The larger volume was used in an attempt to elevate blood levels of isotope. The minor advantage of slightly higher blood levels was outweighed by the smaller changes in the luminal concentration from a 25 ml volume and for this reason, the process was discontinued. Where comparisons between regions and other drugs were made, calculations have been made from the rate constants which can be standardized per length (or weight) of intestinal loop and then also for volume of perfusate; by noting that the empirical rate constant is the product of $DA/\delta V$, D/δ being the permeability of the relevant membrane, A the area and V the perfusate volume.

Data analysis and statistical procedures

Disappearance from the intestinal lumen and appearance in the peripheral blood were calculated by a standard method (Schanker *et al.*, 1958), which assumes that (1) changes in the ^{14}C activity of the perfusate reflect the movement of unlabelled drug and (2) changes in the ^{3}H-activity of labelled PEG 4000 represent changes in perfusate volume. Peripheral blood drug concentrations were calculated by assuming no change in specific activity during absorption. The rates of luminal disappearance of drug and of fluid were calculated and standardized for a loop dry weight of 100 mg. Rate constants, were calculated with a non-linear statistical software (BMDP) programme (Dixon *et al.*, 1981). Luminal concentration values over time were integrated to provide the area under the curve (AUC lumen) as were blood isotope levels, giving a similar blood AUC. Statistical comparisons between control and STa experiments were made by Student's *t* test.

Source of chemicals

All drug radioactive isotopes were ^{14}C-labelled in the aromatic nucleus if possible. Chloroguanide (Proguanil, was obtained as N'[*p*-chlorophenyl-UL-^{14}C]-N^{5}-isopropyldiguanide of specific activity 1.14 mCi $mmol^{-1}$, primaquine as N^{4}-[6-methoxy-8-quinolinyl 2, 4-^{14}C]-pentanediamine of specific activity 1.55 mCi $mmol^{-1}$ and chloroquine as N^{4}-[7-chloro-4-quinolinyl-3-^{14}C]-N',N' diethyl-1,4-pentanediamine of specific activity 1.5 mCi $mmol^{-1}$, from Dr J.A. Kepler, Research Triangle Park, North Carolina, U.S.A., courtesy of Dr P.I. Trigg, WHO, Geneva. Trimethoprim was obtained as 5[(3,4,5-trimethoxyphenyl) methyl]-pyrimidine [2-^{14}C]-diamine of specific activity 3.2 mCi $mmol^{-1}$ and pyrimethamine (as 5-(4-chlorophenyl)-6-ethyl-2, 4-pyrimidine[2-^{14}C]-diamine of specific activity 1.5 mCi $mmol^{-1}$ from Dr W. Gutteridge, Wellcome Research, Beckenham, UK. Quinine required the synthesis of a reactive aldehyde intermediate prior to custom synthesis by Amersham, UK. Quininal was synthesized by Professor Hans Wynberg, University of Groningen, the Netherlands and compared with reference samples of quinidinal obtained from Dr C. Gueremy at Sarl, Gennevilliers, France. This allowed quinine to be synthesized as [vinyl-2^{14}-C]-quinine of specific activity 10 mCi $mmol^{-1}$. [^{3}H]-polyethylene glycol 4000 was obtained from New England Nuclear, Dreieich, West Germany. Unlabelled proguanil was kindly donated by Dr Joseph of ICI, Alderley Park, primaquine and chloroquine from May & Baker, UK, pyrimethamine from Roche Products and trimethoprim from Revlon, UK. Sources of other relevant scintillation counting chemicals are detailed in a previous paper (McEwan & Lucas, 1990). All other chemicals were of reagent grade and purchased from Sigma, U.K. Purified heat stable (STa) *Escherichia coli* enterotoxin from the P16 strain was obtained from Dr Malcolm Burgess, Beecham's, U.K.

Results

The effect of the anti-malarials on intestinal fluid transport and luminal acid-base balance

The absorption of a selection of anti-malarial drugs was studied, mainly in the jejunum after *E. coli* enterotoxin (STa) exposure. Ileal absorption of some of these drugs was studied for comparison. In the proximal jejunum, STa invariably reduced net fluid absorption to zero or caused secretion and the presence of an anti-malarial drug did not prevent this known action of enterotoxin. With some of the anti-malarials (Table 1), net fluid absorption was low and STa then seemed to cause significant secretion e.g. primaquine and quinine. In all cases, STa shifted the balance of fluid movement towards secretion. Therefore, the effect of STa on anti-malarial drug absorption was always investigated under circumstances which would tend to reduce the net amount of drug absorbed, if fluid entrainment is a relevant factor. This was also true for the ileum for chloroquine and trimethoprim but not for pyrimethamine. In the presence of pyrimethamine, the balance of net fluid movement was significantly secretory and STa did not seem to cause further secretion. However, even here, net fluid movement was unfavourable for drug absorption.

Examination of the luminal acid-base changes on perfusion of loops in the absence of STa showed the expected fall in pH which is characteristic of the jejunum and the rise in pH which typifies the ileum. In each case, when STa enterotoxin was perfused, there was a shift toward greater rates of alkalinisation of the luminal fluid in both areas. In the jejunum, in the presence of STa and each of the selected drugs, the usual acidification was no longer seen (Table 1). In the ileum, the rate of alkalinisation was increased by STa despite the presence of the anti-malarial drug. In no case, therefore, did the anti-malarial drug interfere with the action of STa.

The effect of E. coli *STa enterotoxin on anti-malarial drug absorption from the proximal jejunum*

Quinine When 1 mM quinine was perfused, luminal disappearance (Figure 1a) was $6.0 \pm 0.7(6)$ nmol min^{-1} 100 mg^{-1} dry wt, representing about 18% of the dose over 3 h. In jejunum exposed to STa, the rate of disappearance was $15.2 \pm 1.8(6)$ nmol min^{-1} 100 mg^{-1} dry wt which was significantly ($P < 0.001$) elevated above control values. Similarly, the estimated rate constant (Table 2) was significantly higher ($P < 0.001$) in the STa-treated intestine and the integrated

Table 1 The effect of native *E. coli* STa (56 $\mu g\,ml^{-1}$) on net fluid absorption and acidification of luminal contents of rat proximal jejunum or distal ileum *in vivo* in the presence of various weak-electrolyte anti-malarial drugs: acidification is the rate of luminal secretion of hydrogen ion

Weak electrolyte	*Concentration* (mM)	*Net fluid absorption* ($\mu l\,min^{-1}\,100\,mg^{-1}$ dry weight)		*Acidification* ($\mu g\,min^{-1}\,100\,mg^{-1}$ dry weight)	
Jejunum		*Control*	*STa*	*Control*	*STa*
Chloroquine	1.0	3.4 ± 0.3 (10)	−7.4 ± 1.5 (10)***	0.19 ± 0.02 (10)	−0.15 ± 0.05 (10)***
Trimethoprim	1.0	8.1 ± 1.9 (7)	1.4 ± 2.2 (7)*	0.75 ± 0.25 (7)	−0.09 ± 0.10 (6)***
Pyrimethamine	0.1	8.4 ± 1.1 (7)	0.6 ± 1.1 (8)***	0.95 ± 0.08 (8)	0.10 ± 0.11 (8)***
Proguanil	1.0	0.9 ± 0.5 (6)	−3.6 ± 1.7 (8)*	0.35 ± 0.03 (6)	0.12 ± 0.10 (8)**
Quinine	1.0	−4.1 ± 1.7 (6)	−22.9 ± 2.9 (6)***	0.48 ± 0.04 (6)	0.02 ± 0.05 (6)***
Primaquine	1.0	−3.7 ± 1.0 (6)	−21.3 ± 2.5 (6)***	0.47 ± 0.06 (6)	0.06 ± 0.02 (6)***
Ileum					
Chloroquine	1.0	−4.0 ± 1.0 (7)	−9.6 ± 2.0 (7)*	−0.66 ± 0.16 (11)	−1.43 ± 0.22 (7)**
Trimethoprim	1.0	−1.9 ± 0.5 (9)	−4.9 ± 0.7 (10)**	−0.73 ± 0.18 (9)	−1.62 ± 0.09 (10)***
Pyrimethamine	0.1	−27.1 ± 5.9 (7)	−24.2 ± 5.1 (10)	−1.08 ± 0.10 (11)	−1.80 ± 0.09 (10)***

Results expressed as mean ± standard error of the mean with the numbers of animals in parentheses. Statistical significance; $P < 0.05$; **$P < 0.02$; *** $P < 0.01$; NS, not significant.

luminal concentration (AUC) was significantly reduced ($P < 0.05$) compared with the control values. The peripheral blood isotope level was not significantly elevated though (Table 2) some 10% higher.

Primaquine Similarly, the jejunal absorption of primaquine (Figure 1b) after STa exposure was 20.2 ± 3.4(6) nmol min^{-1} 100 mg^{-1} dry wt which was significantly higher ($P < 0.05$) than control values of 10.7 ± 0.9(6). This increase in luminal disappearance was confirmed (Table 2) by an increase in the pooled rate constant mean ($P < 0.001$). As with quinine, this failed to be reflected in the peripheral blood since the AUC was similar in both control and STa treated animals.

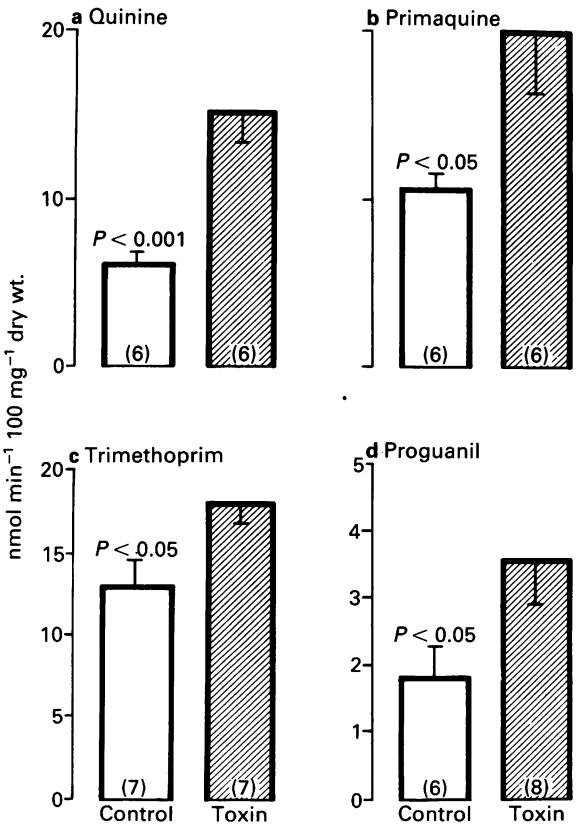

Figure 1 The effect of 56 $\mu g\,ml^{-1}$ of *E. coli* heat stable (STa) enterotoxin (hatched column) on the rate of luminal disappearance of 1 mM ^{14}C-labelled (a) quinine, (b) primaquine, (c) trimethoprim and (d) proguanil expressed as nmol min^{-1} 100 mg^{-1} tissue dry wt of 15 cm loops of rat proximal jejunum *in vivo*. Results are expressed as means (with s.e.mean vertical bars) with the number of animals (equals the number of experiments) in parentheses at the foot of each column.

Trimethoprim and pyrimethamine Enterotoxin, as previously, increased the rate of luminal disappearance of trimethoprim from a 1 mM solution to 18.2 ± 1.1(7) nmol min^{-1} 100 mg^{-1} dry wt which was significantly ($P < 0.05$) higher than the control mean of 13.0 ± 1.6(7) nmol min^{-1} 100 mg^{-1} dry wt (Figure 1c) and the associated rate constant (Table 2) was also significantly ($P < 0.01$) elevated after STa exposure. Blood levels were significantly higher ($P < 0.02$) and luminal AUC was significantly lower ($P < 0.05$) after STa treatment, consistent with increased transfer to the blood.

In contrast, the absorption of pyrimethamine from a 0.1 mM solution was unaffected. This lower concentration had to be used since it represents the upper limit of solubility of this lipophilic compound. In the presence of STa, luminal disappearance of 4.3 ± 0.1(8) nmol min^{-1} 100 mg^{-1} dry wt was not significantly different from the control value of 4.4 ± 0.2(8) nmol min^{-1} 100 mg^{-1} dry wt (not shown in Figure 1). Similarly the rate constants (Table 2) and the luminal AUC were almost identical in the presence and absence of enterotoxin.

Proguanil In the presence of STa, the rate of luminal disappearance of proguanil (Figure 1d) was 3.5 ± 0.6(8) nmol min^{-1} 100 mg^{-1} dry wt which was significantly ($P < 0.05$) greater than the control value of 1.7 ± 0.5(6) nmol min^{-1} 100 mg^{-1} dry wt. Similarly, the absorption rate constant was significantly ($P < 0.05$) elevated (Table 2) and the pooled data estimate confirmed this finding. The luminal AUC was not significantly different after STa exposure but then the amount of drug absorbed was very small, rising only from 4.0 to 6.9% of the dose available. In contrast, the blood AUC was significantly ($P < 0.05$) elevated after STa exposure.

The effect of E. coli *STa enterotoxin on anti-malarial drug absorption from the distal ileum*

Trimethoprim and pyrimethamine In the distal ileum, STa exposure failed to increase the rate of luminal disappearance of the pyrimidine-based anti-malarials. For trimethoprim, the control rate of absorption of 17.7 ± 3.5(9) nmol min^{-1} 100 mg^{-1} dry wt was statistically not different from the rate of 11.7 ± 1.0(10) after STa exposure. However, the absorption rate constant (Table 3) was significantly reduced ($P < 0.02$) after STa exposure. Neither luminal nor the blood AUC was significantly affected.

Similarly with pyrimethamine in the distal ileum, STa had little effect. Luminal disappearance after STa treatment was 1.74 ± 0.16(10) nmol min^{-1} 100 mg^{-1} dry wt which was not significantly different from 1.90 ± 0.13(11) nmol min^{-1} 100 mg^{-1} dry wt in the absence of STa. Likewise, the luminal

Table 2 First order rate constants (k) for luminal disappearance, integrated luminal and blood concentration (AUC_0^t) of weak electrolyte absorbed *in situ* from rat proximal jejunum in the presence and absence of *E. coli* (STa) enterotoxin

Weak electrolyte			10^2k (h^{-1})[a]	10^2k (h^{-1})[b]	*AUC* (lumen) mM.h	*AUC* (blood) μM.h
Chloroquine	Control	(10)	0.9 ± 0.5 (10)	0.9 ± 0.6 (130)	12.6 ± 0.2 (10)	29.0 ± 1.5 (10)
	STa	(10)	6.8 ± 0.9 (11)***	6.6 ± 0.6 (128)***	11.7 ± 0.2 (9)***	29.0 ± 2.2 (9)
Trimethoprim	Control	(7)	3.4 ± 0.4 (7)	3.6 ± 0.3 (85)	12.0 ± 0.3 (7)	9.0 ± 3.0 (7)
	STa	(6)	5.8 ± 0.4 (6)***	6.3 ± 0.1 (78)***	11.1 ± 0.1 (6)*	17.5 ± 1.1 (6)**
Pyrimethamine	Control	(8)	25.8 ± 2.4 (8)	25.5 ± 1.0 (89)	6.6 ± 0.1 (6)	—
	STa	(8)	22.7 ± 1.2 (8)	22.4 ± 0.9 (104)	6.9 ± 0.2 (8)	—
Proguanil	Control	(6)	1.4 ± 0.3 (6)	1.4 ± 0.3 (76)	12.8 ± 0.1 (6)	13.0 ± 3.4 (5)
	STa	(7)	2.3 ± 0.3 (7)*	2.4 ± 0.3 (99)***	12.6 ± 0.1 (7)	29.0 ± 5.3 (6)*
Quinine	Control	(6)	7.0 ± 0.9 (6)	7.0 ± 0.7 (78)	11.8 ± 0.3 (6)	40.0 ± 4.0 (6)
	STa	(5)	15.1 ± 1.9 (6)***	14.1 ± 1.0 (74)***	10.8 ± 0.4 (5)*	44.0 ± 3.0 (5)
Primaquine	Control	(6)	10.1 ± 0.9 (6)	10.3 ± 0.4 (76)	11.3 ± 0.2 (5)	50.0 ± 3.0 (5)
	STa	(6)	14.2 ± 1.9 (6)	13.2 ± 0.8 (76)***	11.0 ± 0.1 (3)	45.0 ± 3.0 (3)

[a] Rate constants calculated from each experiment and averaged; [b] rate constant calculated from pooled experiments. Results are expressed as mean ± s.e.mean; * $P < 0.05$; ** $P < 0.02$, *** $P < 0.01$.

AUC, the absorption rate constants and the peripheral blood levels were unaffected by STa perfusion.

The effect of E. coli *STa enterotoxin on chloroquine absorption from the proximal jejunum and distal ileum*

Exposure to STa had striking effects on the absorption of chloroquine both in the proximal jejunum and distal ileum. In the jejunum, luminal disappearance after STa treatment was 7.0 ± 0.9(10) nmol min^{-1} 100 mg^{-1} dry wt and was significantly greater ($P < 0.001$) than control values of 0.7 ± 0.4(10) nmol min^{-1} 100 mg^{-1} dry wt (Figure 2). The absorption rate constant increased significantly ($P < 0.01$) (Table 2) and the luminal AUC was reduced ($P < 0.01$). These changes were insufficient to increase the peripheral blood drug levels and the AUC remained the same.

In the distal ileum (Figure 2), STa treatment increased significantly ($P < 0.05$) the luminal disappearance rate from 7.7 ± 1.0(10) to 13.2 ± 2.1(7) nmol min^{-1} 100 mg^{-1} dry wt and increased significantly ($P < 0.05$) the associated rate constant (Table 3) from 8.9 ± 1.0(9) to 14.8 ± 2.7(7) × 10^{-2} h^{-1}. The peripheral blood AUC also increased significantly ($P < 0.01$). In addition, mean peripheral blood drug isotope concentrations were significantly ($P < 0.05$ or greater) higher after the first hour of exposure to STa, confirming that both luminal uptake and transfer into the blood were enhanced after enterotoxin treatment.

Physico-chemical constants and absorption parameters of the various anti-malarial weak electrolytes

To facilitate comparison between the changes in absorption as measured by luminal disappearance and the changes in undissociated form as predicted by the dissociation constants, the relevant parameters are presented for the various anti-malarial drugs (Table 4). For the jejunal experiments, the concentration of undissociated weak electrolyte [ni] was calculated for pH 6.2, the mean jejunal surface pH and for pH 7.0, the mean jejunal surface pH after STa exposure. For ileal experiments, the concentration of undissociated weak electrolyte at pH 7.0, the normal ileal surface pH was calculated as well as the concentration at pH 7.6, the ileal surface pH after STa exposure (McEwan *et al.*, 1988). The ratios of STa to normal undissociated concentration were calculated for the jejunum and ileum as were the absorption ratios, which can be derived also from the individual results.

Application of an unstirred layer correction to the experimental data

A proposed (Jackson *et al.*, 1978) equation for flow of charged and uncharged forms across an unstirred layer followed by uptake of the uncharged form only is:-

$$J_{23}^{ni} = \left(\frac{1 + 10^{\alpha_2}}{P_{ni}} + \frac{\delta}{D}\right)^{-1} C_1 \qquad (1)$$

where J_{23}^{ni}, the flux of neutral species past the membrane from compartment 2 (the unstirred layer) into compartment 3 (the mucosa), is related to the bulk phase concentration C_1 and an empirical permeability coefficient; this consisting of an unstirred layer term where D is the diffusion coefficient of both neutral and ionised forms and δ is the unstirred layer thickness, and a membrane permeability term P_{ni} for the neutral form adjusted for the degree of dissociation ($1 + 10^{\alpha_2}$) where $\alpha_2 = pKa\text{-}pH_2$, the dissociation constant of the weak base minus pH_2, the pH within the unstirred layer i.e. the microclimate immediately next to the mucosal membrane.

The unstirred layer contribution to overall permeability can be approximately assessed knowing δ and D, the diffusion coefficient for the weak base. In these calculations, δ was estimated at 580 μm (unpublished observations) and D was calculated to be 0.9 × 10^{-5} cm^2 s^{-1} from quinine conductivity data (Washburn, 1929). The experimentally determined rate constant, was standardized for volume of perfusate and area of perfused intestine to give a permeability term (P*). When corrected for the unstirred layer contribution and for ionisation at the mucosal surface, this gives an estimate of the membrane

Table 3 First order rate constants (k) for luminal disappearance, integrated luminal and blood concentration (AUC_0^t) of weak electrolyte absorbed *in situ* from rat distal ileum in the presence and absence of *E. coli* (STa) enterotoxin

Weak electrolyte			10^2k (h^{-1})[a]	10^2k (h^{-1})[b]	*AUC* (lumen) mM.h	*AUC* (blood) μM.h
Chloroquine	Control	(11)	8.9 ± 1.0 (9)	8.8 ± 0.7 (129)	11.6 ± 0.2 (9)	31.8 ± 1.5 (9)
	STa	(7)	14.8 ± 2.7 (7)*	14.1 ± 1.7 (91)***	11.1 ± 0.6 (7)	39.0 ± 1.5 (7)***
Trimethoprim	Control	(10)	9.2 ± 0.7 (9)	9.2 ± 0.7 (128)	11.6 ± 0.2 (9)	84.0 ± 6.0 (9)
	STa	(10)	7.3 ± 0.4 (10)**	7.3 ± 0.4 (127)**	11.7 ± 0.2 (9)	77.0 ± 3.0 (9)
Pyrimethamine	Control	(11)	21.9 ± 2.8 (11)	21.2 ± 1.3 (137)	0.95 ± 0.04 (10)	10.0 ± 2.7 (10)
	STa	(10)	20.6 ± 2.5 (10)	19.4 ± 1.5 (128)	1.00 ± 0.05 (10)	25.0 ± 16.0 (8)

[a] Rate constants calculated from each experiment and averaged; [b] rate constant calculated from pooled experiments. Results are expressed as mean ± s.e.mean; * $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$.

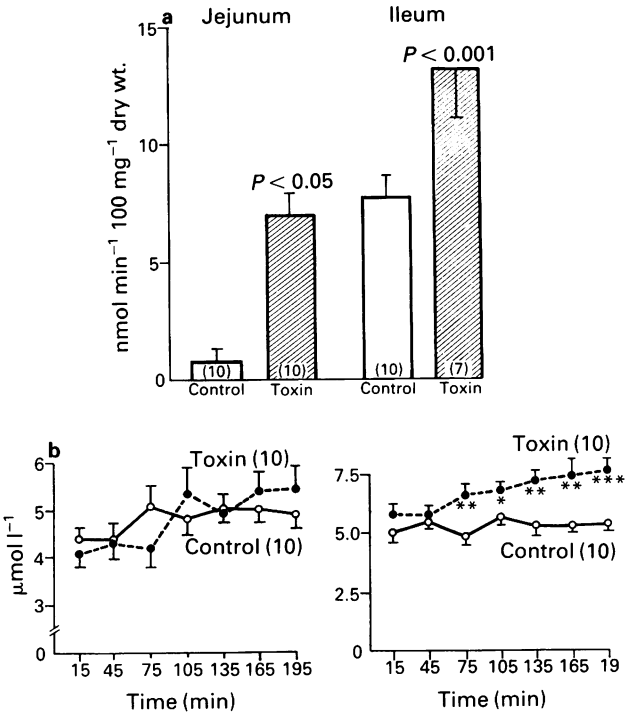

Figure 2 The effect of 56 μg ml^{-1} of *E. coli* heat stable (STa) enterotoxin (hatched column) on (a) the rate of luminal disappearance of chloroquine expressed as nmol min^{-1} 100 mg^{-1} tissue dry wt of 15 cm loops of rat proximal jejunum and distal ileum *in vivo* and on (b) the rate of appearance of drug in the peripheral blood after jejunal (left hand side) or ileal (right hand side) perfusion. Results are expressed as mean (s.e.mean shown by vertical bars) with the number of animals (equals number of experiments) in parentheses. Statistical significance, * $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$.

permeability term for the various anti-malarial drugs, since:-

$$\frac{1}{P^*} = \frac{1}{P_{ni}} + \frac{\delta}{D} \qquad (2)$$

Estimates of P_{ni} for each drug are given in the various circumstances after UWL and dissociation correction (Table 5). In each case, the estimated membrane permeability term was higher for the control values than for the STa-treated intestine.

Discussion

Previous studies have indicated that the enhanced intestinal absorption of some weakly basic electrolytes that occurs after *E. coli* STa enterotoxin exposure, can be explained by the action of the bacterial toxin on the intestinal mucosal surface pH (McEwan & Lucas, 1990). The STa elevates the pH of the acid microclimate which in turn increases the concentration of undissociated weak electrolyte available for permeation as the neutral form. In order to detect further examples of affected weak bases, the intestinal uptake and delivery to the peripheral blood of a series of radiolabelled anti-malarial drugs was studied. These included the pyrimidine anti-malarials, represented by trimethoprim and the more lipophilic pyrimethamine, both with dissociation constants close to neutrality. Proguanil was selected as a representative biguanide with a pKa of 10.9 (Findlay, 1951), close to the pKa of amphetamine, which had previously been shown to undergo enhanced absorption. Primaquine and quinine were chosen as examples of the 8-aminoquinolines, both with a high pKa in the side chain but with a low pKa for the nitrogen in the quinoline ring. Within the physiological range, these compounds would exist predominantly as the monocation. In contrast, chloroquine was studied as it is a 4-aminoquinoline with both dissociable groups of high pKa, making this a dication at physiological pH. The elevation by STa of mucosal surface pH from 6.2 to 6.9 in the jejunum (McEwan *et al.*, 1988) would be expected to convert dication or monocation to the neutral form. Additional experiments were undertaken in ileal loops to see whether a change in mucosal surface pH from 7.0–7.1 to approximately 7.5–7.6 would further enhance absorption in this area.

When perfused through the proximal jejunum, all drugs, with the exception of pyrimethamine, showed enhanced luminal uptake after STa exposure. The degree of increased uptake corresponds reasonably well with the increase in undissociated form, being about four times for the pyrimidines, six times for the 8-aminoquinolines including proguanil and forty times for chloroquine, the 4-amino-quinoline. The relatively modest increase in the amount of neutral form of the pyrimidine anti-malarial is because the pKa values are close to pH 7.0. For the others, the relevant dissociable group has a higher pKa between 8.5 and 10.9, and the difference in microclimate pH after STa exposure caused maximum change in the concentration of undissociated weak-electrolyte, although this amount was small. The very large change for

Table 5 Calculated membrane permeability constant (P_{ni}) for the various anti-malarial drugs after correction for the extent of ionisation and the extent of partition

		Permeability constant (cm s^{-1} × 10^5)	
		Jejunum	*Ileum*
Chloroquine	Control	4.4	1.2
	STa	1.5	0.2
Trimethoprim	Control	0.7	0.1
	STa	0.3	0.05
Pyrimethamine	Control	2.6	0.05
	STa	0.6	0.02
Proguanil	Control	11.2	—
	STa	3.7	—
Primaquine	Control	6.2	—
	STa	1.8	—
Quinine	Control	0.024	—
	STa	0.014	—

Table 4 Dissociation constants (a), molecular weight (b), log octanol: water partition coefficient of undissociated form of weak electrolyte (c), concentration of weak electrolyte at normal mucosal surface pH of 6.2 (d), concentration of weak electrolyte at surface pH of 7.0 after STa treatment (e), concentration-ratio (e/d) of weak electrolyte for STa and control mucosal surface pH (f), mean rate of luminal weak electrolyte absorption after STa treatment relative to rate for untreated intestine (g); (d)–(g) refer to jejunum (h)–(k) have the same meaning but refer to the ileum

					Proximal jejunum				*Distal ileum*			
	(a)		(b)	(c)	(d)	(e)	(f)	(g)	(h)	(i)	(j)	(k)
Weak electrolyte	pKa_1	pKa_2	*MW*	*log P*	[*ni*] *at pH 6.2* (μmol l^{-1})	[*ni*] *at pH 7.0* (μmol l^{-1})	*Ratio* [*ni*]	*Ratio absorption*	[*ni*] *at pH 7.0* (μmol l^{-1})	[*ni*] *at pH 7.6* (μmol l^{-1})	*Ratio* [*ni*]	*Ratio absorption*
Chloroquine	8.10	10.4	320	4.63	0.78 × 10^{-3}	31.6 × 10^{-3}	40.50	10.20	31.6 × 10^{-3}	381 × 10^{-3}	12.04	1.71
Trimethoprim	7.20	—	290	1.55	90.9	386.9	4.25	1.40	386.9	715.2	1.84	0.55
Pyrimethamine	7.34	—	249	2.69	6.75	31.37	4.64	1.00	31.37	64.53	2.05	0.95
Proguanil	2.30	10.9	254	2.04	19.9 × 10^{-3}	125.9 × 10^{-3}	6.32	1.94				
Primaquine	3.50	10.4	259	3.70	6.3 × 10^{-2}	4.0 × 10^{-1}	6.31	2.00				
Quinine	4.14	8.50	324	4.03	4.94	30.60	6.19	3.00				

chloroquine is because the microclimate pH elevation after STa converts monocation to neutral form and also dication to monocation, so that the microclimate effect is amplified. Unlike the low values for quinine, primaquine and proguanil, the value for the second dissociation constant (dication to monocation) for chloroquine is above the microclimate pH value. This multiplicative effect of the two high pKa groups explains the very large change in luminal uptake. When peripheral blood levels are considered, the enhanced luminal absorption translated into higher plasma levels for trimethoprim and proguanil but not for the others. However several of the anti-malarial drugs undergo extensive liver metabolism and what is detected in the peripheral blood appears after the first-pass. It may be that only trimethoprim and proguanil absorption rates after STa are sufficiently high relative to hepatic extraction to cause changes in the peripheral blood. However, the blood data support the conclusion from the luminal uptake rates that STa has increased uptake and that this is attributable to the dissociation changes.

The question arises why the dissociation changes are greater than the absorption changes. Luminal absorption is thought to depend not only on the mucosal membrane but also on the unstirred layer (Thomson & Dietschy, 1984) known to exist at the mucosa. This is usually invoked to explain why apparent permeability changes at the membrane are not fully reflected in the overall permeability constant for the composite unstirred layer and intestinal mucosal membrane. The present data for anti-malarial absorption in the jejunum also show this pattern in that the increase in absorption predicted from ionisation calculations is never fully reflected in the absorption rate constant. The increase in the overall rate of absorption compared with the predicted increase ranged from one half for quinine to an average of one quarter for the remainder. For this reason, it was of interest to try to account for the likely effects of the unstirred water layer in order to relate the experimentally determined changes in rate constants more closely with the anticipated changes predicted from surface pH considerations. This involved estimating roughly the UWL term and calculating the associated membrane permeability term for each drug. When this was done, the estimated membrane permeability coefficients still varied by orders of magnitude between the anti-malarial drugs studied, although this reflected the partition coefficient correction and may only indicate that the octanol:water partition coefficient does not accurately reflect partition into biological membranes. However, even when ionisation and UWL effects were accounted for, the membrane permeability term for each anti-malarial drug was always smaller when STa was present. This implies the presence in control intestine of an additional driving force which when accounted for would allow a consistent estimate of membrane permeability.

Examination of the assumptions implied in the UWL model equation gives some insight into its deficiencies in the case of weak-electrolytes. In the UWL corrected model, absorbed undissociated weak electrolyte is replaced by weak-electrolyte diffusing across the UWL. However, this representation ignores the existence of a large reservoir of unabsorbable dissociated electrolyte at the membrane surface. Disappearing undissociated weak electrolyte is replaced not only by material diffusing down the concentration gradient across the UWL but also by conversion of ionised form at the membrane. The unexpected conclusion from this 'reservoir' model is that the UWL will only determine the rate of absorption for very early times. After the relatively short time needed for the UWL to fill up with ionised and unabsorbable form, the further effect of the UWL will be negligible. Some other factor would have to be incorporated to obtain consistent values for the membrane permeability term and it is likely that this is fluid absorption which entrains the anti-malarials past the membrane.

The experimental support for fluid absorption being the relevant factor comes from the fact that STa reduced net absorption in every case and that the calculated microclimate pH changes are not fully reflected in the absorption changes. In the ileum, advantageous changes in ionisation might have been outweighed by reductions in convection. Similarly, in the jejunum, pyrimethamine absorption is unchanged after STa exposure and ionisation advantages may have exactly balanced entrainment disadvantages.

Translating the acid microclimate model to the ileum requires caution since acidity in the boundary layer is a jejunal phenomenon and cannot be generalised to other areas of the small and large intestine of all species. Electrode measurements of the distal ileum (Lucas, 1983; McEwan & Lucas, 1990) show a neutral microclimate which resembles the colon (McNeil & Ling, 1980). The shift in ileal surface pH by STa from 7.0–7.6 has less effect on absorption when the relevant dissociable group is within this range. In contrast, the effect on chloroquine absorption is still pronounced because the undissociated concentration is still significantly enhanced. Consequently, while the ileum still conforms to a boundary layer model, it is not an acid microclimate boundary layer and enhanced weak base absorption after STa treatment is more difficult to detect.

The increased uptake with STa exposure may be of clinical interest where there is co-existence of malaria and diarrhoeal disease. A concern of tropical medicine has been to determine the extent to which episodes of diarrhoeal disease compromise anti-malarial prophylaxis. In past studies on dysentery and diarrhoea of uncertain aetiology (Maier, 1948), anti-malarial plasma levels were shown to be unaffected. In non-tropical malabsorption syndromes, plasma levels of anti-malarials and other weak bases, are often enhanced (Mattila *et al.*, 1973; Parsons *et al.*, 1975), as predicted by the present studies. The specific effect of STa, on mucosal surface pH, which contrasts with the lack of effect of cholera enterotoxin emphasises that the microclimate will be relevant to weak base absorption, provided the diarrhoeal disease pathogen acts like *E. coli*. A predictable consequence of human small intestinal disease, provided it affects the microclimate pH is the enhanced absorption of certain weak bases, as shown by this present animal model of *E. coli* secretory diarrhoeal disease and by the previous literature reports of clinical cases. This may be an undesirable outcome if a weakly basic drug is given which exceeds the maximum therapeutic level as a consequence. In addition some forms of small intestinal disease may result in increased absorption of dietary carcinogens such as the carcinogenic quinolines, if these resemble the anti-malarial aminoquinolines in their physicochemical and particularly dissociation properties.

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Short-term effects of administration of anticonvulsant drugs on free carnitine and acylcarnitine in mouse serum and tissues

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1 The short-term evolution of concentrations of free carnitine and acylcarnitine was studied in the serum, liver, kidney, heart and skeletal muscle of mice after administration of single therapeutic doses of the anticonvulsant drugs, valproic acid (VPA), carbamazepine (CBZ), phenytoin (PHT) and phenobarbitone (PHB).

2 The effects of the drugs were immediate but transitory, control levels of free carnitine and acylcarnitine having been recovered or almost recovered in serum and in all tissues 8 h post administration (p.a.).

3 VPA was the only drug that significantly reduced free carnitine concentration in serum, which recovered control levels by 4 h p.a.

4 All the drugs studied brought about marked deficits of serum acylcarnitine, which had disappeared 2 h p.a. in the case of VPA and not until 8 h p.a. for CBZ, PHT or PHB.

5 The minimum concentrations of free carnitine and acylcarnitine in serum were invariably associated with the maximum concentration of drug in serum.

6 Free carnitine concentration was not affected by VPA in any tissue, PHT and PHB brought about significant deficits in heart and kidney, and CBZ a significant deficit in muscle.

7 Acylcarnitine concentration was significantly reduced in heart, kidney and muscle by CBZ, PHT and PHB, but in liver the effects of all drugs were very small.

8 These results are compatible with the hypothesis that the primary cause of anticonvulsant-induced alteration of carnitine metabolism is interference with renal reabsorption of carnitine.

Keywords: Valproate; carbamazepine; phenytoin; phenobarbitone; anticonvulsivant drugs; carnitine; acylcarnitine; carnitine deficiency

Introduction

The chief anticonvulsant drugs, including valproic acid (VPA), carbamazepine (CBZ), phenytoin (PHT) and phenobarbitone (PHB), have numerous side effects. In particular, almost all are to some extent hepatotoxic (though fatal hepatic reaction is rare) (Dreifuss & Langer, 1987), and since most epileptics receive long-term treatment with these drugs, it is important to watch for signs of chronic intoxication. The observation (Porter, 1989) that in many cases of anticonvulsant-induced chronic hepatic poisoning, two or more anticonvulsants are being taken simultaneously, with the apparent implication that drug interactions are in many cases largely responsible for the toxic effects, has encouraged the prescription of single-drug treatments (Porter, 1989); unfortunately, monotherapy does not always suffice to control attacks.

VPA treatment has been associated with reductions, in serum (Ohtani *et al.*, 1982; Bohles *et al.*, 1982; Murphy *et al.*, 1985; Laub *et al.*, 1986; Rodriguez-Segade *et al.*, 1989) and liver (Thurston *et al.*, 1983), of the concentration of carnitine, an essential factor in the transport of fatty acid across the inner mitochondrial membrane, where beta-oxidation takes place. Patients treated with VPA exhibit high values of the acylcarnitine/free carnitine ratio (henceforth A/F), and it has been suggested (Laub *et al.*, 1986; Matsuda *et al.*, 1986) that, like other organic acids, VPA may cause loss of carnitine in ester form via the kidney. VPA administration has also been associated with a condition similar to Reye's syndrome, in which carnitine deficiency has also been reported (Glasgow *et al.*, 1980), and it is conceivable that the VPA-induced condition may be caused by the alteration of fatty acid metabolism due to carnitine depletion (Coulter, 1984).

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Though Ohtani *et al.* (1982) found no significant differences between the serum carnitine concentrations of healthy subjects and those of 11 epileptics treated with anticonvulsants other than VPA, we recently found that 21.5% of a group of 149 such patients suffered hypocarnitinaemia (Rodriguez-Segade *et al.*, 1989). This figure was less than the 76.5% hypocarnitinaemia rate observed among patients treated with VPA, but since in most centres epileptics prescribed anticonvulsants other than VPA outnumber those prescribed VPA by about 4:1, the numbers of patients affected in the two groups may be very similar. The work described in this paper was accordingly undertaken with a view to throwing some light on the mechanisms by which administration of CBZ, PHT or PHB alters carnitine metabolism. To this end we determined the evolution of the effects of single therapeutic doses of these drugs on the concentrations of carnitine and acylcarnitine in the serum, liver, kidney, muscle and heart of adult mice, noting in particular whether these effects, like those of VPA (Rozas *et al.*, 1990), were reversible.

Methods

Twenty groups (10 in each) of male Swiss albino mice weighing 35–40 g and fed a standard diet *ad libitum* were used (4 groups for each drug treatment, VPA, CBZ, PHT or PHB and 4 control groups). All were killed by decapitation (following anaesthesia with halothane) after fasting for 8 h and, depending on the group, 30, 120, 240 or 480 min after administration by oral gavage of either a solution containing a therapeutic dose of anticonvulsant (50 mg kg^{-1} of VPA, 20 mg kg^{-1} of CBZ, 10 mg kg^{-1} of PHT or 6 mg kg^{-1} of PHB) or, for the control groups, the same quantity of iso-osmotic sodium chloride solution. Immediately after decapitation, blood was collected from the severed neck vessels and centrifuged at 4°C to

obtain plasma; liver, kidneys, muscle and heart were removed and frozen in liquid nitrogen pending analysis.

The frozen organs were homogenized in a Potter-Elvejhem homogenizer with four times their fresh weight of 50 mM HEPES buffer (pH 7.5) containing 10 mM EDTA. Microcentrifuge tubes containing 0.2–0.5 ml of tissue extract were heated in a water bath at 100°C for 6 min, cooled in ice-water and centrifuged for 10 min at 35,000 g. A volume of 0.2 ml of the supernatant was drawn off, treated with 0.02 ml of 3% H_2O_2 and left for 10 min at room temperature before 0.01 ml of 5×10^4 kg units l^{-1} catalase solution was added to destroy excess H_2O_2. After 30 min the mixture was centrifuged and the concentration of free carnitine in the supernatant was determined.

Free carnitine concentrations in serum and tissue homogenate supernatant were determined by the method of Rodriguez-Segade *et al.* (1985). Total carnitine concentrations (comprising both free and esterified forms) were measured by the same procedure after acylcarnitine had been hydrolysed with 1M KOH as described by McGarry & Foster (1976). Acylcarnitine concentrations were calculated by subtracting free carnitine concentrations measured without alkaline hydrolysis from total carnitine concentrations.

To evaluate the possible interference of the drugs studied with the analytical determination of free carnitine and acylcarnitine in serum, 80 μg of VPA, 10 μg of CBZ, 10 μg of PHT, 12 μg of PHB and a similar volume of iso-osmotic sodium chloride solution were added to different 1 ml samples of a serum pool, and the concentrations of free carnitine and acylcarnitine in the samples were then determined in triplicate (the concentrations of the drugs in the samples were in all four cases greater than those measured in the mouse sera in the main experiment).

The Shapiro-Wilk test was applied to all variables to check for normal distribution. For normally distributed variables, the statistical significance of differences was estimated by approximate parametric tests (Student's t, Snedecor's F, etc.); for non-normally distributed variables, non-parametric tests were used (Wilcoxon's, etc).

Results

In the following account, the words 'deficit' and 'excess' and their derivatives are used to refer to differences between values determined in treated groups and those measured in the control group killed at the same time post administration (p.a.).

Effects of anticonvulsant drugs on serum carnitine concentrations

Figure 1 shows the total carnitine concentration and the concentrations of free carnitine and acylcarnitine in mouse sera 0.5, 2, 4 and 8 h after oral administration of VPA, CBZ, PHT, PHB or iso-osmotic sodium chloride. In all the groups treated with anticonvulsants, total carnitine concentration was significantly deficient 0.5 h p.a.: by 65.8% for VPA ($P < 0.001$), 34.8% for PHB ($P < 0.001$), 18.5% for CBZ ($P < 0.01$) and 15% for PHT ($P < 0.05$). In all these groups, the deficit in total carnitine concentration involved a large deficit of acylcarnitine (81.6% for VPA, 76.1% for PHB, 45.4% for CBZ and 41.7% for PHT), whereas only VPA reduced the free carnitine concentration (by 49.4%).

The effects of CBZ, PHT and PHB that were observed 0.5 h p.a. remained until 2 and 4 h p.a., but by 8 h p.a. acylcarnitine concentrations had reached control levels and free carnitine concentrations exceeded control levels (by 32.7% for CBZ ($P < 0.001$), 34.6% for PHT ($P < 0.01$) and 24.2% for PHB ($P < 0.05$)). The effect of VPA on acylcarnitine concentration had disappeared 2 h p.a., and its effect on free carnitine concentration by 4 h p.a.

Figure 2 shows the concentrations of VPA, CBZ, PHT and PHB in serum 0.5, 2, 4 and 8 h p.a. VPA concentration was maximum 0.5 h p.a. (45.5 ± 11.9 μg ml^{-1}), had fallen sharply to 5.5 ± 2.1 μg ml^{-1} 2 h p.a., and at 0.3 ± 0.2 μg ml^{-1} had practically disappeared 8 h p.a. CBZ concentration peaked at 6.6 ± 1.2 μg ml^{-1} 2 h p.a., and had fallen to near-zero levels (0.6 ± 0.2 μg ml^{-1}) 8 h p.a. PHT concentration was least 0.5 h p.a. (3.1 ± 1.1 μg ml^{-1}), peaked 4 h p.a. at 6.3 ± 1.5 μg ml^{-1} and was still as high as 4.9 ± 0.9 μg ml^{-1} 8 h p.a. PHB concentration fell steadily from 7.6 ± 1.1 μg ml^{-1} 0.5 h p.a. to 3.5 ± 1.5 μg ml^{-1} 8 h p.a.

The possibility that the above results might be due to interference by the drugs in the analytical determination of carnitine was ruled out by the results of the experiments with pooled sera, in which the carnitine concentrations measured in the presence of high concentrations of anticonvulsants differed from those of control samples by less than 5%, a figure within the range of variation of the analytical method.

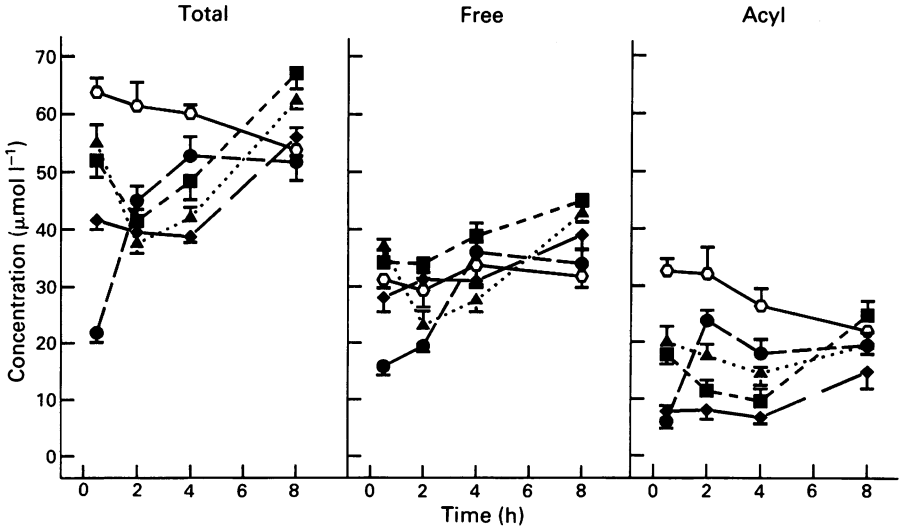

Figure 1 Free carnitine, acylcarnitine and total carnitine concentrations in mouse serum 0.5, 2, 4 and 8 h after oral administration of normal saline (○), 50 mg of valproic acid per kg of body weight (●), 20 mg of carbamazepine per kg of body weight (■), 10 mg of phenytoin per kg of body weight (▲)or phenobarbitone 6 mg per kg of body weight (◆). Samples were obtained when the mice completed 8 h without food; carnitine concentrations were determined as described in Methods. Values plotted are means of 10 mice; vertical bars show ± s.d.

Table 1 Free carnitine and acylcarnitine concentrations (nmol g^{-1}) in mouse liver and kidney 0.5, 2, 4, 8 h after oral administration of normal saline, 50 mg of valproic acid (VPA), 20 mg of carbamazepine (CBZ), 10 mg of phenytoin (PHT), or 6 mg of phenobarbitone (PHB) per kg of body weight

	Liver				*Kidney*			
	0.5 h	2 h	4 h	8 h	0.5 h	2 h	4 h	8 h
Carnitine								
Free								
Saline	331 ± 44	311 ± 51	319 ± 45	276 ± 23	744 ± 89	719 ± 139	680 ± 145	774 ± 59
VPA	364 ± 29	319 ± 13	363 ± 27	287 ± 50	702 ± 59	768 ± 27	567 ± 91	760 ± 72
CBZ	402 ± 25[a]	373 ± 54[c]	407 ± 59[b]	284 ± 17	753 ± 75	619 ± 52	635 ± 90	813 ± 31
PHT	300 ± 49	416 ± 44[a]	399 ± 65[b]	305 ± 54	418 ± 9[a]	412 ± 47[a]	431 ± 56[a]	700 ± 46[b]
PHB	307 ± 32	321 ± 24	311 ± 34	301 ± 46	671 ± 94	609 ± 87	454 ± 69[a]	654 ± 84[b]
Acylcarnitine								
Saline	27 ± 12	30 ± 25	32 ± 15	21 ± 16	81 ± 25	80 ± 38	80 ± 32	60 ± 29
VPA	29 ± 18	25 ± 7	48 ± 17[c]	23 ± 10	25 ± 11[a]	60 ± 12	51 ± 27	58 ± 13
CBZ	58 ± 39[c]	42 ± 6	57 ± 24[c]	20 ± 4	63 ± 42	46 ± 27[c]	38 ± 30[c]	75 ± 22
PHT	28 ± 22	18 ± 4	20 ± 12	10 ± 3	27 ± 5[a]	41 ± 16[c]	42 ± 11[b]	50 ± 8
PHB	12 ± 8[c]	38 ± 26	22 ± 13	19 ± 7	56 ± 31	65 ± 31	28 ± 18[a]	51 ± 13

Results are mean ± s.d.
Superscript letters denote values differing significantly from those of the corresponding controls as the $P < 0.001$ (a) or $P < 0.01$ (b) or $P < 0.05$ (c) levels.

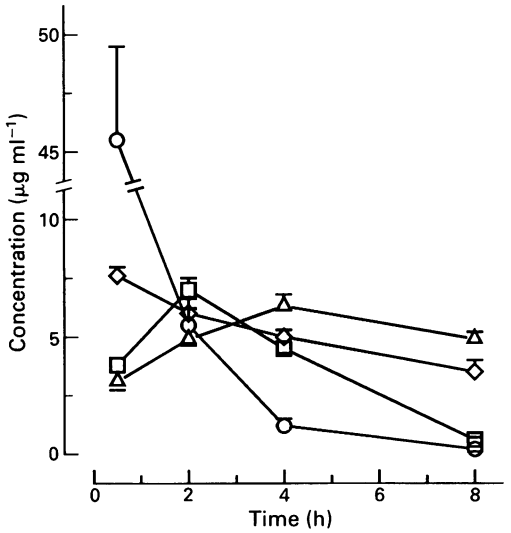

Figure 2 Valproic acid (VPA), carbamazepine (CBZ), phenytoin (PHT) and phenobarbitone (PHB) concentrations in mouse serum 0.5, 2, 4 and 8 h after oral administration of 50 mg of VPA per kg of body weight (○), 20 mg of CBZ per kg of body weight (□), 10 mg of PHT per kg of body weight (△)or 6 mg of PHB per kg of body weight (◇). Samples were obtained when the mice completed 8 h without food; drug concentrations were determined as described in Methods. Values plotted are means of 10 mice; vertical bars span ± s.d.

Effects of anticonvulsant drugs on concentrations of carnitine in tissue

Tables 1 and 2 list the total carnitine concentration and the concentrations of free carnitine and acylcarnitine in mouse liver, kidney, muscle and heart 0.5, 2, 4 and 8 h after oral administration of VPA, CBZ, PHT, PHB or iso-osmotic sodium chloride. The greatest effect in liver (Table 1) was caused by CBZ, with which free carnitine concentrations 0.5, 2 and 4 h p.a. were respectively 21.5%, 19.9% and 27.6% greater than in controls and acylcarnitine concentrations were 114.8% greater than in controls 0.5 h p.a. and 78.1% greater 4 h p.a. With PHT, free carnitine concentration was 33.8% above the control level 2 h p.a. and 25.1% above 4 h p.a., but acylcarnitine was not affected. VPA caused only a slight excess of acylcarnitine 4 h p.a. No significant effects remained in any of the treated groups 8 h p.a.

In kidney (Table 1), free carnitine concentrations were significantly affected by neither VPA nor CBZ, but were reduced by PHT (by 43.8% 0.5 h p.a., 42.7% 2 h p.a., 36.6% 4 h p.a. and 9.6% 8 h p.a., the latter still being statistically significant) and by PHB (by 33.2% 4 h p.a. and 15.5% 8 h p.a.). Acylcarnitine concentrations were also reduced: VPA caused a 69.1% deficit 0.5 h p.a. which had disappeared 2 h p.a.; CBZ significant deficits of 42.5% and 52.5% respectively 2 and 4 h p.a.; PHT deficits of 66.7% 0.5 h p.a., 48.8% 2 h p.a. and 47.5% 4 h p.a.; and PHB a 65% deficit 4 h p.a.

Table 2 Free carnitine and acylcarnitine concentrations (nmol g^{-1}) in mouse muscle and heart 0.5, 2, 4 and 8 h after oral administration of normal saline or 50 mg of valproic acid (VPA), 20 mg of carbamazepine (CBZ), 10 mg of phenytoin (PHT) or 6 mg of phenobarbitone per kg of body weight

	Muscle				*Heart*			
	0.5 h	2 h	4 h	8 h	0.5 h	2 h	4 h	8 h
Carnitine								
Free								
Saline	199 ± 49	215 ± 63	198 ± 61	245 ± 41	1274 ± 124	1237 ± 171	1143 ± 173	1280 ± 94
VPA	160 ± 27[c]	192 ± 57	163 ± 27	260 ± 37	1281 ± 124	1389 ± 142	1325 ± 128[b]	1308 ± 114
CBZ	104 ± 28[a]	124 ± 23[a]	138 ± 50[c]	249 ± 66	1132 ± 28[b]	1109 ± 73	1251 ± 160	1135 ± 103[b]
PHT	198 ± 32	197 ± 20	206 ± 24	261 ± 33	1004 ± 117[a]	1087 ± 90[c]	1020 ± 119	1125 ± 188
PHB	263 ± 33[b]	245 ± 26	236 ± 26	277 ± 50	925 ± 74[a]	1112 ± 34[c]	1101 ± 66	993 ± 60
Acylcarnitine								
Saline	118 ± 16	138 ± 46	138 ± 38	89 ± 24	149 ± 76	250 ± 105	268 ± 93	152 ± 87
VPA	110 ± 17	125 ± 35	121 ± 10	101 ± 40	129 ± 49	119 ± 36[b]	102 ± 42[a]	75 ± 21[c]
CBZ	76 ± 24[a]	71 ± 28[b]	133 ± 32	123 ± 55	36 ± 18[b]	66 ± 29[a]	58 ± 19[a]	83 ± 28[c]
PHT	40 ± 16[a]	41 ± 15[a]	40 ± 13[a]	108 ± 35	118 ± 34	118 ± 47[b]	136 ± 13[a]	102 ± 56
PHB	55 ± 25[c]	46 ± 16[a]	39 ± 7[a]	120 ± 32[c]	93 ± 30	85 ± 22[a]	114 ± 33[a]	137 ± 39

Results are mean ± s.d.
Superscript letters denote values differing significantly from those of the corresponding controls as the $P < 0.001$ (a) or $P < 0.01$ (b) or $P < 0.05$ (c) levels.

In muscle (Table 2), the only significant effect of VPA was a 19.6% deficit of free carnitine 0.5 h p.a., when the concentration of VPA in serum was greatest. CBZ caused free carnitine deficits of 47.7% 0.5 h p.a., 42.3% 2 h p.a. and 30.3% 4 h p.a., and acylcarnitine deficits of 35.6% 0.5 h p.a. and 48.6% 2 h p.a.; control levels were recovered 8 h p.a. for free carnitine and 4 h p.a. for acylcarnitine. PHT and PHB had no effects on free carnitine concentrations except for the 32.2% excess caused by PHB 0.5 h p.a., but both these drugs greatly depressed acylcarnitine concentrations 0.5 h p.a. (PHT by 53.4% and PHB by 66.1%), 2 h p.a. (PHT by 66.7% and PHB by 70.3%) and 4 h p.a. (PHT by 71.7% and PHB by 71%), though 8 h p.a. control levels were restored or exceeded (by a significant 34.8% in the case of PHB).

In heart (Table 2), VPA caused a significant 15.9% excess of free carnitine 4 h p.a. and significant deficits of acylcarnitine of 2 h p.a. (52.4%), 4 h p.a. (61.9%) and 8 h p.a. (50.7%). CBZ mainly affected acylcarnitine concentrations, causing large deficits throughout the study (75.8% 0.5 h p.a., 73.6% 2 h p.a., 78.4% 4 h p.a. and 45.4% 8 h p.a.). With PHT there were free carnitine deficits of 21.2% 0.5 h p.a. and 12.1% 2 h p.a., and acylcarnitine deficits of 52.8% 2 h p.a. and 49.3% 4 h p.a., and PHB behaved similarly, with significant free carnitine deficits of 27.4% 0.5 h p.a., 10.1% 2 h p.a. and 22.4% 8 h p.a., and significant acylcarnitine deficits of 66% 2 h p.a. and 57.5% 4 h p.a.

Discussion

The existence of carnitine deficiency secondary upon VPA treatment is well established (Ohtani *et al.*, 1982; Coulter, 1984; Murphy *et al.*, 1985; Laub *et al.*, 1986; Matsuda *et al.*, 1986; Morita *et al.*, 1986; Rodriguez-Segade *et al.*, 1989), but until a recent study of ours (Rodriguez-Segade *et al.*, 1989) this side effect had not been reported for other anticonvulsants. On that occasion we found that treatment with anti-epileptic drugs other than VPA produced hypocarnitinaemia in 21.5% of the patients taking them.

In the work now described we found that VPA, unlike the other anticonvulsants studied, caused a marked deficit of free carnitine in serum until 4 h p.a., by which time VPA had practically been eliminated from serum. CBZ, PHT and PHB did not reduce serum free carnitine (indeed, they produced a slight excess 8 h p.a.), but like VPA did reduce serum acylcarnitine: 0.5 h p.a. the acylcarnitine deficit was 81.6% with VPA, 76.1% with PHB, 45.4% with CBZ and 41.7% with PHT. While the effect of VPA on acylcarnitine levels had disappeared 2 h p.a., with the other drugs recovery was only achieved 8 h p.a. With all four drugs, the concentrations of both free and acylcarnitine in serum that were observed when the concentration of drug in serum was greatest were not significantly different from the minimum concentrations observed during the experiment, and the recovery of control levels of both free carnitine (if altered) and acylcarnitine tended to coincide with minimum or minimal levels of drug. These findings show that in mice the effects of the drugs studied are immediate but transitory, all serum carnitine deficits having disappeared 8 h p.a., which contrasts with the quite different pharmacokinetics observed in man (Ohtani *et al.*, 1982; Murphy *et al.*, 1985; Matsuda *et al.*, 1986; Rodriguez-Segade *et al.*, 1989).

The fact that in this study the non-VPA anticonvulsants only affected the esterized form of serum carnitine appears to explain the absence of hypocarnitinaemia in most patients treated with these drugs, since the A/F ratio of 0.13 observed in human controls (6.2 μM of acylcarnitine for 47.1 μM of free carnitine; Rodriguez-Segade *et al.*, 1989) implies that in man even the 80% reductions in serum acylcarnitine levels observed in our mice (control A/F ratio of 1.04) would hardly affect the total serum carnitine concentration.

It seems very unlikely that hypocarnitinaemia induced by anticonvulsants is due to a reduction in the rate of transformation of γ-butyrobetaine into carnitine in the liver. Nishida *et al.* (1987) found normal levels of free carnitine in the livers of rats treated with VPA, and in the present study the only significant differences between treated mice and controls as regards hepatic free carnitine and acylcarnitine levels were positive.

It has been suggested that carnitine deficiency in VPA-treated patients may be due to the dietary carnitine deficiency observed in patients on parenteral nutrition (Borum & Bennett, 1986) and some others (Matsuda *et al.*, 1986). In the present study, both controls and drug-treated mice had been fed the same diet, with adequate carnitine and precursor contents.

The high serum A/F ratio in VPA-treated patients (Matsuda *et al.*, 1986; Rodriguez-Segade *et al.*, 1989) has suggested that carnitine deficiency might be due to increased conversion of free carnitine to acylcarnitine. In the present study, however, the VPA-induced deficit in serum free carnitine 0.5 h p.a. was balanced by the deficit in serum acylcarnitine (which was observed with all the drugs used); in no case was the A/F ratio at any time above normal. The deficits in serum free carnitine and acylcarnitine were moreover not offset by any significant excess in any of the tissues examined, and hence cannot, on the basis of our data, be explained in terms of increased uptake of carnitine by tissue.

Another cause of carnitine deficiency is excessive excretion of free carnitine and acylcarnitine associated with reduced renal reabsorption of free carnitine; this is common in Reye's syndrome and similar conditions (Matsuda *et al.*, 1986; Matsuda & Ohtani, 1986), and increased urinary acylcarnitine excretion with insufficient compensatory endogenous carnitine synthesis has also been reported in association with organic acidaemias (Stumpf *et al.*, 1985). In VPA-treated patients, increased urinary excretion of acylcarnitine has been observed (Millington *et al.*, 1985; Matsuda *et al.*, 1986) and valproylcarnitine has been identified in their urine (Millington *et al.*, 1985), though this does not appear to be the acylcarnitine excreted in greatest quantities. The results of the present study are compatible with the possibility that CBZ, PHT and PHB alter renal reabsorption of acylcarnitine, and that VPA alters renal reabsorption of both acylcarnitine and free carnitine; a mechanism of this kind has been put forward on several occasions to explain the action of VPA (Millington *et al.*, 1985; Matsuda *et al.*, 1986; Rozas *et al.*, 1990).

The effects observed in the various tissues examined in this study varied with both drug and tissue. In general, control levels of both free carnitine and acylcarnitine were recovered with time. The only effects on free carnitine concentrations in liver were the slight excesses produced by CBZ and PHT; VPA had no consistent influence on free carnitine concentrations in any of the tissues studied, PHT and PHB caused significant deficits in heart and kidney, and CBZ a significant deficit in muscle.

The various drugs and tissues differed less as regards the observed effects on acylcarnitine concentrations: CBZ, PHT and PHB all brought about more or less marked deficits in heart, kidney and muscle, and in liver acylcarnitine levels were either not affected (by PHT and PHB) or were slightly increased (by VPA and CBZ). Besides increasing acylcarnitine levels in liver, VPA brought about deficits in heart and kidney; it had no effect in muscle.

Most tissues have carnitine concentrations over 10 times serum levels, and must therefore perform active carnitine uptake. The rate of uptake varies widely, however; the turnover times in rat kidney, liver, heart and skeletal muscle are respectively 0.4, 1.3, 21 and 105 h (Brooks & McIntosh, 1975). If similar figures hold for mouse tissues, the differences in carnitine concentration found in this study cannot be due solely to the differences in turnover time.

In conclusion, the results of this study suggest that treatment with all anticonvulsant drugs may be related to alterations of carnitine metabolism. This supports our earlier conclusion (Rodriguez-Segade *et al.*, 1989) that the concentra-

tions of free carnitine and acylcarnitine in the serum of all epileptics treated with these drugs (not just those treated with VPA) should be monitored. Our results are in keeping with the hypothesis that the primary cause of anticonvulsant-induced alteration of carnitine metabolism is interference with renal reabsorption of carnitine; in the case of VPA-induced carnitine deficiency, the situation may be aggravated by excessive acylation of carnitine. The alterations of tissue carnitine levels observed in this study do not appear to exhibit any clear pattern, and are probably a response to the loss of carnitine from serum.

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Evidence that acetylcholine-mediated hyperpolarization of the rat small mesenteric artery does not involve the K^+ channel opened by cromakalim

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1 Acetylcholine causes a concentration-dependent hyperpolarization of the rat small mesenteric artery (diameter at 100 mmHg, 200–400 μm). In the absence of tone the average potential change was from approximately −60 to −75 mV. In the presence of tone induced by endothelin-1 (20 nM), acetylcholine caused vasorelaxation in association with a marked hyperpolarization; from approximately −32 to −71 mV.

2 A number of compounds known to antagonize the actions of cromakalim were tested for their ability to block responses to acetylcholine. Glibenclamide (0.1–3 μM), phentolamine (10–100 μM) and alinidine (1–30 μM) caused a concentration-dependent depolarization of the rat small mesenteric artery which was not dependent on an intact endothelium. Glibenclamide was approximately 10 times more potent than either phentolamine or alinidine, a similar ratio to their potency as antagonists of cromakalim.

3 In the presence of concentrations of the cromakalim antagonists which functionally inhibited responses to cromakalim, only phentolamine and alinidine had a significant effect on the hyperpolarization and functional responses to acetylcholine. Glibenclamide was without effect at the concentrations used.

4 Experiments on pig coronary artery, where acetylcholine causes vasoconstrictor responses, showed that phentolamine and alinidine have some anti-muscarinic activity which could account for their ability to affect vasorelaxant/hyperpolarization responses to acetylcholine in the rat small mesenteric artery.

5 The results suggest that the acetylcholine-mediated hyperpolarization observed in the rat small mesenteric artery does not involve K^+ channels opened by cromakalim. This finding differs from other studies performed on the rabbit middle cerebral artery which show hyperpolarizing responses to acetylcholine to be glibenclamide-sensitive. It is likely therefore that the hyperpolarization response observed to acetylcholine can be initiated through a number of mechanisms, only one of which utilizes K^+ channels opened by cromakalim.

Keywords: Acetylcholine hyperpolarization; EDHF; rat small mesenteric artery; K^+ channels; cromakalim

Introduction

Acetylcholine-induced vasorelaxant responses have been shown to result from the release of vasoactive intermediates from the endothelium (see Angus & Cocks, 1989). The principle vasoactive substance, endothelium-derived relaxing factor (EDRF), is thought to be nitric oxide (NO) or a derivative since the functional actions of acetylcholine are inhibited by compounds known to capture NO, such as haemoglobin (Angus & Cocks, 1989). However, acetylcholine and other cholinomimetics have been shown to hyperpolarize vascular smooth muscle (Bolton *et al.*, 1984; Chen *et al.*, 1988; Feletou & Vanhoutte, 1988). Again, this action is dependent on an intact endothelium which indicates the involvement of an intermediate. However, in contrast to the vasorelaxant effects of acetylcholine, the electrophysiological effects are not blocked by haemoglobin or methylene blue (Chen *et al.*, 1988; Nishiye *et al.*, 1989). This finding has prompted a number of investigators to propose the release of an endothelium derived hyperpolarizing factor (EDHF; see Taylor & Weston, 1988).

The mechanism behind the hyperpolarization observed with acetylcholine may depend on the origin of the vessel. Some studies have shown that the hyperpolarization response is inhibited by ouabain which suggests the involvement of Na^+/K^+ ATPase (Feletou & Vanhoutte, 1988; Brayden & Wellman, 1989). However, the majority of other studies suggest that an increase in K^+ conductance is responsible (see Chen *et al.*, 1989). While there are many types of K^+ channels, recent studies by Standen *et al.* (1989) showed that the hyperpolarizing actions of acetylcholine and a number of other vasodilators, were sensitive to glibenclamide, a compound which antagonizes ATP-sensitive K^+ channels. This result supports the idea that hyperpolarization is the result of increased K^+ conductance. Cromakalim, pinacidil and a number of other compounds termed K^+ channel openers are also thought to produce membrane hyperpolarization by opening the same glibenclamide-sensitive channel (see McPherson & Angus, 1990). Thus it would appear that a number of endogenous ligands use this particular channel to induce membrane hyperpolarization.

In the rat small mesenteric artery we noted that glibenclamide and two other novel antagonists of the actions of cromakalim, phentolamine and alinidine (McPherson & Angus, 1989), caused direct depolarizing effects on the smooth muscle cell. This observation was not made by Standen *et al.* (1989) in their work on the rabbit middle cerebral artery. Consequently we extended our study on the rat small mesenteric artery to examine the actions of the cromakalim antagonists in greater detail and the interaction between these compounds and the vasorelaxant and hyperpolarizing effect of acetylcholine. We found that there was no correlation between the ability of these compounds to block K^+ channels opened by cromakalim and their ability to influence the response to acetylcholine. Thus it would appear that in the rat small mesenteric artery, in contrast to the rabbit middle cerebral artery, acetylcholine-induced hyperpolarization is not mediated through K^+ channels which can be opened by the exogenous ligand, cromakalim.

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Methods

Isolation of resistance blood vessels

Wistar Kyoto (WKY) rats were killed by CO_2 asphyxia. The mesentery was rapidly removed and placed in ice cold Krebs solution (composition in mM: NaCl 119, KCl 4.7, $MgSO_4$ 1.17, $NaHCO_3$ 25, KH_2PO_4 1.18, $CaCl_2$ 2.5 and glucose 11) gassed with 5% CO_2 in O_2. Two mm segments were mounted in a small vessel myograph as previously described (Angus *et al.*, 1988). Briefly, two 40 μm wires were threaded through the lumen of the vessel segment. One wire was attached to a stationary support driven by a micrometer while the other was attached to an isometric force transducer which measured force development. Force development was recorded on a dual flat bed recorder (W&W Scientific Instruments, model 320). Vessels were allowed to equilibrate under zero tension for 30 min. By use of the diameter of the vessel, calculated from the distance between the two mounting wires, a passive diameter-tension curve was constructed as previously described (Mulvany & Halpern, 1977). From this curve the effective transmural pressure could be calculated. The vessel was set at a tension equivalent to that generated at 0.9 times the diameter of the vessel at 100 mmHg. Non-linear curve fitting of the passive length-tension curve was achieved with custom written programmes for the IBM PC (NORMALIZE) which uses the Marquart-Levenberg modification of the Gauss-Newton technique (McPherson, 1985). Vessel diameters at an equivalent transmural pressure of 100 mmHg (D_{100}) are given in the text.

Electrophysiology

In experiments where the intracellular resting membrane potential was monitored, the vessel was mounted as just described. A conventional glass electrode (1 mm blanks, World Precision Instruments Inc., New Haven, U.S.A.) filled with 0.5 M KCl (tip resistance approximately 100 MΩ) was used to impale a single smooth muscle cell. The microelectrode was mounted on a Burleigh Inchworm motor controlled by a 6000 series controller (Burleigh, U.S.A.). The microelectrode was advanced by 0.5 μm steps until impalement was achieved.

The bath containing the vessels (7 ml volume) was part of a 25 ml recirculating system which contained a jacketed organ bath where the Krebs solution was warmed and oxygenated. Drugs could also be added at this site. This design allowed cumulative concentration-effect curves to be constructed when assessing the electrophysiological effects of the various drugs. In some experiments the vessel was activated with a submaximal concentration of endothelin-1 ($\approx$20 nM). In these experiments membrane potential and active tension development changes were recorded simultaneously.

In some experiments the effect of acetylcholine was assessed on vessels which had their endothelium removed mechanically. Thus a polyethylene suture (6/0 prolene, Ethicon) was advanced into the lumen of vessel mounted on the myograph wires. The wires were then separated slightly to bring the vessel into contact with the suture. The suture was moved along the lumen and back eight times. The vessel was then relaxed again and rotated through 90 degrees and this procedure repeated. The viability of the vessel after this procedure was assessed by measuring the response to KPSS before and after rubbing. The effectiveness of the procedure in removing the endothelium was assessed functionally by monitoring the response to acetylcholine (10 μM).

Studies of the pig large coronary artery

Pig right coronary artery was dissected from hearts obtained at an abattoir. Ring segments, 4 mm in length, were mounted in a large vessel myograph and placed under 4 g resting force. A single concentration-effect curve was then constructed to acetylcholine, which in this tissue causes a vasoconstrictor response, in the absence or in the presence of either phentolamine (100 μM) or alinidine (30 μM).

Data collection

Tension and membrane potential data were captured by use of the custom written programme DIGISCOPE (G.A. McPherson) for the IBM PC. This programme uses a DASH16 A/D card (Metrabyte, U.S.A.) which collected and displayed the data at 200 Hz. The data were saved on hard disk and reproduced on a Hewlett Packard 7470A plotter.

Statistics and data analysis

Statistical comparisons between two groups were made by Student's t test. Multiple comparisons between independent samples were made by Scheffe's test (see Wallenstein *et al.*, 1980). Results in the text are the mean $\pm$ s.e.mean for the specified number of experiments.

Drugs

The following drugs were used: acetylcholine bromide (Sigma); endothelin-1 (ET-1, Austpep, Australia); phentolamine mesylate (Ciba-Geigy); alinidine bromide (Boehringer-Ingelheim); glibenclamide (Hoechst).

Results

Characteristics of the electrophysiological and functional effects of acetylcholine on the rat small mesenteric artery

The rat small mesenteric artery ($D_{100} = 312 \pm 32$, $n = 7$) had a resting membrane potential of -60 ± 1 mV ($n = 7$, 7 different vessels) in the absence of any vasoactive substance. Acetylcholine (0.01–1 μM) caused a concentration-dependent hyperpolarization of the smooth muscle in the absence of active tone. The membrane potential in the presence of supramaximal concentrations (>0.1 μM) of acetylcholine was -75 ± 2 mV; a hyperpolarization of approximately 15 mV.

Figure 1 shows representative traces of tension and membrane potential, recorded simultaneously, in a mesenteric vessel ($D_{100} = 297$ μm) which had previously been contracted with endothelin-1 (20 nM). Endothelin-1 caused a depolarization to -32 ± 2 mV ($n = 3$) of the vessel in association with a contractile response. Acetylcholine again caused a concentration-dependent hyperpolarization to -71 ± 4 mV in the presence of the highest concentration of acetylcholine used (10 μM); a hyperpolarization of 39 ± 4 mV. The absolute value for membrane potential in the presence of acetylcholine (10 μM) was similar whether in the absence or in the presence of tone induced by endothelin-1 (i.e. -75 versus -71 mV, respectively). The average results show a close correlation between concentrations of acetylcholine required to cause membrane hyperpolarization and relaxation (Figure 2). Since it was experimentally difficult to maintain impalements of cells that were undergoing active tension changes the majority of the studies examining the effects of acetylcholine were performed in vessels which possessed no active tone.

Direct effect of the antagonists of cromakalim on membrane potential and the role of the endothelium

All three cromakalim antagonists studied (glibenclamide 0.1–3 μM, alinidine 1–30 μM and phentolamine 1–100 μM) caused a direct membrane depolarizing effect. The direct membrane depolarizing action of phentolamine is shown in Figure 3. The maximum depolarization produced by glibenclamide (3 μM),

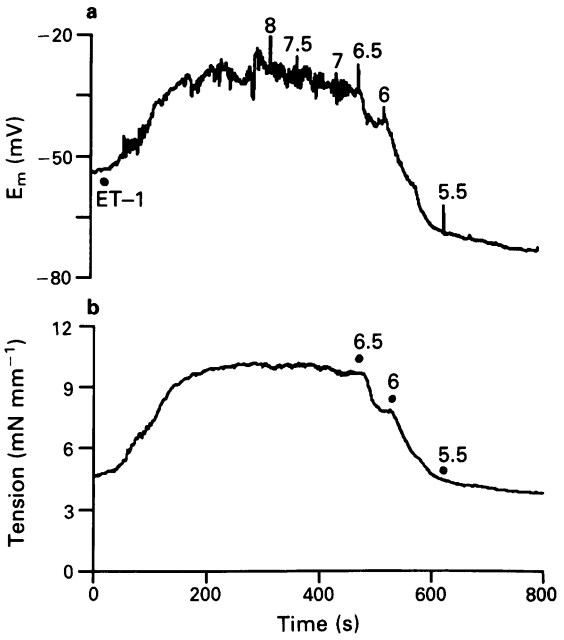

Figure 1 Representative trace obtained in a rat small mesenteric artery in which membrane potential (a) and tension development (b) were recorded simultaneously. Tone was induced with endothelin-1 (ET-1; 20 nM) and the electrical and vasonconstrictor response were followed until they reached a plateau. Thereafter a cumulative concentration-effect curve to acetylcholine was constructed. Concentrations of acetylcholine are marked on the figure as −log (M).

alinidine (30 μM) and phentolamine (100 μM) was 9 ± 1 mV (n = 6), 7 ± 1 mV (n = 7) and 9 ± 2 mV (n = 7) respectively (Figure 4a). The concentration of antagonist required to cause 50% (pD_2 = −log EC_{50}) of the maximum depolarization was calculated graphically (Figure 4b). Glibenclamide (pD_2 = 6.32 ± 0.07) was significantly more potent ($P < 0.05$, Scheffe's test) at causing depolarization by a factor of 5–10 than alinidine (pD_2 = 5.58 ± 0.1) or phentolamine (pD_2 = 5.35 ± 0.19) which were equipotent (Figure 4b).

We observed some dependence of the maximal level of depolarization caused by the antagonists, and the initial resting membrane potential

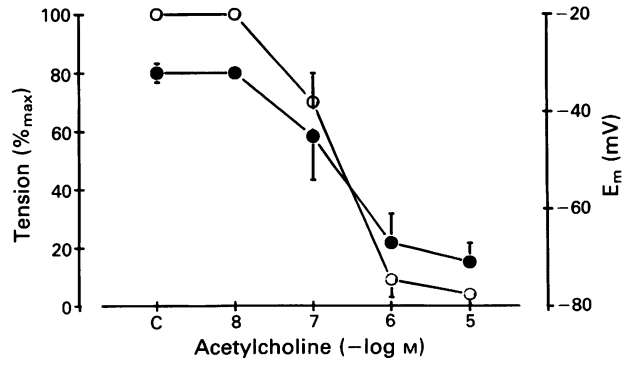

Figure 2 Mean concentration-effect curves for acetylcholine constructed in rat small mesenteric arteries precontracted with endothelin-1 (20 nM). Tension (% E_{max}; left scale, ○) and resting membrane potential (E_m; right scale, ●) were recorded simultaneously. C is the control tension and membrane potential values obtained in the absence of any acetylcholine. Values are the mean for 3 separate determinations; s.e.mean shown by vertical bars.

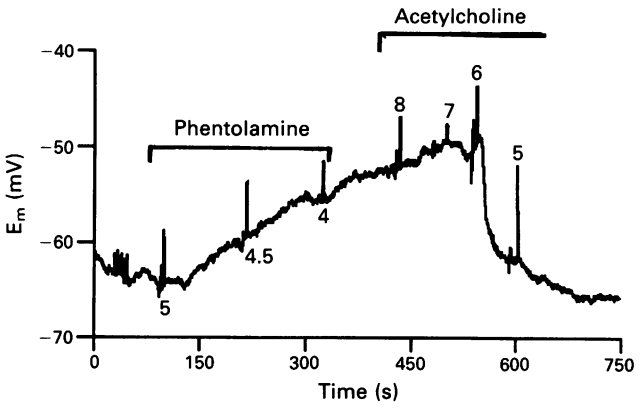

Figure 3 Actual trace showing the effect of increasing concentrations of phentolamine on resting membrane potential (E_m) of a rat small mesenteric artery. At the end of the experiment a cumulative concentration-effect curve was constructed to acetylcholine which hyperpolarized the vessel. Concentrations of drugs are given as −log (M) and indicated by spike marks.

($P < 0.05$, r = 0.67, n = 20). Extrapolation of this regression indicated that the resting membrane potential at which the antagonists had no effect would be approximately −50 mV (see Figure 5a). We also examined whether the degree of depolarization depended on vessel size in this series of experiments and found that there was no correlation ($P > 0.05$, r = 0.09, n = 20) in vessels that ranged in diameter from ≈200–470 μm (Figure 5b).

Vessels were rubbed with the nylon suture to remove the endothelium. In each tissue the maximum response to noradrenaline (10 μM) was assessed before and after the rubbing procedure. The tissue response to noradrenaline after rubbing was 67 ± 1% of the pre-rubbing response indicating some degree of vascular damage. After rubbing, the vessels (D_{100} = 329 ± 15 μM, n = 6) had a resting membrane potential of −51 ± 2 mV (9 impalements from six different vessels) which was significantly different (unpaired t test, $P < 0.05$)

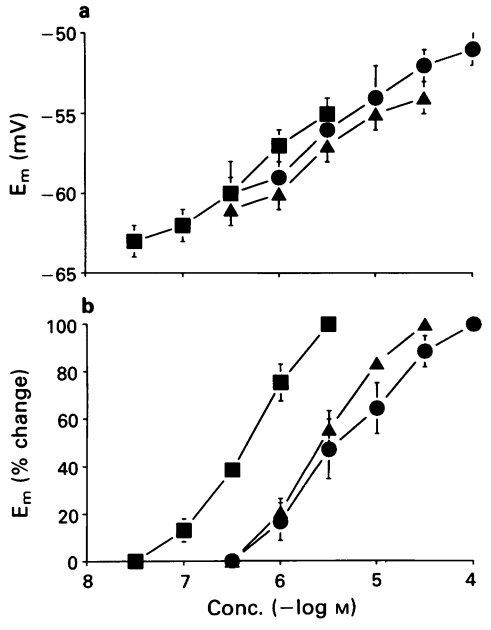

Figure 4 (a) Mean concentration-effect curves constructed in rat small mesenteric arteries assessing the ability of glibenclamide (■), phentolamine (●) and alinidine (▲) to depolarize directly the smooth muscle cell. (b) Standardized mean concentration-effect curves. Membrane potential changes have been expressed as a percentage of the maximum membrane potential change caused by the highest concentration of antagonist used. Results are the mean obtained from 6–7 separate experiments; vertical bars show s.e.mean.

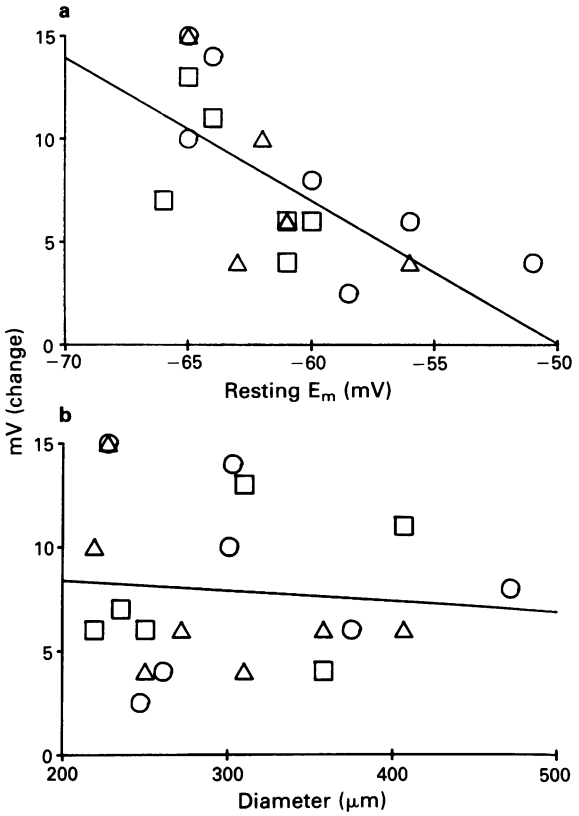

Figure 5 Correlation between the maximum change in resting membrane induced by glibenclamide (□, 3 μM), phentolamine (○, 100 μM) and alinidine (△, 30 μM) and the initial resting membrane potential (a) and also the diameter of the vessel at 100 mmHg transmural pressure (b).

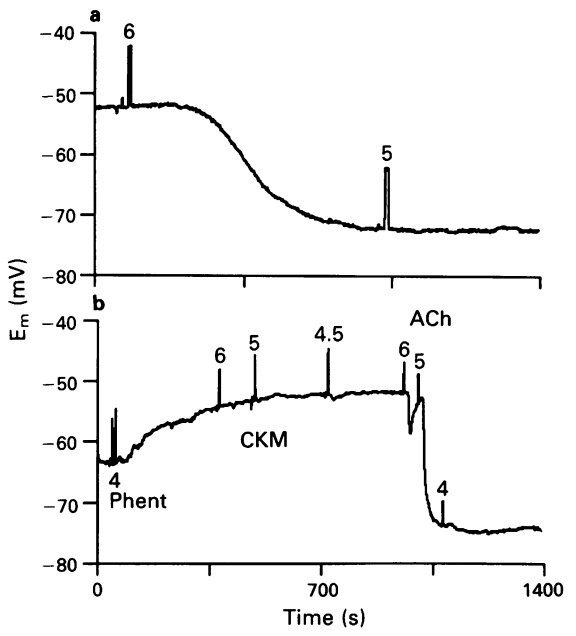

Figure 6 Original records of membrane potential (E_m, mV) in two rat small mesenteric arteries with intact endothelium. (a) Experiment showing the hyperpolarizing effect of cromakalim (−log M) indicated by spike marks. (b) Effect of phentolamine (Phent, 4) on responses to cromakalim (CKM) and acetylcholine (ACh) added cumulatively as indicated. Concentrations are given as −log (M).

from that in unrubbed vessels (−60 ± 1 mV; see earlier). In the absence of endothelium, acetylcholine (10 μM) failed to elicit any hyperpolarization indicating successful removal of the endothelium. However, all cromakalim antagonists still caused significant depolarization (glibenclamide 3 μM, 4.3 ± 0.3 mV; alinidine 30 μM, 3 ± 1 mV and phentolamine 100 μM, 6 ± 1 mV; n = 3–4). None of these values was significantly different from unrubbed vessels although the degree of the depolarization was less than that observed in tissues with an intact endothelium.

Interaction between acetylcholine and antagonists of cromakalim

Figures 6a and b show original traces examining the effects of phentolamine (100 μM) and the subsequent addition of cromakalim and acetylcholine in a vessel with an intact endothelium. In this particular experiment phentolamine caused a large depolarization from −64 to −56 mV. While the effects of cromakalim (1 − 30 μM) were completely inhibited (Figure 6a), acetylcholine-induced hyperpolarization still occurred, although higher concentrations were required (Figure 6b). Further studies were performed examining acetylcholine concentration-effect curves constructed in the absence and presence of glibenclamide (3 μM), alinidine (30 μM) and phentolamine (30 and 100 μM). The tissues were equilibrated with the antagonists for 10 min before construction of the acetylcholine curve. In the absence of the antagonists acetylcholine caused a concentration-dependent hyperpolarization with an EC_{50} of approximately 0.03 μM (Figure 7). Phentolamine (30 μM) and alinidine (30 μM) caused an approximate 10 fold rightward shift in the curve (EC_{50} 0.3 μM) (Figure 7). There was no further shift in the curve when the phentolamine concentration was increased to 100 μM (Figure 7). Glibenclamide failed to affect the EC_{50} of acetylcholine although, due to its direct depolarizing effect, the resting membrane potential was less negative for any given concentration of acetylcholine (Figure 7).

Functional effects of cromakalim antagonists on responses to cromakalim and acetylcholine

The cromakalim antagonists were also tested for their ability to affect the relaxation responses to acetylcholine and cromakalim in mesenteric vessels precontracted with endothelin-1. The responses to cromakalim (0.1–30 μM) were shifted approximately 10 fold to the right in the presence of glibenclamide (3 μM), alinidine (30 μM) and phentolamine (30 μM) (Figure 8). Using acetylcholine as the vasorelaxant however we found gli-

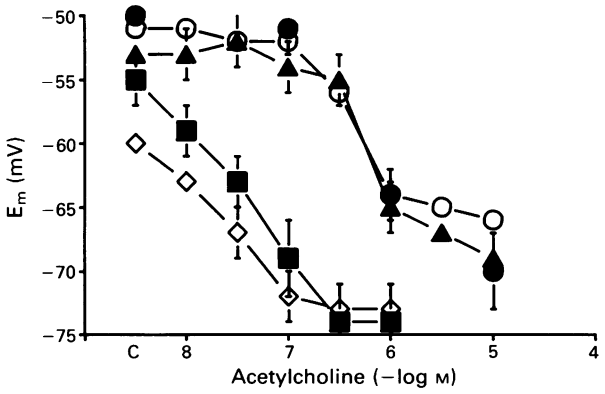

Figure 7 Mean concentration-effect curves for acetylcholine on membrane potential (E_m) in the rat small mesenteric artery. Curves were constructed in the absence (◇) and in the presence of glibenclamide (■, 3 μM), phentolamine (○, 30 and ●, 100 μM) and alinidine (▲, 30 μM). C is the control E_m just prior to the addition of any acetylcholine. Results are the mean for 3 to 6 separate experiments; s.e.mean shown by vertical bars. Each experiment was performed by constructing a concentration-effect curve to acetylcholine in the absence and then in the presence of the antagonist. The control curves were superimposable and consequently only one (n = 6) is included on the graph to aid clarity.

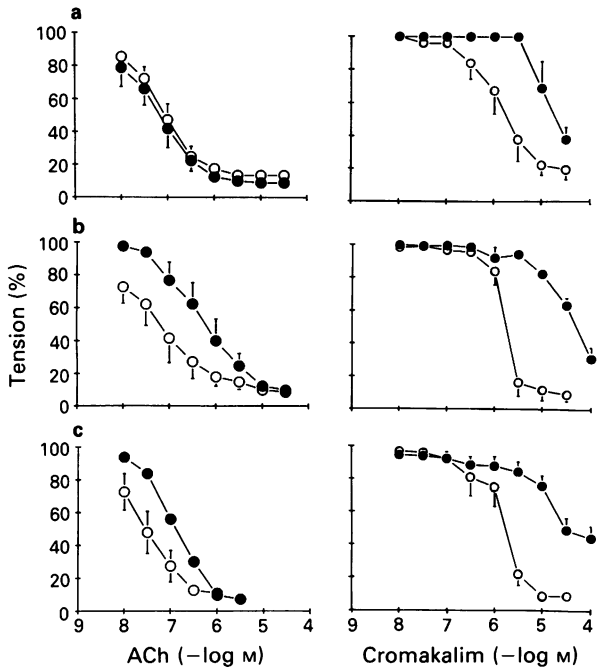

Figure 8 Mean concentration-effect curves constructed for acetylcholine (left panels) or cromakalim (right panels) in the rat small mesenteric artery. Tone was induced with a submaximal concentration of endothelin-1 (10–20 nM). Curves to cromakalim or acetylcholine were constructed in the absence (○) and in the presence (●) of glibenclamide (3 μM; a), phentolamine (100 μM; b) and alinidine (30 μM; c). Relaxation responses, expressed as a percentage of the tone induced by ET-1, are mean from 3 to 6 separate experiments; s.e.mean shown by vertical bars.

benclamide to be ineffective while alinidine and phentolamine caused a 5–10 fold shift to the right of the acetylcholine concentration-effect curve (Figure 8).

Anti-muscarinic effects of phentolamine and alinidine

Anti-muscarinic activity of phentolamine and alinidine were assessed in the pig large coronary artery. In this preparation acetylcholine caused a vasoconstrictor response with an EC_{50} of approximately 60 nM (Figure 9). The concentration-effect curve was shifted to the right (Figure 9) approximately 10 fold by phentolamine (100 μM) and 7 fold by alinidine (30 μM).

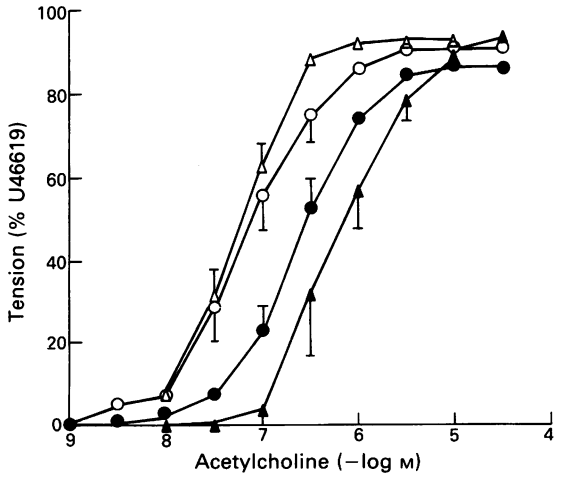

Figure 9 Mean concentration-effect curves for acetylcholine in the pig coronary artery. Curves were constructed for acetylcholine in the absence (open symbols) or in the presence (closed symbols) of phentolamine (▲, 100 μM) or alinidine (●, 30 μM). Contractile responses, expressed as a percentage of the response of the tissue to 11α,9α-epoxymethano-prostaglandin H_2 (U46619, 30 nM, a thromboxane-mimetic), are the mean of 4 to 5 separate experiments; s.e.mean shown by vertical bars.

Discussion

The main finding from this work is that acetylcholine-mediated hyperpolarization does not appear to involve the K^+ channel opened by cromakalim (ATP-sensitive K^+ channels). This result contrasts with that obtained by Standen and his colleagues (Standen *et al.*, 1989) who showed that acetylcholine-induced hyperpolarization in the rabbit middle cerebral artery involved the opening of ATP-sensitive K^+ channels since the response was reversed by glibenclamide. Using very similar microelectrode techniques however, we found that the hyperpolarization and functional vasorelaxant responses to acetylcholine were not susceptible to the effects of glibenclamide at concentrations that blocked the response to cromakalim. This result suggests that there may be two different hyperpolarizing substances released from the endothelium or possibly one substance which utilizes two different coupling mechanisms. In either case one process utilizes ATP-sensitive K^+ channels which are antagonized by glibenclamide (a mechanism activated in the rabbit middle cerebral artery) and at least one other process uses another, as yet unidentified, mechanism.

Whatever the mechanism of the observed hyperpolarization it would appear that it plays little role in the functional vasorelaxant response to acetylcholine since agents which block this response (e.g. methylene blue and haemoglobin) generally fail to affect the hyperpolarization (see Chen *et al.*, 1988; Nishiye *et al.*, 1989). These results have led workers to suggest that at least two factors are released by acetylcholine. EDRF (which is NO or related to NO) and EDHF (see Taylor & Weston, 1988). It is apparent from the literature and the results obtained in the present study that the electrophysiological effects (i.e. hyperpolarization) of acetylcholine are complex and possibly multifactorial. In some vessels such as the rabbit ear artery (Suzuki, 1988) and femoral artery (Huang *et al.*, 1988), rat pulmonary (Chen *et al.*, 1988) and canine mesenteric artery (Komori *et al.*, 1988) acetylcholine causes a small and transient (approximately 5 mV) hyperpolarization. In other studies such as those using the rat mesenteric artery (this study), cat middle cerebral artery (Brayden & Wellman, 1989), dog coronary artery (Chen *et al.*, 1989) and guinea-pig basilar artery (Nishiye *et al.*, 1989) the hyperpolarization can be as much as 25 mV and longer lasting. That the degree and type of hyperpolarization appear to fall into two categories may suggest differing underlying mechanisms. There is already some evidence to support this idea. For example, some reports have suggested that acetylcholine-induced hyperpolarization results from the activation of Na^+/K^+ ATPase since responses are antagonised by ouabain (Feletou & Vanhoutte, 1988; Brayden & Wellman, 1989) while others have shown ouabain to be ineffective (Suzuki, 1988; Chen *et al.*, 1989). The work of Standen and co-workers (1989) suggests that the ATP-sensitive K^+ channel is important but there are several other possibilities which may account for acetylcholine hyperpolarization of smooth muscle. First the work of Tare and co-workers (1990) has shown that authentic NO can indeed affect membrane potential in contrast to earlier studies which have suggested that this is not the case (Komori *et al.*, 1988; Huang *et al.*, 1988). Tare and co-workers (1990) showed that in the guinea-pig uterine artery, acetylcholine and NO both hyperpolarized membrane potential back to control levels in vessels activated with phenylephrine. This response is some what different from that seen in other studies in that the membrane potential did not hyperpolarize beyond that in the inactivated vessel, while most studies involving acetylcholine have shown a true hyperpolarization rather than just repolarization. For example in the present study, acetylcholine hyperpolarized the cell from the rat small mesenteric artery to the same absolute value (approximately −70 mV) irrespective of

whether the vessel was initially depolarized by a vasoconstrictor or not. In any case the observation that acetylcholine and NO can influence membrane potential suggests that the contribution of NO to changes in membrane potential should be examined more thoroughly. Lastly Busse and co-workers (1988) have shown that acetylcholine can also hyperpolarize endothelial cells directly. They raised the possibility that electrical coupling between the endothelial and underlying smooth muscle cell may well account for some of the hyperpolarizing response to acetylcholine. To summarize then, acetylcholine can produce varying degrees of hyperpolarization which depends on the vessel being studied. Such hyperpolarization can result from a number of mechanisms including Na^+/K^+ ATPase activation, K^+ channel opening and possibly other, as yet unidentified, mechanisms. Clearly this area of research requires much more work.

We also tested two other compounds, phentolamine and alinidine, which we have shown (McPherson & Angus, 1989) block the potassium channel opened by cromakalim. In contrast to the result that we obtained with glibenclamide, these two agents did shift the acetylcholine-mediated concentration-dependent vasorelaxant and hyperpolarization response curves to the right.

There may be a number of reasons for the shift in both functional and electrophysiological responses to acetylcholine observed with phentolamine and alinidine. First these agents may possess some anti-muscarinic activity which would affect the response to acetylcholine post-junctionally rather than by interfering with the actions of the intermediates released from the endothelium. We examined this possibility in the pig large coronary artery where acetylcholine causes a constrictor response through the direct activation of the smooth muscle. Use of this preparation avoided interpreting the complicated effects of acetylcholine on the rat small mesenteric artery. Responses to acetylcholine were antagonized by concentrations of alinidine (30 μM) and phentolamine (100 μM) which inhibited the functional and hyperpolarizing response in the rat mesenteric artery. In addition radioligand binding studies (Brunner & Kukovetz, 1988) have shown that alinidine displaces specific [^{3}H]-QNB binding in membranes prepared from guinea-pig heart muscle over the concentration-range of 10–100 μM. Lastly, in our previous studies assessing the selectivity of glibenclamide, phentolamine and alinidine in a number of vascular and non-vascular smooth muscle preparations (McPherson & Angus, 1990) we found that the relative potency of the antagonists in blocking the response to cromakalim did not vary significantly. As a consequence we think is unlikely that, in the rat small mesenteric artery, glibenclamide would not block a K^+ channel susceptible to the actions of phentolamine and alinidine. On the bases of these results we feel that phentolamine and alinidine are not appropriate tools for studying the involvement of K^+ channels in mediating the effects of acetylcholine.

Of greater interest was the finding that all three compounds (glibenclamide, phentolamine and alinidine) had a direct depolarizing effect on resting membrane potential. Previous studies in our laboratory (McPherson & Angus, 1989; 1990) have shown glibenclamide to be approximately 10–30 times more potent than phentolamine and alinidine in blocking the effects of the response to cromakalim. Glibenclamide is active at approximately 1 μM while phentolamine and alinidine are active at 30 μM. This relative potency coincides almost exactly with their ability to induce depolarizing responses in the rat small mesenteric artery. There may be several reasons for this phenomenon. Firstly it is possible that in this artery there is a basal release of a hyperpolarizing factor which tonically influences resting membrane potential. The basal release of EDRF has previously been considered (Moncada & Palmer, 1990) which prompted us to examine the endothelial-dependence of the response. However, in vessels which had the endothelium removed, the compounds were still able to depolarize to some extent. This result would exclude the basal release of an endothelium-derived factor but it does not exclude the release of such a factor from other sites in the blood vessel wall. Another possibility is that the K^+ channel is partially opened in the rat small mesenteric artery under the conditions in which we assessed membrane potential. It is possible that the three compounds, rather than being antagonists of the compounds that open the channel (i.e. occupy the receptor site but do not initiate a response), are actually 'inverse agonists' in that they actively close the K^+ channels if they are open. The interaction between these compounds and K^+-channel openers such as cromakalim and pinacidil is not then a matter of competitive but rather functional antagonism. If this idea proves to be correct then there is obviously some variation in the percentage of K^+ channels spontaneously open since in vessels such as the rat femoral artery (McPherson & Angus, 1989) and rabbit middle cerebral artery (Standen *et al.*, 1989) the compounds do not display direct depolarising effects on the resting membrane potential. Whatever the case may be, the results from this study show that, in the rat mesenteric artery, the activity of K^+ channels sensitive to glibenclamide, phentolamine and alinidine increase the negativity of the membrane potential by approximately 10 mV. Clearly much more work is required in this area.

In summary the results from this study using glibenclamide to block ATP-dependent K^+ channels suggest that this channel plays no role in the hyperpolarizing response to acetylcholine in the rat small mesenteric artery. This result contrasts with those obtained in the rabbit middle cerebral artery described by other workers (Standen *et al.*, 1989). Taken collectively, the results show that acetylcholine-induced hyperpolarization can result from a number of mechanisms, the physiological significance of which has yet to be determined.

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Facilitatory effects of tachykinins and guanethidine on the acetylcholine output stimulated by nicotine from guinea-pig bladder

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1 Contractile responses and acetylcholine release evoked by nicotine in guinea-pig detrusor strips were determined by isotonic transducer and radioimmunoassay, respectively. Nicotine stimulated acetylcholine release and a contractile response in guinea-pig detrusor strips treated with the cholinesterase inhibitor, methanesulphonyl fluoride (MSF). Both actions evoked by nicotine were antagonized by the nicotinic receptor antagonist, hexamethonium but were insensitive to tetrodotoxin.

2 A sympathetic nerve blocker, guanethidine and a tachykinin antagonist, [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]-substance P (rpwwL-SP) partially inhibited the acetylcholine release evoked by nicotine to much the same degree. The inhibitory effects of guanethidine and rpwwL-SP on acetylcholine release were significantly greater than corresponding effects on the contraction evoked by nicotine.

3 In preparations treated with rpwwL-SP to block the tachykinin receptors, guanethidine had no effect on the response to nicotine. Conversely, after treatment with guanethidine to block release of a mediator from sympathetic nerve endings, nicotine-induced responses were not affected by rpwwL-SP.

4 Nicotine-induced contraction was reduced to 30% by the muscarinic cholinoceptor antagonist, atropine and completely abolished after desensitization of P_2-purinoceptors with α,β-methylene ATP in the presence of atropine.

5 A concentration-contractile response curve to neurokinin A (NKA) was shited to the left after cholinesterase inhibition with MSF. Atropine abolished the facilitatory effect of MSF and partially inhibited contractions induced by NKA at 100 nM to 1 μM. The contractile responses to substance P methyl ester (SPOMe) and Tyr^0-neurokinin B (Tyr^0-NKB) were not influenced by MSF or atropine.

6 After desensitization of NK_1 tachykinin receptors with SPOMe or preincubation with senktide, the cholinergic component of the nicotine-induced contraction was the same as the control value (100%).

7 Our findings give further support to our previous results: nicotine stimulates acetylcholine release in a tetrodotoxin-resistant manner in guinea-pig bladder and acetylcholine release evoked by nicotine is increased by the coordinated action of sympathetic nerves and tachykinin(s). It is suggested that the tachykinin receptor subtype involved in acetylcholine release is NK_1.

Keywords: Guinea-pig bladder; nicotine; radioimmunoassay; acetylcholine release; sympathetic nerve; tachykinin(s)

Introduction

Substance P (SP)-like immunoreactivity (SP-LI) and immunoreactive nerves (SP-IR nerves) have been identified throughout the bladder (Alm *et al.*, 1978; Hokfelt *et al.*, 1978). SP causes contraction of the guinea-pig bladder (Falconieri Erspamer *et al.*, 1980; Hunter & Maggio, 1984) and functional NK_1 and NK_2 tachykinin receptor subtypes are present in this tissue (Shinkai & Takayanagi, 1990).

We have shown that nicotine produces a transient, tetrodotoxin-resistant contraction of isolated detrusor strips of guinea-pig, and suggested that the drug might interact with the presynaptic nicotinic receptors located on (1) parasympathetic cholinergic (atropine sensitive), (2) sympathetic non-adrenergic (guanethidine-sensitive, but resistant to bunazosin and yohimbine) and (3) non-sympathetic purinergic (sensitive to desensitization of P_2-purinoceptors by α,β-methylene adenosine 5'-triphosphate) nerves to induce a release of two excitatory final transmitters, acetylcholine and a purine nucleotide (Hisayama *et al.*, 1988a). The potentiation of acetylcholine output from cholinergic neurones by the coordinated action of sympathetic nerve and tachykinin(s) following nicotine stimulation has been shown indirectly through the effect of the tachykinin antagonist [D-Arg^1,D-Pro^2,D-Trp^{7,9}, Leu^{11}]-SP (rpwwL-SP) and the sympathetic nerve blocking agent, guanethidine on the nicotine-induced contraction (Hisayama *et al.*, 1989).

In this study, we determined the role of the sympathetic nerves and tachykinin(s) on nicotine-induced acetylcholine release directly by radioimmunoassay.

Methods

Female Hartley strain guinea-pigs, weighing between 300 and 500 g, were reared on a standard diet and given tap water to drink. The guinea-pigs were stunned by a blow on the head and exsanguinated from the femoral artery. The urinary bladder was rapidly removed and a longitudinal strip (about 2 mm × 20 mm) of the detrusor muscle was prepared. Each strip was suspended vertically under a resting load of 1 g in a 5 ml organ bath which contained Krebs solution of the following composition (mM): NaCl 118, KCl 4.75, $CaCl_2$ 2.50, $MgSO_4$ 1.20, KH_2PO_4 1.20, $NaHCO_3$ 25.0 and glucose 10.0. The organ bath was maintained at 37°C and constantly gassed with carbogen (95% O_2 + 5% CO_2). The response to drugs was recorded isotonically.

The experiments were started after the preparation had been allowed to equilibrate for about 60 min. After priming twice or three times with 300 nM carbachol, the first control dose of nicotine (0.1 mM) was applied; after incubation for 60 min with the appropriate treatments, the second test dose of nicotine was applied.

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Table 1 Effect of drugs on acetylcholine release and on the contractile responses induced by nicotine in guinea-pig bladder treated wtih methanesulphonyl fluoride (MSF)

Treatment	*Acetylcholine release* (%)	*Contraction* (%)
Nicotine 0.1 mM	100	100
+ hexamethonium, 10 μM	15.6 ± 3.5* (4)	26.1 ± 4.6* (4)
+ tetrodotoxin, 30 μM	112.9 ± 17.9 (4)	97.3 ± 7.7 (4)
+ guanethidine, 3 μM	31.8 ± 9.7* (4)	81.3 ± 2.7* (4)
+ rpwwL-SP, 10 μM	27.6 ± 5.8* (4)	77.6 ± 3.4* (4)
Nicotine, 0.1 mM in the presence of rpwwL-SP, 10 μM	100	100
+ guanethidine, 3 μM	99.5 ± 20.3 (4)	101.5 ± 1.7 (4)
Nicotine, 0.1 mM in the presence of guanethidine, 3 μM	100	100
+ rpwwL-SP, 10 μM	94.6 ± 8.8 (4)	112.7 ± 6.3 (4)

* Significant difference from 100% at $P < 0.05$.
rpwwL-SP: [D-Arg[1],D-Pro[2],D-Trp[7,9],Leu[11]]-substance P.

When we determined acetylcholine release and contractile responses simultaneously, each strip was incubated with 1 mM methanesulphonyl fluoride (MSF) for 30 min in order to inhibit acetylcholinesterase activity irreversibly. After priming twice or three times with 300 nM carbachol, Krebs solution containing various concentrations of drugs were superfused at a rate of 0.4 ml min^{-1} from the bottom and collected by aspiration at the top of an organ bath, and the effects of these drugs on acetylcholine output from the strips as well as contractile responses were studied. The superfusate was collected continuously on ice and divided into fractions for each 3 min period, and then assayed for acetylcholine as described below.

Acetylcholine was determined by radioimmunoassay with rabbit antiserum raised against choline hemiglutarate-bovine serum albumin conjugates and tritiated acetylcholine with a specific activity of 75.1 Ci mmol^{-1} according to the method of Kawashima *et al.* (1988). Assays were performed in duplicate at 4°C. To avoid overestimation by cross-reactivity with nicotine, the standard curve for acetylcholine was derived in the presence of nicotine as appropriate.

A 200 μl portion of the superfusate was incubated overnight with 50 μl of the diluted antiserum (1:350) in Tris-HCl buffer (0.15 M, pH 7.4) containing 0.4% bovine gamma-globulin, 0.05% isofluorophosphate and 50 μl of tritiated acetylcholine (about 12.1 pg, 4500 c.p.m.). The same volume of superfusion fluid containing 0.1 mM nicotine served as a blank. Antibody-bound tritiated acetylcholine was separated from the free tritiated acetylcholine by the ammonium sulphate method (Farr, 1958), and the radioactivity of the precipitates was quantitated in a liquid scintillation counter. Acetylcholine release induced by nicotine was calculated by subtracting the value for spontaneous acetylcholine release from that for the total.

Desensitization to α,β-methylene adenosine 5'-triphosphate (α,β-MeATP) and to substance P methyl ester (SPOMe) was produced by the methods of Kasakov & Burnstock (1983) and Laufer *et al.* (1985), respectively (Figure 3).

The pD_2 value (the negative logarithm of the molar concentration which produced 50% of its maximum responses) for a drug was calculated by graphic analysis. Statistical analyses were performed by Student's *t* test. A *P* value of <0.05 was considered a significant difference.

Drugs used were nicotine bitartrate (Nakarai Chemicals, Ltd., Kyoto, Japan), carbachol chloride, atropine sulphate, ATP, MeATP, gamma-globulins, diisopropyl fluorophosphate, bicuculline (Sigma Chemical Co., MO., U.S.A.), hexamethonium dibromide (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan), tetrodotoxin (Sankyo Co., Ltd., Tokyo, Japan), NKA, Tyr^0-NKB, senktide, SPOMe, rpwwL-SP (Peninsula Laboratories, Inc., Belmont, CA, U.S.A.), acetyl[methyl-^{3}H]-choline chloride (Amersham Japan Co., Tokyo, Japan) and methanesulphonyl fluoride (Aldrich Chemical Co., Inc., U.S.A.). Guanethidine sulphate was donated by Ciba-Geigy (Japan), Ltd. (Hyogo, Japan). All drugs used were of analytical grade.

Results

In the isolated detrusor muscle of guinea-pig treated with MSF (1 mM, 30 min) to inhibit acetylcholinesterase irreversibly, a concentration-response curve for the contractile response to nicotine was obtained at concentrations between 10 μM to 30 mM (Figure 1). The pD_2 value for nicotine was 4.21 ± 0.06 ($n = 6$) and the maximum response compared with that to isotonic 120 mM K-solution was 91.6 ± 3.90% ($n = 6$). The contractile response induced by K-solution was not influenced by MSF (95.6 ± 4% of control value, mean of 6 experiments).

Simultaneous measurements of mechanical activity and acetylcholine release

The effects of some drugs on contractile response and acetylcholine release induced by nicotine (0.1 mM) are summarized in Table 1. Acetylcholine release evoked by nicotine (0.1 mM) was 84.9 ± 7.6 pg mg^{-1} tissue per 3 min ($n = 16$). Both the nicotine-induced contraction and acetylcholine release were greatly reduced by 10 μM hexamethonium, and resistant to 3 μM tetrodotoxin (Table 1). The nicotine-induced acetylcholine release was also reduced by 3 μM guanethidine or 10 μM rpwwL-SP to much the same degree (about 30%). On the other hand, the contractile response to nicotine was only weakly reduced by guanethidine or rpwwL-SP (Table 1).

In preparations treated with rpwwL-SP to block the tachykinin receptors, guanethidine had no effect on the responses to

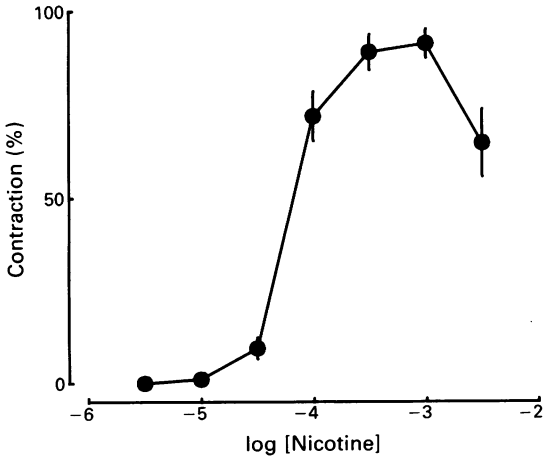

Figure 1 Concentration-response curves for nicotine in guinea-pig detrusor strips treated with methanesulphonyl fluoride (1 mM). Abscissa scale: log molar concentration of nicotine. Ordinate scale: % of contraction induced by isotonic 120 mM K-solution. Each value is presented as a mean with s.e. (vertical line) of 6 experiments.

Table 2 Effects of drugs on the contractile responses to nicotine in guinea-pig bladder treated with methanesulphonyl fluoride

Treatment	*Contraction* (%)
Nicotine, 0.1 mM	100
+ atropine, 1 μM	28.3 ± 4.6* (6)
+ atropine, 1 μM and α,β-MeATP, 50 μM	5.8 ± 3.1*† (6)
+ bunazosin, 0.1 μM	93.7 ± 2.7 (6)
+ yohimbin, 0.3 μM	90.6 ± 5.1 (6)
+ methysergide, 1 μM	97.9 ± 3.7 (6)
+ naloxone, 1 μM	105.6 ± 4.1 (6)
+ bicuculline, 1 μM	100.4 ± 3.8 (6)

* Significant difference from 100% at $P < 0.05$.
† No significant difference from 0% at $P < 0.05$.
α,β-MeATP: α,β-methylene ATP.

nicotine. Conversely, after treatment with guanethidine to prevent release of a mediator from sympathetic nerve endings, the nicotine-induced response was not affected by rpwwL-SP (Table 1).

Effects of some drugs on nicotine-induced contractions in methanesulphonyl fluoride-treated preparations

In the MSF-treated preparation the nicotine-induced contraction was reduced to about 30% of control value by atropine (1 μM). Simultaneous treatment with atropine and α,β-MeATP desensitization to inactivate muscarinic receptors and P_{2X}-purinoceptors respectively, abolished the nicotine contraction. The nicotine-induced contraction was not influenced by pretreatment with bunazosin (1 μM), yohimbine (300 nM), naloxone (1 μM), methysergide (1 μM) or bicuculline (1 μM) (Table 2).

Cholinergic component of the tachykinin-induced contraction

The concentration-response curve for NKA was shifted to the left by treatment with MSF to inhibit acetylcholinesterase activity irreversibly. The pD_2 values for NKA before and after MSF treatment were 6.65 ± 0.11 ($n = 6$) and 7.21 ± 0.03 ($n = 6$), respectively. Atropine (1 μM) abolished the facilitatory effect of MSF and partially inhibited contractions by NKA at 100 nM to 1 μM (Figure 2), suggesting a cholinergic component of the NKA-induced action. In contrast, the pD_2 values of Tyr^0-NKB (water soluble analogue of NKB) and SPOMe were not influenced by MSF or atropine (Table 3). The NK_3 receptor selective agonist, senktide did not cause a contraction in guinea-pig bladder.

Effects of NK_1 and NK_3 selective agonists on the cholinergic component of the nicotine contraction

After desensitization with α,β-MeATP, contractile responses to nicotine (0.1 mM) were not influenced by desensitization by

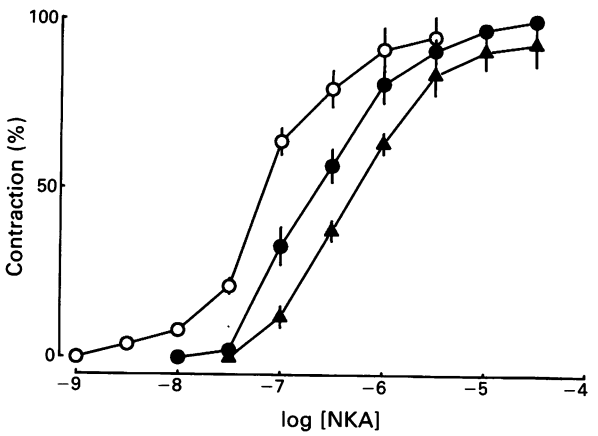

Figure 2 Effect of methanesulphonyl fluoride (MSF) and atropine on the concentration-response curves to neurokinin A (NKA). (●) NKA alone; (○) treated with MSF 1 mM; (▲) in the presence of atropine 1 μM in preparations treated with MSF. Concentration-response curve of NKA was shifted to the left by MSF. Application of atropine abolished the facilitatory effect of MSF and partially inhibited the NKA-induced contraction at 100 nM to 1 μM.

Table 4 Effect of drugs on the contractile responses to nicotine after desensitization with α,β-methylene ATP (α,β-MeATP)

Treatment	*Contraction* (%)
Nicotine, 0.1 mM after desensitization with α,β-MeATP (50 μM)	100
+ SPOMe, 1 μM	93.6 ± 4.16 (6)
+ senktide, 1 μM	94.9 ± 3.40 (6)
+ atropine, 1 μM	0.0 ± 0.00* (6)

* Significant difference from 100% at $P < 0.05$.
SPOMe; substance P methyl ester.

SPOMe (1 μM) or preincubation with senktide (1 μM), but abolished by atropine (1 μM, Table 4). Desensitization with α,β-MeATP and SPOMe was carried out as illustrated in Figure 3.

Discussion

Acetylcholine output evoked by nicotine was determined by radioimmunoassay with rabbit antiserum according to the method of Kawashima *et al.* (1988). They reported that the antiserum was specific for acetylcholine and that the cross-reactivity with choline, phosphatidylcholine and phosphorylcholine was less than 0.012%. To avoid influence of the cross-reactivity with nicotine, the standard curve for acetylcholine was derived in the presence of nicotine.

In order to inhibit acetylcholinesterase activity irreversibly, the strips were incubated at 37°C for 30 min in 1 mM MSF.

Table 3 The pD_2 values of neurokinin A (NKA), Tyr^0-NKB and substance P methyl ester (SPOMe) before and after treatment with methanesulphonyl fluoride and atropine

	pD_2 value		
		After pretreatment	
	Before treatment	*MSF*	*MSF + atropine*
NKA	6.65 ± 0.11 (6)	7.21 ± 0.03* (6)	6.34 ± 0.20 (6)
Tyr^0-NKB	6.48 ± 0.13 (6)	6.50 ± 0.14 (6)	6.22 ± 0.20 (6)
SPOMe	7.43 ± 0.14 (6)	7.40 ± 0.12 (6)	7.24 ± 0.10 (6)

* Significant difference from before treatment value at $P < 0.05$.
MSF: After treatment with methanesulphonyl phluoride 1 mM for 30 min.
The pD_2 value of NKA was increased by the irreversible acetylcholinesterase inhibitor, MSF. This facilitatory effect of MSF was abolished by application of atropine (1 μM).

The pD_2 value for nicotine in the MSF-treated preparation (4.21 ± 0.06, $n = 6$) was significantly greater than in the untreated strips (3.61 ± 0.14, $n = 6$) obtained in our previous study (Hisayama *et al.*, 1988a). Nicotine increased acetylcholine release in guinea-pig derusor strips in this study. This nicotine-evoked response was markedly reduced by hexamethonium (10 μM), indicating that the effect resulted from an interaction with autonomic nicotinic receptors. On the other hand, nicotine-induced acetylcholine release was not influenced by tetrodotoxin (300 nM). It has been suggested that two mechanisms are involved in the transmitter release induced by nicotine, one dependent on sodium action potentials and the other independent (Takayanagi *et al.*, 1984; Hisayama *et al.*, 1988b). In this study it was clearly shown that nicotine evoked acetylcholine release through the latter mechanism.

We confirmed the previous finding that the final effect of the sympathetic nerve and tachykinin(s) is an increased acetylcholine release evoked by nicotine (Hisayama *et al.*, 1988a). Guanethidine and rpwwL-SP inhibited the nicotine-induced acetylcholine release to much the same degree. This inhibitory effect on the release was significantly greater than on the contraction evoked by nicotine. Application of guanethidine to block the release of mediator from the sympathetic nerve, completely abolished the inhibitory effect of rpwwL-SP on nicotine-induced acetylcholine release. Conversely, when the preparation was treated with rpwwL-SP, the inhibitory effect of guanethidine was abolished (Table 1). In other words, when the function of the sympathetic nerve was blocked, the effect of tachykinin(s) was abolished.

Nicotine-induced contraction was not antagonized by bunazosin or yohimbine although a sympathetic blocking agent, guanethidine was effective. It was known that exogenously applied noradrenaline did not cause a contraction even in the presence of noradrenaline uptake mechanisms (Hisayama *et al.*, 1988a). It seems unlikely that the effect of guanethidine was nonspecific, since chemical denervation with 6-hydroxydopamine abolished the inhibitory effect of guanethidine, and the drug did not inhibit the muscarinic receptor and purinoceptor mechanisms (Hisayama *et al.*, 1988a). The sympathomimetic effect of nicotine was non-adrenergic in nature, even if noradrenaline was released from the sympathetic nerve.

Stimulation of acetylcholine release from the cholinergic nerve by tachykinin(s) has previously been shown in guinea-pig ileum (Laufer *et al.*, 1985), spinal cord (Otsuka & Konisihi, 1975) and guinea-pig bladder (Shirakawa *et al.*, 1989). In guinea-pig ileum, NK_3 receptors on enteric neurones may play a functional role in the regulation of motility by promoting the release of acetylcholine (Laufer *et al.*, 1985; Guard & Watson, 1987). Tachykinin(s) facilitate the release of transmitter from the sympathetic nerves as a result of an interaction with NK_2 receptors (Regoli *et al.*, 1990; Tousignant *et al.*, 1987). We recently found that both NK_1 and NK_2 receptors, but not NK_3 receptors, do play an important role in the tachykinin-induced contraction in the guinea-pig urinary bladder (Shinkai & Takayanagi, 1990).

To determine the tachykinin receptor subtype involved in the cholinergic component of nicotine contraction (resistant to the desensitization of P_{2x}-purinoceptors by α,β-MeATP), the effects of two selective agonists were examined. Desensitization of NK_1 receptors by SPOMe or preincubation with the NK_3 selective agonist, senktide had no effect on the cholinergic component of nicotine contraction. NK_2 but not NK_1 or NK_3 tachykinin receptor subtypes might therefore be involved in the increased acetylcholine output evoked by nicotine. Consistent with this result, concentration-response curves to NKA were shifted to the left by treatment with the cholinesterase inhibitor, MSF and were partially inhibited by atropine, indicating that NKA directly stimulated release of acetylcholine from the nerve ending. Sensitivity to SPOMe and Tyr^0-NKB was not influenced by MSF or atropine, because the selectivity of these drugs for NK_2 receptors is relatively low (Table 4).

When applied to the neurones of isolated inferior mesenteric ganglia of guinea-pig, SP caused a membrane depolarization (Dun & Karczmer, 1979). Treatment of rats with 6-hydroxydopamine which selectively destroys principal sympathetic neurones, profoundly decreased substance P in the superior ganglion neurones (Black, 1985). Pernow (1983) suggested in his review that, in general, the level of tachykinin(s)

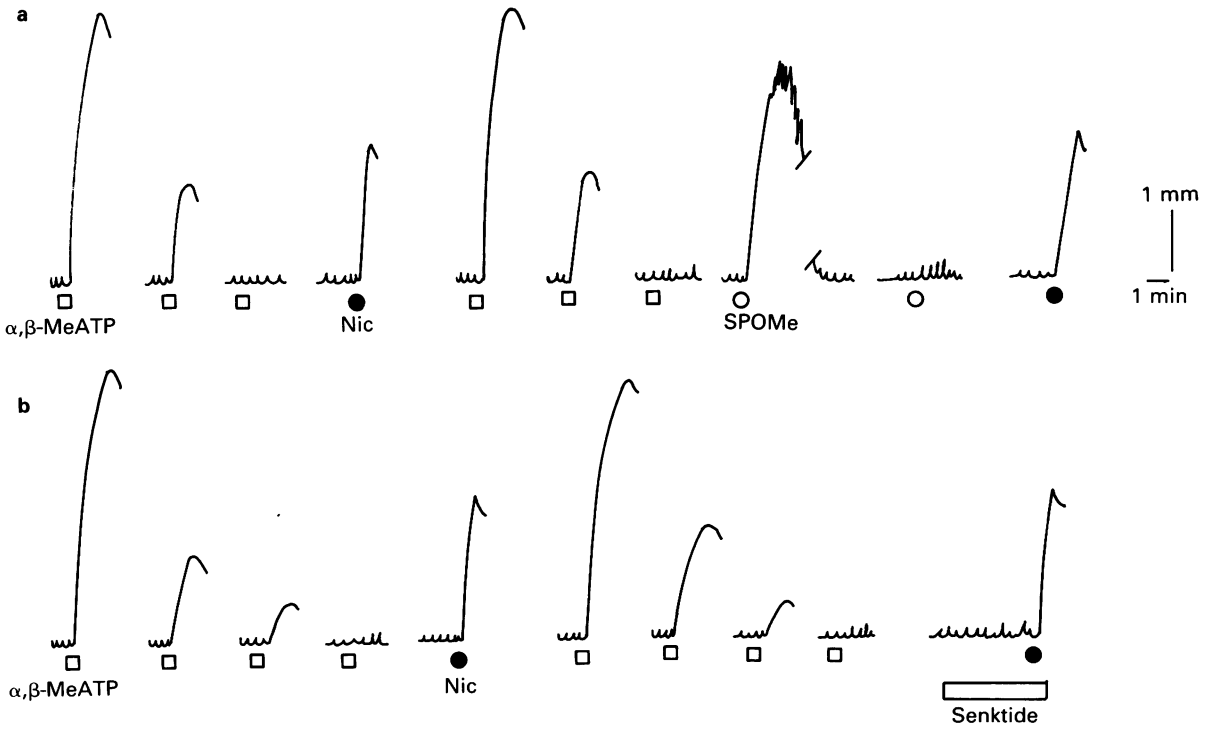

Figure 3 Typical tracings illustrating the effect of substance P methyl ester (SPOMe) desensitization (a) or preincubation with senktide (b) on the cholinergic component of the nicotine-induced contraction. α,β-Methylene ATP (α,β-MeATP) desensitization was achieved by 3 to 5 successive applications (□) at approximately 4 min intervals. Under these conditions, only the cholinergic component of nicotine contraction was observed: (●) nicotine 0.1 mM, (○) SPOMe 1 μM; open bar, senktide 1 μM.

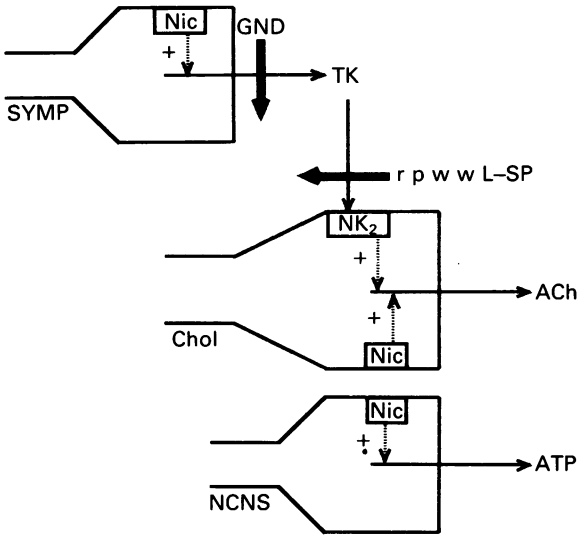

Figure 4 Postulated schemes of mechanisms of action of nicotine in guinea-pig detrusor. Nicotine may interact with the presynaptic nicotinic receptors located on (1) parasympathetic cholinergic, (2) sympathetic non-adrenergic, (3) non-cholinergic non-sympathetic purinergic nerves to induce a release of excitatory transmitters, acetylcholine and a purine nucleotide. Tachykinin(s) from capsaicin-insensitive sites (probably sympathetic nerves) causes facilitation of release of acetylcholine through activation of NK_2 tachykinin receptors. The possibility remains that a purine nucleotide is colocated with acetylcholine in parasympathetic cholinergic nerves. Further evidence for the action of nicotine in this model can be found in Hisayama *et al.* (1988a; 1989). SYMP: sympathetic nerves; Chol: parasympathetic cholinergic nerves; NCNS: non-cholinergic non-sympathetic purinergic nerves; Nic: nicotinic receptors; NK_2: NK_2 tachykinin receptors; GND: guanethidine; TK: tachykinin(s); rpwwL-SP: [D-Arg[1],D-Pro[2],D-Trp[7,9], Leu[11]]-substance P; ACh: acetylcholine.

is regulated by the sympathetic impulse activity.

Capsaicin is known to be a drug that releases and depletes tachykinins from primary sensory neurones (Hua *et al.*, 1986). We showed earlier the lack of effect of capsaicin-treatment on the nicotine-induced contraction, suggesting that sensory nerves are not involved in the action of nicotine as a site from which tachykinin(s) is released (Hisayama *et al.*, 1988a).

Nicotine-induced contraction was reduced to 30% by a muscarinic cholinoceptor antagonist, atropine. In the presence of atropine the contraction was abolished after desensitization of P_2-purinoceptors with α,β-MeATP (Table 2). Consistent with our previous data (Hisayama *et al.*, 1988a), not only acetylcholine but purine nucleotide is involved in the action of nicotine as a transmitter in guinea-pig detrusor strips treated with MSF. Since the final effect of the coordinated action of the sympathetic nerve and tachykinin(s) was only an increased acetylcholine release, the degree of inhibition of the contractile response was less than that of acetylcholine release (Table 1). Though tachykinin(s) could release acetylcholine but not purine nucleotide in our study, it is not clear whether purine nucleotide is in separate purinergic nerves, or is located with acetylcholine in cholinergic nerves.

We present postulated schemes of mechanisms of action of nicotine in guinea-pig detrusor in Figure 4. In conclusion, we have shown that nicotine stimulates acetylcholine output in a tetrodotoxin-resistant manner in guinea-pig detrusor strips, and that acetylcholine release evoked by nicotine is increased by the coordinated action of sympathetic nerves and tachykinin(s). It is further suggested that the tachykinin receptor subtype involved in the increased acetylcholine release is the NK_2 subtype in guinea-pig bladder.

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An electrophysiological study of the actions of angiotensin II at the sympathetic neuroeffector junction in the guinea-pig vas deferens

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1 The effects of angiotensin II on sympathetic neuroeffector transmission in the guinea-pig vas deferens have been investigated by the use of intracellular and focal extracellular recording techniques to measure indirectly, the release of adenosine 5'-triphosphate (ATP).

2 Angiotensin II (10–100 nM) did not alter the amplitude of the first excitatory junction potential (e.j.p.) in a train but increased the amplitude of subsequent e.j.ps. There was a corresponding increase in the probability of occurrence of extracellularly recorded evoked excitatory junction currents (e.j.cs). Spontaneous quantal transmitter release was unaffected by angiotensin II.

3 The enhancement of transmitter release produced by angiotensin II was prevented by the angiotensin receptor antagonist, saralasin.

4 The increase in transmitter release produced by angiotensin II was due to an increase in the probability of transmitter release from individual varicosities and not due to any detectable change in the configuration of the nerve terminal impulse or to the induction of repetitive firing.

5 There was no overall enhancement of e.j.ps or e.j.cs by angiotensin II in reserpinized tissues. Surprisingly, the predominant effect of angiotensin II in reserpinized vasa deferentia was to inhibit evoked transmitter release, an effect reversed by indomethacin (3 μM).

6 The results show that angiotensin II increases the release of sympathetic transmitter by activating prejunctional angiotensin II receptors. However, when the co-transmitter noradrenaline was depleted, angiotensin II now inhibited transmitter release indirectly, presumably by stimulating prostaglandin formation in the smooth muscle cells which then inhibited release transjunctionally.

Keywords: Angiotensin II; Electrophysiology; ATP; Sympathetic nerves; Transmitter release; Vas deferens

Introduction

Many substances can modulate transmitter release from sympathetic nerve terminals by activating specific prejunctional receptors (see Starke, 1977; Westfall, 1977). However, surprisingly little is known about the precise mechanism of action of many of these agents at the level of the individual varicosity. In part this is because methods have not been available to study transmitter release mechanisms in sympathetic nerve terminals on an impulse-to-impulse basis. Novel electrophysiological approaches have shown that electrically evoked transmitter release from individual varicosities in the vas deferens occurs intermittently and is monoquantal (Blakeley & Cunnane, 1979; Cunnane & Stjärne, 1982; Cunnane, 1984). Focal extracellular recording techniques have further shown that intermittence is not caused by a failure of the nerve impulse to invade the varicosity each time the parent axon is stimulated but is due to a low probability of release in the invaded varicosity (Brock & Cunnane, 1987; 1988). Hirst & Nield (1980) also concluded from studies of junction potentials in guinea-pig mesenteric arterioles that evoked transmitter release occurs intermittently. With electrophysiological techniques it is now possible to define more precisely the site of drug action at the sympathetic neuroeffector junction. In particular, the effects of drugs applied to just a few varicosities in the terminal aborization can be studied. In the present investigation the effects of angiotensin II on sympathetic transmission in the guinea-pig vas deferens have been investigated.

Angiotensin II, an octapeptide which plays a role in the regulation of blood pressure (Martin, 1985), is known to enhance sympathetic neuroeffector transmission (Ziogas *et al.*, 1984) by activating prejunctional angiotensin II receptors (reviewed by Starke, 1977; Westfall, 1977; Ziogas, 1991) but the mechanisms involved are unclear. For example, angiotensin II may alter the configuration of the action potential in the nerve terminals or induce repetitive firing. Alternatively, angiotensin II may modify one of the steps involved in depolarization-secretion coupling. Focal extracellular recording techniques can distinguish unequivocally between these possibilities. Furthermore, it is possible to determine whether the enhancement of sympathetic neurotransmission by angiotensin II involves an alteration in the size of the transmitter quantum or in the probability of a varicosity releasing a quantum of transmitter, effects which have not previously been considered.

Earlier studies of the effects of angiotensin II on transmitter release largely involved measurements of noradrenaline overflow from electrically stimulated tissues (see Starke, 1977; Westfall, 1977). However, it is now widely believed that the electrical changes recorded in smooth muscle cells following sympathetic nerve stimulation are due to the action of adenosine 5'-triphosphate (ATP) or a related purine nucleotide, released as a co-transmitter with noradrenaline (Stjärne, 1986; White, 1988). Thus, it is important to remember that in the present study the effects of angiotensin II on ATP rather than noradrenaline release have been investigated. The effects of angiotensin II on purinergic transmission when the noradrenaline stores of sympathetic nerves were depleted by reserpine were also investigated.

Methods

General

Male Duncan-Hartley guinea-pigs (250–350 g) were killed by a blow to the head and bled. Vasa deferentia and the associated

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hypogastric nerve trunks were removed and placed in Krebs solution of the following composition (mM): NaCl 118, KCl 4.7, NaH_2PO_4 0.4, $NaHCO_3$ 25.0, $MgCl_2$ 1.2, $CaCl_2$ 1.8 and glucose 11.1. The Krebs solution was continuously bubbled with 95% O_2/5% CO_2 to pH 7.4.

Reserpine pretreatment

Guinea-pigs were given a single intraperitoneal injection of reserpine (5 mg kg^{-1}) 18 h before removal of the vasa deferentia. The extent of the noradrenaline depletion was assessed by Falck-Hillarp fluorescence histochemistry or by high performance liquid chromatography (h.p.l.c.) analysis of the noradrenaline content of the vas deferens (Allcorn *et al.*, 1986). This reserpine pretreatment protocol routinely produced depletion of catecholamines below detectable levels.

Extracellular recording

Vasa deferentia were pinned out on the Sylgard (Dow-Corning) covered base of a 4 ml organ bath and the outer smooth muscle layer exposed by careful removal of the surface connective tissue in a small region. The organ bath was mounted on the stage of a Zeiss ACM microscope and the tissues perfused at a rate of 2–3 ml min^{-1} with Krebs solution maintained at 36–37°C. The postganglionic sympathetic nerves innervating the vas deferens were stimulated through a suction electrode placed close to the prostatic end (Ferry, 1967; Brock & Cunnane, 1988). A digital stimulator (Applegarth Electronics) was used to deliver rectangular pulses of 0.05–0.1 ms duration and 5–20 V through an optically isolated stimulation unit (Square One Instruments).

A bevelled glass electrode (tip diameter $\leqslant 50\,\mu$m) filled with Krebs solution was positioned on the surface of the vas deferens and slight suction applied (seal resistance < 1 MΩ). A sintered Ag/AgCl pellet in the bath served as the indifferent electrode. The electrode holder was modified to enable a polyethylene cannula to be passed down the barrel of the recording electrode. In this way it was possible to perfuse (75–100 μl min^{-1}) the tip of the electrode with ordinary Krebs solution or Krebs solution containing the appropriate drugs. Approximately 5 min was required for drug solutions to reach the tip as determined by local perfusion with tetrodotoxin to block nerve impulse conduction or by the use of fluorescent dyes. Signals were amplified through an a.c. amplifier (Neurolog NL 104, low frequency cut off 0.1 Hz) and stored on a Panasonic video recorder (AG6200) after digitization (20 kHz) through a Sony digital audio processor unit (PCM-701ES).

Intracellular recording

Vasa deferentia were pinned out on the Sylgard (Dow-Corning) covered base of a 2 ml organ bath and a small window cut in the surface connective tissue in order to expose the outer smooth muscle cells. The bath was perfused at a rate of 2–3 ml min^{-1} with Krebs solution maintained at 36–37°C. In this case the sympathetic nerves were stimulated by gently pulling the hypogastric nerve trunk through a Ag/AgCl ring electrode positioned close to the prostatic end of the vas deferens. A stimulator was used to deliver rectangular pulses as described above.

Changes in membrane potential of smooth muscle cells, on or near the surface of the vas deferens, were recorded by conventional intracellular recording techniques (see Cunnane & Manchanda, 1988a). Capillary-glass micro-electrodes filled with 5 M potassium acetate (resistances 40–80 MΩ) were positioned over the vas deferens with a Leitz micromanipulator and observed through a Wild dissecting microscope. A sintered Ag/AgCl pellet in the bath served as the indifferent electrode. Signals were amplified through a d.c. amplifier (Neurolog NL 102), digitized and stored as described for extracellular recording.

Analysis

A system based on a Zenith.386 microcomputer and an analogue-to-digital converter card (Data Translation – DT2801A) was used to analyse data previously stored on tape. Records were filtered (d.c. – 3 kHz, Neurolog NL 125) and amplified (Neurolog NL 105) as required for analysis by the computer programme SCAN and DVIEW kindly supplied by John Dempster (Strathclyde University).

A paired t test was used to compare data within individual experiments because of the variability in the amplitude and number of events which occurred from attachment to attachment. This variability arises from such factors as the seal resistance of the electrode, the number of functional varicosities in each attachment and their relationship to individual smooth muscle cells in a three dimensional functional syncytium.

Drugs

Angiotensin II (Sigma) and saralasin (Sigma) were prepared as 10^{-3} M stock solutions in distilled water, frozen as 100 μl aliquots, and serially diluted in Krebs solution as required. Reserpine (Sigma) was dissolved in 200 mg ml^{-1} ascorbic acid immediately prior to use. Indomethacin (Sigma) was dissolved in 0.1 M Na_2CO_3 and used immediately.

Results

Effects of angiotensin II on electrical activity recorded with a focal extracellular electrode

The pattern of electrical activity recorded when a focal extracellular electrode is placed on the surface of the vas deferens is shown in the left panel of Figure 1. Following electrical stimulation of the hypogastric nerve trunk with trains of 25 stimuli at 1 Hz, stimulus locked excitatory junction currents (e.j.cs) are evoked which are preceded by a non-intermittent nerve impulse. The e.j.c. is the response of the smooth muscle to evoked quantal release of transmitter and the preceding bi- or tri-phasic spike is the extracellular equivalent of the action potential in the nerve terminals A detailed account of the intermittent nature of the transmitter release mechanism has been given elsewhere (Brock & Cunnane, 1987; 1988).

Control trains of 25 pulses at 1 Hz were delivered every 2 min until a relatively stable pattern of release was obtained, 10–15 trains usually being sufficient. When angiotensin II (10–100 nM) was perfused into the tip of the recording electrode for 25 min (12 trains) a clear enhancement of evoked electrical activity was recorded. Representative trains of 25 pulses at 1 Hz from an experiment in which 30 nM angiotensin II was applied are shown in Figure 1. To improve the signal to noise ratio, the traces were averaged (Figure 1b) and the records show that angiotensin II does not produce any detectable change in the configuration of the nerve terminal impulse, nor induce repetitive firing. The effect on transmitter release was reversed by wash.

In each experiment, negative deflections of sufficient magnitude (twice baseline noise) and duration (> 50 ms) were counted as e.j.cs and the number of e.j.cs occurring during 4 successive trains of 25 pulses at 1 Hz were summed to minimize variability between individual trains. The data fron one such experiment is shown in Figure 2 which clearly indicates that angiotensin II increased the number of e.j.cs occurring per 100 stimuli. The pooled data from 4 experiments are summarized in Table 1.

It was important to establish that there was no change in the postjunctional sensitivity of the smooth muscle membrane to angiotensin II which might have accounted for the 'apparent' increase in e.j.c. probability. In the absence of stimulation spontaneous excitatory junction currents (s.e.j.cs) are recorded, which are a measure of the spontaneous quantal release of

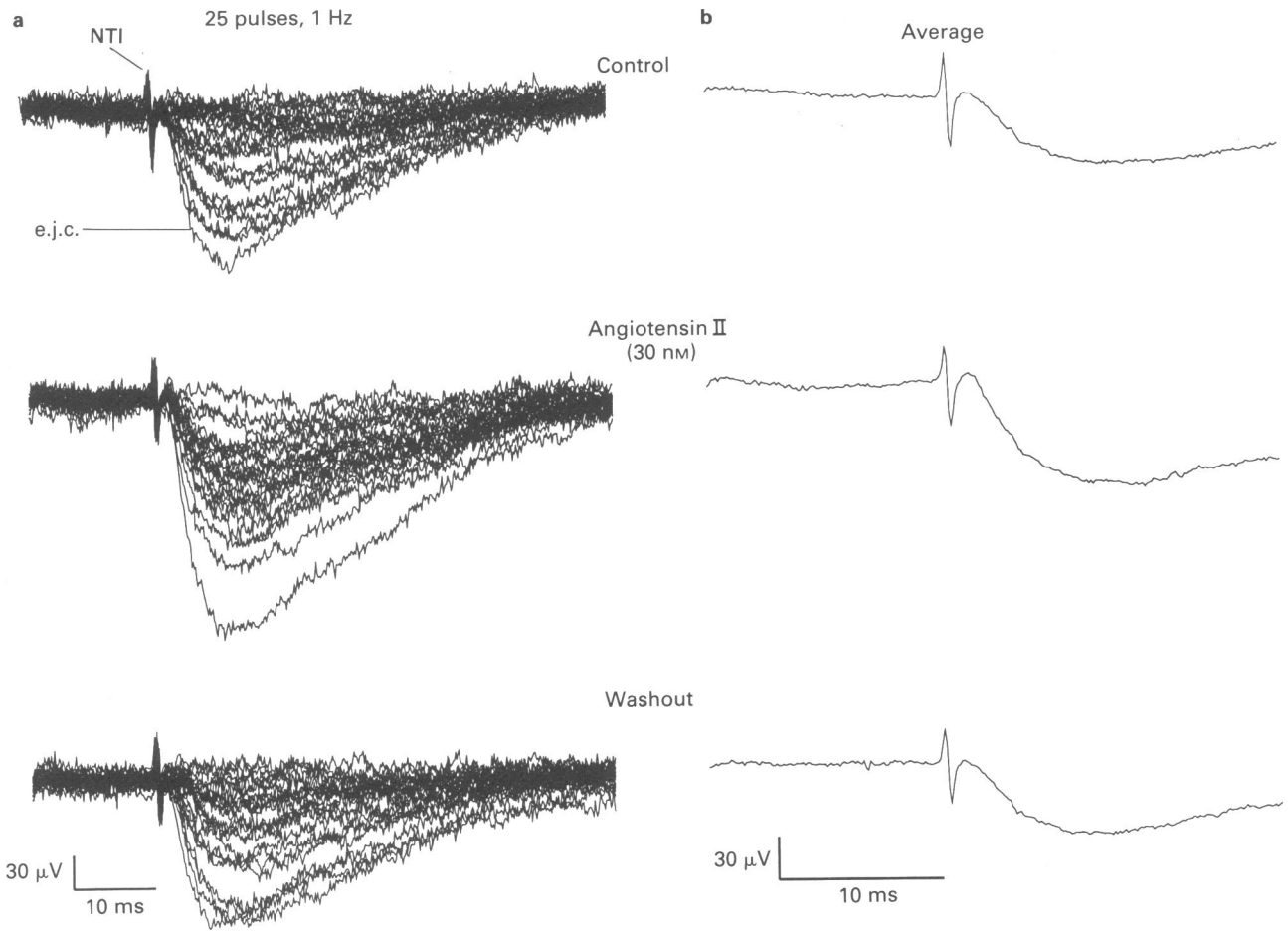

Figure 1 Effects of locally applied angiotensin II on the nerve terminal impulse and transmitter release in the guinea-pig vas deferens. (a) Nerve terminal impulse (NTI) and excitatory junction currents (e.j.cs) evoked by 25 pulses at 1 Hz, before, during 20 min perfusion with angiotensin II (30 nM) and 30 min after wash. Single attachment (b). Averages of the records shown in (a).

transmitter. The amplitude distributions of e.j.cs and s.e.j.cs obtained in the absence and presence of angiotensin II in the same attachment are compared in Figure 3. The e.j.cs evoked by the last 8 control trains of 25 pulses at 1 Hz (200 pulses) before the addition of angiotensin II were analyzed together with the last eight trains of stimuli in the presence of angiotensin II. S.e.j.cs were counted for periods of 5 min before and after the application of angiotensin II. In the absence of angiotensin II, both the e.j.c. and s.e.j.c. amplitude distributions were skewed, a characteristic which makes interpretation of frequency and amplitude changes less certain. Nevertheless, it is clear that angiotensin II increased the number of evoked e.j.cs per train of stimuli and shifted the amplitude distribution to the right, and occasional e.j.cs larger than those seen in the absence of angiotensin II were observed. In contrast, the amplitude distribution of s.e.j.cs was largely unaffected by angiotensin II. These changes indicate that angiotensin II increases the probability of evoked transmitter release.

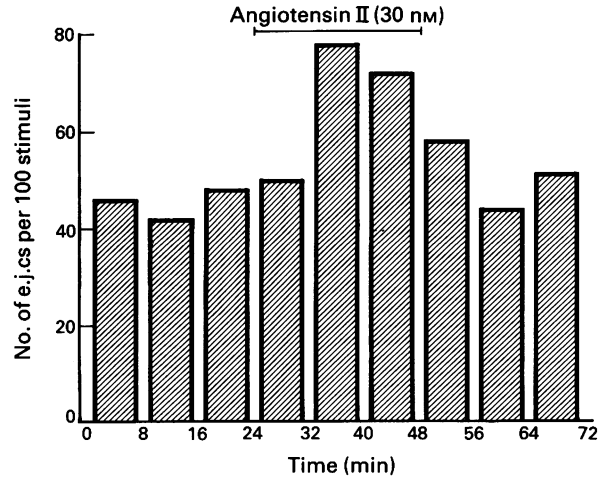

Figure 2 Effects of local application of angiotensin II on electrically evoked transmitter release in the guinea-pig vas deferens. The histogram shows the number of e.j.cs recorded during 4 successive trains of 25 pulses at 1 Hz before, during local perfusion with angiotensin II (30 nM) and after wash in the same attachment.

Effects of saralasin

In three experiments, the angiotensin II receptor antagonist saralasin (100 nM) was added to both the Krebs solution perfusing the recording electrode and the organ bath. Saralasin alone did not significantly alter the configuration of the nerve terminal impulse or the pattern of transmitter release. It should be noted that on one occasion, an increase in transmitter release was observed. This finding was not rigourously pursued in the present study and may be due to the partial agonist properties of saralasin (Ziogas *et al.*, 1984). The local application of 30 nM angiotensin II in the presence of saralasin did not now enhance transmitter release (Table 1).

Effect of angiotensin II on extracellular activity recorded in reserpinized vasa deferentia

Since ATP and not noradrenaline probably mediates the e.j.c. (Brock & Cunnane, 1988) it was of interest to study the effects of angiotensin II in catecholamine-depleted vasa deferentia. In an earlier study, the noradrenaline content of untreated vasa deferentia was $10.1 \pm 1.1\,\mu g\,g^{-1}$ wet weight ($n = 6$) (Cunnane

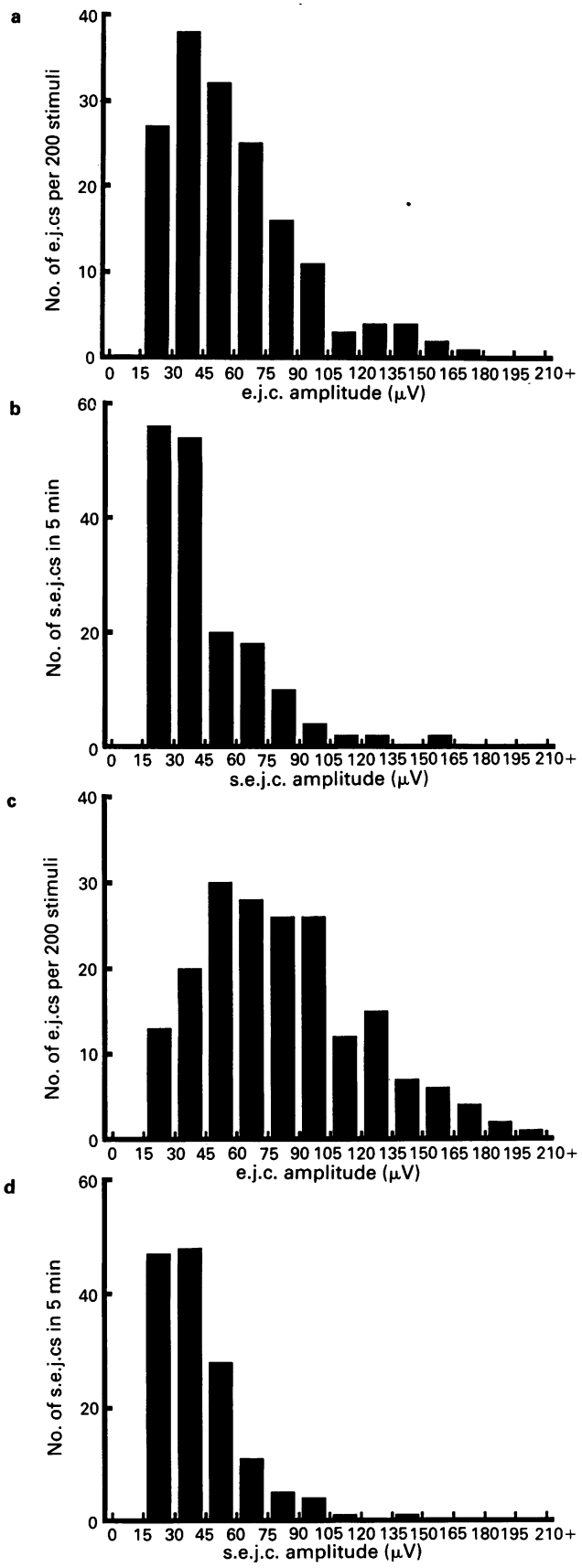

Figure 3 Effect of angiotensin II on the amplitude distribution of s.e.j.cs and e.j.cs recorded in the same attachment. (a) Histograms showing the amplitude distribution of e.j.cs recorded in 8 consecutive trains of 25 pulses at 1 Hz before (left panel) and during the addition (10–24 min) of angiotensin II (30 nM) (right panel). Events less than 15 μV were not discernible from the noise level and were classified as failures of tramsmitter release. (b) Histograms showing the amplitude distribution of s.e.j.cs recorded in a 5 min period before and during local perfusion with angiotensin II (30 nM).

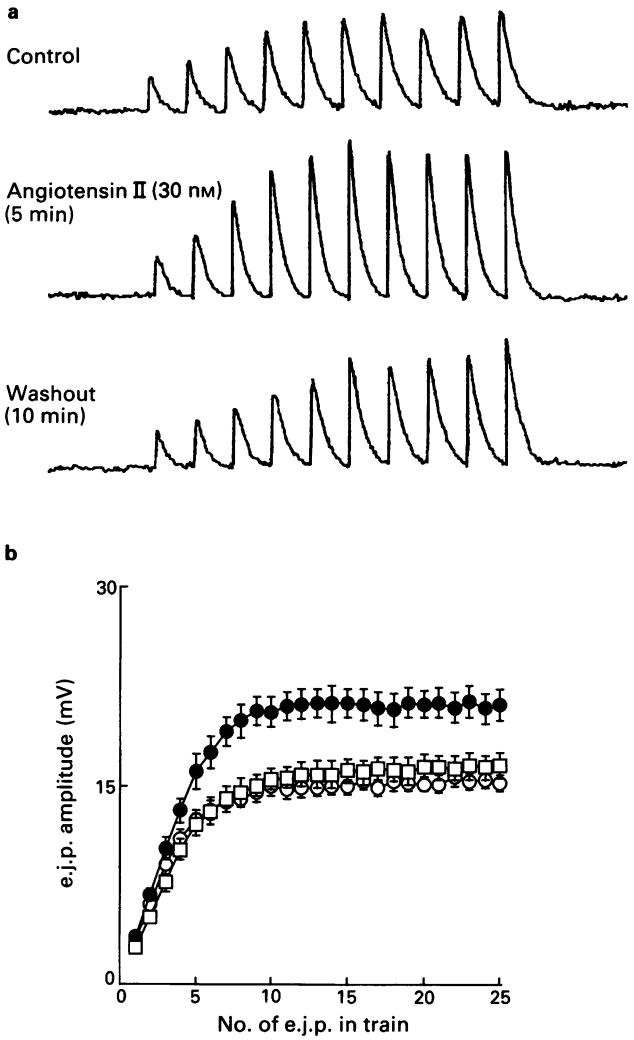

Figure 4 Effects of angiotensin II on e.j.p. amplitude in the guinea-pig vas deferens. (a) E.j.ps evoked by trains of 10 pulses at 1 Hz before and during the addition of angiotensin II (30 nM) in a single cell. (b) The effects of angiotensin II on a population of cells ($n \geqslant 5$ for each group) recorded in single vas deferens before (○), during the application of angiotensin II (10 nM) (●) and after wash (□).

et al., 1989). In the present study, pretreatment of guinea-pigs with reserpine reduced the noradrenaline content of vasa deferentia to undetectable levels by either h.p.l.c. analysis or by Falck-Hillarp fluorescence histochemistry. Spontaneous and evoked electrical activity was recorded in vasa deferentia taken from animals pretreated with reserpine. Surprisingly, in reserpinized tissues, when angiotensin II was applied locally there was no overall increase in the probability of occurrence of e.j.cs (Table 1). Table 1 shows the pooled data from three experiments in which an inhibitor of prostaglandin synthesis, indomethacin (3 μM) was present. Indomethacin alone did not significantly affect transmitter release. However, in the presence of indomethacin, angiotensin II enhanced transmitter release in a manner analogous to that in non-reserpinized vasa. That is, angiotensin II increased the number of e.j.cs evoked per train of stimuli without affecting the configuration of the nerve terminal impulse. In untreated vasa deferentia the average increase in e.j.c. number was 51 $\pm$ 8.6% ($n = 4$) and in indomethacin-treated reserpinized vas deferentia the increase was 44.7 $\pm$ 10.5% ($n = 3$).

Effect of angiotensin II on electrical activity recorded with intracellular electrodes

The resting membrane potential of individual smooth muscle cells lay in the range −65 to −75 mV. Figure 4 shows the

Table 1 The effect of angiotensin II on the number of e.j.cs evoked when trains of 25 pulses at 1 Hz were given at 2 min intervals in untreated and reserpinized vasa deferentia

			Number of e.j.cs/100 pulses		
		n	*Pre-AII*	*AII* (30 nM)	*Mean difference*
Untreated vasa deferentia	Control	4	50 ± 9	74 ± 10	24 ± 4*
	Saralasin (100 nM)	3	58 ± 12	55 ± 12	−3 ± 6
Reserpine-treated vasa deferentia	Control	4	36 ± 8	39 ± 7	3 ± 3
	Indomethacin (3 μM)	3	37 ± 8	54 ± 9	16 ± 4*

Four successive trains were summed to minimize variability between trains (Number of e.j.cs/100 pulses). *Significantly different from pre-angiotensin II (AII) values ($P < 0.05$ paired t test).

characteristic pattern of facilitation of e.j.ps evoked by a train of 10 stimuli at 1 Hz. E.j.ps increased in amplitude during the train until a constant amplitude was reached after about 5–10 stimuli. As previously reported (Bell, 1972; Cunnane *et al.*, 1989), there was no clear effect of angiotensin II on the first e.j.p. in a train but subsequent e.j.ps were enhanced (Figure 4). The resting membrane potential and spontaneous excitatory junction potentials (s.e.j.ps) were unaffected by angiotensin II (not shown).

Effect of angiotensin II on intracellularly recorded activity in reserpinizied vasa deferentia

In reserpinized vasa deferentia the resting membrane potential was the same as in untreated tissues (65–75 mV) and e.j.ps of normal amplitude were recorded during trains of stimuli at 1 Hz. However, the pattern of facilitation was prolonged compared with untreated vasa deferentia (Figure 4 cf. Figure 5), presumably because of removal of α_2-adrenoceptor autoinhibition (Brock *et al.*, 1990). Angiotensin II (30 nM) produced variable effects on e.j.p. amplitude in reserpinized vasa deferentia. The predominant effect of angiotensin II was to depress e.j.p. amplitude ($n = 4$). Figure 5a shows the effects of angiotensin II (30 nM) on e.j.ps recorded in a single cell. The effects of angiotensin II on the mean e.j.p. amplitude measured in a population of cells is shown in the left panel of Figure 5b. In 4 experiments when indomethacin (3 μM) was present in the bathing solution, angiotensin II always enhanced e.j.p. amplitude. The results of an individual experiment in which the effects of angiotensin II were assessed in a population of cells, first in the absence and then in the presence of indomethacin are shown in Figure 5b. The initial inhibition of e.j.ps produced by angiotensin II in reserpinized tissues was clearly reversed to an enhancement in the presence of indomethacin, suggesting that prostaglandins are involved in transjunctional modulation of purinergic transmission. It should be noted that the enhancement of transmitter release produced by angiotensin II in untreated vasa deferentia was observed once and no effect of angiotensin II was observed on another occasion, in experiments with reserpinized vasa deferentia.

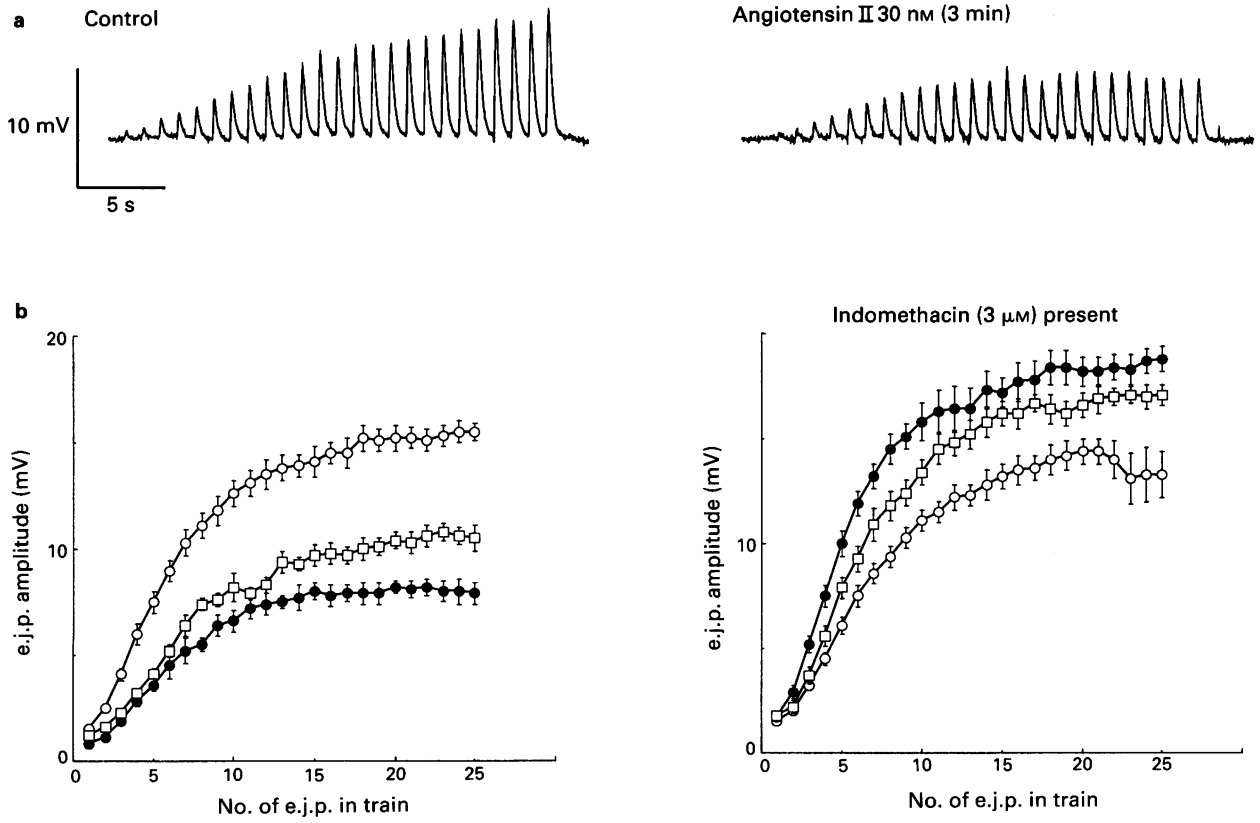

Figure 5 Inhibition of e.j.p. amplitude by angiotensin II in vasa deferentia taken from reserpine pretreated guinea-pigs. (a) Single cell showing the rapid inhibition of e.j.ps by angiotensin II (30 nM). (b) Effect of angiotensin II (30 nM) on a population of cells in the same preparation. The effects of angiotensin II are shown in the absence (left panel) and in the presence (right panel) of indomethacin (3 μM) ($n \geq 4$ cells for each group). Control (○); angiotensin II (●); after washout (□). It is clear that angiotensin II reduces e.j.p. amplitude in reserpinized vasa deferentia and that indomethacin treatment reverses this inhibition to an increase.

Discussion

The finding that angiotensin II increased the amplitude of e.j.ps evoked by trains of stimuli in the guinea-pig vas deferens agrees with the earlier report of Bell (1972). Angiotensin II enhanced e.j.ps without affecting the amplitude or frequency of occurrence of s.e.j.ps which indicates a prejunctional site of action. Surprisingly, angiotensin II did not increase the amplitude of the first e.j.p. in a train of stimuli at 1 Hz, a result not found with other agonists which alter transmitter release. For example, clonidine which inhibits (Blakeley *et al.*, 1981), and tetraethylammonium which enhances (Stjärne *et al.*, 1990) e.j.p. amplitude each have marked effects on the first e.j.p. in a train. It would seem that the varicosity has first to be invaded by a nerve impulse before angiotensin II can enhance release to subsequent stimuli. Similar conclusions can be drawn from the studies with focal extracellular electrodes in which the relationship between the arrival of the nerve terminal impulse and transmitter release was examined. The number of e.j.cs recorded per train of stimuli was increased in the presence of angiotensin II with no marked effect on the configuration of the nerve terminal impulse or without the induction of repetitive firing. Once again, there was no obvious effect on the e.j.cs evoked by the first pulse in a train, even when many trains of stimuli were averaged to minimise variability because of intermittence. The enhancing effect on transmitter release of angiotensin II was blocked by the angiotensin II receptor antagonist saralasin which supports the idea that activation of prejunctional angiotensin II receptors modifies the mechanisms involved in depolarization-secretion coupling.

Starke (1977) concluded that the prejunctional action of angiotensin II involves an increase in the amount of transmitter released per nerve impulse. Based on the assumption that every varicosity released transmitter on stimulation, the enhancement by angiotensin II would require an increase in the amount of transmitter released from each varicosity. It is now evident that transmitter release from individual varicosities in response to trains of low frequency stimuli is intermittent and monoquantal (see Introduction). Therefore, other mechanisms have to be considered to explain the facilitatory effects of angiotensin II on transmitter release mechanisms. Transmitter release may be enhanced by increasing the probability of release from active varicosities or by recruiting previously silent varicosities. Angiotensin II increased the number of e.j.cs recorded per train of stimuli and produced a shift to the right of the amplitude distribution of e.j.cs. If angiotensin II was solely affecting the quantal content of the same number of active varicosities, a shift to the right of the amplitude distribution without a change in the number of e.j.cs would have been expected. Caution needs to be exercised when interpreting changes in shape of skewed distributions, but it is worth noting that the effects of angiotensin II are similar to the effects of increasing the frequency of stimulation (Brock & Cunnane, 1988) or removing α_2-autoinhibition (Brock *et al.*, 1990), procedures which also increase transmitter release.

E.j.ps and e.j.cs were also recorded from reserpinized vasa deferentia suggesting that the transmitter responsible for the generation of the e.j.p. and the e.j.c. is not noradrenaline. Furthermore e.j.ps and e.j.cs are abolished by α,β-methylene ATP which desensitizes P_2-purinoceptors and can be mimicked by the local application of ATP (Brock & Cunnane, 1988; Cunnane & Manchanda, 1988a). ATP is likely to be the transmitter generating the e.j.p. in the vas deferens and is thought to be co-released with noradrenaline from sympathetic nerve terminals (Burnstock, 1986; White, 1988).

A surprising finding in the present study was that following reserpine pretreatment angiotensin II did not significantly enhance e.j.ps or e.j.cs in the guinea-pig vas deferens. Rather, an inhibitory effect on evoked transmitter release was the predominant effect. This inhibitory effect of angiotensin II in reserpinized tissues was reversed to an enhancement by indomethacin treatment suggesting an increased role for stimulation of prostaglandin production by angiotensin II in noradrenaline-depleted preparations. Previous studies indicate that angiotensin II may stimulate prostaglandin synthesis. In tissues such as the, rat mesenteric bed (Jackson & Campbell, 1981), rat heart (Lanier & Malik, 1982) and dog heart (Lanier & Malik, 1983), stimulation of prostaglandin production by angiotensin II has an opposing inhibitory effect on sympathetic noradrenergic transmission, similar to that reported in the present study for sympathetic purinergic transmission. An inhibitory effect of angiotensin II on purinergic transmission has previously been reported. In the rabbit vas deferens, angiotensin II and angiotensin III inhibit the initial purinergic contraction evoked by electrical stimulation, an effect which is also reversed by inhibition of prostaglandin synthesis (Saye *et al.*, 1986; Trachte, 1988).

Interestingly, in the rabbit vas deferens angiotensin II and angiotensin III inhibited the purinergic but enhanced the noradrenergic component of contraction evoked by electrical stimulation. These effects were presumed to be prejunctional since the responses to exogenous noradrenaline and ATP were unaffected by the angiotensins. It has been suggested that this could represent indirect evidence that ATP and noradrenaline are stored and released from different locations in sympathetic nerve terminals (Trachte *et al.*, 1989). This suggestion was also made by Ellis & Burnstock (1989) on the basis of the findings that angiotensin II and angiotensin III could differentially modulate [^{3}H]-noradrenaline release and ATP release as determined by the luciferen-luciferase technique. In the present inquiry it is clear that, when neuronal noradrenaline stores are depleted by reserpine pretreatment, the predominant effect of angiotensin II is inhibition of purinergic transmission. It is not possible to draw any definitive conclusions from the present data about the question of co-storage and co-release of noradrenaline and ATP.

In conclusion, our electrophysiological studies indicate that at low frequencies of stimulation, angiotensin II enhances purinergic transmission. Angiotensin II increased the probability of release from individual varicosities which normally release transmitter intermittently. This prejunctional action of angiotensin II does not involve a change in the configuration of the nerve terminal impulse and is likely to involve changes in the intracellular processes activated by depolarization. In addition, our findings show that agents which modulate transmitter release by activating prejunctional receptors can have additional postjunctional actions which may become important after certain drug treatments or in specific pathophysiological conditions. Thus, following reserpine pretreatment, angiotensin II inhibited rather than enhanced purinergic transmission presumably by triggering prostaglandin synthesis in the smooth muscle which then transjunctionally inhibited transmitter release. Several questions remain unanswered including: (1) whether depletion of noradrenaline from the sympathetic nerve terminals *per se* or an effect of reserpine on smooth muscle cells produces the increased involvement of prostaglandins as transjunctional modulators of transmitter release; and (2) whether the pharmacological profile of the pre- and postjunctional angiotensin II receptor is different.

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Increased flow-induced ATP release from isolated vascular endothelial cells but not smooth muscle cells

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1 Freshly harvested smooth muscle cells and endothelial cells isolated from the rabbit aorta were perfused (0.5 ml min^{-1}) and stimulated twice by an increase of flow rate (3.0 ml min^{-1}) in order to compare their ability to release adenosine 5'-triphosphate (ATP).

2 In smooth muscle cells, the basal release of ATP (0.0265 ± 0.0033 pmol ml^{-1} per 10^6 cells) was not increased during periods of increased flow ($P = 0.2$).

3 In endothelial cells, the concentration of ATP in the perfusate during periods of low flow (0.0335 ± 0.0038 pmol ml^{-1} per 10^6 cells) was significantly increased by 14 times and 5 times during the first and second periods of increased flow, respectively.

4 The release of ATP by endothelial cells did not appear to be caused by the lysis of cells during the period of increased flow because it can be reproduced several times and because there was no difference between lactate dehydrogenase activity in perfused cells and that in non-perfused cells.

5 These results show that, of the two major cell types of the vascular wall, only endothelial cells react to shear stress by releasing ATP.

Keywords: ATP; aorta; endothelial cells; smooth muscle cells; ATP release

Introduction

Adenosine triphosphate (ATP) is a transmitter released upon nerve stimulation of a large variety of sympathetically innervated tissues, including blood vessels (Burnstock *et al.*, 1970; Su, 1975; White, 1988). Upon vascular wall stimulation, there is an overflow of ATP that includes release from both neuronal as well as extraneuronal sites (Levitt & Westfall, 1982; Westfall *et al.*, 1987). Although ATP is not taken up by endothelial cells and smooth muscle cells, these vascular cells incorporate adenosine which is rapidly converted into ATP (Su, 1975; Pearson *et al.*, 1978; Pearson & Gordon, 1979). In this way, it seems natural to consider the smooth muscle cells and the endothelial cells of the vascular wall as a likely postjunctional source of ATP.

Because postjunctional release of ATP from vessels can occur in conditions such as hypoxia (Paddle & Burnstock, 1974) it might play an important role in some pathophysiological states. Recently, it has been shown that isolated segments of rabbit aorta submitted to transmural stimulation or exposed to α_1-adrenoceptor agonists released ATP. Endothelial cells were mainly responsible for this release (Sedaa *et al.*, 1990). During shear stress induced by fast flow, endothelial cells from different vascular beds have also been shown to release ATP (Milner *et al.*, 1990a,b) but it is not known if vascular smooth muscle cells express the same property in these experimental conditions.

In the present study, we investigated the release of ATP by smooth muscle cells that were freshly isolated from the rabbit aorta and submitted to increased flow. The results were compared with the release of ATP from endothelial cells isolated from the same animals.

Methods

Cell cultures

Endothelial and smooth muscle cells were obtained from the thoracic aorta of 12-month-old male rabbits (New Zealand strain). Vessels were dissected from heart to diaphragm under sterile conditions. The aortae were placed into cold (4°C) Hanks medium. Connective tissue was removed and the aortae were everted by use of a glass Pasteur pipette with a hook at the end. The everted vessels were incubated for 10 min in M199 medium at 37°C, then pulled onto the end of a 1 ml plastic pipette and incubated for 5 min at 37°C in 0.1% crude collagenase. Endothelial cells were dislodged with a stream of M199 medium supplemented with 10% foetal calf serum and centrifuged (180 *g* for 10 min).

The aorta was cut longitudinally and incubated in a solution of collagenase and elastase (1 mg ml^{-1} and 0.25 mg ml^{-1} respectively) for 40 min. The adventitia was removed and the remaining muscle medial layer was split into rings with forceps. The tissue was then incubated for 2 h in collagenase-elastase (1 mg ml^{-1} and 0.5 mg ml^{-1} respectively). Smooth muscle cells were suspended in M199 supplemented with 10% serum and then centrifuged (180 *g* for 10 min).

After harvesting, endothelial and smooth muscle cells were resuspended in M199 supplemented with 10% foetal calf serum, placed in a 25 mm diameter filter holder containing a 3 μm pore size Millipore filter (MF Millipore membrane, type SS, Millipore, U.S.A.) and allowed to equilibrate for 2 h at 37°C in a 5% CO_2 atmosphere.

Release experiments

Cells were perfused through the Millipore filter at low flow rate (0.5 ml min^{-1}) with oxygenated Krebs buffer at 37°C (composition (mM): NaCl 122, KCl 5.2, $CaCl_2$ 2.4, $MgSO_4$ 1.2, $NaHCO_3$ 25.6, KH_2PO_4 1.2, Na_2EDTA 0.03 and glucose 11) using a variable peristaltic pump (LKB 12000 varioperspex). After a 15 min equilibration, the perfusate was collected every 30 s for 32 min. During the time course of the experiment, cells were stimulated twice by a high flow rate (3.0 ml min^{-1}) for 3 min.

Determination of the number of cells

The protein content of endothelial cells was determined after an overnight incubation of the filter at 4°C in a sterile solution of 0.1% Triton X100 in phosphate-buffered saline according to the method developed by Bradford (1976). The number of cells was estimated by comparing the given value with a standard curve correlating the protein content of the cells with the number of released nuclei obtained after treatment of the

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filters with a hypotonic solution of citric acid containing 0.1% (w/v) crystal violet (Van Wezel, 1973). The number of smooth muscle cells was estimated, prior to the experiment, by counting.

Lactate dehydrogenase (LDH) activity measurements

Endothelial cells from a rabbit aorta were placed on two filters. One filter was processed for perfusion and the other was incubated in oxygenated Krebs solution. After the experiment, the two filters were incubated in 1 ml of a solution of 0.1% Triton X100 for 2 h at 20°C and homogenized; 0.5 ml of the homogenate was then incubated at 37°C in 1 ml of phosphate-buffered saline containing pyruvate (0.75 mM) and NADH (1.28 mM). After 30 min, 1 ml colour reagent was added and the filters were incubated for 20 min at 20°C; 5 ml of 0.4 M NaOH was then added to each sample. Absorbance was read at 442 nm with a spectrophotometer. LDH activity in the Krebs perfusate (0.5 ml samples) was quantified in the same way.

ATP measurements

After the experiment, 100 μl of the perfusate from each fraction was transferred into polypropylene tubes and left at 4°C for 2 h. ATP quantification was performed on a Packard luminometer by addition of 200 μl of luciferase-luciferin (3.33 mg ml^{-1}) to the sample as previously described (Kirkpatrick & Burnstock, 1987).

Drugs

M199 medium and Hanks balanced salt solution were from Gibco, U.K. Foetal calf serum was from Flow Laboratories, U.K. Collagenase (crude) was from Boehringer-Mannheim, West Germany. Elastase (Type III), luciferase-luciferin and the LDH colour reagent were from Sigma, U.S.A.

Statistics

Results are expressed as pmol of ATP released per ml per million cells and are presented as mean values ± s.e.mean. Results were analysed and compared by a Wilcoxon-Mann-Whitney test and interpreted as significantly different at $P < 0.005$. n refers to the number of experiments performed.

Results

Figure 1 shows results on release of ATP from endothelial and smooth muscle cells during periods of increased flow. During low flow rate (0.5 ml min^{-1}), ATP is released from both endothelial (Figure 1a) and smooth muscle cells (Figure 1b). This basal release (first 5 min) is not significantly different ($P = 0.08$) in endothelial cells (0.0335 ± 0.0038 pmol ml^{-1} per 10^6 cells) and in smooth muscle cells (0.0265 ± 0.0033 pmol ml^{-1} per 10^6 cells). During periods of increased flow rate (3.0 ml min^{-1}), ATP release from endothelial cells was rapidly and significantly increased. This release of ATP was significantly greater during the first period of increased flow (0.4662 ± 0.0435 pmol ml^{-1} per 10^6 cells) than in the second one (0.16375 ± 0.0192 pmol ml^{-1} per 10^6 cells). In smooth muscle cells the amounts of ATP released during the first (0.0333 ± 0.0023 pmol ml^{-1} per 10^6 cells) and second periods of stimulation (0.0280 ± 0.0024 pmol ml^{-1} per 10^6 cells) were not significantly different ($P = 0.2$) from the basal release and were not significantly different ($P = 0.1$) from each other.

Figure 2 represents the effects of three consecutive periods of increased flow on the release of ATP by freshly isolated endothelial cells. The basal release of ATP (first 5 min, 0.0278 ± 0.0028 pmol ml^{-1} per 10^6 cells) was significantly increased during these three periods. However, the amount of

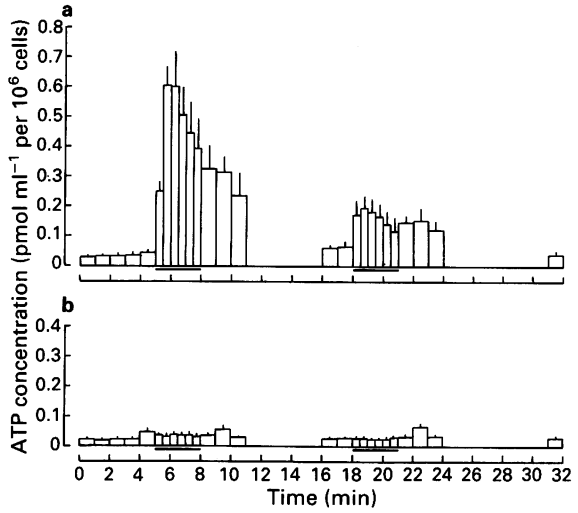

Figure 1 ATP release in (a) endothelial cells and (b) smooth muscle cells freshly isolated from rabbit thoracic aorta. Periods of increased flow (3 ml min^{-1}) are indicated by a horizontal bar. Results are expressed as picomol of ATP released ml^{-1} per 10^6 cells. $n = 4$.

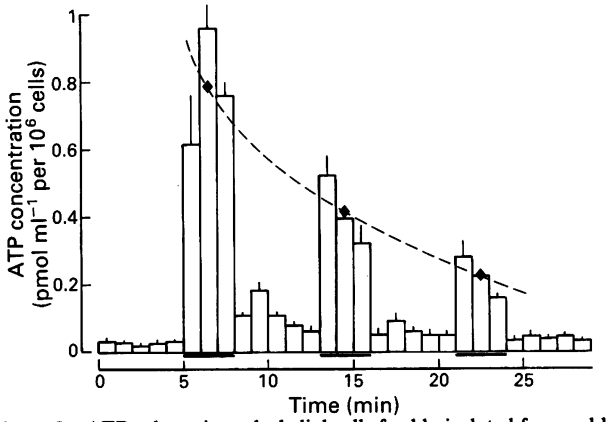

Figure 2 ATP release in endothelial cells freshly isolated from rabbit thoracic aorta. Periods of increased flow (3 ml min^{-1}) shown by a horizontal bar. (◆) Mean of ATP released during stimulation; (– – –) exponential regression. $n = 3$. Results are expressed as picomol of ATP released ml^{-1} per 10^6 cells.

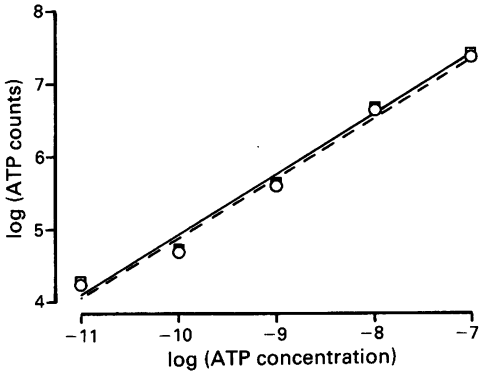

Figure 3 Activity of exonucleases. Endothelial cells were perfused at low flow rate (0.3 ml min^{-1}) for 5 min with Krebs solution containing increasing concentrations of ATP. Continuous line: ATP concentration in Krebs solution before passage over the cells; broken line: ATP concentrations in Krebs solution after passage over the cells. $n = 3$.

ATP released during the first stimulation was greater than that released during the second, which itself was greater than the amount released during the third stimulation. Statistical analysis showed that this decrease was exponential (correlation coefficient = 0.999).

In order to assess the activity of exonucleases, endothelial cells were perfused at low flow rate (0.3 ml min^{-1}) in the presence of different concentrations of ATP in the Krebs buffer. Results, presented on Figure 3, show that ATP concentration

is not significantly different (−2%) in the Krebs buffer collected before perfusion of the cells from that in the buffer collected after passage over the cells.

LDH activity was measured in the perfusate (Krebs buffer) and was not detectable at fast flow or low flow rate. LDH activity measured after treatment of endothelial cells on the filter with Triton X100 was slightly smaller (95.6 ± 1.2%, $n = 4$) in perfused cells than in control cells (100%).

Discussion

In this study we have shown that smooth muscle cells freshly isolated from the rabbit thoracic aorta are unable to release ATP when subjected to increased flow. In contrast, endothelial cells freshly isolated from the same vascular bed of the same animals released ATP when stimulated.

This feature is unlikely to be due to the different enzymatic treatments of the two cell types, given that the same differences have been observed in preliminary experiments on cultured endothelial and smooth muscle cells (data not shown). The release of ATP by freshly isolated endothelial cells during periods of increased flow does not seem to result from cell lysis since release of this agent may be reproduced several times and since the lactate dehydrogenase activity is not detectable in the perfusate and only slightly reduced (−4.4%) after the experiment, in perfused cells. It is also improbable that this release results from non-specific mechanisms since vasopressin, which is present in the cells, is not released during periods of fast flow (Milner *et al.*, 1990a).

There was a larger amount of ATP released by endothelial cells during the first period of increased flow than during the second. In fact, successive stimulations of endothelial cells by fast flow showed that the decrease in the release of ATP was exponential. The main source of ATP is adenosine (Pearson *et al.*, 1978). Since there is no adenosine in the Krebs perfusion medium, the concentration of this substrate may be limiting so that the synthesis of ATP during the experiment is reduced. This may, at least partly, explain this decrease.

ATP is released by nerve varicosities, hypoxic erythrocytes (Bergfeld & Forester, 1989) and aggregating blood platelets. The vascular receptors mediating responses to ATP have been distinguished into two subtypes: P_{2X}-purinoceptors are located on smooth muscle cells and mediate vasoconstriction, P_{2Y}-purinoceptors are present on endothelial cells and mediate vasodilatation (Burnstock & Kennedy, 1985). These opposing P_2-purinoceptor responses can occur in the same tissue, as has been demonstrated, for example, in the aorta (White *et al.*, 1985) and coronary arteries (Houston *et al.*, 1987). In this way, ATP released by nerves is able to generate a vasoconstriction acting directly on smooth muscle cells via P_{2X}-purinoceptors. On the other hand, ATP released by platelets or by erythrocytes provokes the relaxation of the vessel via the release of endothelium-derived relaxing factor (EDRF) acting on P_{2Y}-purinoceptors on endothelial cells.

An increase in blood flow provokes a vasodilatation and this is not due to passive distension of the vessel wall (Hull *et al.*, 1986). After removal of the endothelium, vessels do not dilate to increased flow. It has already been shown that this mechanical stimulation acts by releasing EDRF from the endothelium (Hull *et al.*, 1986). Our results indicate that during periods of fast flow. ATP is released by endothelial cells but not by smooth muscle cells. This suggests that, of these two major cell types in the vascular wall, it is only endothelial cells which react to shear stress by releasing ATP.

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Endothelium-dependent and endothelium-independent vasodilatation of the hepatic artery of the rabbit

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1 The isolated hepatic artery of the rabbit contracted to exogenously applied noradrenaline (NA). There was no significant difference in the maximal contraction or the EC_{50} value in vessels where the endothelium was present and in endothelium-denuded preparations.

2 Acetylcholine (ACh) induced a vasodilatation of vessels preconstricted with NA which was entirely dependent on the endothelium.

3 Adenosine 5'-triphosphate (ATP), 2-methylthio ATP, adenosine and sodium nitroprusside induced concentration-dependent, sustained relaxations of vessels in which tone had been induced with NA. The relaxation responses were not reduced after removal of the endothelium. 8-Phenyltheophylline antagonized the relaxation response produced by adenosine, but not that due to ATP at lower concentrations. The maximum response to ATP was reduced in the presence of 8-phenyltheophylline.

4 α,β-Methylene ATP produced further contraction of vessels preconstricted with NA in both endothelium-denuded preparations and in vessels where the endothelium remained intact.

5 Immunohistochemical analysis was used to show the presence of nerve fibres containing substance P (SP), calcitonin gene-related peptide (CGRP) and vasoactive intestinal polypeptide (VIP) in the hepatic artery. Application of SP induced a concentration-dependent relaxation which was entirely dependent on the presence of an intact endothelium. CGRP and VIP, however, elicited concentration-dependent relaxations which were independent of the endothelium.

7 It is concluded that in the rabbit hepatic artery, responses to ACh are dependent on the presence of intact endothelium. P_1-, P_{2x}- and P_{2y}-purinoceptors, mediating relaxation to adenosine, vasoconstriction to ATP and vasodilatation to ATP respectively, are located on vascular smooth muscle. Furthermore, CGRP and VIP mediate a direct vasodilatation of smooth muscle both in the absence and the presence of the endothelium, whereas SP produces a relaxation via receptors located on the endothelium.

Keywords: Hepatic artery; endothelium; vasodilatation

Introduction

The involvement of sympathetic noradrenaline (NA)-containing nerves in the control of vascular smooth muscle is well known (Bevan *et al.*, 1980). During recent years, the results from several studies suggest that perivascular nerves of man and other mammals also contain regulatory substances other than NA; these include purines and peptides (Lundberg & Hökfelt, 1983; Ganten *et al.*, 1984; Burnstock, 1988). Purine nucleosides and nucleotides have been shown to have widespread vascular actions (Drury & Szent-Györgyi, 1929; Burnstock & Brown, 1981). In 1978, Burnstock proposed that the receptors mediating the responses to purines should be categorized as P_1- and P_2-purinoceptors, with selectivity for adenosine and adenosine 5'-triphosphate (ATP) respectively. ATP can be released as a cotransmitter with NA from sympathetic nerves and acts on a P_{2x}-purinoceptor subtype on vascular smooth muscle to produce a contraction (Burnstock & Kennedy, 1985); this has been demonstrated in both the hepatic and the saphenous artery of the rabbit (Burnstock & Warland, 1987; Brizzolara & Burnstock, 1990). In addition, since the discovery that relaxation to acetylcholine (ACh) in a number of vessels is dependent on the presence of an intact endothelium (Furchgott & Zawadzki, 1980; Furchgott *et al.*, 1981; De Mey & Vanhoutte, 1982; Vanhoutte & Rimele 1983; Peach *et al.*, 1985), it has been shown that, in all vessels, except the rabbit portal vein (Kennedy & Burnstock, 1985a) and the rabbit mesenteric artery (Mathieson & Burnstock, 1985), ATP induces vasodilatation wholly or partly via P_{2y}-purinoceptors located on the endothelium (De Mey & Vanhoutte, 1980; 1982; Cocks & Angus, 1983; Furchgott, 1983; Kennedy *et al.*, 1985; Burnstock & Kennedy, 1985; Liu *et al.*, 1989). In contrast, adenosine has been shown to mediate a vasodilatation via P_1-purinoceptors that is dependent on the presence of an intact endothelium in only a few cases (Gordon & Martin, 1983; Kennedy & Burnstock, 1985b).

Endothelium-independent vasodilatation is produced by several peptides including calcitonin gene-related peptide (CGRP) (Brain *et al.*, 1985; Hanko *et al.*, 1985; Uddman *et al.*, 1986; Kawasaki *et al.*, 1988; Marshall & Craig, 1988), and vasoactive intestinal polypeptide (VIP) (Hand *et al.*, 1984; Lee *et al.*, 1984; Schoeffter & Stoclet, 1985; Varga *et al.*, 1986; Fazekas *et al.*, 1987). Other neuropeptides, including substance P (SP), vasopressin and angiotensin II, have been shown to be stored in and released from endothelial cells (Lincoln *et al.*, 1990). Furthermore, SP has been shown to produce a relaxation of the vasculature that is dependent on the presence of an intact endothelium (Mione *et al.*, 1990).

These experiments were designed to examine the role of the endothelium in the local control of the vascular tone of the rabbit isolated hepatic artery. The vascular location of P_1- and P_2-purinoceptors was also investigated by use of ATP, 2-methylthio ATP, α,β-methylene ATP and adenosine in the presence and absence of endothelium. The responses to ACh and sodium nitroprusside (which acts directly on the smooth muscle) (Murad *et al.*, 1979) were also investigated in the presence and absence of the endothelium and acted as controls of endothelial and smooth muscle cell integrity. Immunocytochemical staining was used to examine the presence of nerve fibres containing SP, CGRP and VIP (Costa *et al.*, 1980), while the relaxant action of these peptides was investigated by pharmacological methods.

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Methods

Tissue preparation

Male New Zealand White rabbits (2.3–3.3 kg) were killed by an overdose of pentobarbitone sodium (Sagatal), which was injected via the ear vein, and exsanguinated. The proper hepatic artery, which runs from the gastroduodenal artery to the porta hepatis, was cleaned of excess connective tissue and fat. Two 4 mm ring segments were cut and the endothelium of one of the rings was removed by pulling a braided silk suture through the lumen of the vessel. Each ring was then mounted horizontally under isometric conditions in a 5 ml organ bath by inserting a tungsten wire through the lumen of the vessel ring, which was anchored to a stationary support. Another wire, similarly inserted, was connected to a Grass FT03C force-displacement transducer. The responses were recorded on a Grass ink-writing polygraph. The preparations were placed under a resting tension of 0.75–1.0 g and allowed to equilibrate for 1.5–2 h in Bülbring-modified Krebs solution of the following composition (mM): NaCl 133, KCl 4.7, NaH_2PO_4 1.35, $NaHCO_3$ 16.3, $MgSO_4$ 0.61, glucose 7.8 and $CaCl_2$ 2.52, pH 7.2 (Bülbring, 1953). The solution was maintained at 37°C and aerated 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 peptide degradation and adhesion to the surfaces of the glassware.

Pharmacology

A cumulative concentration-response curve for NA was established for each segment in order to find the maximum contractile response. The absence of endothelium was assessed by lack of relaxation to ACh (Furchgott & Zawadzki, 1980). Before tissues were exposed to any test drug, they were contracted to approximately 75% of maximal tension with NA. After contractions reached a plateau, ACh was administered. If tissues did not relax to ACh, the endothelium was considered removed. In some cases, rubbed and unrubbed preparations were opened longitudinally and stained with silver nitrate as described by Caplan *et al.* (1974). Briefly, the preparations were immersed successively in the dark at room temperature in: (1) HEPES (20 mM) buffered (pH 7.4) solution containing 4.6% glucose for 150 s; (2) 0.4% $AgNO_3$ in 4.2% glucose solution for 60 s, and (3) 4.6% glucose solution for 60 s. The arteries were then fixed at room temperature in 0.1 M sodium cacodylate containing 7.5% sucrose and examined under the light microscope.

Application of purines and sodium nitroprusside

In the presence and absence of endothelium, vessels were preconstricted to 75% of maximal tension with NA. Cumulative concentration-response curves to ATP, 2-methylthio ATP, α,β-methylene ATP, adenosine and sodium nitroprusside were constructed.

Construction of cumulative concentration-response curves to ATP and adenosine was repeated for the preparations preincubated with 8-phenyltheophylline (10 μM) for 20 min.

Application of peptides

In the presence and absence of endothelium, the preparations were constricted to 75% of their maximal tension with NA. SP, CGRP, and VIP were added to the bath as single additions, each at 30 min intervals. The peptides 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.

Immunocytochemistry

Vessels were cleaned of excess connective tissue and fat. Vessel segments were slit open longitudinally and stretched out, adventitial side uppermost, onto strips of Sylgard silicone rubber. The segments were then processed for immunofluorescent localization of SP, CGRP, and VIP according to the method of Costa *et al.* (1980).

The preparations were immersion-fixed in 4% paraformaldehyde for 1–1.5 h. They were then washed three times in phosphate buffered saline (PBS) for 10 min and then placed for 30 min in 80%, 90% and 100% alcohol to dehydrate the tissues. Following a 20 min period of immersion in Histoclear, the tissues were rehydrated by placing them for 30 min in 100%, 90% and 80% alcohol. Following three washes in PBS/Triton X solution at 10 min intervals, the primary antibody to SP, CGRP or VIP (1:200 in each case) was placed on a segment and incubated for 12–18 h at room temperature in a moist atmosphere. After three 10 min washes in PBS/Triton X, the tissue was incubated with the second goat anti-rabbit fluorescein-isothiocyanate (FITC)-conjugated antibody (1:50) for 1 h. The tissue was then washed for 10 min in PBS, followed by PBS containing 0.05% pontamine sky blue and 1% dimethylsulphoxide for 15 min. After washing in PBS twice for a further 20 min, the tissues were stretched out on slides and left to dry before being mounted in Citifluor and viewed under a Zeiss microscope.

Drugs and chemicals

(−)-Noradrenaline bitartrate (NA), α,β-methylene adenosine 5′-triphosphate (α,β-methylene ATP) (lithium salt), adenosine (hemisulphate salt), adenosine 5′-triphosphate (ATP) (disodium salt), acetylcholine bromide (ACh), sodium nitroprusside (sodium nitroferricyanide), 8-phenyltheophylline, bacitracin and bovine serum albumin were obtained from Sigma Chemical Co. Ltd; Sagatal was supplied by May and Baker; 2-methylthio adenosine 5′-triphosphate (2-methylthio ATP) (Research Biochemicals Inc., U.S.A.), substance P (SP), calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP), antibody for SP, CGRP and VIP were obtained from Cambridge Research Biochemicals; goat anti-rabbit IgG conjugated to FITC was obtained from Nordic and Citifluor was obtained from Citifluor Ltd, London.

NA was dissolved and diluted in 100 μM ascorbic acid. 8-Phenyltheophylline was dissolved in a solution of 80% methanol and 0.1% sodium hydroxide. All other drugs were dissolved in distilled water.

Statistical analysis

Data are given as a mean ± s.e.mean. Results were analysed by Student's t test (paired or unpaired data as appropriate) and a probability of less than or equal to 0.05 was considered significant.

Results

Both in the absence and presence of the endothelium, NA caused a concentration-dependent contraction of the rabbit hepatic artery. The maximum response to NA and the EC_{50} in those preparations where the endothelium was intact (2.06 ± 0.13 g and 5.39 ± 3.75 μM respectively, $n = 8$) were comparable with those in preparations denuded of endothelium (1.96 ± 0.17 g and 6.0 ± 0.91 μM respectively, $n = 8$). This demonstrates that the tissue had not been damaged by the mechanical removal of the endothelium. Addition of NA (10 μM) produced a sustained contractile response of the vessel that was approximately 75% of the maximum NA contraction.

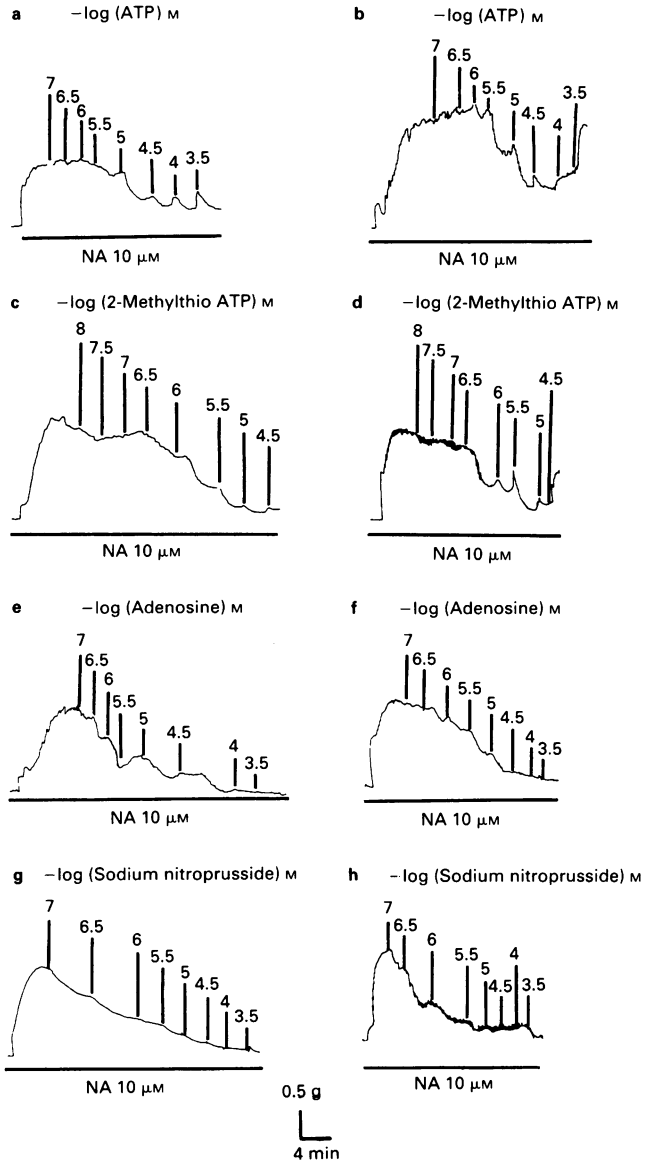

Figure 1 Isolated ring-preparations of the rabbit hepatic artery preconstricted with noradrenaline (NA) to 75% of the maximal constriction. Response to ATP: (a) endothelium intact; (b) endothelium removed, 2-methylthio ATP; (c) endothelium intact; (d) endothelium removed, adenosine; (e) endothelium intact; (f) endothelium removed, sodium nitroprusside; (g) endothelium intact; (h) endothelium removed.

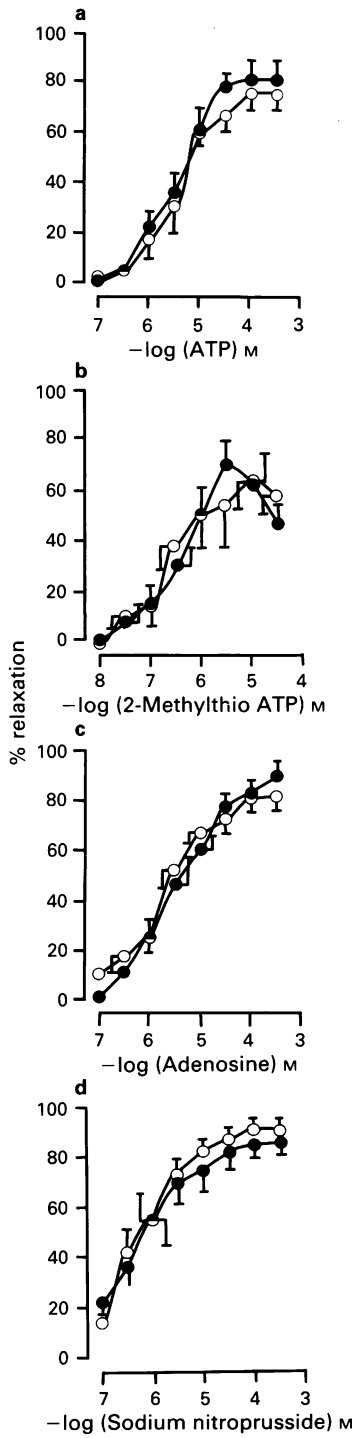

Figure 2 Isolated ring-preparations of the rabbit hepatic artery preconstricted with noradrenaline (NA) (10 μM). (a) Concentration-response curve to ATP in the presence (●) ($n = 6$) and in the absence (○) ($n = 6$) of endothelium. (b) Concentration-response curve to 2-methylthio ATP in the presence (●) ($n = 5$) and in the absence (○) ($n = 6$) of endothelium. (c) Concentration-response curve to adenosine in the presence (●) ($n = 10$) and absence (○) ($n = 6$) of endothelium. (d) Concentration-repsonse curve to sodium nitroprusside in the presence (●) ($n = 6$) and absence (○) ($n = 5$) of endothelium. Each point represents the mean percentage relaxation of the NA-induced contraction and vertical bars denote the s.e.mean.

In the presence of endothelium, addition of ACh to preconstricted vessels produced concentration-dependent, sustained relaxant responses. When the endothelium was removed, the relaxant response to ACh was abolished.

Responses to ATP, 2-methylthio ATP, adenosine, α,β-methylene ATP and sodium nitroprusside

In the presence of endothelium, ATP, 2-methylthio ATP, adenosine and sodium nitroprusside produced concentration-dependent, sustained relaxant responses (Figures 1 and 2). Occasionally, a small contraction preceded the relaxant response to ATP and 2-methylthio ATP. Removal of the endothelium did not significantly affect the response of the vessel to these agents. In preconstricted vessels, α,β-methylene ATP elicited concentration-dependent contractions in vessels where the endothelium remained intact and in those preparations where the endothelium had been removed by mechanical rubbing (Figure 3). Occasionally, a relaxation was observed at higher concentrations of α,β-methylene ATP.

Preincubation of the tissues with 8-phenyltheophylline (10 μM), a potent antagonist at P_1-purinoceptors (Smellie *et al.*,

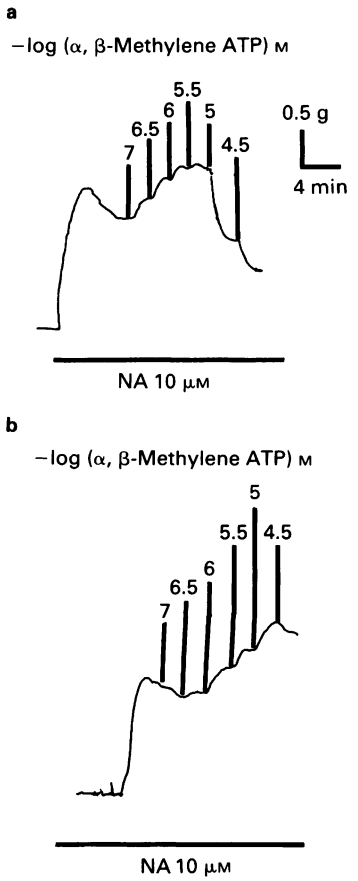

Figure 3 Rabbit isolated hepatic artery preconstricted with noradrenaline (NA) to 75% of the maximal constriction. Effect of α,β-methylene ATP in (a) the presence and (b) the absence of endothelium.

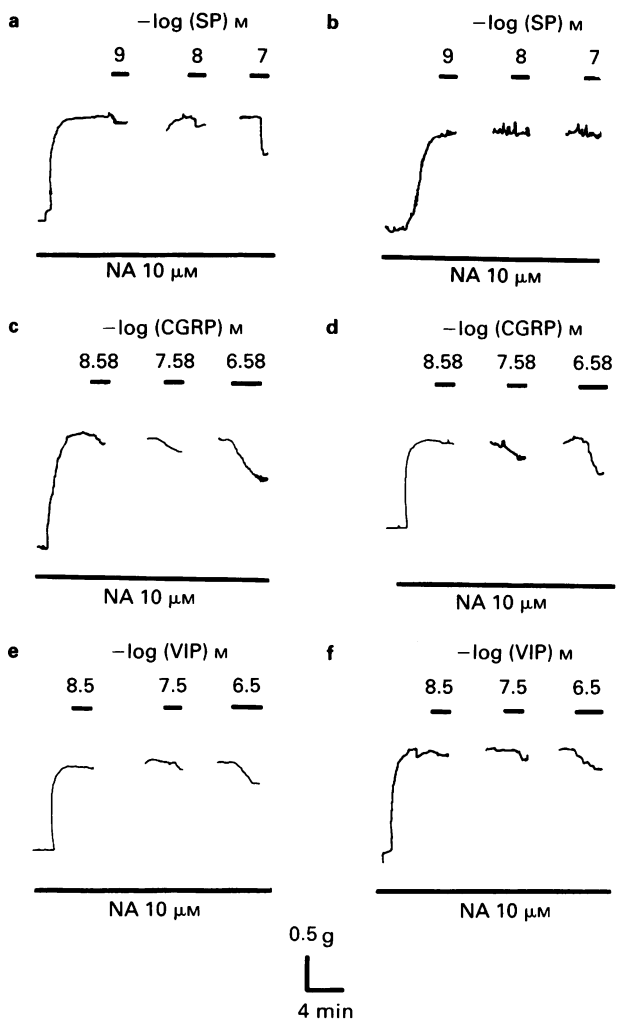

Figure 4 Rabbit isolated hepatic arterial ring preparations preconstricted with noradrenaline (NA) to 75% of the maximal constriction. Responses to single additions of substance P (SP) (a) in the presence and (b) in the absence of endothelium; calcitonin gene-related polypeptide (CGRP) (c) in the presence and (d) in the absence of endothelium; vasoactive intestinal polypeptide (VIP) (e) in the presence and (f) in the absence of endothelium.

1979; Griffith *et al.*, 1981), significantly antagonized relaxations to adenosine (Table 1). The maximum response of the vessel to ATP was reduced in the presence of 8-phenyltheophylline. The EC_{50} value for ATP in vessels with endothelium was not affected by 8-phenyltheophylline while vessels without endothelium were more sensitive to the purine in the presence of 8-phenyltheophylline. Relaxations to adenosine were not affected by the solvent for 8-phenyltheophylline.

Responses to peptides

SP produced a concentration-dependent relaxation of the vessel that was entirely dependent on the presence of an intact endothelium (Figures 4a,b). A maximum response of 45 ± 11.4% ($n = 7$) (Table 2) relaxation of the NA-induced contraction was observed after application of 0.1 μM SP to the organ bath. In the presence of endothelium, CGRP and VIP elicited a maximum relaxation of the preparation of 51 ± 6.6% ($n = 7$) and 34 ± 7.5% ($n = 5$) respectively. In endothelium-denuded preparations, CGRP and VIP induced a vasodilatation with a maximum of 58 ± 12.0% ($n = 6$) and 46 ± 11.2% ($n = 5$) respectively. The magnitude of the responses to CGRP and VIP were not significantly different in

Table 1 Effects of ATP and adenosine on the rabbit isolated hepatic artery in the absence (control) and presence of 8-phenyltheophylline (8-PT) (10 μM) in endothelium intact (+e) and endothelium denuded (−e) preparations

	Maximal relaxation (%)		EC_{50}	
Agonist	*Control*	*+8-PT*	*Control*	*+8-PT*
ATP				
+e	81 ± 6.46 (6)	52 ± 9.34 (6)*	2.7 ± 0.32 (6)	12.2 ± 7.25 (6)
−e	75 ± 7.09 (6)	52 ± 6.22 (7)*	2.1 ± 0.59 (6)	0.91 ± 0.10 (6)*
Adenosine				
+e	90 ± 4.09 (10)	69 ± 9.77 (6)*	6.4 ± 3.06 (10)	89 ± 25.5 (6)***
−e	83 ± 6.75 (6)	64 ± 10.95 (6)*	2.0 ± 0.63 (6)	12.1 ± 4.51 (6)**

All values are given as mean ± s.e.mean with the number of observations (n) in parentheses.
* $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$ indicate significant differences between control responses and those in the presence of 8-PT.

Table 2 Effects of substance P (SP), calcitonin gene-related peptide (CGRP) and vasoactive intestinal polypeptide (VIP) on the rabbit hepatic artery preconstricted with noradrenaline in the presence (+e) and absence (−) of endothelium

Peptide	*% relaxation*		
SP	1 nM	10 nM	100 nM
+e	3.7 ± 2.44 (7)	13 ± 7.0 (7)	45 ± 11.4 (7)
−e	0 (7)	0 (7)	0 (7)
CGRP	2.63 nM	26.3 nM	263 nM
+e	16 ± 9.8 (7)	28 ± 11.6 (7)	51 ± 6.6 (7)
−e	5.7 ± 5.7 (6)	37 ± 6.1 (6)	58 ± 11.9 (6)
VIP	3.0 nM	30 nM	300 nM
+e	3.6 ± 2.2 (5)	9.4 ± 3.2 (5)	34 ± 7.5 (5)
−e	8.3 ± 7.6 (5)	16 ± 10.5 (5)	46 ± 11.2 (5)

All values are expressed as mean ± s.e.mean with the number of observations (n) in parentheses.
Responses are measured as the percentage relaxation of the noradrenaline-induced contraction.

the absence and presence of the endothelium (Figure 4, Table 2).

Immunocytochemistry

All vessels showed positive SP-like immunoreactivity (SP-LI), positive CGRP-LI and positive VIP-LI.

Discussion

In this study, the endothelial dependence of the responses of the hepatic artery of the rabbit to several vasoactive agents was assessed by examining their reactivity before and after the intimal surface was rubbed to remove the endothelium.

The ability of the hepatic artery to constrict in response to NA was not significantly affected by the removal of the endothelium. This result not only demonstrates that hepatic arterial vasoconstriction in response to NA is independent of the endothelium, but also that the mechanical removal of the endothelium did not damage the smooth muscle of the preparation.

The endothelium has been shown to have an obligatory role in the relaxations of isolated arteries to ACh (Furchgott & Zawadzki, 1980; Furchgott *et al.*, 1981; De Mey & Vanhoutte, 1982; Vanhoutte & Rimele, 1983; Peach *et al.*, 1985). Our experiments show that ACh-induced vasodilatation of the rabbit hepatic artery also depends on the presence of an intact endothelium. In most peripheral blood vessels, ATP also induces a relaxation that is endothelium-dependent (Furchgott *et al.*, 1981; De Mey *et al.*, 1982; Rapoport *et al.*, 1984; Martin *et al.*, 1985; Houston *et al.*, 1987). Furthermore, endothelium-dependent relaxation of the perfused rabbit hepatic arterial bed by ATP has also been demonstrated (R. Mathie, personal communication). In this study, however, relaxation to ATP and 2-methylthio ATP was not significantly affected by the removal of the endothelium. In the rabbit portal vein (Kennedy & Burnstock, 1985a) and the rabbit mesenteric artery (Mathieson & Burnstock, 1985), ATP also caused relaxation via P_2-purinoceptors by an endothelium-independent mechanism. The physiological relevance of the different locations of P_{2y}-purinoceptors in the isolated hepatic artery and in the perfused hepatic arterial bed preparation has yet to be established. Vasodilatation of the hepatic artery to adenosine was independent of the endothelium in common with other vessels (Hardebo *et al.*, 1983; Kennedy & Burnstock, 1985b; Mathieson & Burnstock, 1985). 8-Phenyltheophylline, a selective P_1-purinoceptor antagonist (Smellie *et al.*, 1979; Griffith *et al.*, 1981), significantly increased the EC_{50} value for adenosine but not that for ATP, indicating that adenosine but not ATP is acting via P_1-purinoceptors. However, it should be noted that the maximum response to high concentrations of ATP is reduced in the presence of 8-phenyltheophylline. This result implies that ATP may have some action via P_1-purinoceptors as a result of its breakdown to adenosine.

In contrast to ATP, 2-methylthio ATP and adenosine, α,β-methylene ATP did not produce a relaxation of the rabbit hepatic artery in which tone had been induced by NA. Indeed, a concentration-dependent contraction was observed both in the absence and presence of endothelium. Similar results have been reported in other blood vessels (Kennedy *et al.*, 1985; Kennedy & Burnstock, 1985a; Mathieson & Burnstock, 1985; Houston *et al.*, 1987). In 1985, Burnstock & Kennedy proposed a subdivision of the P_2-purinoceptor into P_{2x} and P_{2y} subtypes and suggested that the P_{2x}-purinoceptor mediates vasoconstriction and the P_{2y}-purinoceptor mediates vasodilatation. Brizzolara & Burnstock (1990) have demonstrated that in the hepatic artery of the rabbit, α,β-methylene ATP induces a concentration-dependent contraction and that ATP and NA act as cotransmitters from sympathetic nerves, the purinergic component being mediated by ATP acting through postjunctional P_{2x}-purinoceptors. Thus, it would appear that in the rabbit hepatic artery, three sub-populations of purincoeptor exist on the smooth muscle, namely a P_1-purinoceptor mediating a vasodilatation to adenosine, a P_{2x}-purinoceptor mediating a vasoconstriction to ATP and a P_{2y}-purinoceptor mediating a vasodilatation to ATP.

Immunocytochemical studies have shown a wide distribution of SP, (Edvinsson *et al.*, 1981; Furness *et al.*, 1982; Barja *et al.*, 1983; Goehler *et al.*, 1988), CGRP (Rosenfeld *et al.*, 1983; Hanko *et al.*, 1985; Sasaki *et al.*, 1986; Goehler *et al.*, 1988) and VIP (Larsson *et al.*, 1976; Uddman *et al.*, 1981; Malencik & Andersson, 1983; Varga *et al.*, 1986) in both the central and peripheral nervous systems. In the rabbit hepatic artery, nerve fibres containing SP, CGRP and VIP were identified. CGRP and VIP have been shown to be potent vasodilators of several blood vessels, and in this study, both CGRP and VIP induced a relaxation that was independent of the endothelium. This result is consistent with the results reported for most other vessels where removal of the endothelium does not prevent CGRP- or VIP-induced vasodilatation (Duckles & Said, 1982; Brum *et al.*, 1985; Brain *et al.*, 1985; Girgis *et al.*, 1985; Hanko *et al.*, 1985; Schoeffter & Stoclet, 1985; Varga *et al.*, 1986; Edvinsson *et al.*, 1989).

SP has also been demonstrated to be a powerful vasodilator of several blood vessels and in all cases its action requires the presence of an intact endothelium (Furchgott, 1983; D'Orleans-Juste *et al.*, 1985; Edvinsson *et al.* 1985; Bolton & Clapp, 1986; Stewart-Lee & Burnstock, 1989). The presence of SP-like immunoreactivity has been demonstrated in the hepatic arteries of the rat and human (Burt *et al.*, 1987; Tanikawa *et al.*, 1988) and its potent vasodilator action observed in the hepatic artery of the dog (Withrington, 1987). Removal of the endothelium in this study, completely abolished any response of the rabbit hepatic artery to SP thus demonstrating the obligatory role of an intact endothelium in SP-mediated vasodilatation.

Constant blood flow to the liver must be maintained since the hepatic clearance of many blood-borne drugs and hormones is blood-flow limited. Adenosine has been shown to be an important mediator of the compensatory hyperaemic response of the hepatic artery in response to portal vein occlusion (Lautt, 1981; Mathie & Blumgart, 1990). The results of this study provide further evidence for the involvement of adenosine in the control of hepatic arterial tone via P_1-purinoceptors which are located on the smooth muscle. Whilst there is strong evidence for the role of adenosine in the regulation of hepatic arterial resistance (Lautt, 1981; Mathie & Blumgart, 1990), it does not appear to be the sole mediator of hepatic arterial dilatation. The results from this study provide evidence for the putative role of ATP as a vasodilator of the hepatic artery of the rabbit, acting through postjunctional P_{2y}-purinoceptors located on the vascular smooth muscle. Furthermore, previous studies have demonstrated a

hepatic arterial constriction in response to ATP via smooth muscle P_{2x}-purinoceptors following sympathetic nerve stimulation (Brizzolara & Burnstock, 1990). In most vessels, the P_{2y}-purinoceptor is located on the endothelium (De Mey & Vanhoutte, 1980; 1982; Cocks & Angus, 1983; Furchgott, 1983; Kennedy *et al.*, 1985; Burnstock & Kennedy, 1985; Liu *et al.*, 1989). Although the response mediated via the two P_2-purinoceptors in the hepatic artery are in opposition, the location of both receptors on the smooth muscle may be of physiological significance in the control of vascular tone. It could be that, depending on the tone of the vessel, responses of the artery to ATP may be either constriction or relaxation and that in some pathological situations, one or other of these responses may dominate.

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Characterization of endogenous noradrenaline release from intact and epithelium-denuded rat isolated trachea

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1 Overflow of endogenous noradrenaline (NA) from the *in vitro* incubated rat trachea evoked by two periods of electrical field stimulation (S1, S2, at 3 or 15 Hz) or by high potassium (60 mM) was determined by high performance liquid chromatography (h.p.l.c.) with electrochemical detection.

2 In the presence of the neuronal uptake inhibitor desipramine, the α_2-adrenoceptor antagonist, yohimbine, enhanced the overflow of NA evoked by stimulation at 3 Hz by about 100% suggesting the presence of presynaptic inhibitory autoreceptors on the sympathetic nerves innervating the trachea.

3 When desipramine and yohimbine were present throughout the experiments, the overflow of NA evoked by the second period of electrical stimulation (S2) was significantly smaller than that evoked by the first (S1). This decline of overflow was prevented when the NA precursor, tyrosine, was additionally present throughout the experiments.

4 After removal of the epithelium, the tissue content of NA was reduced by about 30%, suggesting that part of the NA may be present and released within the epithelium. However, the overflow of NA evoked by stimulation at 3 Hz or 15 Hz was reduced by 70–80%, indicating that the epithelium may additionally exert a permissive role on the release of NA within the airways, possibly by suppressing inhibitory factors.

5 Stimulation by high potassium (60 mM for 10 min) caused a large overflow of NA (about 45% of the tissue NA), both from epithelium-free and epithelium-denuded tracheae. Thus the 'endogenous inhibition' of NA release after removal of the epithelium is surmountable when a high potassium stimulus is applied.

6 An enhanced muscarinic inhibition of NA release in epithelium-denuded tracheae may not be responsible for the reduction in NA overflow, since the muscarinic receptor antagonist, scopolamine, did not significantly affect the overflow of NA evoked by 3 or 15 Hz, either in the presence or the absence of the epithelium.

7 The muscarinic receptor agonist, oxotremorine, inhibited the overflow of NA evoked by stimulation at 3 Hz almost completely, an effect antagonized by scopolamine, demonstrating specific, inhibitory muscarinic receptors.

8 Since damage to the epithelium is known to occur in obstructive bronchial disease, the present experiments suggest that an impaired sympathetic (broncho-relaxant) neurotransmission could be involved in the pathogenesis of bronchoconstriction.

Keywords: Noradrenaline release; trachea; airway; epithelium; sympathetic neurotransmission; muscarinic receptor; α-adrenoceptor

Introduction

The airways are innervated by cholinergic and peptidergic nerves (for reviews see Coburn, 1987; Lundberg & Saria, 1987). In addition, by use of histofluorescence techniques, catecholaminergic nerve fibres have also been demonstrated in different parts of the airways of many mammals (see Gabella, 1987). Noradrenaline (NA) is very effective in relaxing the smooth muscle of the airways (e.g. McDougal & West, 1953; Carswell & Nahorski, 1983; Zaagsma *et al.*, 1987; Lemoine *et al.*, 1989) and there is indirect evidence suggesting that neuronally released NA plays an important role in relaxing airway smooth muscle tone (Foster, 1964; Lemoine *et al.*, 1989). Therefore, impairment of the sympathetic neurotransmission might be involved in the pathogenesis of obstructive bronchial disease. Despite this great significance of NA in the airways, studies in which the release of NA from isolated airway tissue has been measured directly, have not yet been carried out. Therefore, the aim of the present study was first, to establish an *in vitro* model which allows direct measurement of the release of endogenous NA from airway tissue (rat trachea) and second, to start experiments which will elucidate regulatory mechanisms involved in the control of NA release in this tissue. A possible role of the epithelium in the regulation of NA release was investigated, since there is increasing evidence that the airway epithelium plays an important part in the regulation of the airway smooth muscle tone (see Goldie *et al.*, 1990) and has pronounced inhibitory effects on the release of acetylcholine (Wessler *et al.*, 1990a,b). Preliminary accounts of the present results have been given (Racké *et al.*, 1989a; 1990).

Methods

Preparation and incubation of the trachea

Female Sprague-Dawley rats weighing 190–210 g (Charles River Wiga, Sulzfeld, F.R.G.) were used. The animals were kept at a constant temperature (21°C) and a regular light (06 h 30 min–19 h 30 min) dark (19 h 30 min–06 h 30 min) cycle with food and water *ad lib.* for at least 1 week before use. The animals were killed between 09 h 00 min and 11 h 30 min by stunning followed by exsanguination. The whole trachea (from the larynx to the carina) was dissected and transferred into a petri dish. It was then opened by a cut along the ventral side, fixed between two platinum wire field electrodes and finally incubated in 1.7 ml Krebs-HEPES solution of the following composition (mM): NaCl 118.5, KCl 5.7, $CaCl_2$ 1.25, $MgCl_2$ 1.2, sodium EDTA 0.03, (+)-ascorbic acid 0.06, HEPES 20.0 (adjusted to pH 7.4 with NaOH) and D-glucose 11.1. The medium contained in addition the neuronal uptake inhibitor, desipramine (1 μM) and in most experiments the NA precursor, tyrosine (10 μM) and α_2-adrenoceptor antagonist, yohimbine (1 μM). The medium was kept at 37°C and continuously gassed with 100% O_2. The bath fluid was changed every 10 min. The medium of the first 40 min was discarded, then it was collected

for measurement of NA into glass tubes which contained 100 μl of a solution containing EDTA (2.8%, v/v) and Na_2SO_3 (2.8%) and 100 μl 1 M $HClO_4$ (resulting in a total volume of 1.9 ml; subsequently referred to as 'samples').

In some experiments the epithelium was removed prior to the start of incubation by gently rubbing the luminal surface of the trachea with a pipe cleaner. Removal of the epithelium was confirmed at the light microscopic level after staining with haematoxylin and eosin. At the light microscopic level significant lesions to the submucosal tissue were not observed.

At the end of incubation each trachea was blotted and weighed and homogenized in 2 ml 0.1 M $HClO_4$ by means of a hand operated glass homogenizer. After extraction for 1 h at 0–4°C the homogenates were centrifuged at 20000 g. The supernatant was stored at 0–4°C until analysed later the same day. The mean weight (mg) of the tracheae with intact epithelium was 47.6 $\pm$ 1.2 (n = 167) and was reduced to 42.5 $\pm$ 1.4 (n = 32) after removal of the epithelium.

Stimulation of noradrenaline release

Electrical field stimulation (S1, S2) was carried out after 60 and 110 min of incubation, respectively (see Figure 1). Biphasic pulses were delivered from a Grass S6 stimulator. During each period of stimulation pulses of 0.4 ms, 250 mA, 3 Hz (unless specified otherwise) were applied 3 times for 1 min with 1 min intervals. The current flow was determined by the voltage drop across a resistance by use of an oscilloscope.

In one series of experiments high potassium stimulation was used instead of electrical stimulation. In these experiments the tissue was incubated during two periods (S1, 60–70 min; S2, 110–120 min; see Figure 3) with medium containing 60 mM potassium (sodium correspondingly reduced to 64.2 mM).

Analytical procedure

First, NA in the samples and tissue extracts was adsorbed onto alumina as described previously (Racké et al., 1989b): after addition of 0.5 ng dihydroxybenzylamine (DHBA) as internal standard, 1.4 ml portions of the samples or 1 ml portions of the tissue extracts were added to 20 mg alumina, adjusted to pH 8.4–8.5 (by addition of Tris-HCl) and shaken for 20 min. The alumina was washed three times with 1 ml H_2O. Then the catechols were eluted from the alumina by adding 150 μl 0.1 M $HClO_4$ and shaking for 5 min. After centrifugation, 100 μl portions of the clear supernatant were injected into a high performance liquid chromatography (h.p.l.c.) apparatus with electrochemical detection (for details see Racké & Muscholl, 1986). Briefly, the separation of NA and DHBA was achieved on a reverse phase column (length 250 mm, inner diameter 4.6 mm, prepacked with Shandon ODS-Hypersil, 5 μm) using as mobile phase a 0.1 M phosphate buffer (adjusted to pH 3.0), which contained octane sulphonic acid sodium salt (250 mg ml^{-1}), sodium EDTA (0.3 mM) and methanol (4%, v/v). Quantitation was carried out with an electrochemical detector (Waters 460) equipped with a glassy carbon working electrode and an Ag/AgCl reference electrode. The potential was set at +0.70 V. The limit of detection was about 15 and 50 fmol for NA and DHBA, respectively. The recovery of the extraction procedure, determined by use of the added DHBA as internal standard, was between 65 and 75%. The values given under Results were corrected for the recoveries obtained in the individual analysis.

Calculations and statistical analysis

The overflow of NA is expressed as pmol per g wet weight of tissue and per collection period (i.e. pmol g^{-1} 10 min^{-1}). The evoked overflow of NA was calculated by summing the overflow of NA observed in the two samples collected during and immediately after the stimulation and by subtracting twice the spontaneous overflow determined immediately before the respective stimulation. The evoked overflow of NA is expressed in absolute terms (pmol g^{-1} per stimulation), as ratio S2/S1 or as % of the tissue NA determined at the end of the incubation experiments. EC_{50} values were calculated with the help of the computer programme of Tallarida & Murray (1981). Mean values of n observations are given $\pm$ s.e.mean. The significance of differences was evaluated by Student's t test. For multiple comparisons the modified t test according to Bonferroni was used (see Wallenstein et al., 1980).

Drugs

Desipramine hydrochloride (Serva, F.R.G.); oxotremorine sesquifumarate (Sigma, F.R.G.); scopolamine hydrobromide (Sigma), tetrodotoxin (Sigma); tyrosine (Sigma) and yohimbine hydrochloride (Boehringe Ingelheim, F.R.G.) were used.

Results

The spontaneous overflow of NA in the presence of 1 μM desipramine alone was 4.3 $\pm$ 0.9 pmol g^{-1} 10 min^{-1} (n = 14). It was not significantly altered when 1 μM yohimbine alone (3.8 $\pm$ 0.8 pmol g^{-1} 10 min^{-1}, n = 8) or in combination with 10 μM tyrosine was additionally present from the onset of incubation (in several series of experiments between 4.9 $\pm$ 1.8 and 5.9 $\pm$ 1.5 pmol g^{-1} 10 min^{-1}). In addition, none of the other drugs tested in the present study significantly altered the spontaneous overflow of NA (not shown).

In the presence of 1 μM desipramine, electrical field stimulation caused a clear increase in the overflow of NA (Figure 1) which returned to basal value in the subsequent sample (Figure 1, shaded part). The overflow of NA evoked by the electrical stimulation (Table 1) corresponded to 2.1 $\pm$ 0.38% of the tissue NA, determined at the end of the incubation experiments (see Table 1). In control experiments, a second stimulation (S2) caused a similar overflow of NA resulting in a ratio S2/S1 of 1.13 (Table 1). Tetrodotoxin (1 μM), added 30 min before S2, inhibited almost completely the evoked overflow of NA, as did omission of calcium from the incubation medium (Figure 2).

Addition of yohimbine (0.1 to 10 μM), 30 min before S2 enhanced the evoked overflow of NA by 58% at 0.1 μM and by 82% at 1 μM (Figure 2). At 10 μM the effect of yohimbine declined, possibly because of some local anaesthetic effects of yohimbine at this high concentration (Azuma et al., 1977;

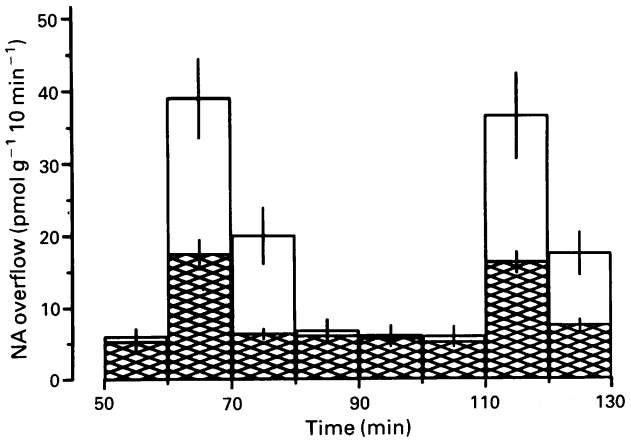

Figure 1 Effects of electrical stimulation on the overflow of noradrenaline (NA) from rat tracheae incubated *in vitro*. The incubation medium contained desipramine (DMI, 1 μM) either alone (hatched part of the columns, n = 5) or in combination with yohimbine (1 μM) and tyrosine (10 μM) (total height of the columns, n = 8) from the onset of incubation. Two periods of electrical field stimulation (S1, S2) were carried out starting at 60 min and 110 min respectively. Abscissa scale: time after start of incubation. Ordinate scale: overflow of NA, expressed as pmol g^{-1} 10 min^{-1}, means with s.e.mean shown by vertical lines.

Table 1 Effects of yohimbine and tyrosine on the electrically evoked overflow of noradrenaline (NA) from the rat trachea and the NA tissue concentration

	NA overflow (pmol g^{-1}/stim)	(S2/S1)	*Tissue NA* (pmol g^{-1})
DMI	26 ± 4.0	1.13 ± 0.05	1323 ± 221
n	9	5	9
DMI + Yoh	54 ± 7.0**	0.65 ± 0.09*	1071 ± 97
n	8	4	8
DMI + Yoh + Tyr	49 ± 3.6	0.94 ± 0.04†	1347 ± 69
n	49	8	49

The isolated tracheae were incubated and stimulated electrically as described in Methods. Desipramine (DMI, 1 μM) alone or in combination with yohimbine (Yoh, 1 μM) and tyrosine (Tyr, 10 μM) was present in the medium from the onset of incubation, as indicated. Values are means ± s.e.mean of *n* experiments of the overflow of NA evoked by the first electrical stimulation (S1, see Figure 1) in the absence of additional drugs (pmol g^{-1}/stimulation), the ratio S2/S1 in the control experiments (i.e. absence of additional drugs during S2) and the tissue NA determined at the end of the incubation experiments. Significance of differences: from the corresponding value in the absence of Yoh, * $P < 0.05$; ** $P < 0.01$; from the corresponding value in the absence of Tyr † $P < 0.05$.

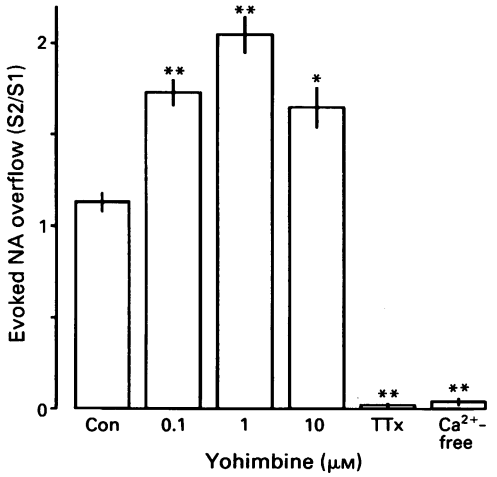

Figure 2 Effect of yohimbine, tetrodotoxin (TTx) or omission of calcium on the overflow of noradrenaline (NA) from the rat trachea evoked by electrical stimulation (3 Hz). The medium contained 1 μM desipramine from the start of incubation. Yohimbine (at the concentrations indicated below the columns) or TTx (1 μM) were added to the incubation medium or calcium was omitted from the medium 30 min before S2. Height of columns: evoked NA overflow, expressed as ratio S2/S1, means of 3–5 experiments; vertical lines show s.e.mean. Significance of differences from the controls (Con.): * $P < 0.05$; ** $P < 0.01$.

Briggs & Meyer, 1986). When 1 μM yohimbine was present from the onset of incubation the electrically induced increase in the overflow of NA was substantially higher and persisted into the next collection period (see Figure 1). The total amount of NA released by S1 was enhanced to 54 pmol g^{-1} (Table 1), corresponding to an increase of 119%. However, in the presence of yohimbine the overflow of NA evoked by S2 was significantly lower than that evoked by S1 (Table 1). When tyrosine (10 μM) was added, in addition to yohimbine, to the incubation medium from the onset of incubation the overflow of NA evoked by S1 was similar to that observed in the presence of yohimbine alone (Table 1), but the decline of overflow during S2 was prevented (Table 1). Therefore, these incubation conditions (presence of desipramine, yohimbine and tyrosine) were used in the following experiments.

When the epithelium was removed prior to the start of the incubation experiments, the overflow of NA evoked by stimulation at 3 Hz was reduced by about 80% compared with the overflow observed in tissue with intact epithelium (Table 2). After removal of the epithelium, the tissue NA concentration, determined at the end of the incubation experiments, was also significantly reduced, but only by about 30% (Table 2).

Increasing the stimulation frequency from 3 to 15 Hz increased the fractional release of NA from epithelium-containing tracheae by 59 ± 14% and that from epithelium-denuded preparations by 147 ± 35% (Table 2) even though overflow in the latter preparation remained depressed compared with intact controls. Strikingly, in the experiments with 15 Hz stimulation the ratio S2/S1 observed in intact controls was significantly below unity (0.57 ± 0.04, $n = 5$, Figure 3), whereas in epithelium-free preparations the ratio S2/S1 was 0.92 ± 0.09 ($n = 5$).

Incubation of the trachea for 10 min with the K^+ 60 medium caused a very large overflow of NA, both from epithelium-containing (Figure 3) and epithelium-denuded preparations (Table 2). The overflow of NA evoked by S1, expressed as a percentage of the tissue content at the end of the experiments was similar in epithelium-free and epithelium-containing tracheae (Table 2). The S2/S1 ratios were 0.67 ± 0.02 and 0.71 ± 0.05, in the presence and absence of the epithelium, respectively. The total amount of NA released by the two high potassium stimulations was equivalent to about 75% of the total tissue NA content. Nevertheless, the tissue concentration of NA was not significantly reduced in these experiments indicating that an effective NA *de novo*-synthesis can occur in the isolated trachea.

Table 2 Effects of removal of the epithelium on the overflow of noradrenaline (NA) from the rat trachea evoked by different stimuli and on the NA tissue concentration

	NA overflow (pmol g^{-1}/stim)	(%)	*Tissue NA* (pmol g^{-1})	*n*
3 Hz				
DMI + Yoh + Tyr	52 ± 3.0	3.33 ± 0.19	1595 ± 47	80
DMI + Yoh + Tyr epithelium-denuded	12 ± 2.2*	0.96 ± 0.13*	1193 ± 110*	17
15 Hz				
DMI + Yoh + Tyr	91 ± 8.6	5.38 ± 0.44	1697 ± 88	17
DMI + Yoh + Tyr epithelium-denuded	24 ± 3.7*	2.37 ± 0.34*	995 ± 52*	10
K^+ & 60 mM				
DMI + Yoh + Tyr	558 ± 105	44.6 ± 2.0	1234 ± 194	4
DMI + Yoh + Tyr epithelium-denuded	363 ± 34	44.6 ± 3.4	827 ± 91	5

The isolated tracheae were incubated and stimulated electrically either at 3 Hz or at 15 Hz or stimulated by incubation with high potassium medium (K^+ 60 mM) for 10 min. Desipramine (DMI, 1 μM), yohimbine (Yoh, 1 μM) and tyrosine (Tyr, 10 μM) were present in the medium from the start of incubation. Values are means ± s.e.mean of *n* experiments of the overflow of NA evoked by the first stimulation (S1) expressed either as pmol g^{-1}/stimulation or as % of the tissue NA determined at the end of the experiments. Significance of differences from the corresponding value with intact epithelium: * $P < 0.01$.

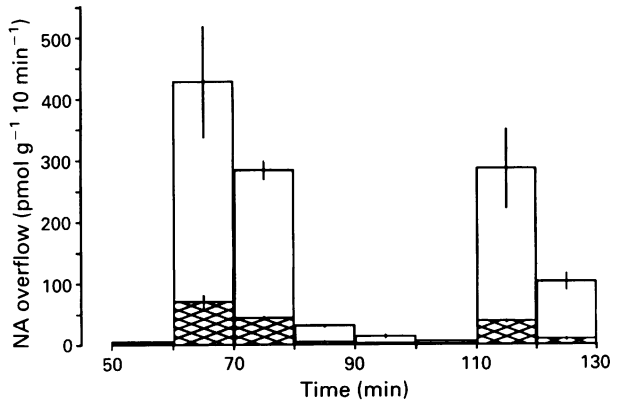

Figure 3 Effects of electrical stimulation (hatched columns) or high potassium stimulation (total column height) on the overflow of noradrenaline (NA) from rat tracheae incubated *in vitro*. The incubation medium contained desipramine (1 μM), yohimbine (1 μM) and tyrosine (10 μM) from the start of incubation. Two periods of stimulation were carried out, at 60 and 110 min respectively, either electrical field stimulation (as described in Figure 1, except that the stimulation frequency was 15 Hz, $n = 6$) or incubation for 10 min with medium containing 60 mM potassium ($n = 4$). Abscissa scale: time after start of incubation. Ordinate scale: overflow of NA, expressed as pmol g^{-1} 10 min^{-1}, means with s.e.mean shown by vertical lines.

In epithelium-free preparations, scopolamine (0.1 or 1 μM, added 30 min before S2) did not affect significantly the overflow of NA evoked by stimulation at 3 Hz (S2/S1 1.16 $\pm$ 0.18, $n = 5$ and 1.09 $\pm$ 0.27, $n = 3$, respectively; versus 0.96 $\pm$ 0.23 $n = 5$, of the controls) nor that evoked by stimulation at 15 Hz (S2/S1, 1.02 $\pm$ 0.22, $n = 4$, in the presence of 1 μM scopolamine; versus 0.92 $\pm$ 0.09, $n = 5$, of the controls). Scopolamine (0.1 or 1 μM) also failed to affect the overflow of NA from epithelium-containing preparations evoked by stimulation at either 3 or 15 Hz (each $n = 4$–5, data not shown).

The muscarinic receptor agonist, oxotremorine, inhibited in a concentration-dependent manner the overflow of NA from the epithelium-containing trachea evoked by stimulation at 3 Hz, the block being almost complete at a concentration of 1 μM (Figure 4). The EC_{50} of oxotremorine was 41 nM. Scopolamine antagonized the effect of oxotremorine in a manner suggesting a competitive interaction (Figure 4). Oxotremorine (1 μM and 10 μM) reduced the overflow of NA by only 70 $\pm$ 3.5% ($n = 4$) and 60 $\pm$ 12% ($n = 3$), respectively in tissue stimulated at 15 Hz.

Finally, oxotremorine (1 μM) inhibited the overflow of NA from epithelium-denuded tracheae evoked by stimulation at 3 Hz by 94% (S2/S1 0.06 $\pm$ 0.02, $n = 4$; versus 1.00 $\pm$ 0.15, $n = 7$ of the controls).

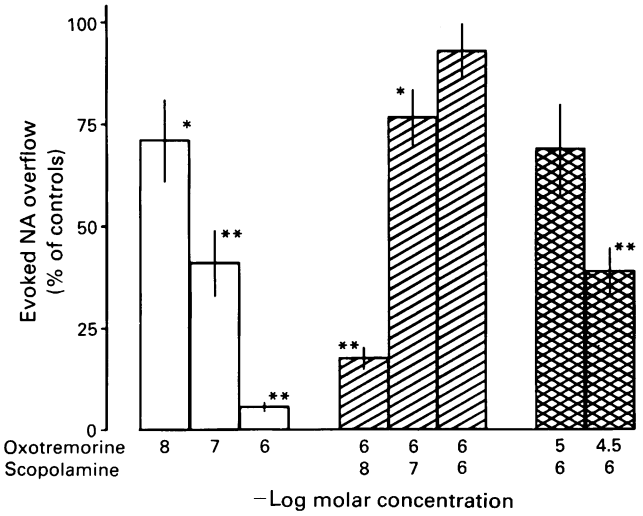

Figure 4 Effects of oxotremorine in the absence (open columns) or presence (filled columns) of scopolamine in different concentrations (as indicated below the columns) on the overflow of noradrenaline (NA) from the rat trachea evoked by electrical stimulation (3 Hz). The medium contained 1 μM desipramine, 1 μM yohimbine, 10 μM tyrosine and, in the respective experiments, scopolamine (10 nM–1 μM) from the start of incubation. Oxotremorine, at the concentrations indicated was added to the medium 10 min before S2. Height of columns: evoked NA overflow, expressed as percentage of the mean ratio S2/S1 of the respective control experiments in the absence or presence of scopolamine at the different concentration (S2/S1 ratios between 0.76 and 0.89). Given are means of 4–8 experiments with s.e.mean shown by vertical lines. Significance of differences from the respective control experiments: * $P < 0.05$; ** $P < 0.01$.

Discussion

The present experiments showed that the overflow of endogenous NA from *in vitro* incubated rat isolated tracheae can be measured by h.p.l.c. with electrochemical detection. The overflow of NA evoked by electrical field stimulation was calcium-dependent and blocked in the presence of tetrodotoxin, indicating that it reflects NA release from sympathetic nerves triggered by a sodium-dependent depolarization (i.e. propagated neuronal activity).

The α_2-adrenoceptor antagonist, yohimbine, markedly enhanced the evoked release of NA indicating that sympathetic nerve terminals in the trachea, like in other tissues studied so far (see Starke *et al.*, 1989), are endowed with inhibitory α-adrenoceptors which may belong to the α_2-subtype. The pronounced autoinhibition, as indicated by the large stimulatory effect by yohimbine, under the present *in vitro* conditions may be the consequence of blockade of neuronal NA uptake and the resulting large elevation of the NA biophase concentration in the incubated tissue.

The significant fall in the ratio S2/S1 after simultaneous blockade of NA reuptake and the inhibitory autoreceptors suggests that the enhanced release of NA (during S1) resulted in a significant depletion of a functionally important NA pool, although the released amounts of NA corresponded to only about 5% of the tissue content of NA. This depletion could, however, be prevented by the addition of tyrosine, which may allow a sufficient *de novo* synthesis of NA under the present *in vitro* conditions.

There is increasing evidence that the airway epithelium plays an important role in the regulation of airway smooth muscle tone (see Goldie *et al.*, 1990). This action of the epithelium may also involve effects on neurotransmission, as recent experiments showed a strong inhibitory effect of the epithelium on the *in vitro* release of [^{3}H]-acetylcholine from the guinea-pig (Wessler *et al.*, 1990a,b) and also rat (Wessler, unpublished observations) trachea. The present experiments show that the epithelium affects sympathetic neurotransmission in the airways. Removal of the epithelium was accompanied by a significant reduction of the NA tissue levels (about 30%), indicating that some of the sympathetic fibres may terminate in the epithelium. This is supported by observations from fluorescence histochemical studies (Zussman, 1966). However, removal of the epithelium resulted also in a marked reduction of the release of NA from the remaining nerve endings as indicated by the pronounced fall in the fractional release of NA (Table 2). As removal of the epithelium enhanced the release of acetylcholine (see above) it seems unlikely that these effects are attributable to non-specific tissue damage. Moreover, the inhibition of NA release was surmountable by increasing the stimulus intensity (compare low and high stimulation frequency electrical stimulation and high potassium stimulation, see Table 2).

An augmented muscarinic inhibition of NA release is not involved, as scopolamine did not significantly enhance the release of NA, either in the presence or absence of the epithelium. The present experiments showed, however, that the release of NA from the rat trachea can be largely inhibited by the muscarinic receptor agonist oxotremorine, more effectively at 3 than at 15 Hz. The inhibition is antagonized by scopolamine in a manner that suggests a competitive interaction,

indicating the involvement of specific muscarinic receptors. Thus, sympathetic nerves innervating the airways, like those in the cardiovascular system (see Muscholl, 1980), are endowed with inhibitory muscarinic receptors.

Although the muscarinic receptor antagonist, scopolamine, failed to cause a significant increase in NA release under the present *in vitro* conditions, the present experiments can not exclude the possibility that endogenous acetylcholine released from parasympathetic nerves in the trachea may inhibit NA release. There are several reasons for which an endogenous cholinergic inhibition of NA release might have been missed under the present *in vitro* conditions. For example, blockade of muscarinic autoreceptors which can cause an enhanced release of acetylcholine from the parasympathetic nerves in the airways (Fryer & Maclagan, 1984; Faulkner *et al.*, 1986; Kilbinger *et al.*, 1990) may also facilitate the release of peptides, particularly those co-stored with acetylcholine, such as vasoactive intestinal polypeptide (VIP) or peptide histidine isoleucine (PHI) (see Barnes, 1987; Lundberg & Saria, 1987). Thus, a possible facilitation of NA release in the presence of a muscarinic receptor antagonist could have been masked by an augmented peptidergic inhibition of NA release. An increased release of inhibitory peptides could also be involved in the reduction of NA release after removal of the epithelium. Therefore, it seems very important that future experiments will elucidate the role of peptidergic mechanisms.

Finally, it should be considered that, particularly in rats, a large fraction of sympathetic nerve fibres within the airways is associated with vascular walls (Zussman, 1966; El-Bermani *et al.*, 1970, 1978). The present experiments cannot differentiate between NA released within airway smooth muscles and NA deriving from blood vessels. However, it appears not unlikely that NA released around blood vessels not only regulates blood flow, but may also significantly affect the airway smooth muscle, particularly because of the syncytial structure of the airway smooth muscle.

In conclusion, the present experiments show that the release of NA in the trachea, like that in other tissues, is inhibited by α-adrenergic autoreceptors, and muscarinic heteroreceptors. Of particular importance is the demonstration that sympathetic neurotransmission in the trachea is facilitated by an epithelium-dependent mechanism. Since epithelial damage is a common feature of obstructive bronchial disease (Dunnill, 1960; Boushey & Holtzman, 1985; Laitinen *et al.*, 1985), these observations suggest that an impaired sympathetic (bronchorelaxant) neurotransmission, together with an enhanced cholinergic (broncho-constrictor) neurotransmission (see Wessler *et al.*, 1990a,b), could be an important factor in the pathogenesis of the bronchial obstruction.

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The effect of inhibitors of the L-arginine/nitric oxide pathway on endotoxin-induced loss of vascular responsiveness in anaesthetized rats

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1 The effects on blood pressure and on pressor responses to noradrenaline (NA), of N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine methyl ester (L-NAME), inhibitors of the L-arginine/nitric oxide pathway, were investigated in anaesthetized rats receiving an infusion of bacterial endotoxin (*E. coli* lipopolysaccharide, LPS).

2 Infusion of LPS ($10\,mg\,kg^{-1}\,h^{-1}$) for 50 min had no effect on mean arterial blood pressure (MABP) but induced a reduction in responsiveness to noradrenaline ($100\,ng$–$1\,\mu g\,kg^{-1}$). L-NMMA ($30\,mg\,kg^{-1}$), but not D-NMMA, caused an increase in MABP of approximately 30 mmHg and restored responses to NA. This effect was reversed by L- but not D-arginine ($100\,mg\,kg^{-1}$).

3 In LPS-treated rats, blood pressure responses to NA were only marginally increased by the cyclo-oxygenase inhibitor, indomethacin ($5\,mg\,kg^{-1}$). L-NAME ($1\,mg\,kg^{-1}$) caused a similar increase in MABP and restored pressor responses to NA both in the presence and absence of indomethacin.

4 Co-infusion of vasopressin ($100\,ng\,kg^{-1}$, for 10 min) with LPS ($10\,mg\,kg^{-1}\,h^{-1}$) in order to reproduce the hypertensive effect of L-NMMA and L-NAME increased pressor responsiveness to 100 and $300\,ng\,kg^{-1}$ NA but not to $1\,\mu g\,kg^{-1}$ NA.

5 Infusion of sodium nitroprusside ($30\,\mu g\,kg^{-1}\,min^{-1}$) decreased responsiveness to NA even when the hypotension was corrected by co-infusion of vasopressin ($50\,ng\,kg^{-1}\,min^{-1}$).

6 These results demonstrate that the restoration of vascular responsiveness to NA in LPS-treated anaesthetized rats by inhibitors of the L-arginine/nitric oxide pathway is stereospecific and reversible. Furthermore, the experiments involving indomethacin suggest that although cyclo-oxygenase products of arachidonic acid may contribute to the development of LPS-induced hyporeactivity, the effect of L-NAME is unlikely to involve inhibition of the cyclo-oxygenase pathway. Comparison of NA responsiveness during vasopressin and L-NMMA/L-NAME-induced hypertension shows that increasing the blood pressure may modify LPS-induced hyporeactivity, but cannot account for the complete restoration of responses to NA by L-NMMA and L-NAME. These observations suggest that activation of nitric oxide formation from L-arginine makes a direct contribution to the production of vascular hyporeactivity by LPS *in vivo*.

Keywords: Endotoxin; vascular reactivity; L-arginine pathway; cyclo-oxygenase pathway; nitric oxide; L-NMMA; L-NAME; indomethacin

Introduction

Loss of vascular responsiveness to vasoconstrictor agents develops following administration, or release, of bacterial lipopolysaccharide (LPS) in animal models and may be an important factor contributing to eventual circulatory collapse. Cardiovascular hyporeactivity can be observed both *in vivo*, and *ex vivo* in vessels isolated from animals after LPS treatment. It affects the responses to a variety of constrictor substances including those acting independently of receptors (Parratt, 1989).

LPS is known to activate many cell types and administration to the whole animal sets in train the release of a variety of factors, which may be implicated as mediators of hyporeactivity. Among these we have recently proposed that nitric oxide (NO), derived from the amino acid L-arginine (Palmer *et al.*, 1988a) is a major mediator of the hyporeactivity seen both *in vivo* and *ex vivo* (Julou-Schaeffer *et al.*, 1990; Gray *et al.*, 1990a). This proposal was based largely upon the observation that responsiveness to constrictor agents in both systems was restored by N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of the L-arginine/NO pathway (Rees *et al.*, 1989a). Although the activity of L-NMMA and other related inhibitors has now been relatively well characterized *in vitro* (Moncada *et al.*, 1989; Rees *et al.*, 1990) and *in vivo* (Gardiner *et al.*, 1990a,b), their actions under pathological conditions like shock, have been less widely studied.

Underlying vascular tone is one of the factors which determines the response to vasoactive agents *in vivo* and this may be of particular importance after LPS administration when many vasodilator substances including prostacyclin, prostaglandins of the E series, plasma kinins and histamine, are liberated (Schlag & Redl, 1987). L-NMMA, in the dose required to restore responsiveness to noradrenaline also increased the blood pressure (Gray *et al.*, 1990b; Julou-Schaeffer *et al.*, 1990) probably through constriction of several vascular beds (Gardiner *et al.*, 1990a). At present the contribution of this increase in vascular tone compared to the inhibition of NO synthesis *per se* in the restoration of responsiveness to NA is unclear.

In a previous study L-NMMA prevented the development of LPS-induced hyporeactivity in anaesthetized rats, supporting our conclusion from *ex vivo* studies that activation of the L-arginine/nitric oxide pathway contributed to the induction of hyporesponsiveness by LPS (Julou-Schaeffer *et al.*, 1990). However, the possible contribution of indirect effects of L-NMMA to the restoration of responsiveness *in vivo*

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was not considered in this initial investigation. The aims of this present study were to characterize further the effect of L-NMMA in restoring responsiveness to noradrenaline *in vivo*, to examine its stereospecificity and reversibility and its potency compared to N^G-nitro-L-arginine methyl ester (L-NAME, a novel inhibitor of the L-arginine/nitric oxide pathway, Moore *et al.*, 1990), to investigate the role, if any, of a change in basal vascular tone and to consider the possibility of an interaction with the cyclo-oxygenase pathway of arachidonic acid metabolism. A preliminary account of some of these results has been given to a meeting of the British Pharmacological Society (Gray *et al.*, 1990b).

Methods

Anaesthesia was induced in male Wistar rats (12–15 weeks, 250–300 g) by intraperitoneal injection of sodium pentobarbitone (60 mg kg^{-1}) and maintained by intravenous injection as required. Arterial blood pressure was monitored with a pressure transducer (Gould Statham P23 ID) connected, via a cannula containing heparinised saline (100 iu ml^{-1}) to the right carotid artery. The output from the pressure transducer was displayed on a Beckman R511A pen recorder. Cannulae were also placed in the right and left femoral veins for administration of drugs, anaesthetic and LPS. The animals were allowed to breath spontaneously via a tracheal cannula. Body temperature was maintained at 37 ± 0.5°C with a heated underblanket controlled by a rectal thermistor probe.

Experimental protocols

After a stabilisation period of 20 min, pressor responses were obtained to noradrenaline (NA) 100 ng, 300 ng and 1 μg kg^{-1} (in 0.5 ml kg^{-1}). To assess the effect of LPS on NA-induced increases in blood pressure, responses were again obtained after 50 min of infusion of either LPS (10 mg^{-1} kg^{-1} h^{-1}) or its vehicle (saline, 0.4 ml h^{-1}). During continued infusion of LPS or saline the effects of various drug interventions were then investigated according to the protocols described below. The blood pressure was allowed to return to pre-NA levels between each drug.

Protocol 1 L-NMMA and L-arginine Pressor responses to NA were obtained 10 min after administration of L-NMMA (30 mg kg^{-1}), D-NMMA (30 mg kg^{-1}) or solvent (saline), again 5 min after D-arginine (100 mg kg^{-1}) or saline and then finally 5 min after L-arginine or saline.

Protocol 2 Indomethacin and L-NAME Pressor responses to NA were obtained 15 min after administration of indomethacin (5 mg kg^{-1}) or its solvent (4% bicarbonate) and then subsequently 10 min after L-NAME (1 mg kg^{-1}).

Protocol 3 Vasopressin Pressor responses to NA were obtained during a 10 min infusion of vasopressin (100 ng^{-1} kg^{-1} min^{-1} in LPS-treated and 50 ng^{-1} kg^{-1} min^{-1} in control rats) when the blood pressure had stabilised at approximately 30 mmHg above the pre-infusion level and then subsequently when blood pressure had returned to control levels on the cessation of infusion.

Protocol 4 Sodium nitroprusside In the absence of LPS or saline infusion, pressor responses to NA were obtained before infusion of sodium nitroprusside (SNP, 30 μg^{-1} kg^{-1} min^{-1}), during a 10 min infusion of SNP and then again on re-infusion of SNP with vasopressin (50 ng^{-1} kg^{-1} min^{-1}), which corrected its blood pressure lowering effect (see Results).

Drugs

Noradrenaline bitartrate, heparin sodium, N^G-nitro-L-arginine methyl ester hydrochloride, D-arginine hydrochloride and [Arg^8]-vasopressin acetate (all Sigma); L-arginine hydrochloride (Calbiochem); L- and D-N^G-monomethylarginine citrate (Salford Ultrafine chemicals); sodium nitroprusside (Merck) and lipopolysaccharide (*E. coli* 055:B5, Difco) were dissolved in 0.9% saline. Indomethacin (Sigma) was dissolved in a 4% solution of bicarbonate. Doses of drugs were calculated as g of salt per kg body weight.

Statistical analysis

Results are expressed throughout as arithmetic mean ± s.e.mean. Pressor responses to NA were compared by analysis of variance (ANOVA). Where ANOVA showed significant differences ($P < 0.05$) the results were further analysed with an *a posteriori* Student Newman-Keuls test.

Results

The effect of E. coli *lipopolysaccharide on mean arterial blood pressure and on pressor responses to noradrenaline*

Infusion of LPS (10 mg^{-1} kg^{-1} h^{-1}) over 50 min caused no significant change in mean arterial blood pressure (MABP) of anaesthetized rats (122 ± 3 mmHg before infusion and 117 ± 3 mmHg at 50 min, $n = 26$). Dose-dependent pressor responses to NA in these rats were, however, significantly reduced (Figure 1a and b). In control rats neither the MABP

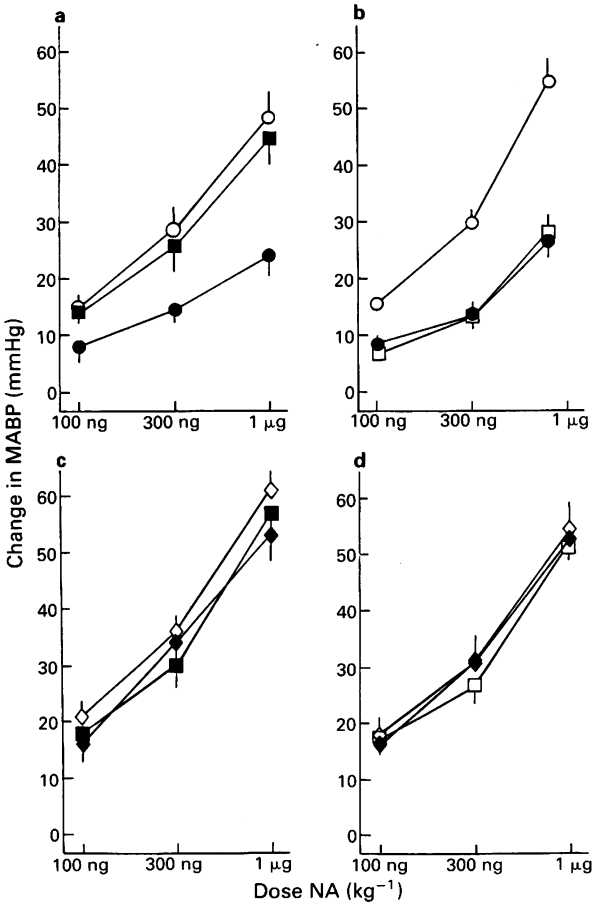

Figure 1 Effects of N^G-monomethyl-L-arginine (L-NMMA) and D-NMMA on increases in mean arterial blood pressure (MABP) elicited by noradrenaline (NA, 100 ng–1 μg kg^{-1}) during continuous infusion of *E. coli* lipopolysaccharide (LPS, 10 mg kg^{-1} h^{-1}, a and b) or saline (c and d) in anaesthetized rats. NA-induced pressor responses are shown before LPS (○) or saline (◇) infusion, after 50 min of LPS (●) or saline (◆) infusion and 10 min after administration of L-NMMA (■, 30 mg kg^{-1}, a and c) or D-NMMA (□, 30 mg kg^{-1}, b and d). Values are the mean of 6–7 experiments; vertical lines show s.e.mean.

(100 ± 5 mmHg before infusion and 103 ± 5 mmHg at 50 min, $n = 15$) nor pressor responsiveness to NA (Figure 1c and d) were altered by infusion of saline.

Effects of L-NMMA, D-NMMA, L- and D-arginine on mean arterial blood pressure and pressor responses to noradrenaline

Administration of L-NMMA (30 mg kg^{-1}) to LPS-treated rats resulted in a significant ($P < 0.01$) increase in MABP of 33 ± 5 mmHg ($n = 8$) and in the restoration of pressor responses to NA (Figure 1a). An equivalent dose of D-NMMA (Figure 1b) or saline (1 ml kg^{-1}, results not shown) had no significant effect either on MABP or on NA-induced pressor responses.

In control rats, infusion of L-NMMA increased the MABP to a similar degree as in LPS-treated rats (34 ± 6 mmHg, $n = 6$) while D-NMMA and saline were without effect. NA-induced increases in MABP were not altered by any of these interventions in control rats (Figure 1c and d, saline not shown).

During the continuous infusion of LPS (Figure 2), neither the L-NMMA induced increase in MABP nor the restored pressor responses to NA could be reversed by D-arginine (100 mg kg^{-1}) but both were reduced by the subsequent administration of L-arginine (100 mg kg^{-1}, Figure 2a). Both effects of L-NMMA were sustained until the end of the experiment if saline was injected in place of D- and L-arginine (Figure 2b). In the absence of L-NMMA, administration of L-arginine (100 mg kg^{-1}) alone caused no change in MABP or in pressor responsiveness to NA (Figure 2c). In control animals the L-NMMA-induced increase in blood pressure was reversed by L-arginine (MABP reduced from 142 ± 9 mmHg to 107 ± 14 mmHg, $P < 0.05$) but was unaffected by D-arginine or solvent (results not shown).

Effects of L-NAME and indomethacin on mean arterial blood pressure and pressor responses to noradrenaline

Because there is evidence of a common pathway for stimulation of nitric oxide and prostacyclin release in endothelial cells (de Nucci *et al.*, 1988), and for a role for cyclo-oxygenase products in mediating vascular hyporesponsiveness following LPS administration (Gray *et al.*, 1990c), indomethacin (5 mg^{-1} kg^{-1}) was administered during LPS infusion. This resulted in a significant increase in MABP (from 110 ± 6 to 125 ± 4 mmHg, $P < 0.02$). In addition, responses to NA 300 ng and 1 μg kg^{-1}, but not to 100 ng kg^{-1} were slightly but significantly ($P < 0.05$) increased after indomethacin (Figure 3a). The solvent for indomethacin (4% bicarbonate) was without effect on either MABP (105 ± 2 mmHg pre vs 109 ± 3 mmHg post) or on reduced responses to NA (Figure 3b). Subsequent administration of L-NAME (1 mg kg^{-1}) caused a similar increase in MABP in indomethacin pretreated (17 ± 1 mmHg) and in solvent pretreated rats (20 ± 4 mmHg) and restored pressor responses to NA to pre-LPS infusion values in both groups (Figure 3b). In saline-infused rats, neither indomethacin nor its solvent affected MABP or pressor responses to NA (results not shown). In these control rats, L-NAME increased the MABP (22 ± 4 mmHg in indomethacin pretreated, $n = 5$, 24 ± 3 mmHg in control, $n = 5$) but, like L-NMMA (Figure 1), had no effect on NA-induced pressor responses (see sample trace in Figure 4). For example, in rats not receiving indomethacin pretreatment increases in MABP in response to NA 100 ng, 300 ng and 1 μg kg^{-1} were 16 ± 2, 36 ± 3 and 52 ± 6 mmHg before L-NAME and 16 ± 4, 36 ± 3 and 53 ± 3 mmHg after L-NAME respectively ($n = 5$).

Effect of vasopressin on pressor responses to noradrenaline

In LPS-treated and in control rats vasopressin (VP) was infused at rates which induced similar increases in MABP to those observed with L-NMMA and L-NAME (see above). As in the previous groups LPS induced a reduction in pressor responsiveness to NA (Figure 5a), but no significant change in the MABP. During continuous LPS infusion a dose of 100 ng^{-1} kg^{-1} min^{-1} of VP was required to induce a change in MABP of 28 ± 2 mmHg. In control rats a similar increase in MABP (29 ± 7 mmHg) was induced by infusion of 50 ng^{-1} kg^{-1} min^{-1} of VP. This indicates that pressor responsiveness to VP as well as to NA was reduced by LPS infusion. During the infusion of VP in LPS-treated rats, pressor responses to 100 ng and 300 ng kg^{-1} of NA were slightly increased whereas those to 1 μg kg^{-1} NA were unaffected

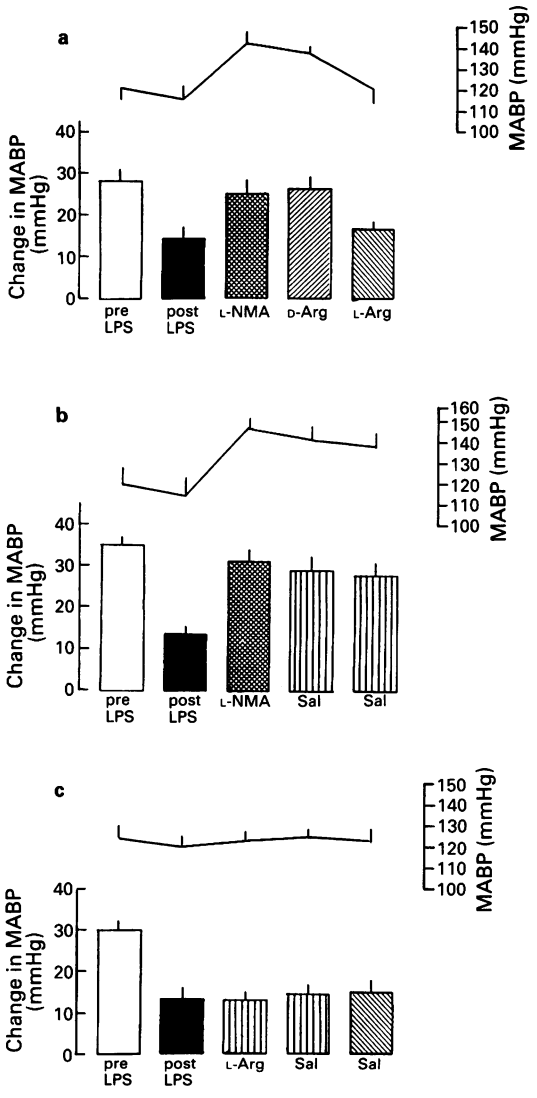

Figure 2 Stability of the effects of N^G-monomethyl-L-arginine (L-NMMA, 30 mg kg^{-1}) on MABP (line graphs) and pressor responses to noradrenaline (NA, 300 ng kg^{-1}, histograms) and their reversal by L- but not D-arginine (100 mg kg^{-1}) during continuous infusion of *E. coli* lipopolysaccharide (LPS, 10 mg kg^{-1} h^{-1}) in anaesthetized rats. The increase in MABP and restoration of responsiveness by L-NMMA (L-NMA) in (a) are both reversed by subsequent administration of L (L-Arg) but not D-arginine (D-Arg). (b) Shows them both maintained during saline (Sal) admininstration. In (c) both the MABP and LPS-induced depression of responsiveness are maintained during the time period of the experiment and MABP is not reduced, nor the hyporesponsiveness to NA enhanced, by provision of additional L-arginine (100 mg kg^{-1}). Values are the mean of 6–7 experiments; vertical lines show s.e.mean. * $P < 0.05$ compared to pre-infusion value.

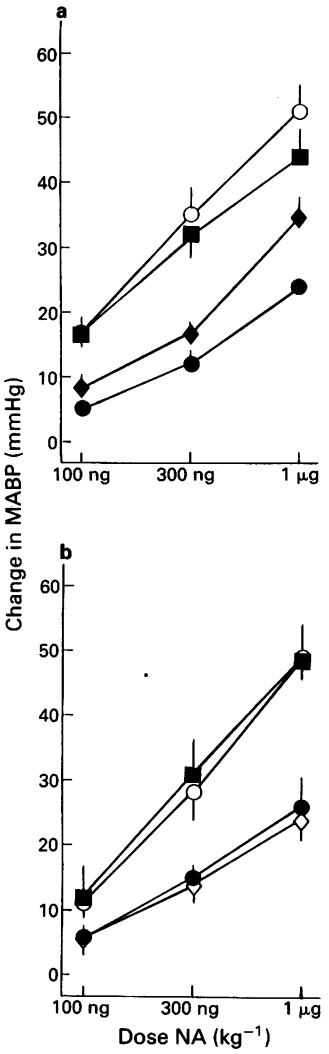

Figure 3 The effect of indomethacin (5 mg kg^{-1}) or its solvent (4% bicarbonate), and N^G-nitro-L-arginine methyl ester (L-NAME, 1 mg kg^{-1}) on pressor responses to noradrenaline (NA, 100 ng–1 μg kg^{-1}) in anaesthetized rats receiving a continuous infusion of *E. coli* lipopolysaccharide (LPS, 10 mg kg^{-1} h^{-1}, i.v.). NA-induced increases in mean arterial blood pressure (MABP) are shown before infusion (○), after 50 min of LPS infusion (●), 10 min after administration of indomethacin (◆, a) or its solvent (◇, b) and 5 min after the subsequent administration of L-NAME (■, a and b). Values are the mean of 6 experiments; vertical lines show s.e.mean.

(Figure 5a). VP was without effect on NA responses in control animals (Figure 5b).

Effect of sodium nitroprusside infusion on mean arterial blood pressure and pressor responses to noradrenaline

As in the experiments described above, NA produced a dose-dependent increase in the MABP of anaesthetized rats (Figure 6). Infusion of sodium nitroprusside (SNP, 30 μg^{-1} kg^{-1} min^{-1}) lowered MABP (from 120 ± 5 mmHg to 59 ± 1 mmHg, $P < 0.01$) and induced a significant reduction in these NA-induced pressor responses (Figure 6). On cessation of the SNP infusion both the MABP and NA responsiveness returned to normal (results not shown). To counteract the influence of the SNP-induced hypotension, it was co-infused with vasopressin (50 ng^{-1} kg^{-1} min^{-1}). Although the MABP was now similar to the pre-infusion level (118 ± 4 mmHg), pressor responsiveness to NA remained significantly depressed (Figure 6).

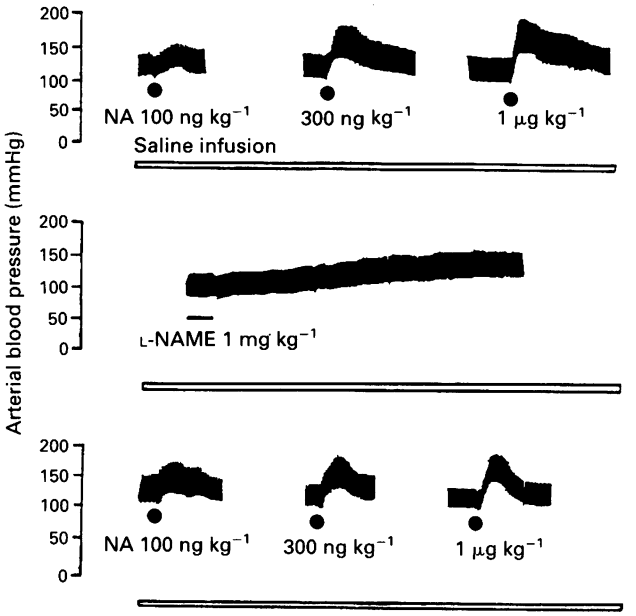

Figure 4 A representative trace showing the effect of N^G-nitro-L-arginine methyl ester (L-NAME, 1 mg kg^{-1}) on continuously recorded arterial blood pressure and pressor responses to noradrenaline (NA, 100 ng–1 μg kg^{-1}) in anaesthetized rats during infusion of saline (0.4 ml h^{-1}). Infusion of L-NAME over 1 min induced an increase in the basal blood pressure which was maintained during the administration of NA. Pressor responses to NA obtained 10 min after infusion of L-NAME were not altered from those obtained before L-NAME. This trace is representative of the data from 5 experiments, values are given in the text.

Discussion

In agreement with our previous findings (Gray *et al.*, 1990b; Julou-Schaeffer *et al.*, 1990) L-NMMA increased the mean arterial blood pressure and restored pressor responses to noradrenaline after infusion of LPS. The reversibility and stereospecificity of this effect is shown by the re-establishment of hyporesponsiveness by L- but not D-arginine and by the lack of effect of the D-enantiomer of NMMA, which does not inhibit synthesis of NO by endothelial cells (Palmer *et al.*, 1988b). Responsiveness was also restored by another analogue of L-arginine, L-NAME which has recently been shown to inhibit vascular nitric oxide synthesis (Rees *et al.*, 1990). It is of interest that this inhibitor was at least 30 times more potent than L-NMMA in both elevating blood pressure and in restoring reactivity to NA. This can probably be explained by the fact that L-NMMA but not L-nitroarginine undergoes substantial metabolism by the vascular endothelium to yield L-arginine, thus essentially becoming a substrate rather than an inhibitor (Hecker *et al.*, 1990). We have previously found that hyporesponsiveness to NA (Julou-Schaeffer *et al.*, 1990) and to calcium (Gray *et al.*, 1990a) in aortae removed from rats given LPS is enhanced in the presence of additional L-arginine. This implies that L-arginine is a rate limiting factor and that endogenous tissue supplies of the amino acid are depleted following LPS administration. However, in the present *in vivo* studies provision of L-arginine had no potentiating effect, suggesting that supplies of L-arginine are sufficient to allow full expression of the L-arginine/NO pathway. In this context, it is noteworthy that plasma levels of arginine are reported to be increased in the later stages of severe clinical septic shock (Cerra *et al.*, 1979).

Activation of the L-arginine/NO pathway was recently implicated in the induction of hypotension by endotoxin (Thiemermann & Vane, 1990) and by tumour necrosis factor, a cytokine released from macrophages in response to endotoxin (Kilbourn *et al.*, 1990). However, despite evidence for

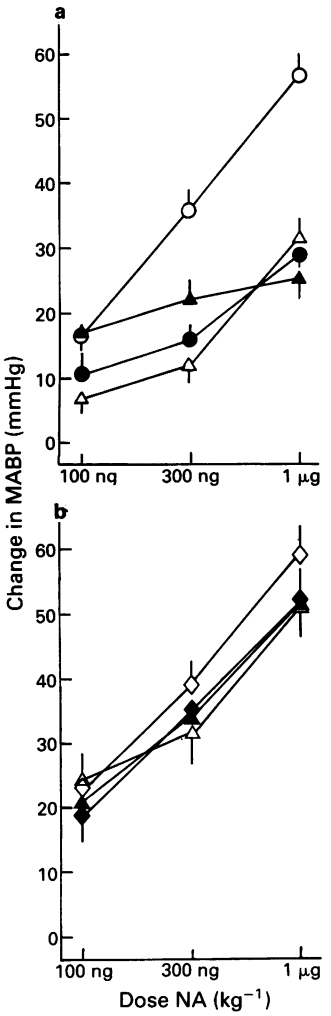

Figure 5 The effect of vasopressin, infused at doses to increase the mean arterial blood pressure (MABP) by approximately 30 mmHg, on pressor responses to noradrenaline (NA, 100 ng–1 $\mu g\,kg^{-1}$). (a) Increases in MABP elicited by NA before infusion of *E. coli* lipopolysaccharide (LPS) (○), after 50 min of LPS infusion (●, 10 $mg\,kg^{-1}\,h^{-1}$), during co-infusion of LPS with vasopressin (▲, 100 $ng^{-1}\,kg^{-1}\,min^{-1}$) and on cessation of vasopressin infusion (△). (b) Increases in MABP elicited by NA before infusion of saline (◇), after 50 min of saline infusion (◆), during co-infusion of saline with vasopressin (▲, 50 $ng^{-1}\,kg^{-1}\,min^{-1}$) and on cessation of vasopressin infusion (△). Values are the mean of 6–7 experiments; vertical lines show s.e.mean.

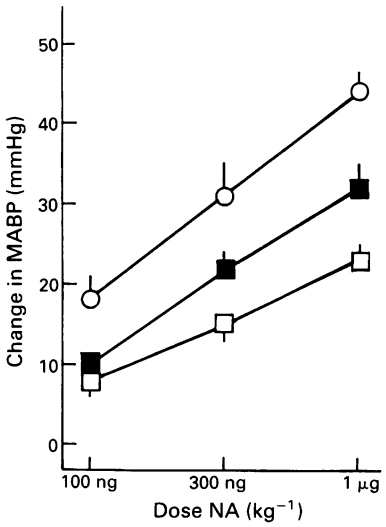

Figure 6 The effect of sodium nitroprusside (SNP, 30 $\mu g\,kg^{-1}\,min^{-1}$) alone, or co-infused with vasopressin (50 $ng\,kg^{-1}\,min^{-1}$, ■) on noradrenaline (NA, 100 ng–1 $\mu g\,kg^{-1}$)-induced increases in mean arterial blood pressure (MABP) in anaesthetized rats. Pressor responses are shown before SNP infusion (○), during infusion of SNP (□), and on co-infusion of SNP with a dose of vasopressin which corrected the SNP-induced hypotension. Values are the mean of 6 experiments; vertical lines show s.e.mean.

increased activation of this pathway by LPS infusion in the present study, there was no associated change in basal blood pressure. Hypotension can be produced by use of larger doses of LPS in anaesthetized rats, but a non-hypotensive dose was specifically chosen for the present study to simplify the comparison of NA-induced pressor responses between control and LPS-treated rats. It is likely that reflex compensatory mechanisms (McKechnie *et al.*, 1985) play a role in the prevention of hypotension. In pithed rats, which lack such reflex responses, hypotension is induced by much lower doses of LPS than that used here (Gray *et al.*, 1990c).

A paradoxical feature of the present results is that although L-NMMA and L-NAME caused equivalent increases in mean arterial blood pressure in control rats they had no effect on pressor responses to NA. Functional (Palmer *et al.*, 1988b) or mechanical (Furchgott & Zawadski, 1980) removal of the endothelium *in vitro* results in both an enhanced sensitivity and an increase in the maximal contractile responses to α-adrenoceptor agonists. This is believed to be due to inhibition of a basal release of relaxing factor/NO from the endothelium (Martin *et al.*, 1985). The pressor effect of L-NMMA *in vivo* has also been attributed to inhibition of this basal release (Rees *et al.*, 1989b; Aisaka *et al.*, 1989). Consequently, one might expect to see potentiation of *in vivo* responses to NA when there is functional impairment of the endothelium. The failure to demonstrate such an enhanced pressor response may be explained in several ways. Since responses to NA were measured in terms of changes in arterial blood pressure, it is possible that any potentiating effects of L-NMMA and L-NAME at the vascular level were masked by depression of cardiac output (through negative inotropic and chronotropic actions, Gardiner *et al.*, 1990b). However, the present dose of L-NAME was 10 times lower than that which reduced the cardiac output in the study of Gardiner *et al.* (1990b). Moreover, it remains true that L-NMMA and L-NAME enhanced NA-induced pressor responses in LPS-treated rats, in which they would also have caused a reduction of cardiac output. The results could also therefore be interpreted as showing that the basal release of NO from the endothelium is less important in determining responsiveness *in vivo* than *in vitro* or that L-NMMA and L-NAME are acting at a site removed from the endothelium to induce systemic hypertension. An examination of the effects of these inhibitors on NA-induced changes in regional vascular blood flow would be necessary to resolve the question of the role of basal NO release in determining vascular reactivity *in vivo*.

Whatever the mechanism, hypertension developed over a period of several min in response to both L-NMMA and L-NAME and was maintained for at least 30 min. Surprisingly the hypertension was of the same magnitude in both LPS-treated and control rats. These results contrast with those of a recent study in which L-NMMA-induced hypertension was significantly enhanced in anaesthetized dogs treated with tumour necrosis factor (TNF), a cytotoxic protein produced by macrophages on activation with bacterial endotoxin (Kilbourn *et al.*, 1990). A possible explanation for the discrepancy is that administration of TNF in the latter study was associated with a profound systemic hypotension whereas a non-hypotensive dose of LPS was chosen for the current experiments. The difference may reflect differences in degree of activation of the L-arginine/NO pathway. Alternatively, the doses of inhibitors used in the present study may have only submaximally inhibited NO production; certainly the dose of L-NMMA used in the present study (30 $mg\,kg^{-1}$), was found

to be submaximal in increasing the blood pressure in anaesthetized rabbits (Rees *et al.*, 1989b).

Because the hypertensive effects of L-NMMA and L-NAME are mediated largely by constriction in the renal, mesenteric and hindquarters vascular beds (Gardiner *et al.*, 1990a) we chose vasopressin, which causes a similar profile of vascular bed constriction (Gardiner *et al.*, 1989), to examine the role of the increase in basal blood pressure in restoring vascular responsiveness to NA during LPS infusion. Whilst this degree of hypertension had no effect on NA-induced pressor responses in control rats it slightly increased responses to the two lower doses of NA during LPS infusion. This result shows that although an increase in vascular tone may exert some influence in LPS-treated rats, it alone cannot account for the restoration of reactivity to NA by L-NMMA and L-NAME.

Prostaglandins have been extensively studied in models of endotoxaemia and septic shock and have been implicated in many of the pathophysiological sequelae (reviewed by Ball *et al.*, 1986). Indeed treatment with cylo-oxygenase enzyme inhibitors before either the induction of sepsis (Fink *et al.*, 1985) or infusion of LPS (Gray *et al.*, 1990c) prevents the development of hyporeactivity. In the present study, indomethacin given during LPS infusion in a dose which inhibits prostaglandin synthesis (Higgs & Flower, 1981) resulted in a marginal increase in the blood pressure and incomplete restoration of pressor responses to NA. This implies a limited contribution from vasodilator prostaglandins in determining vascular hyporesponsiveness, at least in this model. It does not exclude the possibility that indomethacin was indirectly blocking the release of NO. Prostaglandin E_2 (PGE_2) has recently been shown to increase LPS-stimulated NO synthesis by macrophage and Kupffer cell cytosol by increasing adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Mülsch *et al.*, 1990). The possibility that prostaglandins potentiate, or even initiate, the events leading to activation of the L-arginine/NO pathway by LPS accommodates the previous observations with inhibitors of cyclo-oxygenase (Fink *et al.*, 1985; Gray *et al.*, 1990c) and the current results with inhibitors of NO synthase. However, we found no evidence for the possibility that L-NAME increases blood pressure, or restores reactivity, through inhibition of prostaglandin synthesis in LPS-treated rats. Both these effects were the same after indomethacin or solvent pretreatment.

NO is believed to modulate vascular tone through stimulation of the guanylate cyclase enzyme in the cytosol of smooth muscle cells (Arnold *et al.*, 1977; Schultz *et al.*, 1977). The resulting accumulation of guanosine 3':5'-cyclic monophosphate (cyclic GMP), acting through several mechanisms (reviewed by Lincoln, 1989) reduces the availability of intracellular calcium to contractile proteins. The hyporesponsiveness to NA which occurred during infusion of sodium nitroprusside (which spontaneously releases NO and stimulates guanylate cyclase, Katsuki *et al.*, 1977) can be taken as further evidence for NO release by LPS. This has been confirmed in *ex vivo* experiments where we have found an increased concentration of cyclic GMP in aortae from LPS-treated, compared to control rats (Fleming *et al.*, 1990a). Moreover, contractile responses to NA (Julou-Schaeffer *et al.*, 1990) and to calcium in depolarizing solution (Gray *et al.*, 1990a) were restored by methylene blue, an inhibitor of soluble guanylate cyclase (Martin *et al.*, 1985). A role for cyclic GMP in production of hyporeactivity by LPS is a particularly attractive hypothesis given that it was proposed that the lesion produced by LPS involved post-receptor disruption of intracellular calcium regulation (Bigaud *et al.*, 1990).

While the present results suggest that NO production from L-arginine is increased after LPS administration, the cell type responsible remains to be determined. Evidence is now available for the presence of NO synthase in several of the many cell types activated by LPS, including macrophages (Hibbs *et al.*, 1987), neutrophils (Rimele *et al.*, 1989), endothelial cells (Mayer *et al.*, 1989; Mülsch *et al.*, 1989; Salvemini *et al.*, 1990a), mast cells (Salvemini *et al.*, 1990b), platelets (Radomski *et al.*, 1990), hepatocytes (Billiar *et al.*, 1990) and Kuppfer cells (Billiar *et al.*, 1989) all of which are potential sources *in vivo*. However, the demonstration of LPS-induced hyporeactivity in endothelium-denuded rat aortae (Fleming *et al.*, 1990b; Julou-Schaeffer *et al.*, 1990; Gray *et al.*, 1990a) would seem to exclude many of these cells and suggests a vascular source of NO. Indeed several authors have recently concluded that the L-arginine/NO pathway can occur in smooth muscle cells on the basis of experiments with endothelium-denuded aorta, either after L-arginine depletion (Wood *et al.*, 1990) or after removal from endotoxin-treated rats (Knowles *et al.*, 1990).

In summary, the present results show that neither the hypertensive effects of L-NMMA and L-NAME *per se* nor an interaction with cyclo-oxygenase products account entirely for the reversal of the LPS-induced vascular hyporeactivity by these inhibitors of the L-arginine/NO synthase pathway. These results and recent reports that NO contributes to TNF—(Kilbourn *et al.*, 1990) and endotoxin—(Thiemermann & Vane, 1990) induced hypotension therefore support our previous hypothesis that NO is a key mediator in endotoxaemia (Fleming *et al.*, 1990b; Julou-Schaeffer *et al.*, 1990).

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Effects of pretreatment with caffeine or ryanodine on the myocardial response to simulated ischaemia

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1 The cytoplasmic calcium concentration ($[Ca]_c$) of rat isolated atrial myocardium was assessed with the dye indo-1. Dye-loaded atria were superfused with physiological salt solution and excited with radiation at 360 nm, while epifluorescence emissions were collected simultaneously at 400 nm and 500 nm. The ratio of these emissions was used as a measure of $[Ca]_c$.

2 Dye-loaded atria showed a phasic rise and fall in $[Ca]_c$ with each applied electrical pacing stimulus. The amplitude of these oscillations was reduced by the presence of caffeine (10^{-3}–10^{-2} M) or of ryanodine (10^{-8}–10^{-6} M) in a concentration-dependent manner.

3 Atria superfused with a solution the composition of which resembled that found extracellularly in regions of myocardial ischaemia rapidly lost systolic increments in $[Ca]_c$, while end-diastolic $[Ca]_c$ values gradually rose.

4 Pretreatment with caffeine (10^{-2} M) or ryanodine (10^{-7} M) protected atria against the rise in end-diastolic $[Ca]_c$ that occurred when the tissue was exposed to conditions of simulated ischaemia.

Keywords: Rat atrial myocardium; cytoplasmic calcium concentration; indo-1 fluorescence; simulated ischaemia; caffeine; ryanodine

Introduction

The concentration of calcium in the cytoplasm of superfused myocardial cells ($[Ca]_c$) has been shown to rise in response to conditions that simulate those which prevail during ischaemia (Northover, 1990). Depletion of the cellular store of adenosine 5'-triphosphate (ATP) which occurs under these conditions (Northover, 1989) probably is responsible for the rise in $[Ca]_c$. Moreover, the rise in $[Ca]_c$ appears to be directly responsible for the deleterious electrical, mechanical and structural changes that occur during ATP depletion (Wexler *et al.*, 1986; Northover, 1987; 1989; Leijendekker *et al.*, 1990). Whether the Ca which accumulates in the myoplasm under these circumstances is derived from the extracellular space via the sarcolemma, or is released from an intracellular store such as the sarcoplasmic reticulum (SR), however, is uncertain. Caffeine and ryanodine were used in the present study to examine the role of the SR in this connection. Both drugs deplete the cardiac SR of its Ca, albeit by different mechanisms (Hess & Wier, 1984; Kondo, 1988; Sitsapesan & Williams, 1990). Moreover, both drugs protect the heart against disturbances of rhythm associated with loading of the myocardium with Ca (Hajdu & Leonard, 1961; Kahn *et al.*, 1964; DiGennaro *et al.*, 1983; 1984; Golovina *et al.*, 1988; Thandroyen *et al.*, 1988; Boutjdir *et al.*, 1990). If the SR contributes to myoplasmic Ca during simulated ischaemia (SI), then one might expect caffeine and ryanodine to reduce the rise in $[Ca]_c$ under these conditions.

Methods

Atria were isolated from rat hearts and mounted on a plastic holder as previously described (Northover, 1990). Atria were bathed, unless otherwise stated, with normal superfusate (NS) of the following composition (mM): NaCl 138, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, NaH_2PO_4 0.5, $NaHCO_3$ 10, glucose 10, and gassed with 95% O_2 plus 5% CO_2, Atria were stimulated throughout an experiment at 4 Hz with 2 ms square wave pulses, each of 10 V. A thread sutured to the left atrial appendage was connected to a force displacement transducer (type SB-IT, Nihon Khoden) at a diastolic tension of 100 mg. Tension was recorded via a d.c. amplifier (type 3552, Cardiovascular Instruments) coupled to a heated stylus recorder (type 5041, Lectromed). Atria were allowed to equilibrate in NS for 1 h before dye-loading.

Dye-loading and fluorescence measurement

Atria were loaded with the acetoxymethyl ester of indo-1 (Sigma) as described previously (Northover, 1990).

Dye-loaded atria, on the plastic holder, were positioned diagonally across a standard 1 cm square-section quartz cuvette and maintained at 34°C in a specially designed fluorimeter equipped with one excitation monochromator and with two emission monochromators, each with a photon-counting photomultiplier tube (Alphascan System, Photon Technology International). Epifluorescence emissions were collected digitally at 60 data points s^{-1} from the front face and one side face of the cuvette. Excitation was at 360 nm, and the two emission monochromators were set at 400 nm and 500 nm respectively. Grynkiewicz *et al.* (1985) showed that the intensity of fluorescence (F) emitted by indo-1 is increased by calcium in a concentration-dependent manner at 400 nm, but diminished at 500 nm, and that the ratio of emission intensities at these two wavelengths (F400/500) provides a measure of the free calcium concentration.

Prior to dye-loading, the combined tissue and instrumental autofluorescence signals were recorded and filed in computer memory, and then routinely deducted from signals obtained later with atria in the dye-loaded state. Tissue autofluorescence needed to be recorded during each experimental intervention, however, as it varied under different conditions of superfusion. Autofluorescence corrected signals from dye-loaded atria were typically about 5 fold greater than tissue autofluorescence intensity.

Simulated ischaemia

In order to simulate ischaemic conditions the NS was replaced by a superfusate of abnormal composition (Ferrier *et al.*, 1985), containing KCl 7 mM, racemic sodium lactate 20 mM and no glucose. The pH was reduced to 6.4 by reducing the $NaHCO_3$ to 4 mM. Hypoxia was produced by replacing O_2 by N_2 containing 0.0002% or less of O_2 (British Oxygen Company).

Drugs

Ryanodine was purchased from Calbiochem (Lot 910031, assayed to contain 35% ryanodine and 54% dehydroryanodine). Ryanodine and dehydroryanodine have indistinguishable pharmacological properties on the SR (Waterhouse *et al.*, 1987; Ito *et al.*, 1989). Caffeine was purchased from Sigma.

Results

Caffeine

Caffeine in NS at between 10^{-3} and 10^{-2} M produced concentration-dependent changes in the behaviour of dye-loaded atria paced at 4 Hz. Although caffeine at 10^{-3} M failed to alter peak systolic and end-diastolic tensions, it rapidly reduced peak systolic F400/500 ratios (Figure 1). Since end-diastolic fluorescence values did not change significantly, the calculated systolic increment in F400/500 ratios also declined under the influence of caffeine. In contrast to this, caffeine at 10^{-2} M produced a brief increase followed by a persistent and total loss of systolic developed tension. During a 15–20 min period of exposure to this concentration of caffeine a substantial contracture developed. When contracture was established neither the tension values nor the F400/500 ratios showed systolic increments in response to electrical pacing stimuli, but after a small early diastolic rise, all F400/500 ratios declined to approximate end-diastolic values recorded in the absence of caffeine (Figure 2). All of these effects of caffeine were reversed when atria were returned to drug-free NS.

Ryanodine

During pacing of dye-loaded atria at 4 Hz the addition of ryanodine to NS at between 10^{-8} and 10^{-6} M reduced systolic increments in atrial tension and the F400/500 ratios in a concentration-dependent manner. This was due to diminished peak systolic but not end-diastolic values (Figure 3). The effects of ryanodine were slowly progressive during the first 30 min of exposure to the drug and were then not reversible, even after treatment with drug-free NS for up to 2 h.

Stimulation rate-dependent effects of ryanodine and caffeine

For these experiments the dye-loaded atria were exposed initially to drug-free NS and paced for 5 min first at 6 Hz and then for a further 5 min at 3 Hz. Both systolic developed tension values and peak systolic F400/500 ratios at the end of 5 min of pacing at 6 Hz were less than corresponding values at the end of 5 min of pacing at 3 Hz (Figure 4). This constitutes a negative rate-tension staircase of the type that has been extensively investigated by Stemmer & Akera (1986). There was a similar staircase in concurrent F400/500 ratios (Figure 4). Atria were then treated for 30 min with ryanodine at 10^{-8} M. Ryanodine-treated atria paced for 5 min at 6 Hz showed systolic developed tension values and peak systolic F400/500 ratios that were larger than corresponding values seen at the end of 5 min of pacing at 3 Hz (Figure 4). Ryan-

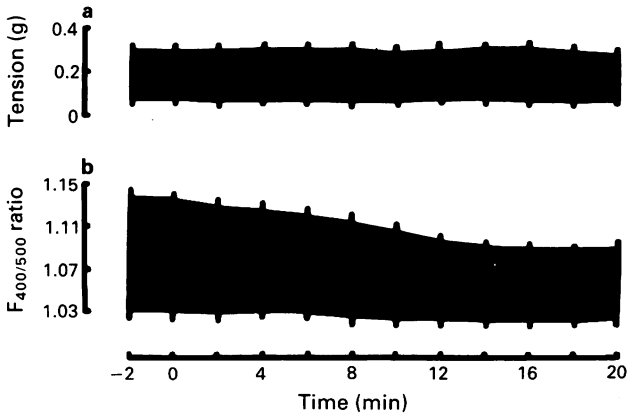

Figure 1 Atrial tension (a) and F400/500 ratios (b) during exposure of rat isolated atria to caffeine 10^{-3} M in normal superfusate from time zero. Upper edges of each panel represent peak systolic values and lower edges end-diastolic values. Vertical bars represent s.e.mean of 20 systolic-diastolic cycles from each of 10 dye-loaded atria.

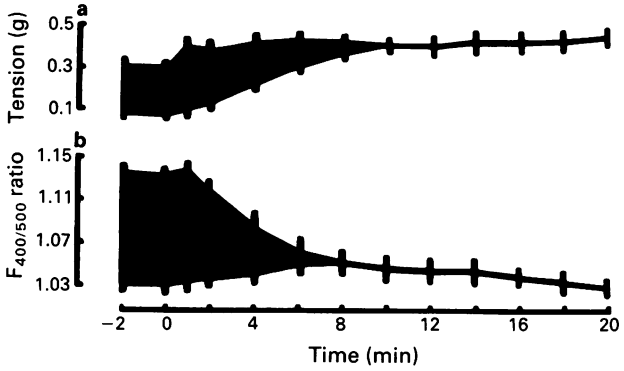

Figure 2 Atrial tension (a) and F400/500 ratios (b) during exposure of rat isolated atria to caffeine 10^{-2} M in normal superfusate from time zero. Upper edges of each panel represent peak systolic values and lower edges end-diastolic values. Vertical bars represent s.e.mean of 20 systolic-diastolic cycles from each of 10 dye-loaded atria.

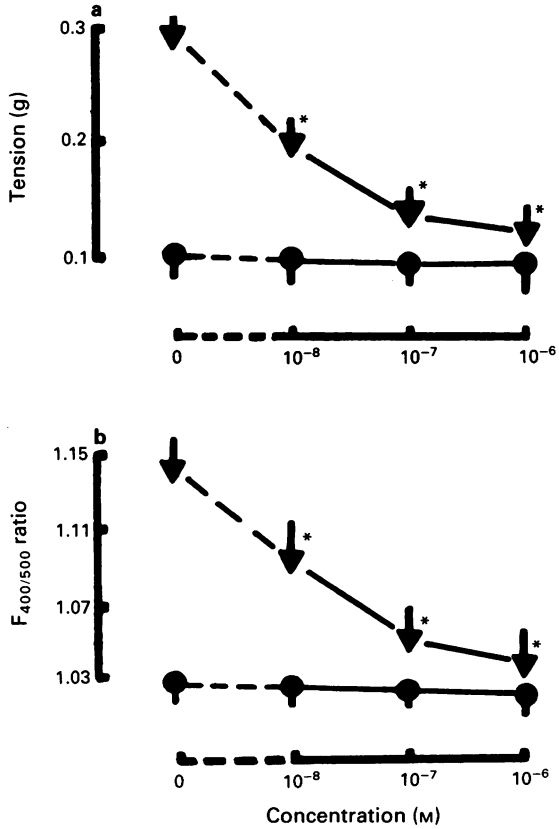

Figure 3 Atrial peak systolic (▼) and end-diastolic (●) tensions (a) and F400/500 ratios (b) after exposure for 30 min to various concentrations of ryanodine in normal superfusate. Vertical bars represent s.e.mean of 20 systolic-diastolic cycles from each of 10 dye-loaded atria. A significant difference exists ($P < 0.05$) between a value marked * and the corresponding peak systolic value of the ryanodine-free control.

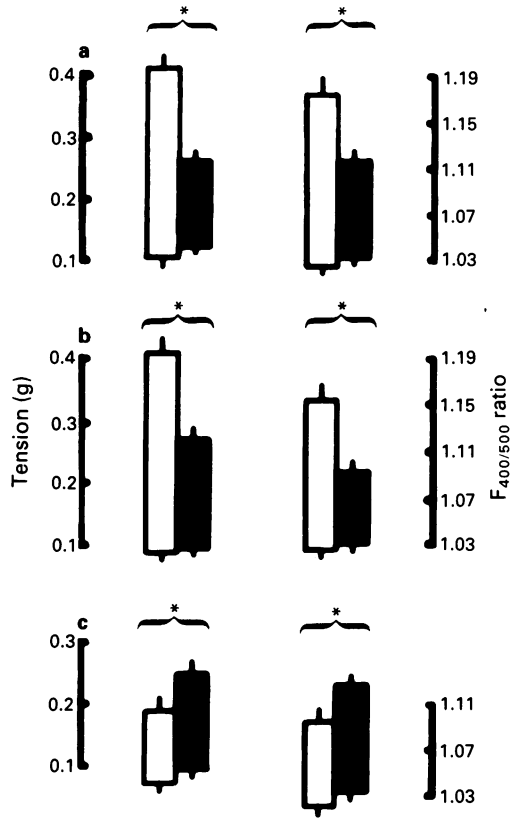

Figure 4 Atrial peak systolic (top of column) and end-diastolic (bottom of column) values of tension and corresponding F400/500 ratios after pacing for 5 min at 6 Hz (solid columns) or at 3 Hz (open columns). Atria were exposed to normal superfusate (NS) alone (a), to NS containing caffeine 10^{-3} M for 15 min (b), or to NS containing ryanodine 10^{-8} M for 30 min (c). The s.e.mean is indicated by vertical lines at the end of each column and was derived from 20 systolic or diastolic values from each of 10 dye-loaded atria. A significant difference exists between the members of a pair of peak systolic values marked *.

odine, therefore, reversed the sign of both rate-dependent staircase effects. In contrast, atria that had been exposed to caffeine at 10^{-3} M in NS for 15 min still showed a negative rate-tension staircase and the systolic increments in F400/500 ratios remained smaller at 6 Hz than at 3 Hz (Figure 4).

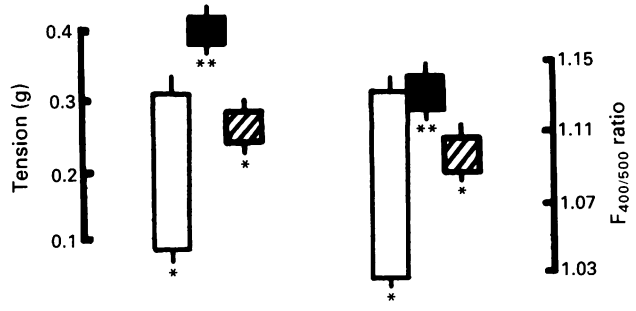

Figure 5 Atrial peak systolic (top of column) and end-diastolic (bottom of column) values of tension and corresponding F400/500 ratios. Atria were exposed to normal superfusate alone (open columns), to simulated ischaemia (SI) for 20 min (solid columns), or to SI for 20 min after prior treatment with ryanodine 10^{-7} M for 30 min (hatched columns). The s.e.mean is indicated at the end of each column by vertical lines and was derived from 20 systolic or diastolic values from each of 10 dye-loaded atria. A significant difference exists ($P < 0.05$) between an end-diastolic value marked ** and corresponding values marked *.

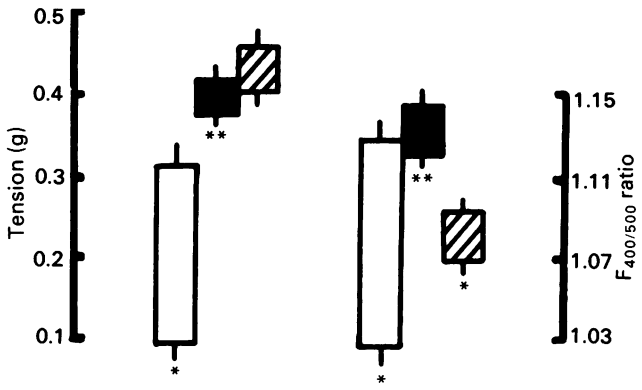

Figure 6 Atrial peak systolic (top of column) and end-diastolic (bottom of column) values of tension and corresponding F400/500 ratios. Atria were exposed to normal superfusate alone (open columns), to simulated ischaemia (SI) for 20 min (solid columns), or to SI for 20 min after prior treatment with caffeine 10^{-2} M for 20 min (hatched columns). The s.e. of the mean is indicated at the end of each column by vertical lines and was derived from 20 systolic or diastolic values from each of 10 dye-loaded atria. A significant difference exists ($P < 0.05$) between an end-diastolic value marked ** and corresponding values marked *.

Simulated ischaemia

Experiments were undertaken next to investigate whether pretreatment of atria with either ryanodine or caffeine would modify the rise in $[Ca]_c$ that occurs during exposure to SI (Northover, 1990). Atria that had been pretreated for 30 min with ryanodine at 10^{-7} M showed smaller SI-induced rises in end-diastolic tension values and end-diastolic F400/500 ratios than atria that had not received ryanodine (Figure 5). Atria that had been pretreated for 20 min with caffeine at 10^{-2} M also had smaller SI-induced rises in F400/500 ratios than atria that had not been exposed to caffeine (Figure 6). Despite this, however, end-diastolic tension rose during SI by a similar amount in the caffeine-pretreated and non-pretreated atria (Figure 6).

Discussion

Several reasons exist for thinking that changes which occur in F400/500 ratios of indo-1-loaded atria accurately reflect contemporary changes taking place in $[Ca]_c$ values (see Northover, 1990). If one accepts those reasons, then the present study suggests that atria exposed to NS containing ryanodine or caffeine possess lower systolic $[Ca]_c$ values than atria exposed to drug-free NS, and that atria pretreated with these drugs show smaller rises in end-diastolic $[Ca]_c$ when exposed to SI than non drug-pretreated atria. The rise in diastolic tension during SI probably is due both to increased rates of Ca-dependent cross-bridge cycling and to rigor bond formation.

The quantity of Ca stored by the SR is the main determinant of the quantity of Ca released into the myoplasm during systole, and hence of systolic force development. In rat ventricular myocardium, in contrast to that of some other species, Na/Ca exchange across the sarcolemma functions in a Ca-entry mode at normal diastolic membrane potentials (Shattock & Bers, 1989). A similar situation prevails in rat atria (unpublished observations). Efflux of Ca from rat hearts, therefore, is unusually ATP-dependent, and $[Ca]_c$ values particularly influenced by the SR store of Ca. Both ryanodine and caffeine interfere with Ca storage in the SR (Nayler, 1963; Frank & Sleator, 1975), which explains why both drugs in NS decreased peak systolic F400/500 ratios in the present experiments, confirming previous findings (Hess & Wier, 1984). Nevertheless, ryanodine and caffeine deplete the SR of Ca by different mechanisms (see Introduction). This explains why they produced contrasting responses during changes in the

frequency of electrical stimulation in the present experiments. Ryanodine-treated atria, but not those exposed to caffeine at 10^{-3} M, showed greater systolic increments in tension during stimulation at 6 Hz than at 3 Hz, confirming previous findings (Bers, 1985; Stemmer & Akera, 1986; Tanaka & Shigenobu, 1989). These results are accounted for by parallel changes that occur in $[Ca]_c$ values. The effects of caffeine at 10^{-3} M, in NS on the rate-tension staircase in the present experiments agree with earlier findings (Henderson *et al.*, 1974), and also may be accounted for by the accompanying changes in $[Ca]_c$ values. In the present experiments, exposure of atria to caffeine at 10^{-2} M in NS caused a contracture while reducing mean $[Ca]_c$ values, confirming previous reports (Hess & Wier, 1984). The ability of caffeine to sensitize the myofibrils to Ca would explain the contracture (Wendt & Stephenson, 1983; Hess & Wier, 1984). The early temporary rise in $[Ca]_c$ in response to caffeine probably reflects a faster early rate of Ca release from the SR into the myoplasm than can be extruded by the sarcolemmal pump. As the SR becomes depleted of Ca, however, the former will be overtaken by the latter.

Uptake of Ca from the myoplasm by the SR requires ATP (Frank & Sleator, 1975), and since ATP depletion occurs during SI (Northover, 1989), depletion of the SR store of Ca would be expected, and was demonstrated to occur by Haworth *et al.* (1987). The Ca lost from the SR may either accumulate in the myoplasm or be pumped out via the sarcolemma, although lack of ATP will impede the latter process. Pretreatment with ryanodine or caffeine inhibited the SI-induced rise in $[Ca]_c$ in the present experiments. A negatively inotropic drug such as ryanodine, however, would tend to conserve ATP, and thus to protect SR stores of Ca. Although Thandroyen *et al.* (1988) found that negatively inotropic concentrations of ryanodine failed to modify ATP depletion in rat hearts during coronary artery occlusion, the total store of ATP may not always reflect changes taking place in small subcompartments of the cell. For the present purpose an alternative tool is needed.

Caffeine at 10^{-2} M aggravated ATP depletion during coronary artery occlusion in the rat (Thandroyen *et al.*, 1988), but still protected against the SI-induced rise in $[Ca]_c$ in the present experiments. Provided that caffeine does not promote Ca-efflux by a direct action on the sarcolemma, or inhibit Ca-influx, the ability of caffeine to reduce the SI-induced rise in $[Ca]_c$ in the present experiments would be attributable to depletion of the SR store of Ca. Under some circumstances caffeine does promote Ca-efflux, but this is due to a rise in $[Ca]_c$ resulting from the release of Ca from the SR (Clusin, 1983; Clusin *et al.*, 1983), and would not reduce the value of caffeine as a tool with which to manipulate the SR in the present experiments. Caffeine also has been reported to reduce Ca-influx, but only after prolonged exposure to the drug, and with an opposite effect initially (Kohlhardt *et al.*, 1974; Eisner *et al.*, 1979; Hess & Wier, 1984). Ohba (1973) failed to detect any inhibition of influx, but confined measurements to the first 8 min of drug exposure. When a late inhibition did occur in previous experiments it was unclear whether slow Ca channel-conductance was reduced or whether reduced Ca entry was due to a build-up of Ca in the myoplasm. The latter is more probable since influx of ^{45}Ca in mammalian myocardium is promoted rather than inhibited by caffeine (Guthrie & Nayler, 1967; Shine & Langer, 1971). If caffeine did enhance slow Ca channel-conductance in the present experiments, any resulting effects on $[Cal]_c$ must have been more than counterbalanced by concomitant effects upon the SR. The apparent contribution of the SR to the SI-induced rise in $[Ca]_c$ would then have been under-estimated.

In conclusion, the SR appears to provide some of the rising myoplasmic Ca concentration between 8 min and 20 min after the start of SI. Caffeine, and perhaps ryanodine, by depleting the SR of Ca may inhibit some of this rise. Influx of Ca across the sarcolemma also may contribute, but the magnitude of this is presently unknown. During the first 3–4 min of SI the SR retains substantial stores of releasable Ca (Allen *et al.*, 1988; Stern *et al.*, 1988). At that early stage of SI the contribution of the SR to $[Ca]_c$ may be insignificant.

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Effects of intravenous μ and κ opioid receptor agonists on sensory responses of convergent neurones in the dorsal horn of spinalized rats

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1 Electrophysiological experiments have been performed to assess the effects of intravenously administered μ and κ opioid agonists on the responses to noxious thermal and mechanical and non-noxious tactile stimuli of single convergent neurones in laminae III-VI of the dorsal horn of spinalized rats anaesthetized with α-chloralose.

2 The μ receptor agonists tested were fentanyl (1–16 μg kg^{-1}) and morphine (0.5–16 mg kg^{-1}) and the κ-receptor agonists U-50,488 (1–16 mg kg^{-1}) and tifluadom (0.1–1.6 mg kg^{-1}). Multiple drug tests were made on each cell so that compounds could be compared under closely comparable conditions.

3 In one protocol, thermal and mechanical nociceptive responses of matched amplitudes were elicited alternately. Both μ and κ agonists dose-dependently reduce the neuronal responses. Thermal nociceptive responses were as sensitive to the κ agents as were the mechanical nociceptive responses; the μ agonists similarly reduced both types of response in parallel.

4 In another protocol, nociceptive and non-nociceptive responses were elicited alternately to permit the degree of selective antinociception to be assessed. The μ agonists were scarcely selective, fentanyl reducing nociceptive only slightly (but significantly at 4–16 μg kg^{-1}) more than non-nociceptive responses. The κ-opioid agonist U50,488 reduced tactile responses somewhat more than nociceptive responses.

5 The spontaneous discharge of these cells with ongoing activity was reduced to a significantly greater degree than the evoked responses; this is likely to have contributed to the non-selectivity of the reduction of the evoked responses.

6 The results are discussed with respect firstly to previous reports that κ opioids are ineffective in tests of thermal nociception, and secondly to the likely spinal mechanisms by which opioid receptor agonists mediate antinociception.

Keywords: Opioids; μ-opioid receptor agonists; κ-opioid receptor agonists; spinal cord; intravenous; convergent neurones; sensory responses

Introduction

There is plentiful evidence that opioid analgesics have direct spinal actions that reduce the nociceptive responses of spinal neurones; this applies both to the motoneurones that mediate reflex and other motor outputs and to the neurones in the dorsal horn that are related to ascending projection pathways. There has however been considerable confusion as to which opioid receptors may be involved, which types of nociceptive input may be preferentially reduced, and which neuronal elements are the most affected.

Many reports have indicated that κ opioids are relatively ineffective on thermally-induced, as compared with mechanically or chemically-induced, spinal nociceptive reflexes (for reviews see Yaksh & Noueihed, 1985; Millan, 1990). We have recently shown in electrophysiological tests that such selectivity does not occur when care is taken to match the intensities of the peripheral stimuli used to elicit the reflexes, and when access to all spinal receptor sites is ensured by the use of systemic rather than topical administration of opioid agonists (Parsons & Headley, 1989a). Similar non-selectivity between thermal and non-thermal nociceptive reflexes is seen in standard behavioural reflex tests when similar care is taken to match the stimulus intensities (Millan 1989, 1990), at all but the strongest of stimulus intensities. There is however no direct evidence concerning the relative effectiveness of systemic κ opioids on thermal versus non-thermal nociceptive responses of neurones in the spinal dorsal horn, although the data of Calthrop & Hill (1983) and of Fleetwood-Walker *et al.* (1988) suggest that there would be no great difference.

Studies in man indicate that morphine, whether administered systemically (Wikler, 1950) or spinally (Willer *et al.*, 1988; Chabal *et al.*, 1989), reduces pain sensations/nociceptive reflexes whilst having minimal effects on functions mediated by non-nociceptive afferents. It remains unclear how this selective analgesia is mediated, in terms of the precise spinal neuronal elements upon which the opioids may act. A specific problem arises from the finding that most dorsal horn neurones that respond to noxious stimuli also respond to non-nociceptive inputs: they are 'convergent', 'wide dynamic range', or 'multireceptive' in nature. The role of this population of convergent neurones in pain sensation remains under discussion (Besson & Chaouch, 1987; Dubner, 1989).

Various authors have reported that opioid receptor agonists can selectively reduce the nociceptive responses of such convergent neurones whilst leaving the responses to low threshold inputs relatively unaffected (see Kitahata & Collins, 1981; Duggan & North, 1984; Besson & Chaouch, 1987). Several such studies were performed with electrical rather than with 'natural', or adequate, stimulation of primary afferents, but it should be remembered that such stimulation of a subset of primary afferents cannot be expected to mimic the activation of receptors by adequate stimuli. Moreover many of the reports were of local rather than systemic administration of the opioid agonists, but as nociceptive and non-nociceptive afferents terminate, broadly speaking, in different laminae of the dorsal horn, local administration could result in selective effects as a result simply of access to some but not other neuronal elements. Of studies testing systemic opioids on

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responses to adequate peripheral stimuli, some have shown a rather poor degree of selectivity between nociceptive and non-nociceptive responses (Yaksh, 1978; Einspahr & Piercey, 1980). Notably, however, the systemic studies performed to date have been with opioid agonists acting primarily at μ opioid receptors; there is no information on the selectivity of systemic κ opioids on convergent dorsal horn neurones in the spinal cord (but see Calthrop & Hill, 1983). Consequently the degree of selectivity between nociceptive and non-nociceptive responses of convergent spinal neurones, particularly in relation to the doses of opioid agonists that mediate behavioural analgesia, remains unclear.

For these reasons we have now examined the degree to which μ and κ opioid receptor agonists, administered systemically to spinalized rats, distinguish between sensory responses of convergent neurones in laminae III–VI of the rat spinal dorsal horn: potential selectivity was examined firstly between alternating thermal and mechanical nociceptive responses, and secondly between nociceptive and non-nociceptive responses. Some of the data have been presented elsewhere in preliminary form (Headley *et al.*, 1984; 1987; Headley & Dong, 1990).

Methods

Animal preparation

Experimental methods were similar to those detailed previously (Parsons & Headley, 1989a). Data were analysed from experiments on 40 spinalized Wistar-style male rats. Briefly, arterial, venous and tracheal cannulae were inserted under halothane anaesthesia, and a laminectomy performed between Th9-L4 vertebrae. The spinal cord was sectioned at Th10; where the dorsal vein was substantial, it was left intact together with a small wedge of dorsal column tissue.

After surgery, halothane was discontinued and anaesthesia was maintained with α-chloralose (50–60 mg kg^{-1}, i.v. initially, supplemented with doses of 20 mg kg^{-1} approximately every hour). Some animals were ventilated after paralysis with pancuronium (1.5 mg kg^{-1}, i.v. initially, supplemented as required to maintain an adequate (but not complete) level of muscle relaxation); in these animals end tidal CO_2 was monitored and maintained close to 4%. The adequacy of anaesthesia in paralysed animals was ensured by (a) the use of the relatively long-lasting anaesthetic α-chloralose, administered strictly in the same dose regime used in non-paralysed animals; (b) the selection of pancuronium which, as well as causing less hypotension than gallamine in our rats, is relatively short-lasting in this species (Durant *et al.*, 1980), thereby permitting intermittent but direct monitoring of the degree of anaesthesia; and, most importantly, (c) by monitoring cardiovascular (blood pressure and heart rate) responses to noxious stimuli applied rostral to the spinalization (Flecknell, 1987). In all cases body temperature was maintained close to 37°C; blood pressure was monitored and experiments were terminated if systolic pressure fell below 100 mmHg.

Peripheral stimuli and neuronal recording

Noxious thermal stimuli were applied to one hindlimb with a 1.5 cm^2 flat contact thermode with feedback control; the thermode was held at 37°C and then ramped to 46.0–48.8°C where it was held until the end of the 30 s stimulus. Noxious pinch stimuli were administered with pneumatically controlled forceps; in later experiments this was both feedback-controlled and calibrated to give 1–3.5 N applied over a tip area of 3 mm^2 for 15 s. Non-noxious mechanical stimuli, lasting 15–20 s, consisted of indenting the skin or moving the toes with a relay-operated device working at 3–20 Hz. In all cases stimulus intensity and duration were constant between applications and repetition rate was controlled in an automated cycle of two (lasting 3–3.5 min) or occasionally three stimuli (lasting 4–5 min). If inflammatory changes in response to the repeated noxious stimulation became detectable, the experiment was terminated.

Extracellular recordings of convergent neurones in laminae III–VI were made with three barrel glass micropipettes that contained 3.5 M NaCl for recording; quisqualate Na (5 mM in 200 mM NaCl pH 7.5) to permit activation of neurones and hence a distinction between somata and axons; and pontamine sky blue (2% in 0.5 M Na acetate) to permit histological verification of recording sites.

Neuronal activity, stimulus details and physiological data were recorded continuously on a pen recorder. Counts of evoked spike activity, in epochs related to the sensory stimuli, were analysed on-line to permit drug effects on each type of response to be expressed as a percentage of the last 3 pre-drug control values.

Drugs, drug administration and analysis

The drugs tested were the μ-opioids, fentanyl citrate (Sublimaze, Janssen) and morphine HCl; the κ opioids U-50, 488 (*trans*-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide methane sulphonate; Upjohn) and tifluadom HCl (Sandoz); and the antagonist naloxone HCl (Sigma). (See Parsons & Headley, 1989a, for the reasons for selecting these agonists).

All opioid agonists were administered i.v. Injections were made over 30 s starting 1 min before the next evoked response; maximal effects are nearly always seen on the first subsequent response. Doses were incremented in a cumulative ($\log_2$) regime until evoked responses were reduced to below 25% control or the maximum dose compatible with monitoring subsequent recovery had been administered. The dose ranges used were fentanyl 1–16 μg kg^{-1}; morphine 0.5–16 mg kg^{-1}; U-50,488 1–16 mg kg^{-1}; and tifluadom 0.1–1.6 mg kg^{-1}. The opioid antagonist naloxone was given in doses of 1–50 μg kg^{-1}. (Doses refer to salts except for fentanyl base.) This regime permitted several drugs to be tested on most cells. In these experiments various anaesthetic agents were compared with the opioid agonists; data on the former will be presented elsewhere. Test data were only included for analysis either if (as was usual) recovery from the drug exceeded 50% of the initial drug effect or if the effect was reversed by naloxone (all cases tested).

The fact that the full dose-response range was not tested on all cells precludes constructing standard dose-response curves; most of the pooled data are instead presented in bar graph form.

The quantitative analysis below is performed on the '% control' data for spike counts over the following stimulus-related epochs: heat (the entire 30 s stimulus); late pinch (excluding the first 5 s of the stimulus, which is presumed to contain a higher proportion of low threshold afferent induced activity); tap/vibration (the entire 15–20 s stimulus); and spontaneous activity monitored over 60–80 s between evoked responses.

Results

The results presented below were obtained in experiments on 9 convergent neurones responding to alternating noxious heat and pinch stimuli, and from 42 cells responding to alternating noxious and non-noxious stimuli. At least one μ and one κ receptor agonist was tested under closely comparable conditions on 18 of these cells. The data were not sufficiently extensive to permit a statistically significant comparison between drugs and responses for each of spinal laminae III (5 cells), IV (14 cells), V (21 cells) and VI (11 cells); neurones in these laminae appeared to respond similarly to the test drugs, and data for all laminae have therefore been pooled.

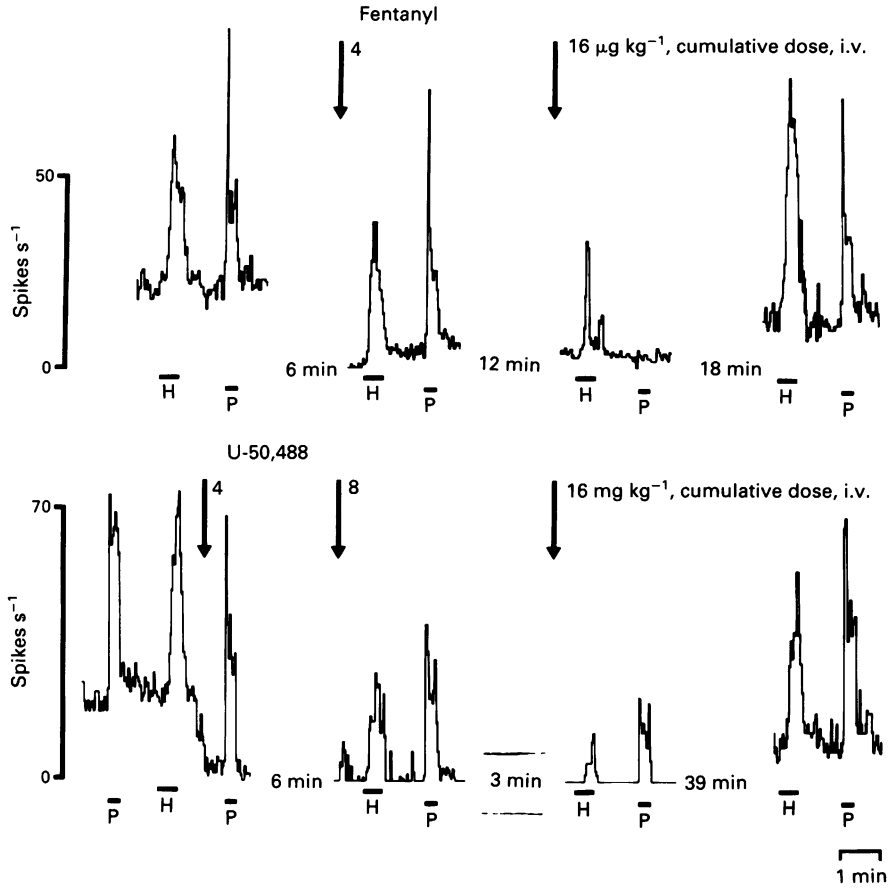

Figure 1 Both the μ opioid receptor agonist fentanyl and the κ agonist U-50,488, reversibly reduced thermal and mechanical nociceptive responses in parallel when tested on a single convergent neurone in lamina VI of the spinal dorsal horn of a chloralose anaesthetized spinalized rat. Note the greater sensitivity of the spontaneous as compared with the evoked activity. The cell was activated alternately by noxious heat (H; ramped from 37 to 48°C, applied to the plantar foot over 30 s every 3 min) and noxious pinch (P; toe 5 for 15 s). Fentanyl was tested at doses of 1, 2, 4, 8 and 16 μg kg^{-1}; some parts of the record have been omitted, as indicated. The lower record was obtained after an interval of 16 min; at this stage the cell had become somewhat more sensitive to both noxious stimuli. This record shows all three doses of U-50,488 tested on this cell.

Effects of μ and κ opioids on spontaneous activity

The protocol followed is illustrated in Figure 1 which shows tests with both fentanyl and U-50,488 on the same convergent neurone recorded in lamina VI. This cell had a relatively high level of spontaneous discharge, and it can be seen that both μ and κ receptor agonists reduced this ongoing activity at doses lower than those that substantially reduced the evoked responses (i.e. the area under the trace for each response). Not all convergent neurones show such high levels, or indeed any, spontaneous activity and consequently the percentage reduction by the agonists was variable between cells. Figures 2, 4 and 6 show pooled data for the effects of the μ and κ receptor agonists on such spontaneous activity and demonstrate that the spontaneous activity of convergent neurones, when present, was reduced by both types of agonist. This reduction occurred at doses appreciably lower than those that had clear effects on either nociceptive or non-nociceptive evoked responses; by the same token the reduction of spontaneous activity was significantly greater than that of evoked responses at all doses tested (Figures 4 and 6).

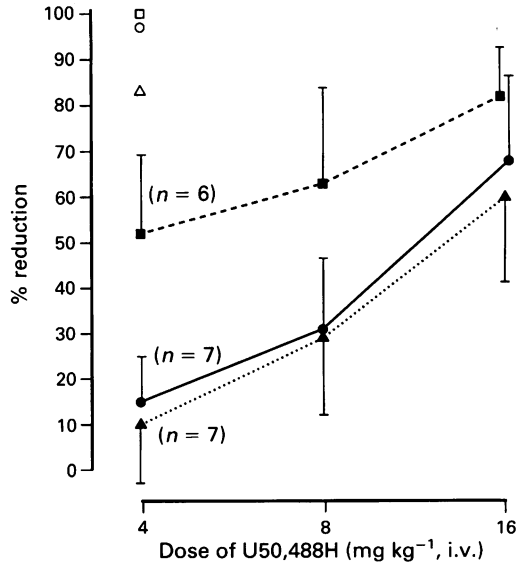

Figure 2 Dose-responses curves of pooled data from a total of 9 neurones tested with U-50,488 on responses to alternating thermal (triangles) and mechanical (circles) noxious stimuli, and on the spontaneous activity (squares), recorded in the manner illustrated in Figure 1. Seven cells (filled symbols) were tested at all three doses; one of these was not spontaneously active. Two other cells (open symbols) were sufficiently sensitive at 4 mg kg^{-1} for further doses not to be administered. Statistical tests (Wilcoxon matched pairs) between the nociceptive response types gave P values greater than 0.10 at all doses.

Effects on alternating thermal and mechanical nociceptive responses

A significant finding is that thermal and non-thermal nociceptive responses, which were carefully matched in terms of evoked firing rate, were reversibly reduced in parallel by the κ as well as the μ opioid. This parallel reduction of thermal and

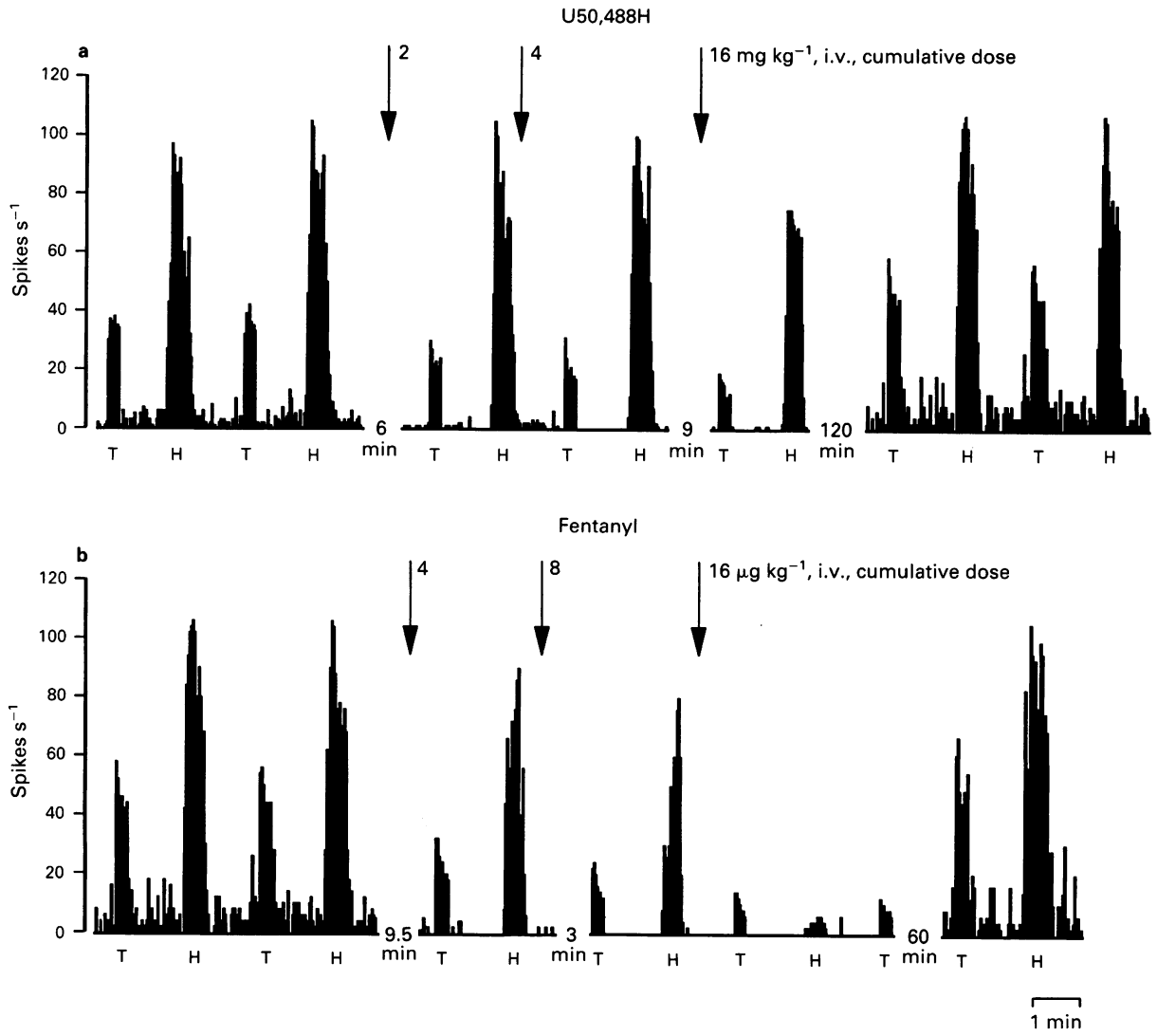

Figure 3 Relative non-selectivity of the κ agonist U50,488 (a) and the μ receptor agonist fentanyl (b) on alternating nociceptive and non-nociceptive responses of a convergent neurone in lamina VI. T: tap stimulus to toes 2–4, at 3 Hz for 15 s; H: heat to toes 2–4 for 30 s, ramped from baseline 37°C and then held at 47.5°C. For clarity the last two stimulus cycles on the upper trace are repeated at the start of the lower trace.

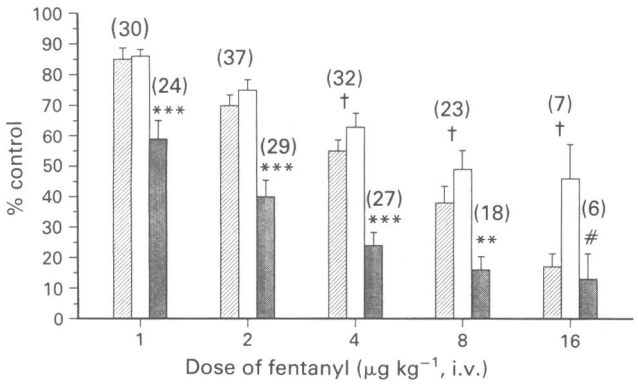

Figure 4 Pooled data from 40 neurones on which fentanyl was tested i.v. on alternating nociceptive (hatched columns) and non-nociceptive responses (open columns) according to the protocol shown in Figure 3. Not all doses were tested on all cells; numbers tested are shown above the bars. The effects on spontaneous activity are also shown (stippled columns) for those cells showing such activity (numbers shown above the stippled columns). Statistical tests: Wilcoxon (two tailed) tests were performed for each dose between the two evoked responses: † $P < 0.05$. Mann-Whitney U tests were performed between spontaneous and evoked activity: ** $P < 0.005$, *** $P < 0.001$ between spontaneous and both evoked responses; # $P < 0.05$ between spontaneous and non-nociceptive responses.

mechanical nociceptive responses was a consistent observation with the κ as well as the μ receptor agonists. This is shown for the pooled data of tests with U-50,488 in Figure 2. The benzodiazepine κ ligand, tifluadom, similarly reduced the thermal and mechanical responses in parallel on the 4 neurones tested; at 0.4 mg kg^{-1} responses were reduced to 38% ± 20 (mean ± standard error of mean) and 28% ± 18 of control respectively. The large standard error values for both κ ligands indicate the variability of drug potency between cells, but, as indicated by the mean values, and in Figure 1 on any one cell, the two types of nociceptive response were reduced to a very similar degree. The μ receptor agonist fentanyl showed a similar pattern on the paired nociceptive responses of the three cells tested: at 4 μg kg^{-1} it reduced thermal and non-thermal nociceptive response to means of 19% control (±12 and ±15 respectively).

Effects on alternating nociceptive and non-nociceptive responses

Whereas care was taken, when noxious stimuli were alternated, to achieve similar firing rates for the two types of response, the properties of many convergent neurones preclude achieving this when noxious and non-noxious stimuli are alternated: many cells will fire more vigorously to the more intense stimulus. Moreover the pattern of the evoked discharge is different for the two types of response (Steedman

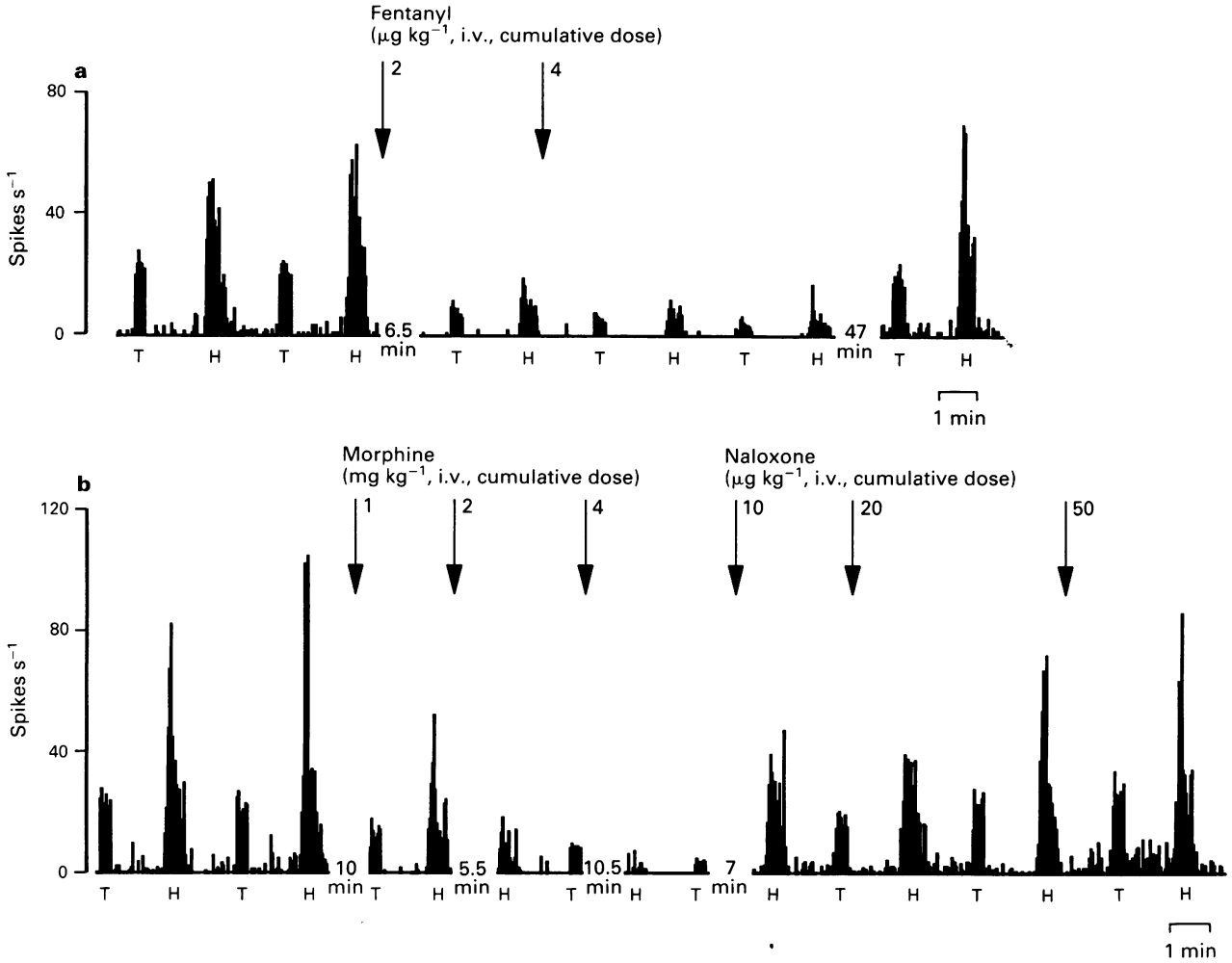

Figure 5 Fentanyl (a) and morphine (b) tested with cumulative i.v. doses on the same cell responding to alternating noxious and non-noxious stimuli. Both μ receptor agonists reduced the responses non-selectively, and the effect of morphine was reversed by naloxone. Noxious heat (H; 37°C baseline ramped to 48.8°C) was applied to toe 2 of the ipsilateral hindlimb; the non-noxious stimulus was tapping toe 5 at 3 Hz. The morphine test was performed 90 min after the fentanyl test; during this period the cell became progressively more sensitive to the noxious stimulus, although no peripheral inflammation was detectable. Cell in lamina V of an α-chloralose anaesthetized and spinalized rat.

& Zachary, 1990). These points should be borne in mind when interpreting the type of test reported below.

When the same group of opioid receptor agonists was tested in a similar preparation on alternating nociceptive and non-nociceptive reflex responses of single motoneurones

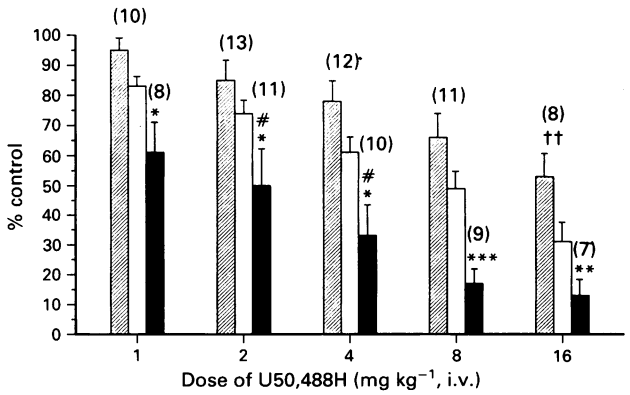

Figure 6 Pooled data from 15 neurones on which U-50,488 was tested on alternating nociceptive and non-nociceptive responses. Data presented as in Figure 4. Wilcoxon matched pairs tests between the two types of evoked response: †† $P < 0.01$. Mann-Whitney u tests (a) between spontaneous and nociceptive responses: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ and (b) between spontaneous and non-nociceptive responses: # $P < 0.05$.

(Parsons & Headley, 1989b), the selectivity shown by both μ and κ opioids varied considerably between units, showing selective antinociception only when relatively lower doses were effective. This pattern was not seen with dorsal horn convergent neurones, which were less sensitive than motoneurones in terms of the reduction of responses at given intravenous doses of the opioid agonists.

Figure 3 shows an example of a test with both μ and κ agonists on a convergent neurone activated alternately by noxious heat and non-noxious tap stimuli. Figure 3b shows a test with fentanyl and indicates that the μ agonist reduced both nociceptive and non-nociceptive responses; that is, it was not selectively antinociceptive on this neurone.

Pooled data for the effects of fentanyl on such alternating nociceptive and non-nociceptive responses are shown in Figure 4. On this population of convergent neurones the μ receptor agonist reduced both responses, showing only weak selectivity which reached significance at only the higher doses tested (4–16 μg kg^{-1}; compare data in Figure 4 with those on motoneurone responses in Table 1 of Parsons & Headley, 1989b). The spontaneous activity of those neurones which had an ongoing discharge was reduced to a significantly greater degree.

In view of this non-selectivity by fentanyl, a limited number of tests were performed with morphine (0.5–16 mg kg^{-1}, i.v.). In each case ($n = 4$) morphine showed the same degree of non-selectivity as did fentanyl; an example of a direct comparison is shown in Figure 5. At 4 mg kg^{-1}, thermal and tactile

responses were reduced to means of 13% ($\pm$6) and 21% ($\pm$6) control respectively. These non-selective effects of morphine were readily reversed by low i.v. doses of naloxone, as is shown in Figure 5.

The κ opioid U-50,488 displayed no selective antinociception at all. Figure 3a shows a test (same neurone as the fentanyl test) in which it reduced the non-nociceptive responses to an even greater degree than the responses to noxious heat. The pooled data shown in Figure 6 indicate that this was a consistent finding; at all doses tested, it reduced non-nociceptive responses somewhat more, although this difference only reached significance at the highest dose ($16\,mg\,kg^{-1}$).

Discussion

The i.v. administration of the opioid receptor agonists used in this study should have ensured that all opioid receptors within the spinal cord were exposed to similar concentrations of the agents, a situation most suited to an assessment of the possible selective effects of opioids between the different types of sensory response of dorsal horn convergent neurones. The fact that μ and κ agonists were often tested on the same neurone responding to the same stimuli permitted a direct comparison to be made between the actions of these agents. The site(s) of action of the opioid agonists should have been restricted by the spinalization to the spinal cord. The effectiveness of systemic κ opioids in such physiological tests contrasts with the low levels of κ binding sites reported in rat spinal cord; technical problems, however, have evidently contributed to the latter findings (Wood *et al.*, 1989).

Thermal vs non-thermal nociceptive responses

To avoid the potential problems associated with using stimuli of differing intensities, care was always taken to adjust the stimulation devices so that firstly the stimuli were applied as close as possible within the peripheral receptive field of the neurone, and secondly the evoked firing rate of the neurone was similar for the two alternating response types. Under these conditions neither the μ nor the κ agonists tested showed any selectivity between thermal and non-thermal nociceptive responses, just as was observed under similar conditions when reflex responses of single motoneurones were tested (Parsons & Headley, 1989a). The present results provide further evidence that the relative spinal inactivity of κ opioids that has been reported by many authors performing standard reflex tests of thermal nociception (see Yaksh & Noueihed, 1985), has resulted primarily from a combination of inadequate stimulus matching and inadequate access of the opioids from the site of topical administration to the relevant spinal receptors (Parsons & Headley, 1989a; Parsons *et al.*, 1989; Millan 1989; 1990).

At high stimulus intensities, even with matched withdrawal latencies, κ agonists are less effective against thermal than against mechanical nociceptive flexion reflexes (Millan, 1989). In the present experiments such high intensity thermal stimuli could not be tested because, under our conditions, repeating stimuli of above 49.5°C results relatively rapidly in inflammatory swelling. All thermal stimuli were therefore kept below this limit. Consequently there remains no electrophysiological evidence concerning this difference between μ and κ opioids seen by Millan (1989) at high stimulus intensities.

Nociceptive vs non-nociceptive responses

The general consensus from previous electrophysiological tests with spinal convergent neurones is that the opioids that have been tested systemically – and that act primarily at μ receptors – showed a selective antinociception: the non-nociceptive responses were relatively unaffected. This is true whether comparisons were between responses to noxious and non-noxious adequate peripheral stimuli, or between responses to electrical stimulation of fast- versus slowly conducting primary afferent fibres. There have however been some exceptions to this general finding. Most clearly, Yaksh (1978) and Einspahr & Piercey (1980) reported that systemic morphine was not selective on convergent neurones in the cat spinal cord, an effect not reported in various other tests of systemic μ opioids in cats (Calvillo *et al.*, 1979; Headley *et al.*, 1987). Equivalent tests with systemic opioids appear not to have been performed either in rat or with κ-selective opioid agonists. Preliminary data with systemic κ agonists on convergent neurones in cats suggested that the only effect was a change in the threshold of the nociceptive response (Piercey *et al.*, 1982).

We now show that in rats, the μ receptor agonists fentanyl and morphine, administered intravenously in spinalized animals, show only very weak selectivity between alternating nociceptive and non-nociceptive responses of convergent neurones in laminae III–VI. The κ agonist U-50,488 was effective on these neurones in that it depressed spontaneous and evoked activity, but it did not cause any degree of selective antinociception. The question therefore arises as to how these electrophysiological results relate to the behavioural data from both animals and man indicating that spinal opioid agonists can indeed be selectively antinociceptive.

Significance of effects on spontaneous activity

A major contribution to the non-selectivity of the opioid agonists on evoked responses is likely to have been the marked reduction of spontaneous activity caused by low doses of the agents: such a decrease in neuronal excitability would result in non-selective reductions of all synaptic responses. It may be pertinent that DNIC (diffuse noxious inhibitory controls), which evidently involve a spinal relay utilizing an opioid peptide (Le Bars *et al.*, 1987), cause a non-selective inhibition of convergent neurone activity (Le Bars *et al.*, 1979). Whilst there is clear evidence for direct depressant effects of opioids on spinal neurones (Zieglgansberger & Bayerl, 1976; Yoshimura & North, 1983), it is not possible to resolve from the current type of experiment whether such a depression was occurring on the cells under study or on cells earlier in the (probably) polysynaptic pathway mediating the ongoing and/or evoked synaptic activity.

Although we take care to keep the area of surgery well outside the receptive fields of the neurones studied, tonic nociceptive input to the recorded cell could still be generated by the surgical preparation of the animal (Duggan & North, 1984; Collins *et al.*, 1987). In acute preparations, nociceptive neurones have indeed been reported to have a higher spontaneous discharge than do non-nociceptive neurones (Surmeier *et al.*, 1989). As implied above, such tonic activity would enhance superimposed phasic responses such as those elicited in this study. It follows that even if the opioid agonists were selective in reducing nociceptive input onto the cell, the resulting decrease in tonic activity would cause the non-nociceptive inputs of these convergent neurones to be reduced as well as the nociceptive responses. Two other findings suggest that the effect of opioid agonists on surgery-induced ongoing activity contributes significantly to the reduction of evoked responses. Firstly, morphine has long been known to depress the spontaneous activity of nociceptive more than that of non-nociceptive cells (Kitahata & Collins, 1981). Secondly, the potency of fentanyl on spinal withdrawal reflexes is increased when the severity of preparatory surgery is increased in a controlled manner (Hartell *et al.*, 1990).

There is an alternative explanation for the apparent discrepancy between the non-selectivity seen with convergent neurones and the selectivity of the spinal analgesia that is seen behaviourally and clinically with opioid analgesics: namely that it is the balance of activity between populations of dorsal horn neurone that permits the interpretation of nociception by higher centres. This postulate has arisen from the proper-

ties of DNIC inhibitions (as discussed by Besson & Chaouch, 1987) and the same considerations would apply to the anti-nociception mediated by opioids. We have recently found that systemically administered opioid agonists can indeed have differential effects between different types of dorsal horn neurone (Headley & Dong, 1990); the data will be presented and the concept discussed in full elsewhere.

Conclusion

The effects of opioid receptor agonists on convergent dorsal horn neurones are qualitatively similar to the effects they have on spinal reflexes tested under closely comparable conditions: both μ and κ opioids are relatively non-selective in reducing responses to noxious thermal, noxious mechanical and light tactile stimuli. The selectivity reported in other types of test (a) between nociceptive and non-nociceptive responses and (b), for κ opioids, between thermal and non-thermal responses, requires other explanations in terms of the technical limitations of the techniques employed, as well, perhaps, as of other sorts of neural interaction.

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Demethoxyviridin and wortmannin block phospholipase C and D activation in the human neutrophil

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1 The fungal metabolite, wortmannin, has recently been shown to inhibit fMet-Leu-Phe-stimulated superoxide production and phospholipase D (PLD) activation in the human neutrophil.

2 We have found that a close structural analogue of wortmannin, demethoxyviridin, has a similar inhibitory profile but in addition blocks phosphatidylinositol 4,5-bisphosphate-specific phospholipase C and hence inositol 1,4,5-trisphosphate (IP_3) formation.

3 Inhibition of fMet-Leu-Phe-stimulated PLD by demethoxyviridin was characteristically non-competitive ($IC_{50} = 31 \pm 10$ nM).

4 Inhibition of fMet-Leu-Phe-stimulated IP_3 formation required concentrations almost 10 times higher ($IC_{50} = 250 \pm 130$ nM).

5 Surprisingly, demethoxyviridin only inhibited fMet-Leu-Phe-induced intracellular calcium mobilization at concentrations 100 times greater than those needed to block IP_3 formation.

6 Demethoxyviridin also inhibited PLD activation induced by sodium fluoride or phorbol myristate acetate (PMA) but the concentrations required were 100 times those needed to block fMet-Leu-Phe-stimulated PLD.

7 These observations support the contention that PLD plays an important role in signal transduction in the human neutrophil and indicate that wortmannin and demethoxyviridin inhibit PLD activation at a common step in the signalling pathway.

8 Furthermore, these results suggest that demethoxyviridin may block the interaction between the chemotactic peptide receptor and a GTP-binding protein that is intimately involved in PLD activation.

Keywords: Neutrophil; phospholipase D; phosphatidylbutanol; phospholipase C; inositol 1,4,5-trisphosphate; diradylglycerol; calcium mobilization; superoxide

Introduction

There is now a considerable amount of evidence suggesting that phospholipase D (PLD) plays an important role in intracellular signal transduction (Pelech & Vance, 1989; Loffelholz, 1989; Exton, 1990). PLD is activated by a variety of stimulatory agonists in many different cell types (Kobayashi & Kanfer, 1987; Bocckino *et al.*, 1987; Pai *et al.*, 1988a; Rubin, 1988; Liscovitch, 1989; Gelas *et al.*, 1989; Ben-Av & Liscovitch, 1989; Qian & Drewes, 1989; Gruchalla *et al.*, 1990). The major substrate for this enzyme appears to be phosphatidylcholine (PC), which is hydrolysed to phosphatidic acid (PA). PA can be further metabolised by phosphatidate phosphatase to yield the second messenger, diacylglycerol, which activates protein kinase C. PA may also serve a second messenger function since it is known (i) to be mitogenic, (ii) to increase intracellular calcium and guanosine 3',5'-cyclic monophosphate (cyclic GMP) concentrations, (iii) to activate phospholipase A_2 and (iv) to inhibit cellular adenylate cyclase (Moolenaar *et al.*, 1986; Yu *et al.*, 1988; Imagawa *et al.*, 1989; Ohsako & Deguchi, 1981; 1983; Harris *et al.*, 1981; Van Corven *et al.*, 1989). The precise mechanism by which PLD is activated is unclear, but it is known that calcium ionophores, phorbol esters, unsaturated fatty acids and guanine nucleotides will increase PLD activity in intact cells and isolated membrane preparations (Bocckino *et al.*, 1987; Qian & Drewes, 1989; Billah *et al.*, 1989; Anthes *et al.*, 1989; Tettenborn & Mueller, 1988). PLD activity can be readily quantitated in intact cells by measuring the formation of phosphatidylalcohols, which are produced by a unique PLD-dependent transphosphatidylation reaction (Kobayashi & Kanfer, 1987; Pai *et al.*, 1988b). Recent work from this laboratory has indicated that in cytochalasin B-primed neutrophils, superoxide production stimulated by the chemotactic peptide, fMet-Leu-Phe, is totally dependent on PLD activation (Bonser *et al.*, 1989).

The fungal metabolite, wortmannin, is a potent inhibitor of neutrophil activation. Wortmannin inhibits fMet-Leu-Phe-stimulated superoxide production without affecting the mobilization of intracellular calcium (Dewald *et al.*, 1988). Further information on its mode of action comes from the studies of Reinhold *et al.* (1990) who have reported that wortmannin blocks PLD activation induced by fMet-Leu-Phe. In this study we have investigated the effects of a close structural analogue of wortmannin i.e. demethoxyviridin, on signal transduction events in the human neutrophil. Like wortmannin, demethoxyviridin potently inhibits the respiratory burst in neutrophils and blocks PLD activation in response to the chemotactic peptide. Furthermore, demethoxyviridin is also able to block fMet-Leu-Phe-stimulated inositol 1,4,5-trisphosphate (IP_3) formation. Evidence is presented which suggests that wortmannin and demethoxyviridin act by a common mechanism that may disrupt the interaction between the chemotactic peptide receptor and a guanosine 5'-triphosphate (GTP)-binding protein(s).

Methods

Human peripheral blood neutrophils were purified (Tateson *et al.*, 1988) and suspended in 30 mM HEPES-buffered Hanks balanced salt solution, pH 7.2 (Hanks & Wallace, 1949). fMet-Leu-Phe- and phorbol ester-stimulated superoxide generation were measured as described by Cohen & Chovaniec (1978). PLD activity was estimated by measuring the incorporation of high specific activity [^{3}H]-butan-1-ol (12 Ci mmol^{-1}, Amersham International, Amersham, Bucks) into [^{3}H]-phosphatidylbutanol (PBut) as described by Randall *et al.* (1990).

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Amounts of IP_3 were measured by the method described by Palmer *et al.* (1988) and levels of diradylglycerol (DRG) were determined according to Preiss *et al.* (1986), except that mixed micelles containing 5 mol% of phosphatidylserine in Triton X-100 (0.3%, w/v) were used in place of β-octylglucoside/cardiolipin. Release of intracellular calcium was measured with the fluorescent probe fluo-3AM (Molecular Probes Inc.) Fluo-3AM-loaded neutrophils were stimulated with fMet-Leu-Phe in the presence of 4 mM EGTA and fluorescence was monitored with a Perkin-Elmer MPF2 spectrofluorimeter with monochromators set at 506 nm excitation and 526 nm emission. A K_d value of 4.5×10^{-7} M for fluo-3 was used to quantify the fluorescence (Minta *et al.*, 1989).

Wortmannin was supplied by Sandoz, Basle, Switzerland and demethoxyviridin was a gift from Dr J. Hanson, Sussex University, Brighton, E. Sussex. Inhibitors were dissolved in dimethylsulphoxide and the final vehicle concentration did not exceed 0.2% by volume.

Results

The effects of wortmannin and demethoxyviridin on human neutrophil activation are compared in Figure 1. Wortmannin was found to be a potent inhibitor of fMet-Leu-Phe-stimulated superoxide production, with an IC_{50} value (concentration causing 50% inhibition) of 7 ± 1 nM. Demethoxyviridin was equally potent and blocked the respiratory burst with an IC_{50} of 5 ± 2 nM. At concentrations up to 1 μM, wortmannin and demethoxyviridin had no effect on PMA-stimulated superoxide production (results not shown). Although wortmannin is a potent inhibitor of superoxide production it is reported to have no effect on fMet-Leu-Phe-induced intracellular calcium mobilization, even at concentrations up to 1 μM (Dewald *et al.*, 1988). Figure 1 illustrates the action of demethoxyviridin on chemotactic peptide-stimulated intracellular calcium mobilization. Release of intracellular calcium was inhibited by demethoxyviridin but only at concentrations several thousand times greater than were required to block superoxide production. All of these observations strongly suggest that wortmannin and demethoxyviridin inhibit receptor-effector coupling by a common mechanism.

This mechanism was investigated further by monitoring the effects of wortmannin and demethoxyviridin on fMet-Leu-Phe-stimulated second messenger-diacylglycerol production. Both inhibitors blocked diradylglycerol (DRG) generation stimulated by the chemotactic peptide (Figure 2). Demethoxyviridin was approximately six times more potent than wortmannin as an inhibitor of DRG production (IC_{50} values for

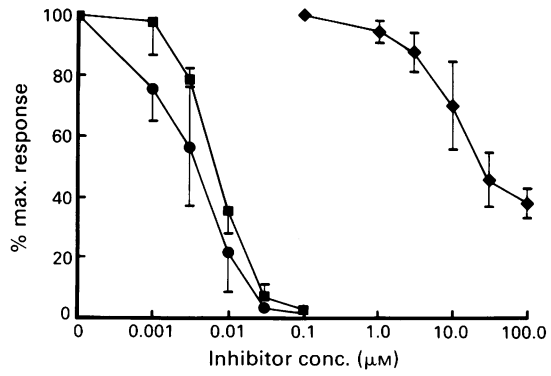

Figure 1 Effect of wortmannin and demethoxyviridin on fMet-Leu-Phe-stimulated superoxide production and intracellular calcium mobilization. Cytochalasin B-treated human neutrophils were stimulated with 100 nM fMet-Leu-Phe in the presence of wortmannin (■) or demethoxyviridin (●, ◆) and superoxide production (●, ■) or intracellular calcium mobilization (◆) measured as described in Methods. Results are the mean from three separate experiments with neutrophils from different donors; vertical bars show s.e.mean.

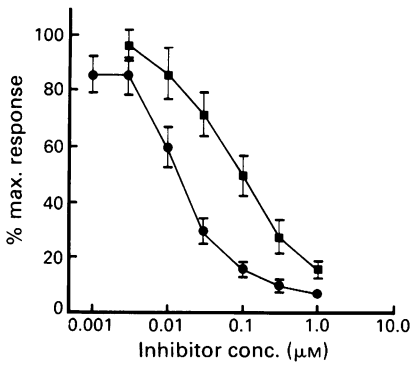

Figure 2 Inhibition of fMet-Leu-Phe-stimulated diradylglycerol (DRG) formation by wortmannin or demethoxyviridin. Human neutrophils pretreated with cytochalasin B were incubated with different concentrations of wortmannin (■) or demethoxyviridin (●) for 5 min at 37°C. fMet-Leu-Phe (100 nM) was then added and DRG production measured by radioenzymatic assay. Results are the mean from three separate experiments with neutrophils from different donors; vertical bars show s.e.mean. Resting DRG levels were 111 ± 10 pmol per 10^7 cells ($n = 36$) and increased after 5 min to 573 ± 44 pmol per 10^7 cells ($n = 36$) in the absence of the inhibitors.

wortmannin and demethoxyviridin were 110 ± 45 nM and 15 ± 3 nM, respectively). Since wortmannin is reported to block fMet-Leu-Phe-induced PLD activation (Reinhold *et al.*, 1990), it was necessary to explore the effect of demethoxyviridin on PLD. Demethoxyviridin inhibited the PLD-dependent formation of phosphatidylbutanol (PBut) in chemotactic peptide-stimulated human neutrophils at concentrations similar to those needed to block DRG formation (Figure 3). This observation provides further evidence that the PLD-phosphatidate phosphatase pathway is the major source of DRG in activated neutrophils. The mode of action and selectivity of demethoxyviridin as an inhibitor of phospholipase activation was examined by measuring its effect on chemotactic peptide-stimulated inositol 1,4,5-trisphosphate (IP_3) production (Figure 3). Surprisingly, phospholipase C-dependent IP_3 formation was blocked by demethoxyviridin at concentrations that were only 10 times higher than those needed to inhibit PLD activation (Figure 3). More importantly, the concentrations of demethoxyviridin that almost totally abolished

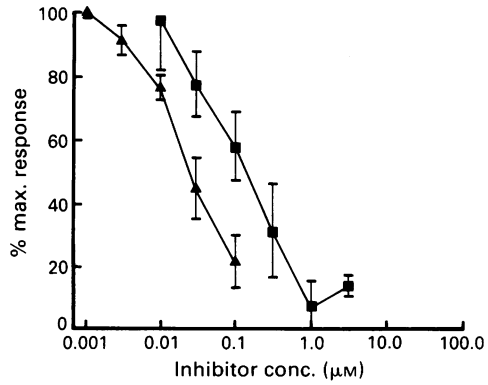

Figure 3 Effect of demethoxyviridin on fMet-Leu-Phe-stimulated 1,4,5-trisphosphate (IP_3) production and [^{3}H]-phosphatidylbutanol ([^{3}H]-PBut) formation. Cytochalasin B-pretreated human neutrophils were incubated with demethoxyviridin for 5 min at 37°C then stimulated with 100 nM fMet-Leu-Phe and phospholipase D (PLD)-dependent [^{3}H]-PBut formation measured after a further 5 min (▲). IP_3 production induced by fMet-Leu-Phe (300 nM) was monitored at 20 s in the absence of cytochalasin B (■). Results are the mean for three separate experiments with neutrophils from different donors; vertical bars show s.e.mean. Basal [^{3}H]-PBut values were 1893 ± 528 d.p.m. ($n = 3$) and increased to 5749 ± 2778 d.p.m. ($n = 3$) in the absence of demethoxyviridin. Resting IP_3 levels were 13.3 ± 2.0 pmol per 10^7 cells ($n = 13$) and reached 50.3 ± 8.1 pmol per 10^7 cells in the absence of the inhibitor.

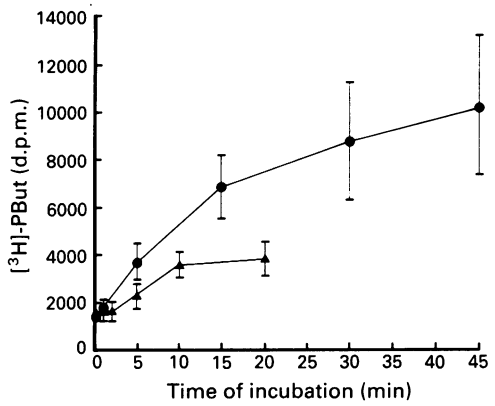

Figure 4 Time courses for phorbol myristate acetate (PMA)- and sodium fluoride-induced [^{3}H]-phosphatidylbutanol ([^{3}H]-PBut) formation in human neutrophils. Neutrophils pretreated with cytochalasin B were incubated with 100 nM PMA (●) or 20 mM sodium fluoride (▲) and phospholipase D-dependent [^{3}H]-PBut formation measured as described in Methods. Results are the mean for three separate experiments with neutrophils from different donors; vertical bars show s.e.mean.

IP_3 formation had no effect on intracellular calcium mobilization (Figures 1 and 3).

The site of interaction of demethoxyviridin with the receptor-linked PLD-dependent signalling pathway in the neutrophil was investigated by monitoring its effects on PMA- and sodium fluoride-stimulated PBut formation. PMA-stimulated transphosphatidylation was slow and still increasing after 45 min (Figure 4). In contrast, PLD activation induced by fluoride displayed a distinct lag period of 1–2 min followed by a rapid response that was complete within 10 min (Figure 4). Demethoxyviridin inhibited PMA-stimulated PLD activity in a concentration-dependent manner ($IC_{50} = 4 \pm 1\,\mu M$) and blocked fluoride-activated PLD at concentrations above 300 nM (Figure 5). It should be noted that these concentrations are much greater than are required to inhibit fMet-Leu-Phe-stimulated PBut formation (Figure 3).

The type of inhibition exhibited by demethoxyviridin in fMet-Leu-Phe-stimulated neutrophils was examined by monitoring its effects on the chemotactic peptide dose-response curve for PBut formation (Figure 6). Increasing concentrations of demethoxyviridin progressively depressed maximum PBut formation in response to fMet-Leu-Phe with little indication of a rightward shift in ED_{50} values, consistent with a non-competitive type of inhibition.

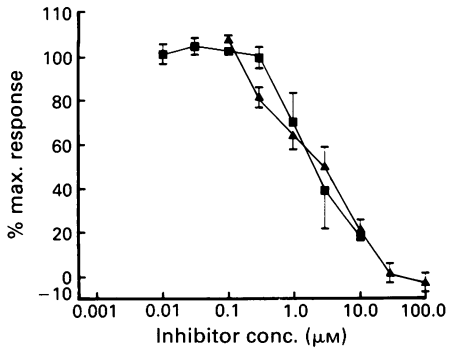

Figure 5 Effect of demethoxyviridin on phorbol myristate acetate (PMA)- and sodium fluoride-stimulated [^{3}H]-phosphatidylbutanol ([^{3}H]-PBut) production in human neutrophils. Neutrophils (0.5 ml at 2×10^7 cells per ml) were preincubated with cytochalasin B and different concentrations of demethoxyviridin for 5 min at 37°C then treated with 100 nM PMA (▲) or 20 mM sodium fluoride (■) and [^{3}H]-PBut formation quantitated. PMA- and sodium fluoride-induced PBut formation was measured after 30 min and 20 min, respectively. Results are the mean from three experiments with neutrophils from different donors; vertical bars show s.e.mean.

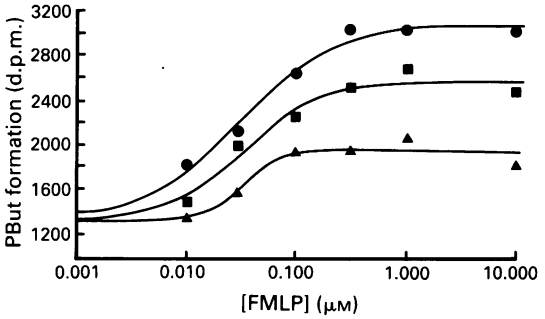

Figure 6 Inhibition of fMet-Leu-Phe (FMLP)-stimulated [^{3}H]-phosphatidylbutanol ([^{3}H]-PBut) formation by demethoxyviridin. Concentration-effect curves for fMet-Leu-Phe-stimulated [^{3}H]-PBut formation were generated in the absence (●) or presence of 20 nM (■) and 50 nM (▲) demethoxyviridin, according to the procedure outlined in the legend to Figure 3. Results are from a single representative experiment.

Discussion

Recent work has suggested that the fungal metabolite, wortmannin, may inhibit chemotactic peptide-stimulated superoxide production by blocking the activation of PLD (Dewald *et al.*, 1988; Reinhold *et al.*, 1990). The formation of phosphatidylalcohols is a specific indicator of PLD activity and provides a simple means of monitoring PLD activity in intact cells (Kobayashi & Kanfer, 1987; Pai *et al.*, 1988b). We have used the formation of [^{3}H]-PBut from high specific activity [^{3}H]-butan-1-ol to investigate the inhibitory action of a close analogue of wortmannin, i.e. demethoxyviridin, in the human neutrophil. We have found that demethoxyviridin and wortmannin inhibit superoxide production at similar concentrations. Furthermore, demethoxyviridin blocked PLD-dependent phosphatidylalcohol formation at concentrations similar to those reported for wortmannin (Reinhold *et al.*, 1990), suggesting that these fungal metabolites act at a common step in the signal transduction pathway. The inhibition of fMet-Leu-Phe-stimulated [^{3}H]PBut formation was characteristically non-competitive, suggesting that demethoxyviridin does not block the interaction of the chemotactic peptide with its receptor. This observation also reinforces the view that these inhibitors block signal transduction events.

Information on the site of action of wortmannin has been obtained from studies that have bypassed receptor events and activated PLD directly. It is reported that wortmannin does not inhibit phosphatidylethanol (PEt) formation stimulated by PMA (Reinhold *et al.*, 1990). In agreement with this, we found that PMA-induced transphosphatidylation was only inhibited by demethoxyviridin at high concentrations. These observations indicate that wortmannin and demethoxyviridin are not active site inhibitors and that inhibition probably occurs at a point along the signalling pathway that lies between the chemotactic peptide receptor and PLD. Interestingly, Reinhold and co-workers (1990) reported that PMA was a weaker stimulus than fMet-Leu-Phe for PLD activation in the neutrophil. It is worthwhile noting that this group measured PMA-induced PEt formation after 5 min. Our data show that this period of incubation would only detect a fraction of the PLD activity stimulated by PMA.

Phorbol esters and fMet-Leu-Phe stimulate the release of large amounts of DRG from cytochalasin B-treated neutrophils (Honeycutt & Niedel, 1986; Bonser *et al.*, 1989; Dougherty *et al.*, 1989). There is now strong evidence indicating that the hydrolysis of phosphatidylinositol 4,5-bisphosphate by PLC cannot be the sole source of this lipid and that the majority of the DRG released is probably derived through the PLD-phosphatidate phosphatase pathway (Pelech & Vance, 1989; Loffelholz, 1989; Bonser *et al.*, 1989; Exton, 1990). The inhibitory effects of wortmannin and demethoxyviridin on DRG production observed in this study provides further

support for this pathway as the major source of second messenger-diacylglycerol in the neutrophil. It has been reported by Reinhold *et al.* (1990) that wortmannin does not block fMet-Leu-Phe-stimulated diacylglycerol production. We can offer no explanation for their failure to observe any effect, since in our experiments significant inhibition of DRG production (i.e. 40–50%) was observed with wortmannin at the concentrations used by these investigators.

Activation of PLD may involve a GTP-binding protein since non-hydrolysable GTP analogues (e.g. GTPγS) have been shown to stimulate PLD in membranes isolated from brain, liver and HL60 cells (Boccckino *et al.*, 1987; Qian & Drewes, 1989; Tettenborn & Mueller, 1988; Anthes *et al.*, 1989). There are several examples where G-protein activation has been mimicked by high concentrations of fluoride (Eckstein *et al.*, 1979; Sternweis & Gilman, 1982; Bigay *et al.*, 1985; Gabig *et al.*, 1987). Fluoride was able to activate PLD in cytochalasin B-treated neutrophils but this response was not inhibited by demethoxyviridin at concentrations that completely inhibited fMet-Leu-Phe-stimulated PBut formation. These observations suggest that demethoxyviridin does not block GTP-binding protein function but may interfere with the interaction between the GTP-binding protein and the chemotactic peptide receptor.

Mobilization of intracellular calcium is mediated by the second messenger IP_3, produced by a receptor-coupled, phosphatidylinositol 4,5-bisphosphate-specific PLC (Berridge & Irvine, 1989). Wortmannin is reported not to inhibit fMet-Leu-Phe-stimulated calcium release at concentrations which completely block superoxide production, suggesting that it does not inhibit IP_3 formation (Dewald *et al.*, 1988). Demethoxyviridin did inhibit IP_3 formation induced by the chemotactic peptide but the concentrations required were 10–20 times greater than those needed to block PLD activation and DRG production. More surprisingly, inhibition of intracellular calcium mobilization by demethoxyviridin occurred at concentrations 100 times greater than those required to inhibit IP_3 formation. It is possible to envisage several explanations for this discrepancy. The most likely possibility is that only a fraction of the IP_3 that is formed is required to mobilize intracellular calcium. Alternatively, a separate mechanism for mobilizing intracellular calcium may exist that is independent of IP_3 production.

PA is reported to increase cellular calcium concentrations and evoke calcium-mediated physiological responses (Salmon & Honeyman, 1980; Ohsako & Deguchi, 1981; 1983; Harris *et al.*, 1981). It has been suggested that PA may function as an endogenous calcium ionophore, although it is also known to enhance the production of IP_3 (Putney *et al.*, 1980; Jackowski & Rock, 1989). Mobilization of intracellular calcium is unaffected by wortmannin and demethoxyviridin at concentrations that totally inhibit PLD activation. It is clear, therefore, that PA generated by PLD activation does not contribute to the release of intracellular calcium in the neutrophil. It is evident from these studies that demethoxyviridin displays little selectivity for PLD over PLC and consequently blocks both PLC- and PLD-derived second messenger production, yet is able to discriminate between the functional responses that are dependent on these second messengers.

In summary we have found that a close structural analogue of wortmannin, demethoxyviridin, inhibits fMet-Leu-Phe-stimulated superoxide production in the human neutrophil by blocking the activation of PLD. Wortmannin and demethoxyviridin probably act at the same site in the signal transduction pathway and may block the interaction between the receptor for fMet-Leu-Phe and a GTP-binding protein that is intimately involved in PLD activation. Interestingly, demethoxyviridin does not inhibit intracellular calcium mobilization at concentrations that almost completely inhibit IP_3 formation.

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Endothelin modulates calcium channel current in neurones of rabbit pelvic parasympathetic ganglia

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1 The effects of endothelin were studied, *in vitro*, on neurones contained in the rabbit vesical pelvic ganglion by use of intracellular and single-electrode voltage clamp techniques under conditions where sodium and potassium channels were blocked.

2 In the current-clamp experiments, endothelin (1 μM) caused a depolarization followed by a hyperpolarization of the membrane potential. In the voltage-clamp experiments, endothelin (0.01–1 μM) caused an inward current followed by an outward current in a concentration-dependent manner.

3 Membrane conductance was increased during the endothelin-induced depolarization and inward current. Membrane conductance was decreased during the endothelin-induced hyperpolarization and outward current.

4 The endothelin-induced inward and outward currents were not altered by lowering external sodium concentration or raising external potassium concentration.

5 The endothelin-induced inward current was depressed (mean 72%) in a Krebs solution containing nominally zero calcium and high magnesium. These results suggest that a predominent component of the endothelin-induced inward current is mediated by calcium ions.

6 The calcium-insensitive component of the inward current was abolished by a chloride channel blocker, 4-acetamide-4'-isothiocyanostilbene-2,2'-disulphonic acid. The mean reversal potential for the calcium-insensitive component of the inward current was −18 mV. This value is near the equilibrium potential for chloride. Thus, it is presumed that the calcium-insensitive component of the inward current is carried by chloride ions.

7 Endothelin caused an initial depression followed by a long lasting facilitation of both rapidly and slowly decaying components of high-threshold calcium channel currents (N- and L-type).

8 In summary, the data show that for neurones in the vesical pelvic ganglia, endothelin causes membrane depolarization and activates an inward current. The ionic mechanisms involve receptor-operated calcium and chloride currents. Also, endothelin causes an initial depression followed by a long-lasting facilitation of the voltage-dependent calcium current.

Keywords: Endothelin-1; parasympathetic ganglia; single neurones; depolarization; calcium current; single electrode voltage-clamp

Introduction

Endothelin is a potent vasoconstrictor peptide recently isolated from porcine and human vascular endothelial cells (Itoh *et al.*, 1988; Yanagisawa *et al.*, 1988). In porcine coronary artery smooth muscle, endothelin augmented the dihydropyridine-sensitive voltage-dependent calcium channel current (Goto *et al.*, 1989; Silberberg *et al.*, 1989) whereas in portal vein smooth muscle, endothelin augmented both the T- and L-type calcium channel current (Inoue *et al.*, 1990).

Endothelin may also function as a neuropeptide (Masaki, 1989) in the central and peripheral nervous systems. Endothelin binding sites and immunoreactivity have been identified and depolarizing responses to endothelin have been reported for neurones (Giaid *et al.*, 1989; Koseki *et al.*, 1989; Yoshizawa *et al.*, 1989a,b; MacCumber *et al.*, 1990). Little is known, however, regarding the action of endothelin on neuronal ionic conductances and how activation of the endothelin receptors alters the operation of neuronal voltage-dependent calcium channels. The present study was undertaken to determine whether endothelin regulates the ionic conductance and voltage-dependent calcium channels of mammalian autonomic neurones, by use of intracellular current clamp and single-electrode voltage clamp techniques. A preliminary account of some of this work has been published (Nishimura *et al.*, 1989; 1990).

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Methods

Male white rabbits weighing 2.0–3.0 kg were anaesthetized with sodium pentobarbitone (40–50 mg kg^{-1}, i.v.). The methods for isolation of vesical pelvic ganglia were described previously (Nishimura *et al.*, 1988b). After removal of the pelvic ganglia, rabbits were killed by a large dose (100–150 mg kg^{-1}, i.v.) of sodium pentobarbitone. Individual ganglia were pinned onto Sylgard at the bottom of a small chamber (0.5 ml) and continuously superfused with Krebs solution (3 ml min^{-1}) having the following composition (mM): NaCl 117, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11. Solutions were gassed with 95% O_2, 5% CO_2 and preheated to 35–37°C.

Microelectrodes were filled with 2 M CsCl unless stated otherwise. Tip resistances of microelectrodes were 20–40 MΩ. Membrane currents were recorded by the single-electrode voltage-clamp method with an Axoclamp 2A (Axon Instruments). Sampling frequency ranged between 3 and 5 kHz with a 70–30 duty cycle. Signals from the microelectrode were displayed on an oscilloscope with digitized memory (Nihon Kohden, VC-11) and recorded on a pen-writing chart recorder (Nihon Kohden, RJG-3022 and RJG-4124). Data were also stored on a video cassette data recorder (SONY KS609) for later analysis.

To block voltage-dependent sodium and potassium currents, a modified Krebs solution was used which contained tetrodotoxin (TTX, 300 nM) and tetraethylammonium (TEA, 50 mM) and where external sodium was reduced from 143.2 mM to 93.2 mM. To suppress potassium currents, caesium

ions (Cs^+) were also ionophoretically injected into neurones through a recording microelectrode filled with 2 M CsCl. In the current clamp experiments, injection of Cs^+ causes membrane depolarization (range, −35 mV to −10 mV; Akasu *et al.*, 1990). To record action potentials from these neurones the depolarization was nullified by injection of a constant hyperpolarizing current through the microelectrode. Efficiency of Cs^+-injection was judged by the broadening of the action potential, the complete abolition of the afterhyperpolarization and the appearance of an afterdepolarization following the action potential (Nishimura *et al.*, 1988b). Voltage-dependent inward calcium and barium currents were recorded where sodium and potassium currents were suppressed (see above). Leak currents were determined by applying hyperpolarizing voltage-commands of magnitudes equal to depolarizing commands used to evoke the inward current. Leak currents were also recorded during depolarizing commands equivalent to those used to evoke inward currents after block of voltage-dependent calcium and barium currents by the removal of calcium or barium or by cobalt application. Leak currents were subtracted from relevant currents to yield the calcium and barium currents by use of a Nicolet memory oscilloscope (Nicolet 4094).

Calcium- and barium-free Krebs solution contained nominally zero calcium and barium and 12 mM magnesium. Nominally zero calcium and high magnesium solutions have been shown to block fast excitatory postsynaptic potentials recorded from neurones in vesical pelvic ganglia (Nishimura *et al.*, 1988a). Drugs were dissolved in Krebs solution and applied by changing the flow with a three-way stopcock.

Drugs used were: endothelin-1 from Peptide Institute (Japan); tetrodotoxin (TTX) from Sankyo; tetraethylammonium chloride (TEA) from Tokyo Kasei (Japan); 4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid disodium salt (SITS) from Research Organics (U.S.A.); (+)-tubocurarine chloride, yohimbine hydrochloride and prazosin hydrochloride from Sigma; atropine sulphate from Merck; [3α-tropanyl]-1H-indole-3-carboxylic acid ester (ICS 205-930) from Sandoz. Results are expressed as the mean ± standard error of the mean (s.e.mean).

Results

Endothelin-induced membrane responses

Superfusion of the pelvic ganglia with endothelin (1 μM for 2–3 min) produced a slow depolarization in neurones with a mean time to peak of 1.1 ± 0.1 min ($n = 5$) and a mean peak amplitude of 19 ± 3 mV (Figure 1a). When endothelin was removed from the Krebs solution the depolarization recovered and was followed by membrane hyperpolarization with a mean peak amplitude of 10 ± 6 mV ($n = 5$). The hyperpolarization occurred for time periods ranging from 20 to 30 min. In three neurones the depolarizing and hyperpolarizing action of endothelin was associated with a decrease and an increase in membrane input resistance, respectively. The endothelin-induced depolarization and the subsequent hyperpolarization after washout and associated changes in membrane input resistance were not altered by the cholinoceptor antagonists, (+)-tubocurarine (10 μM) and atropine (1 μM) ($n = 2$) (Gallagher *et al.*, 1982), by the adrenoceptor antagonists, yohimbine (1 μM) and prazosin (1 μM) ($n = 2$) (Akasu *et al.*, 1985) or by the 5-hydroxytryptamine (5-HT) antagonist, ICS 205-930 (10 nM) ($n = 2$) (Akasu *et al.*, 1987). Thus, cholinoceptors, adrenoceptors and 5-HT_3 receptors were not involved in mediating the response to endothelin.

Endothelin-induced membrane currents

Voltage-clamp methods were used to examine the ionic mechanisms underlying the endothelin-induced responses, under conditions where voltage-dependent sodium and potassium currents were blocked (see Methods). Superfusion of the ganglia with endothelin (10 nM to 1 μM) induced an inward current with amplitudes ranging from 0.2 to 3.3 nA, at holding potentials ranging from −50 to −75 mV (Figure 2a). When endothelin was removed from the superfusing solution the inward current, which slowly declined, was followed by an outward current (amplitude range, 0.1–2.2 nA). The outward current lasted for time periods ranging between 15 to 30 min before recovery. Endothelin induced inward and outward currents in a concentration-dependent manner (Figure 2b). The calculated EC_{50} values were 60 nM and 90 nM for the inward and outward currents, respectively. The current responses were reproducible, when endothelin was applied to neurones for times ranging between 10 to 30 s and intervals between applications were longer than 30 min. Current responses to endothelin showed tachyphylaxis, when applied for a period of 5 to 10 min. Subsequent application of endothelin caused no current response even after neurones were superfused with an endothelin-free Krebs solution for more than 30 min.

Conductance changes associated with endothelin-induced currents

Endothelin-induced inward and outward currents were associated with an increase and a decrease, respectively, in membrane conductance (Figure 3a,b). Membrane conductance was

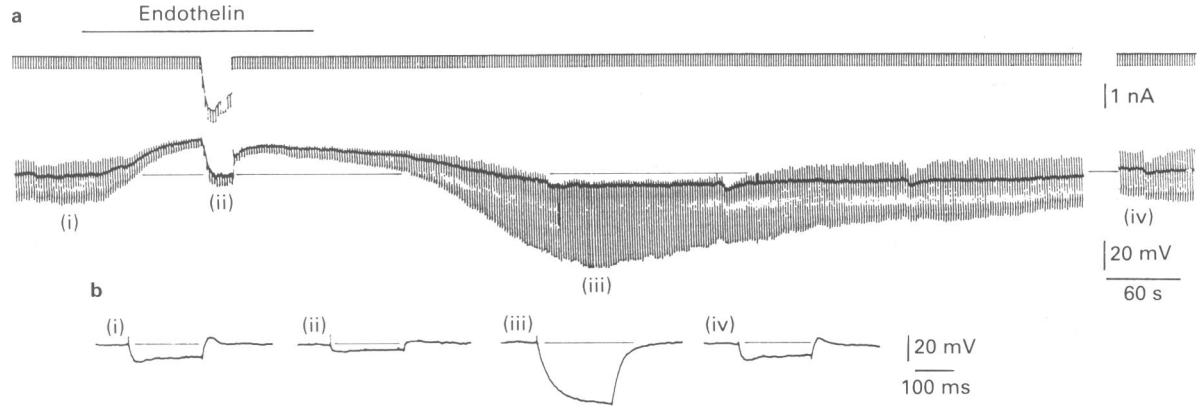

Figure 1 (a) Effect of endothelin on resting membrane potential and membrane input resistance of vesical pelvic neurone. The neurone was superfused with Krebs solution containing 1 mM caesium and impaled by a microelectrode containing 3 M KCl. Endothelin (1 μM) induced a depolarization followed by a hyperpolarization of the membrane potential. Input resistance was calculated from the amplitude of the hyperpolarizing electrotonic potentials (lower trace) evoked by injection of hyperpolarizing current pulses (upper trace) through the recording microelectrode. Endothelin-induced depolarization was partially nullified by hyperpolarizing direct current. Horizontal bar indicates time period of endothelin application. (b) Expanded records of the electrotonic potential. All records were taken at the times marked by numbers in (a). Record (iv) was taken 20 min after removal of endothelin from the perfusate. The resting membrane potential was −56 mV.

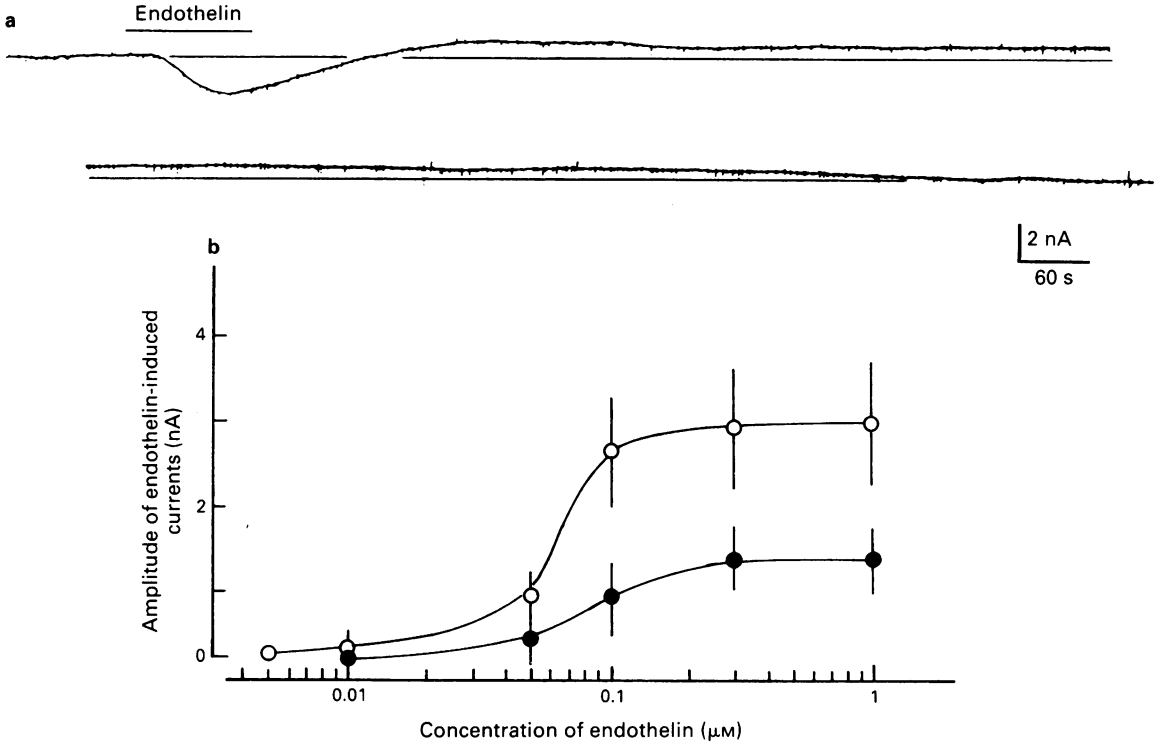

Figure 2 Effect of endothelin on membrane current. The modified Krebs solution contained tetrodotoxin (300 nM) and tetraethylammonium (50 mM) and sodium ions were reduced to 93.2 mM. Caesium ions had been injected into the cell. (a) Endothelin (100 nM) caused inward current followed by outward current. The horizontal bar indicates the time period of endothelin-application. (b) Relationship between concentration of endothelin (abscissa scale) and amplitude of current responses evoked by endothelin (ordinate scale). Points and vertical lines represent mean of 3 to 5 responses and s.e.mean, respectively: (○) inward current; (●) outward current.

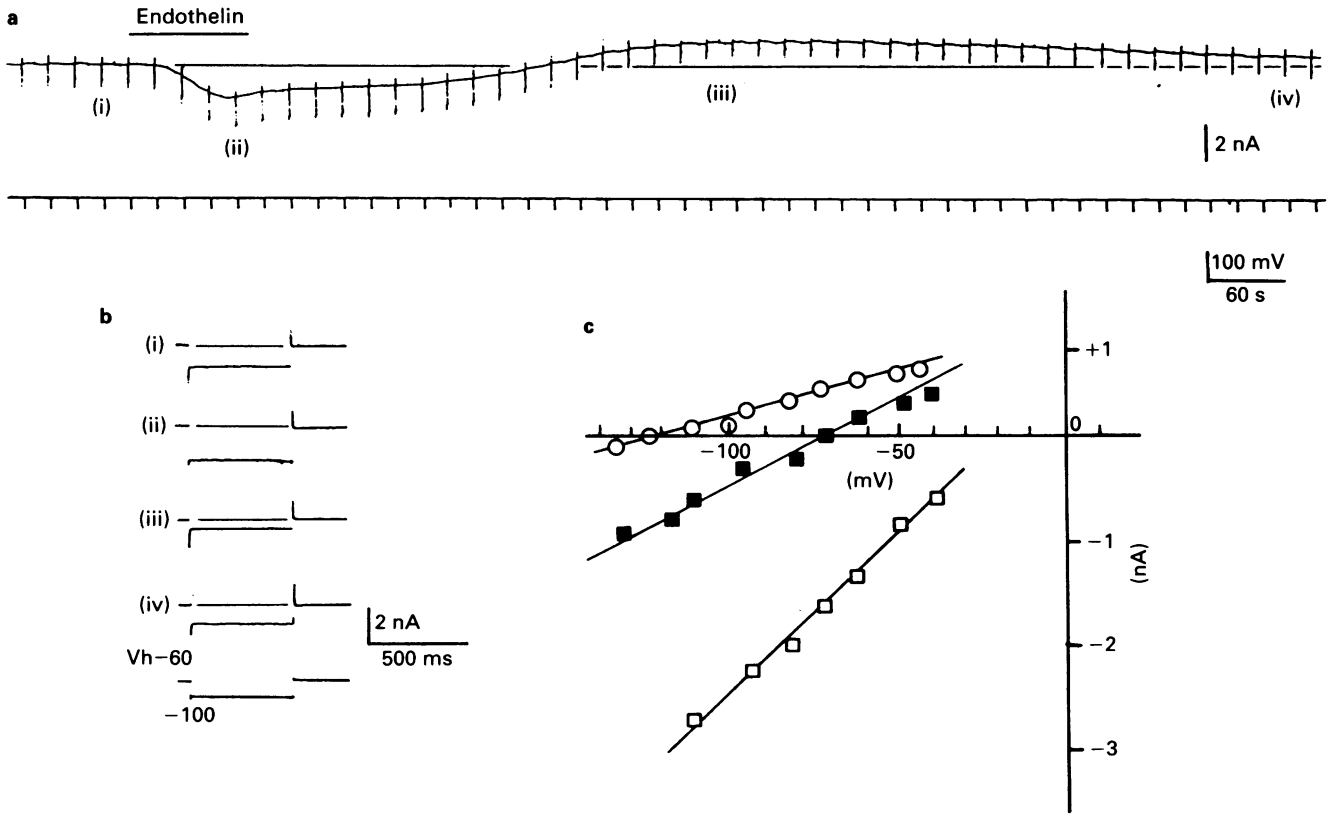

Figure 3 (a) Conductance changes during endothelin-induced (100 nM) current responses in a caesium-loaded neurone. The superfusate contained tetrodotoxin (300 nM) and tetraethylammonium (50 mM). The neurone was voltage-clamped at −60 mV and subjected to hyperpolarizing voltage jumps to −100 mV to measure membrane conductance. (b) Expanded records of inward currents produced by the hyperpolarizing command pulse of 500 ms duration. Records (i)–(iv) were taken at the times marked by numbers in (a). (c) Current-voltage relation (I–V curve) made by step commands with a duration of 200 ms at 0.2 Hz. The holding potential was −72 mV. The perfusate also contained caesium (2 mM) to block inward rectification. I–V curve before application of endothelin (100 nM) (■); I–V curve during endothelin-induced inward (□) and outward (○) currents, respectively.

determined by measuring the steady-state inward current induced by a 500 ms step command from a holding potential of −60 mV to −100 mV. In modified Krebs solution the calculated membrane conductance for this neurone was 25 nS. During the endothelin-induced inward and outward currents calculated membrane conductances were 43 nS and 11 nS, respectively (Figure 3b).

Measurements of membrane conductance (chord conductance) were also obtained from the slope of the linear portion of a current-voltage relation (I–V curve). Figure 3c shows the I–V curve of a neurone in a modified Krebs solution containing 2 mM caesium. The calculated chord conductance was 21 nS at potentials ranging between −50 mV and −110 mV. Endothelin-induced (100 nM) inward current was associated with an increase in the slope of the linear portion of the I–V curve (Figure 3c); the chord conductance was 40 nS in the presence of endothelin. In contrast, the slope of the linear I–V curve was decreased during the endothelin-induced outward current (Figure 3c). The chord conductance was 16 nS during the outward current. The mean chord conductance of four neurones in a modified Krebs solution was 23 ± 4 nS. The mean chord conductance during the endothelin-induced inward and outward currents was 39 ± 6 nS and 12 ± 3 nS, respectively.

Calcium dependency of endothelin-induced inward currents

In caesium-loaded neurones superfused with a modified Krebs solution containing TTX (300 nM) and TEA (50 mM), lowering the external calcium concentration to a nominal zero value did not change the holding current. Under these conditions the amplitude of the endothelin-induced currents was reduced and the outward current was totally inhibited (Figure 4a). The mean decrease in peak amplitude of the inward current for six neurones was 72 ± 3%.

In caesium-loaded neurones superfused with a modified Krebs solution, lowering the external sodium to 26.2 mM (67 mM sodium chloride was replaced with equimolar choline chloride) did not alter the amplitude and the time course of the endothelin-induced inward currents. In three neurones the range of peak amplitudes for the endothelin-induced (100 nM) inward and outward currents in a low sodium (26.2 mM) solution was 2 to 3 nA and 0.5 to 1 nA, respectively. In two other caesium-loaded neurones, raising the external potassium to 10 mM did not alter the holding current or the endothelin-induced inward and outward currents. These results suggest that the endothelin-induced inward current is mediated in part by calcium ions.

Reversal potential for endothelin-induced inward current

Experiments were next conducted to determine the reversal potential for the endothelin-induced inward current in the presence and absence of external calcium. Figure 4b shows that effects of endothelin (100 nM) on a current-voltage relation (I–V curve) for a neurone when the external calcium concentration was 2.5 mM (Figure 4b(i)) and nominally zero (Figure 4b(ii)). In both panels, the reversal potentials of the inward currents were estimated from the intersection of I–V

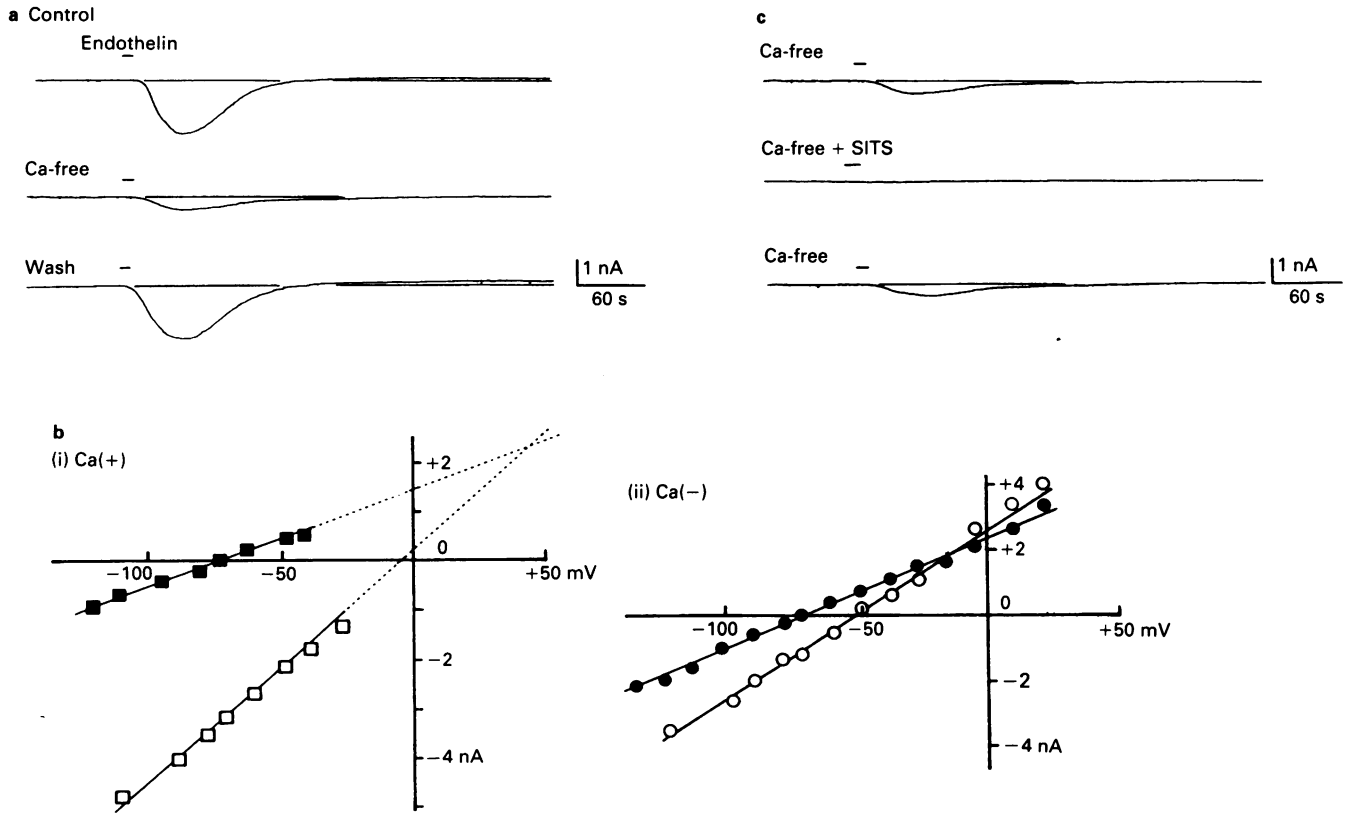

Figure 4 (a) Effects of removal of calcium ions on endothelin-induced (100 nM) current responses. The holding potential was −60 mV. Modified Krebs solutions contained tetrodotoxin (300 nM) and tetraethylammonium (50 mM). The calcium-free solution contained nominally zero calcium and 12 mM magnesium. Horizontal bars above each trace represent the time period of endothelin-application. (b) Effect of endothelin (100 nM) on the steady state I–V curve obtained in the presence (i) and absence (ii) of calcium. Step commands with a duration of 200 ms were applied from a holding potential of −70 mV at 0.2 Hz. Filled and open symbols were obtained before and during application of endothelin, respectively. I–V curves were obtained from two cells. (c) Effect of SITS (500 μM) on calcium-insensitive inward current produced by endothelin. Holding potential was −62 mV. Endothelin (100 nM) was applied to the perfusate at a time period indicated by short horizontal bars.

curves. In modified Krebs solution containing 2.5 mM calcium the estimated reversal potential for the endothelin-induced inward current was +45 mV. The mean reversal potential was +42 ± 3 mV ($n = 4$). In the calcium-free solution, endothelin induced an inward shift of membrane current and increased the slope of the I–V curve. The estimated reversal potential for the calcium-insensitive component for the cell was −14 mV. The mean reversal potential was −18 ± 4 mV ($n = 4$). The reversal potential for the calcium-insensitive component for the endothelin-induced inward current was near the equilibrium potential for chloride ions (Akasu *et al.*, 1990).

Effect of SITS on the endothelin-induced inward current

Pharmacological experiments were next conducted to determine whether chloride ions mediate the calcium-insensitive component of the endothelin-induced inward current. 4-Acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid (SITS) has been reported to block chloride current in squid axon (Inoue, 1985), cultured astrocytes (Gray & Ritchie, 1986), sensory neurones (Bader *et al.*, 1987), endocrine cells (Korn & Weight, 1987) and parasympathetic neurones (Akasu *et al.*, 1990). Addition of SITS (0.5 mM) to a calcium-free solution completely inhibited the calcium-insensitive component of the inward current (Figure 4c) ($n = 3$). The results are consistent with chloride being a charge carrier for a component of the inward current.

Voltage-dependent calcium (I_{Ca}) and barium (I_{Ba}) current

Voltage-dependent I_{Ca} and I_{Ba} were recorded from caesium-loaded neurones superfused with a Krebs solution containing Ca^{2+} (2.5 mM) or Ba^{2+} (2.5 mM), TTX (300 nM) and TEA (50 mM) (see Methods). When calcium was the charge carrier, application of a step command from a holding potential of −55 mV to −5 mV evoked an inward current with amplitudes ranging between 1–3 nA followed by an inward tail current (Figure 5). Tail currents are due to deactivation of calcium-dependent chloride channels (Akasu *et al.*, 1990). In contrast when barium was the charge carrier, application of a step command from a holding potential of −60 to −10 mV evoked inward currents with slower decay time than that of I_{Ca} and the absence of tail currents (Figure 6). Both the I_{Ca} and I_{Ba} were blocked by application of either cobalt (1 mM) or ω-conotoxin (500 nM) (Akasu *et al.*, 1990). During the endothelin-induced inward current, the I_{Ca} and tail current were reduced (Figure 5). When endothelin was removed from the perfusate, both I_{Ca} and the tail current recovered and then increased in amplitude as long as the outward current occurred.

Figure 6 shows the effect of endothelin (100 nM) when barium was the charge carrier for the calcium channel current. Endothelin also caused both inward and outward currents. The amplitude of the I_{Ba} was also depressed and then facilitated by endothelin. Partial recovery of the I_{Ba} was evident in the continued presence of endothelin (Figure 6c). The facilitation of the I_{Ba} lasted for approximately 20 min in an endothelin-free modified Krebs solution. The action of endothelin on the I_{Ba} was concentration-dependent (Figure 7).

Current-voltage relation of the I_{Ba}

Figure 8a(i) shows the current-voltage relation (I–V curve) of net membrane currents obtained before and during the endothelin-induced inward current. The neurone was initially clamped at −60 mV and subjected to step commands which ranged between −100 mV and +50 mV. Endothelin (100 nM) caused a 2 nA shift of the holding current at −60 mV and depressed the amplitude of the I_{Ba}. This resulted in a nearly linear I–V curve for the net membrane currents during the endothelin-induced inward current. Amplitudes of the net I_{Ba} obtained by subtraction of leak currents were plotted against command voltage in Figure 8a(ii). The I_{Ba} activated near −30 mV and the peak of the I_{Ba} occurred at −10 mV to +10 mV (see Akasu *et al.*, 1990). Endothelin depressed the amplitude of I_{Ba} at all potentials tested without producing any change in the threshold and the peak voltage of the I_{Ba} (Figure 8a(ii)). Figure 8b(i) shows the I–V curve of net membrane currents obtained during the outward current. Endothelin increased the amplitude of the I_{Ba} at each potential tested, but did not change the threshold membrane potential and the peak voltage of the I_{Ba} (Figure 8b(ii)).

Effect of endothelin on two types of voltage-dependent calcium current

Two types of high-threshold calcium currents, comparable to N- and L-type of calcium currents observed in cultured sensory neurones of the chick embryo (Nowycky *et al.*, 1985; Fox *et al.*, 1987), were recorded in neurones of rabbit vesical pelvic ganglia (Akasu *et al.*, 1990). At holding potentials between −70 and −50 mV, the I_{Ba} evoked at potentials more positive than −30 mV consists of the sum of two components of decay; a rapid decay with a mean time constant of 150 ms and a slow decay with a mean time constant of 560 ms (Akasu *et al.*, 1990). In addition, a slowly decaying component of

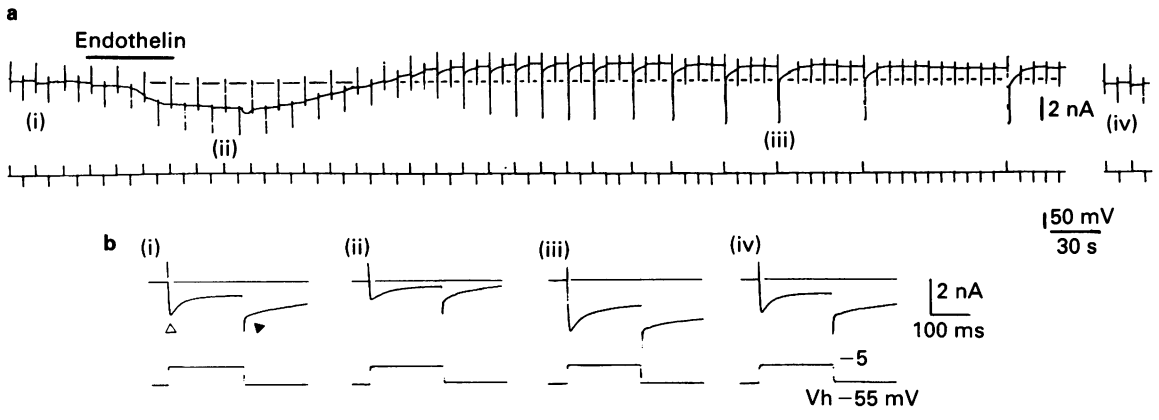

Figure 5 Effect of endothelin (100 nM) on voltage-dependent calcium currents (△) and tail currents (▲) recorded from caesium-loaded neurone. Modified Krebs solution contained tetrodotoxin (300 nM) and tetraethylammonium (50 mM). (a) Calcium and tail currents were evoked by a depolarizing step command (duration of 200 ms) from a holding potential of −55 mV to −5 mV. Upper and lower traces represent membrane current and holding voltage, respectively. Horizontal bar above current trace indicates time period of application of endothelin. (b) Expanded records (i)–(iv) were taken at the times marked by numbers in (a). Record (iv) was taken 20 min after withdrawal of endothelin from the perfusate.

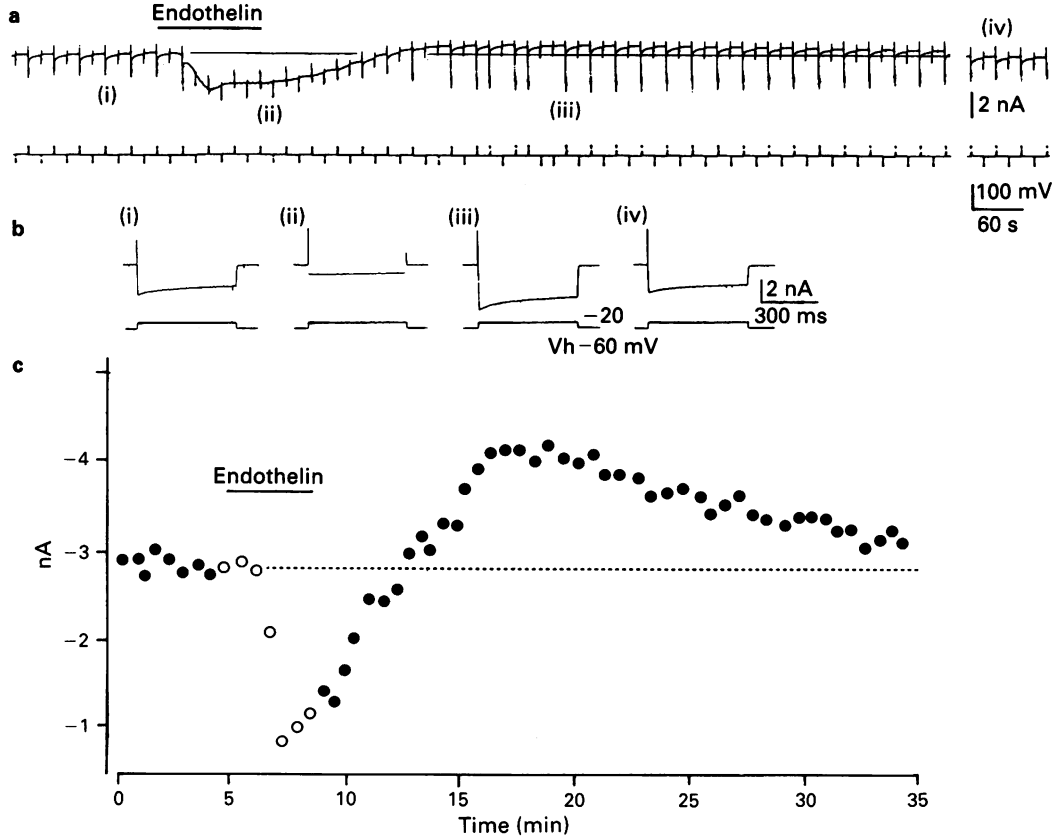

Figure 6 Effect of endothelin (100 nM) on barium inward current (I_{Ba}) recorded from a caesium-loaded neurone. Modified Krebs solution contained tetrodotoxin (300 nM) and tetraethylammonium (50 mM). Calcium (2.5 mM) was replaced with equimolar barium. (a) Upper and lower traces represent membrane current and holding voltage, respectively. The I_{Ba} was evoked by a depolarizing step command to −20 mV (duration of 500 ms) from a holding potential of −60 mV. Horizontal bar indicates the time period of endothelin-application. Downward and upward deflections in lower trace represent step commands to −100 mV and −20 mV, respectively. (b) Expanded records of depolarizing step command (lower trace) and I_{Ba} (upper trace) taken at times marked by numbers in (a). Record (iv) was obtained 20 min after withdrawal of endothelin. Leak currents obtained by hyperpolarizing step command were subtracted (see Methods). (c) Time course of inhibition and facilitation of I_{Ba} caused by endothelin (100 nM). Ordinate scale: peak amplitude of I_{Ba} in nA. Abscissa scale: time in min.

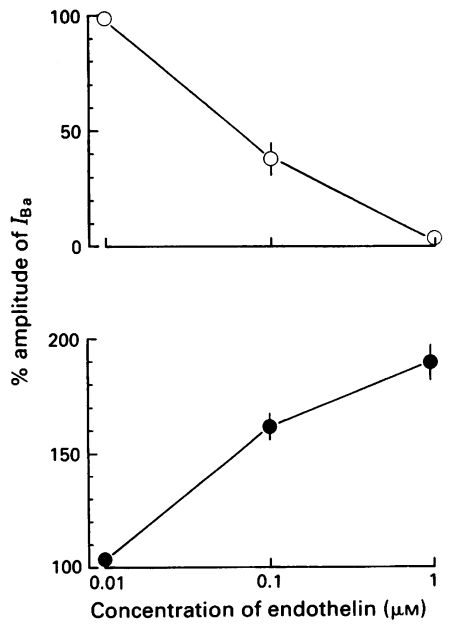

Figure 7 Relationship between concentration of endothelin (abscissa scale) and amplitude of I_{Ba} (ordinate scale). I_{Ba} was evoked by step commands to between −20 to +10 mV from a holding potential of −60 mV. Amplitude of I_{Ba} is expressed as a percentage of control amplitude. Upper and lower graphs represent endothelin-induced depression and facilitation of I_{Ba}, respectively. Circles and vertical lines represent mean and s.e.mean, respectively. Number of neurones tested were 3 for 10 nM, 8 for 100 nM and 2 for 1 μM of endothelin.

calcium current can be recorded in isolation from the rapidly decaying component by use of a pulse protocol where neurones are held at −45 to −40 mV and subjected to step commands to potentials more positive than −10 mV (Figure 9b). The time constant of these decays are similar to N- and L-type calcium currents (Fox *et al.*, 1987) or rapidly and slowly inactivating components of N-type calcium channel current (Plummer *et al.*, 1989; Seward & Henderson, 1990).

Endothelin (100 nM) depressed the amplitude of the I_{Ba} measured at the beginning (mean 70 ± 6%, $n = 5$) and the end (mean 40 ± 4%) of the 500 ms step command (Figure 9a). Figure 9a also shows that during the endothelin-induced inward current the initial rapid component of I_{Ba} inactivation was not apparent whereas the slow component of inactivation still remained although it had been reduced. During the endothelin-induced outward current, the I_{Ba} increased in amplitude at the beginning (mean 58 ± 6%, $n = 4$) and the end (53 ± 5%, $n = 4$) of the 500 ms command pulses. Figure 9b shows the effect of endothelin (100 nM) on slowly decaying current (similar to the L-type current). The peak amplitude of the I_{Ba} was depressed (mean 37 ± 6%, $n = 3$) during the endothelin-induced inward current. In contrast, the amplitude of the I_{Ba} was increased (mean, 67 ± 5%, $n = 3$) at the peak of the outward current. These results indicate that both rapidly and slowly decaying components of the calcium channel currents were depressed and facilitated by endothelin.

Discussion

The results of the present study indicate that endothelin causes membrane depolarization associated with a decrease in membrane input resistance followed by membrane hyperpo-

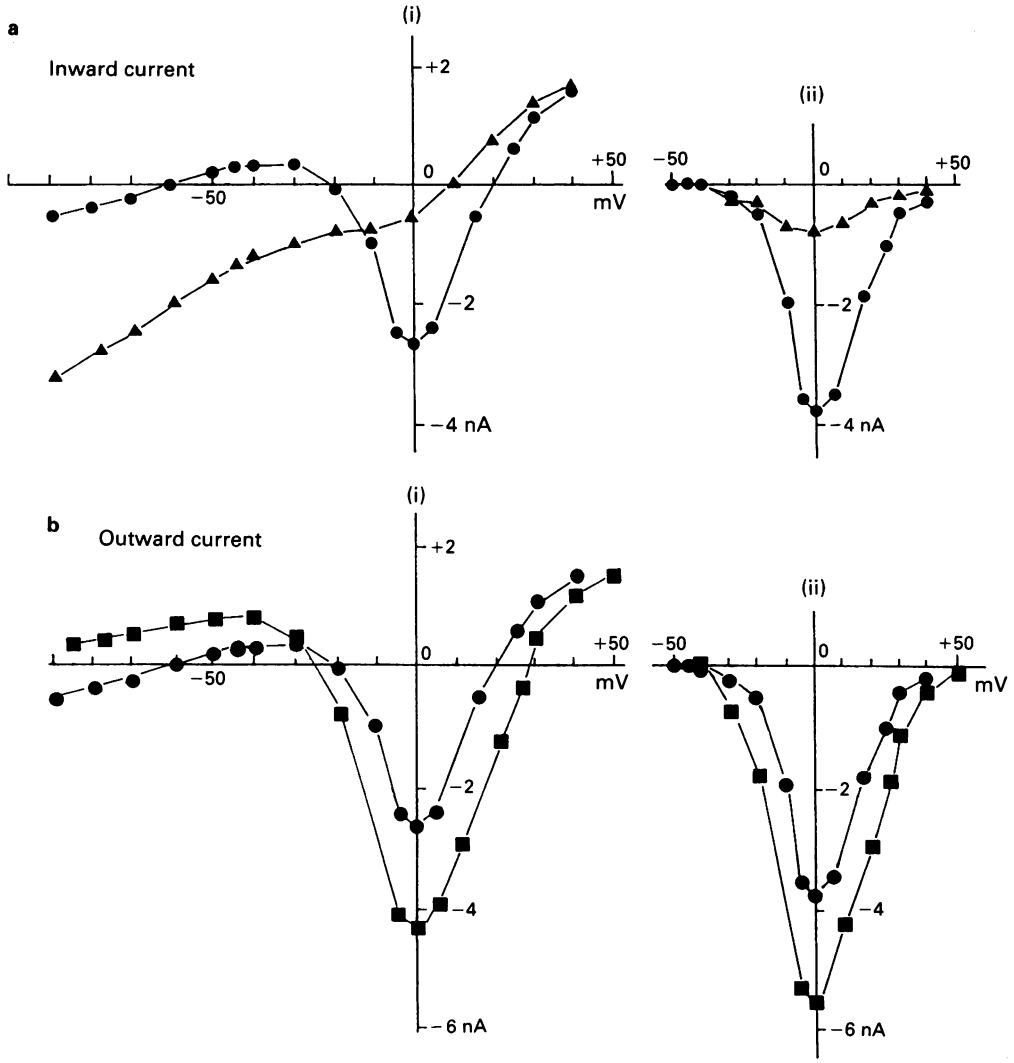

Figure 8 Current-voltage relation (I–V curve) for I_{Ba} obtained from a caesium-loaded neurone during inward current (a) and outward current (b) produced by endothelin (100 nM). I–V curves were obtained by step commands (duration of 200 ms) to potentials between −120 and +50 mV from a holding potential of −60 mV. Perfusate contained tetrodotoxin (300 nM), tetraethylammonium (50 mM) and caesium (2 mM). Graphs (ii) show net I_{Ba} obtained by subtraction of leak currents. Leak currents were obtained in barium-free solution containing 1 mM cobalt. Control I–V curve obtained before application of endothelin (100 nM) (●); I–V curves obtained during endothelin-induced inward (▲) and outward (■) currents, respectively. Data were obtained from one neurone.

larization associated with an increase in membrane input resistance of neurones of rabbit vesicle pelvic ganglia. The membrane effects of endothelin were concentration-dependent. It is likely that the effects of endothelin were due to a direct action on the postsynaptic neurone and did not involve cholinoceptors, 5-HT receptors (5-HT_3 subtypes) or adrenoceptors. Both the magnitude and time course of the depolarization and hyperpolarization and the associated changes in membrane input resistance were not altered after blockade of these three types of receptor. Furthermore, a component of the endothelin-induced inward current which was coincident with the endothelin-induced depolarization was observed after blocking presynaptic release of cholinergic transmitter (modified Krebs solution containing nominally zero calcium and high magnesium; Nishimura *et al.*, 1988a).

It is possible, however that endothelin may also release neuropeptides (i.e. substance P, vasoactive intestinal polypeptide (VIP) and enkephalin) from presynaptic sites. For example in rat spinal cord the effect of endothelin was considered to be mediated by substance P (Yoshizawa *et al.*, 1989a). A prejunctional release of substance P and/or VIP by endothelin is, however, unlikely for neurones in vesical parasympathetic ganglia as in these neurones the administration of substance P (unpublished observations) and VIP (Akasu *et al.*, 1986) caused membrane depolarization associated with an increase in membrane input resistance. In contrast, endothelin-induced depolarization was associated with a decrease in membrane input resistance. Also, for neurones in parasympathetic colonic ganglia the administration of δ, μ and κ opioid receptor agonists did not cause membrane depolarization and changes in membrane input resistance (Kennedy & Krier, 1987).

Endothelin caused an initial inward followed by an outward current in caesium-loaded neurones superfused with modified Krebs solution containing TTX (300 nM) and TEA (50 mM). The inward current was blocked by approximately 72% by removal of calcium from the perfusate, while it was not altered by lowering external sodium ions or by increasing extracellular potassium ions. These results suggest that endothelin-induced inward current is primarily carried by calcium ions. Furthermore, the reversal potential of the calcium-insensitive component of the inward current obtained by I–V curves was near the equilibrium potential for chloride ions (Akasu *et al.*, 1990). A chloride channel blocker, SITS, completely eliminated the calcium-insensitive component of the inward current produced by endothelin. It is presumed that the endothelin-induced inward current also involves activation of chloride current.

The results of the present study show that endothelin also modulates both rapidly and slowly decaying components of

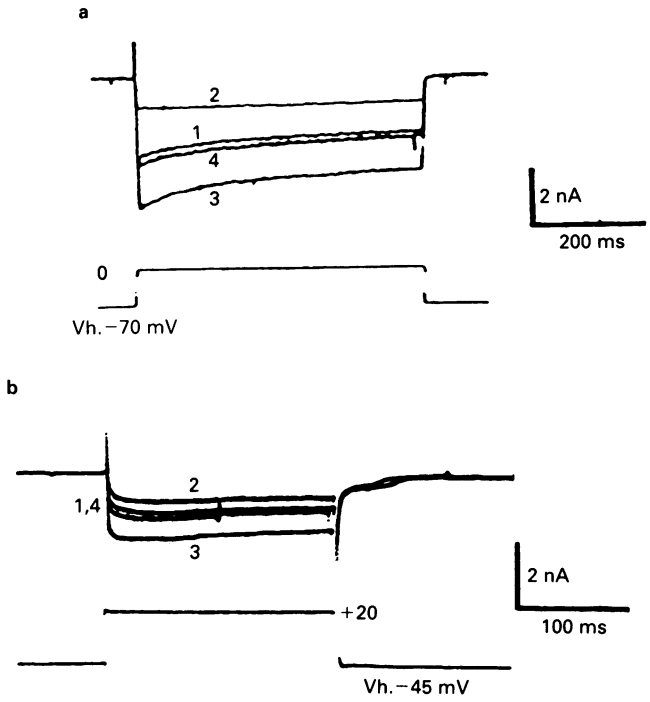

Figure 9 Effect of endothelin (100 nM) on the high-threshold calcium currents, sum of N- and L-type (a) and isolated L-type (b) I_{Ba}, obtained from caesium-loaded neurone in the presence of tetrodotoxin (300 nM) and tetraethylammonium (50 mM). In (a) N- and L-type currents were evoked by step commands to 0 mV from a holding potential of −70 mV. In (b) the L-type current was evoked by step commands to +20 mV from a holding potential of −45 mV. Duration of step commands were 500 ms and 200 ms for (a) and (b), respectively. Leak currents were determined by hyperpolarizing step commands (see Methods). Records (1) in (a) and (b) were obtained before application of endothelin (100 nM). Records (2) and (3) were taken during the inward and outward currents produced by endothelin, respectively. Records (4) were taken 20 min after withdrawal of endothelin. Data in (a) and (b) were obtained from different cells.

voltage-dependent calcium channel currents. These two components were comparable to N- and L-type calcium currents (Nowycky *et al.*, 1985; Fox *et al.*, 1987; Akasu *et al.*, 1990). Endothelin depressed the peak of the I_{Ba} evoked at the beginning of the depolarizing command pulses and to a lesser extent depressed the I_{Ba} measured at the end of voltage command pulse with duration of 500 ms.

In mammalian parasympathetic neurones, the depression of the voltage-dependent calcium channel current by endothelin may be related to elevation of intracellular calcium. In the present study, endothelin activates receptor-operated calcium channels and this may increase the intracellular concentration of calcium (or barium). Alternatively, endothelin may increase intracellular calcium by mobilizing the release of calcium from intracellular stores via the formation of inositol triphosphate; an action reported for glioma cells (Zhang *et al.*, 1990) and anterior pituitary cells (Stojiković *et al.*, 1990). Increased intracellular calcium may cause suppression of the voltage-dependent calcium channels through a 'calcium-induced calcium block' (Brehm & Eckert, 1978; Tillotson, 1979; Lee *et al.*, 1985). In addition the influx of barium ions also leads to an elevation of intracellular calcium due to activation of a calcium-induced calcium release mechanism (Scott *et al.*, 1988).

Endothelin also produced a long-lasting facilitation of voltage-dependent high threshold calcium channel currents (N- and L-type) in neurones of vesical pelvic ganglia. This effect was consistently observed when endothelin was removed from the external Krebs solution. A similar augmentation of dihydropyridine-sensitive L-type calcium channel current by endothelin has been reported for porcine coronary artery (Goto *et al.*, 1989; Silberberg *et al.*, 1989). In the hepatic portal vein, endothelin augments both T- and L-type calcium currents (Inoue *et al.*, 1990). Thus, vesical pelvic neurones represent another site where endothelin facilitates the voltage-dependent calcium channel current.

The mechanism for the endothelin-induced facilitation of the voltage-dependent calcium channel current is not known for either smooth muscle cells or for peripheral autonomic neurones. It is likely that the facilitating action of endothelin in the present study is due to an indirect action involving second messengers in view of the long period required to observe facilitation of the calcium channel current.

In summary, the present data show that endothelin causes membrane depolarization and hyperpolarization of mammalian autonomic neurones. These actions are associated with alterations in receptor-operated calcium and chloride currents and also the voltage-gated calcium channel current. If endothelin is released from urinary bladder vascular endothelial cells, it may modulate the calcium-dependent release of neurotransmitters in autonomic ganglia (Miller, 1987).

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Subclassification of the presynaptic α_2-autoreceptors in rabbit brain cortex

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1 α_2-Adrenoceptor binding sites have been subclassified into α_{2A} sites of which a main characteristic is very low affinity for prazosin, and α_{2B} sites with relatively high affinity for prazosin. The presynaptic α_2-autoreceptors in rabbit brain cortex were studied in order to classify them in terms of α_{2A} and α_{2B}. Release of [^{3}H]-noradrenaline in cortical slices was elicited by trains of 4 pulses delivered at 100 Hz.

2 Clonidine caused concentration-dependent inhibition of the stimulation-evoked overflow of tritium, with an EC_{50} of 7.5 nM and a maximal inhibition by 96%.

3 The following α-adrenoceptor antagonists shifted the concentration-response curve of clonidine to the right (antagonist-receptor dissociation constants K_D in brackets): yohimbine (14 nM), 2-[2H-(1-methyl-1,3-dihydroisoindole)methyl]-4,5-dihydroimidazole (BRL 44408; 15 nM) and 1,2-dimethyl-2,3,9,13b-tetrahydro-1H-dibenzo[c,f]imidazo[1,5-a]azepine (BRL 41992; 630 nM). Prazosin 1 μM and 2-[2-[4-(o-methoxyphenyl)piperazine-1-yl]-ethyl]-4,4-dimethyl-1,3(2H,4H)-isoquinolinedione (AR-C 239) 1 μM failed to antagonize the effect of clonidine. Higher concentrations of prazosin and AR-C 239 greatly accelerated the basal efflux of tritium.

4 The method used permits the functional determination of antagonist affinities undistorted by endogenous α_2-autoinhibition. A comparison with affinities derived from radioligand binding experiments indicates that the presynaptic α_2-autoreceptors in rabbit brain cortex are markedly different from the α_{2B}-subtype and probably belong to the prazosin-insensitive α_{2A}-subtype.

Keywords: Rabbit brain cortex; noradrenaline release; presynaptic α_2-autoreceptors; α_{2A}-adrenoceptors; α_{2B}-adrenoceptors; clonidine; yohimbine; prazosin

Introduction

Presynaptic α-autoreceptors of noradrenaline-releasing neurones were the prototype of α_2-adrenoceptors. It was within this group that non-homogeneity of α_2-adrenoceptors was first noticed (Doxey & Everitt, 1977; Dubocovich, 1979). Later subclassifications were mainly based on radioligand binding experiments (reviewed by Docherty, 1989; McGrath *et al.*, 1989). Extensive evidence now indicates that the α_2-selective (Starke *et al.*, 1975; Weitzell *et al.*, 1979) ligands [^{3}H]-yohimbine and [^{3}H]-rauwolscine label at least two α_2 sites which differ, above all, in their affinity for prazosin. The prazosin-insensitive site was called α_{2A} (K_i of prazosin about 300 nM). The relatively prazosin-sensitive site was called α_{2B} (K_i of prazosin about 5 nM; Bylund, 1985; 1988; Nahorski *et al.*, 1985; Bylund *et al.*, 1988). The existence of further α_2 binding sites (see Discussion) is less well established.

It has been difficult to correlate the α_{2A} and α_{2B} binding sites with functioning receptors (see Docherty, 1989; McGrath *et al.*, 1989). From data in the literature, Bylund *et al.* (1988) suggested that 'it is possible that the *alpha*-2 receptors on noradrenergic axon terminals are of the B subtype'. This identification, however, is questionable. The presynaptic α_2-autoreceptors of rat vas deferens, suggested to be α_{2B} by Bylund *et al.* (1988), are classically prazosin-insensitive, with functionally measured K_D values of 1 μM or higher (Dubocovich, 1979; Doxey & Roach, 1980; Table 19 of Docherty, 1989); a recent functional study in fact indicates that the rat vas deferens autoreceptors are α_{2A} (Connaughton & Docherty, 1990). The same prazosin-insensitivity holds true for the presynaptic α_2-autoreceptors of rat brain cortex (Dubocovich, 1979; Hedler *et al.*, 1981; Frankhuyzen & Mulder, 1982; the latter study on rat hippocampus), also suggested to be α_{2B} (Bylund *et al.*, 1988; see Gobbi *et al.*, 1990, for critical discussion of the suggestion); again a recent functional study classified the receptors as α_{2A} (Gobbi *et al.*, 1990).

One functionally observed heterogeneity of α_2-adrenoceptors is a difference between presynaptic α_2-autoreceptors in the rat and the rabbit (Starke, 1981; Reichenbacher *et al.*, 1982; Ennis, 1985; Lattimer & Rhodes, 1985; Alabaster *et al.*, 1986; Limberger *et al.*, 1989). Are the α_2-autoreceptors of rabbit brain cortex α_{2B}, in contrast to the α_{2A}-autoreceptors of rat brain? We studied the question in brain slices, using brief trains of electric pulses to release noradrenaline, a method that avoids the development of autoinhibition which otherwise may falsify autoreceptor characterization (Limberger *et al.*, 1989; for review see Starke, 1987; Singer, 1988). With clonidine as agonist we determined the antagonist-autoreceptor dissociation constants K_D of five antagonists: yohimbine (non-selective in terms of α_{2A} and α_{2B}), prazosin, 2-[2-[4-(*o*-methoxyphenyl) piperazine-1-yl]-ethyl]-4,4-dimethyl-1,3(2H,4H)- isoquinolinedione (AR-C 239; α_{2B}-selective; Bylund *et al.*, 1988), 1,2-dimethyl-2,3,9, 13b-tetrahydro-1H-dibenzo[c,f]imidazo[1,5-a]azepine (BRL 41992; α_{2B}-selective; Young *et al.*, 1989) and 2-[2H-(1-methyl-1,3-dihydroisoindole)methyl]-4,5-dihydroimidazole (BRL 44408; α_{2A}-selective; Young *et al.*, 1989).

Methods

The experiments were carried out as described previously (Limberger *et al.*, 1989), with the differences mentioned below. Briefly, slices of the rabbit occipito-parietal cortex were preincubated with (−)-[^{3}H]-noradrenaline 0.1 μM for 30 min, then superfused with [^{3}H]-noradrenaline-free medium containing desipramine 1 μM, one slice per superfusion chamber, and stimulated by a train of 4 pulses, delivered at a frequency of 100 Hz, after 60, 100, 140 and 180 min of superfusion (S_1–S_4). Desipramine was always added throughout superfusion in order to block the re-uptake of [^{3}H]-noradrenaline and thus increase the electrically evoked overflow of tritium. α-Adrenoceptor antagonists, when used, were also present throughout superfusion at a fixed concentration. Cumulative

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concentration-response curves of clonidine were determined by addition of increasing concentrations 20 min before S_2, S_3 and S_4. The superfusate was collected in 5 min periods. The outflow of tritium was expressed as fractional rate (min^{-1}). The overflow elicited by electrical stimulation was expressed as a percentage of the tritium content of the tissue at the time of stimulation. For further evaluation of basal tritium efflux, ratios were calculated of the fractional rate of outflow in the collection periods immediately before S_2, S_3 and S_4, and the outflow in the collection period immediately before S_1 (b_2/b_1, b_3/b_1 and b_4/b_1). For further evaluation of the electrically evoked overflow, ratios were calculated of the overflow elicited by S_2, S_3 and S_4, and the overflow elicited by S_1(S_2/S_1, S_3/S_1 and S_4/S_1).

K_D values were determined as follows. Six slices were used in a single experiment. One or two slices were superfused with medium containing neither clonidine nor an antagonist (controls), or containing an antagonist only (at the highest concentration used). One or two other slices received only clonidine at three increasing concentrations before S_2, S_3 and S_4. The remaining slices were exposed to the antagonist as well as to clonidine. The percentage inhibition caused by clonidine in each single brain slice was calculated, taking the respective average control (no clonidine, no antagonist) S_2/S_1, S_3/S_1 and S_4/S_1 ratio (line 1 of Table 2 below) as reference value. [One might object that the appropriate reference S_n/S_1 ratio in the presence of an antagonist would have to be obtained in experiments with the antagonist alone, given at the same concentration. This, however, was not considered necessary, because the S_n/S_1 ratios in controls (no clonidine, no antagonist) were very close to those obtained in the presence of the highest concentration of each antagonist used (no clonidine) and were all close to unity (see Table 2 below).] In contrast to our previous study, agonist concentrations causing less than 10 or more than 90% inhibition were included.

The K_D value of yohimbine was calculated as described by Waud (1975). Sigmoid curves were simultaneously fitted to all percentage inhibition values obtained in clonidine/yohimbine experiments (i.e., to all values on which Figure 1 below is based), using equation 52 of Waud (1975) which yields the EC_{50} of clonidine, its maximal effect, the K_D of yohimbine and a parameter Q corresponding to the slope of the Arunlakshana-Schild plot (Arunlakshana & Schild, 1959). Since Q was close to unity for yohimbine (0.87), it was constrained to 1 for the final calculation of K_D. The K_D of BRL 44408 was calculated in the same manner (i.e., sigmoid curves were fitted simultaneously to all percentage inhibition values on which Figure 2 below is based; Q was 0.96). Prazosin, AR-C 239 and BRL 41992 caused no or only a small shift of the clonidine concentration-response curve, and in these cases the curve-fitting calculations did not converge. Therefore, one sigmoid curve was fitted to the percentage inhibition values for clonidine alone and, separately, one curve to the values obtained in the presence of each antagonist concentration (for instance, one curve was fitted to the 'clonidine alone' data and another curve was fitted separately to the 'clonidine in the presence of BRL 41992' data of Figure 3).

Results are expressed as arithmetic means ± s.e.mean except when derived from sigmoid curve fitting (EC_{50} value and maximal effect of clonidine and antagonist K_D values). Statistical comparisons of group means were done with the Mann-Whitney test if Kruskal-Wallis analysis indicated a significant difference. In the case of multiple comparisons, the significance levels to be exceeded were adjusted according to Bonferroni (Wallenstein et al., 1980). In some cases, two concentration-response curves obtained by sigmoid curve fitting were tested for a significant difference using the procedure of Motulsky & Ransnas (1987; pp. 371–372). n is the number of brain slices.

(−)-[ring-2,5,6-^{3}H]-noradrenaline, specific activity 41 Ci $mmol^{-1}$, was purchased from Du Pont (Dreieich, FRG). Yohimbine HCl was purchased from Roth (Karlsruhe, FRG). Clonidine HCl was a gift from Boehringer (Ingelheim, FRG), desipramine HCl from Ciba-Geigy (Basel, Switzerland), prazosin HCl from Pfizer (Karlsruhe, FRG), BRL 44408 and BRL 41992 from Dr M.A. Cawthorne, SmithKline Beecham (Great Burgh, Epsom, Surrey), and AR-C 239 HCl from Dr J.C.A. van Meel, Thomae (Biberach, FRG). Desipramine, clonidine, yohimbine, prazosin and AR-C 239 were dissolved in water. BRL 44408 and BRL 41992 were dissolved in 10 mM HCl. Control slices were always run with the respective solvent.

Results

The time course of the outflow of tritiated compounds has been shown previously (Mayer et al., 1988; Limberger et al., 1989). The basal outflow of tritium, expressed as fractional rate in the collection period before S_1 (b_1), was not greatly changed when α-adrenoceptor antagonists were present throughout superfusion, except for a 2.6 fold increase by prazosin 1 μM and a 1.7 fold increase by AR-C 239 1 μM (Table 1). Yohimbine and AR-C 239 did not change the overflow of tritium elicited by S_1, whereas prazosin caused a 30% increase and both BRL substances caused a decrease (Table 1).

Table 1 Basal outflow and evoked overflow of tritium from slices of rabbit brain cortex

Drug present throughout superfusion (μM)	b_1 (min^{-1})	S_1 (% of tissue tritium)	n
Desipramine 1	0.00114 ± 0.00004	0.622 ± 0.030	53
Desipramine 1 + yohimbine 0.03	0.00097 ± 0.00007	0.546 ± 0.038	16
Desipramine 1 + yohimbine 0.1	0.00085 ± 0.00003**	0.559 ± 0.032	14
Desipramine 1 + yohimbine 0.3	0.00094 ± 0.00006	0.585 ± 0.049	15
Desipramine 1 + yohimbine 1	0.00114 ± 0.00007	0.653 ± 0.040	22
Desipramine 1 + BRL 44408 0.03	0.00122 ± 0.00005	0.416 ± 0.048*	12
Desipramine 1 + BRL 44408 0.1	0.00135 ± 0.00007	0.371 ± 0.031**	15
Desipramine 1 + BRL 44408 0.3	0.00125 ± 0.00003	0.353 ± 0.026**	15
Desipramine 1 + BRL 44408 1	0.00118 ± 0.00004	0.334 ± 0.045**	22
Desipramine 1 + BRL 41992 1	0.00154 ± 0.00008**	0.384 ± 0.022**	21
Desipramine 1 + prazosin 1	0.00296 ± 0.00013**	0.807 ± 0.045*	25
Desipramine 1 + AR-C 239 0.1	0.00125 ± 0.00006	0.548 ± 0.033	8
Desipramine 1 + AR-C 239 1	0.00191 ± 0.00008**	0.514 ± 0.030	26

Slices of rabbit brain cortex were preincubated with [^{3}H]-noradrenaline and then superfused with medium containing the drugs indicated. b_1 represents the outflow of tritium in the collection period immediately before the first period of electrical stimulation (55–60 min of superfusion) and is expressed as fractional rate (min^{-1}); the outflow in absolute terms, in slices superfused with desipramine only, was 0.86 ± 0.03 nCi per 5 min. S_1 represents the overflow of tritium elicited by the first period of electrical stimulation (4 pulses/100 Hz) and is expressed as a percentage of the tritium content of the tissue; the overflow in absolute terms, in slices superfused with desipramine only, was 0.88 ± 0.05 nCi. Means ± s.e.mean of n brain slices. Significant differences from desipramine only: * $P < 0.05$; ** $P < 0.01$.

Table 2 Stability of baseline conditions: S_n/S_1 ratios in the absence of clonidine

Drug present throughout superfusion (μM)	*Stimulation-evoked overflow of tritium* S_2/S_1	S_3/S_1	S_4/S_1	n
Desipramine 1	1.09 ± 0.02	1.13 ± 0.02	1.16 ± 0.03	27
Desipramine 1 + yohimbine 1	1.10 ± 0.07	1.10 ± 0.07	1.12 ± 0.08	7
Desipramine 1 + BRL 44408 1	1.07 ± 0.04	1.08 ± 0.05	1.06 ± 0.04	9
Desipramine 1 + BRL 41992 1	1.10 ± 0.06	1.11 ± 0.06	1.04 ± 0.05	9
Desipramine 1 + prazosin 1	0.95 ± 0.08	1.07 ± 0.04	1.19 ± 0.05	14
Desipramine 1 + AR-C 239 1	1.11 ± 0.02	1.10 ± 0.04	1.14 ± 0.04	8

Slices of rabbit brain cortex were preincubated with [^{3}H]-noradrenaline and then superfused with medium containing the drugs indicated. They were stimulated four times with 4 pulses/100 Hz (S_1–S_4). S_n/S_1 values represent the ratios of the overflow elicited by S_2, S_3 and S_4, and the overflow elicited by S_1. Means ± s.e.mean of *n* brain slices. There were no significant differences between S_n/S_1 ratios.

The electrically evoked overflow of tritium was reproducible in the absence of clonidine, independently of whether the superfusion medium contained only desipramine 1 μM or desipramine combined with the highest concentrations of the α-adrenoceptor antagonists tested (Table 2).

Clonidine, when added before S_2, S_3 and S_4 at increasing concentrations, reduced the evoked overflow of tritium. Concentration-response curves are shown in Figures 1–5. The EC_{50} in the absence of antagonists was 7.5 nM, and the maximal inhibition 96%.

Both yohimbine (Figure 1) and BRL 44408 (Figure 2), applied at concentrations of 30 nM to 1 μM, shifted the clonidine concentration-response curve increasingly to the right. Sigmoid curve fitting yielded K_D values of 14.4 ± 1.2 nM ($pA_2 = 7.84$) and 15.1 ± 1.5 nM ($pA_2 = 7.82$) for yohimbine and BRL 44408, respectively.

The three other antagonists were very weak. BRL 41992 1 μM caused only a small shift to the right (Figure 3), corresponding to a K_D value of 630 nM ($pA_2 = 6.2$) when calculated from equation 4 of Furchgott (1972). No shift at all was

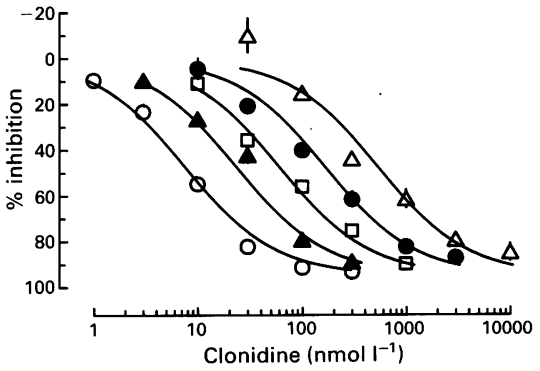

Figure 1 Interaction between clonidine and yohimbine on the evoked overflow of tritium. Brain slices were stimulated four times with 4 pulses/100 Hz (S_1–S_4). Yohimbine, when used, was present throughout superfusion at a fixed concentration. Clonidine was added before S_2, S_3 and S_4 at increasing concentrations. Abscissa scale, concentration of clonidine. Ordinate scale, percentage inhibition caused by clonidine, calculated from S_n/S_1 overflow ratios. Clonidine alone (○); in the presence of yohimbine 30 nM (▲); 100 nM (□); 300 nM (●) and 1 μM (△). Means of 3–11 (clonidine alone 7–17) brain slices; vertical lines show s.e.mean. pA_2 of yohimbine: 7.84.

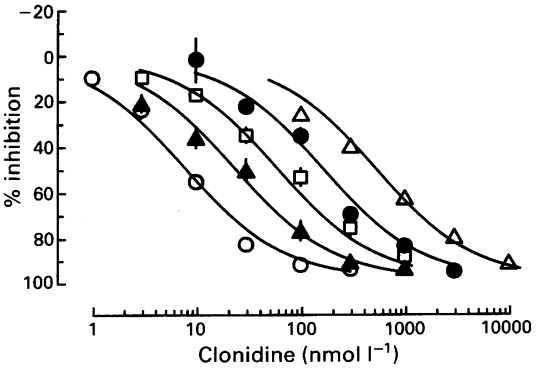

Figure 2 Interaction between clonidine and BRL 44408 on the evoked overflow of tritium. Brain slices were stimulated four times with 4 pulses/100 Hz (S_1–S_4). BRL 44408, when used, was present throughout superfusion at a fixed concentration. Clonidine was added before S_2, S_3 and S_4 at increasing concentrations. Abscissa scale, concentration of clonidine. Ordinate scale, percentage inhibition caused by clonidine, calculated from S_n/S_1 overflow ratios. Clonidine alone (○); in the presence of BRL 44408 30 nM (▲); 100 nM (□); 300 nM (●) and 1 μM (△). Means of 4–10 (clonidine alone 7–17) brain slices; vertical lines show s.e.mean. pA_2 of BRL 44408: 7.82.

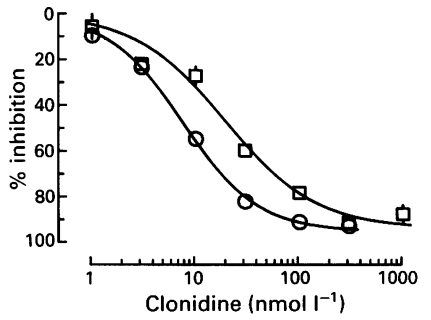

Figure 3 Interaction between clonidine and BRL 41992 on the evoked overflow of tritium. Brain slices were stimulated four times with 4 pulses/100 Hz (S_1–S_4). BRL 41992, when used, was present throughout superfusion at a fixed concentration. Clonidine was added before S_2, S_3 and S_4 at increasing concentrations. Abscissa scale, concentration of clonidine. Ordinate scale, percentage inhibition caused by clonidine, calculated from S_n/S_1 overflow ratios. Clonidine alone (○); in the presence of BRL 41992 1 μM (□). Means of 3–10 (clonidine alone 7–17) brain slices; vertical lines show s.e.mean. pA_2 for BRL 41992: 6.2.

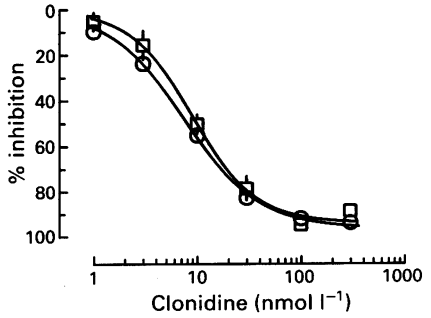

Figure 4 Interaction between clonidine and prazosin on the evoked overflow of tritium. Brain slices were stimulated four times with 4 pulses/100 Hz (S_1–S_4). Prazosin, when used, was present throughout superfusion at a fixed concentration. Clonidine was added before S_2, S_3 and S_4 at increasing concentrations. Abscissa scale, concentration of clonidine. Ordinate scale, percentage inhibition caused by clonidine, calculated from S_n/S_1 overflow ratios. Clonidine alone (○); in the presence of prazosin 1 μM (□). Means of 3–8 (clonidine alone 7–17) brain slices; vertical lines show s.e.mean. pA_2 for prazosin: < 6.

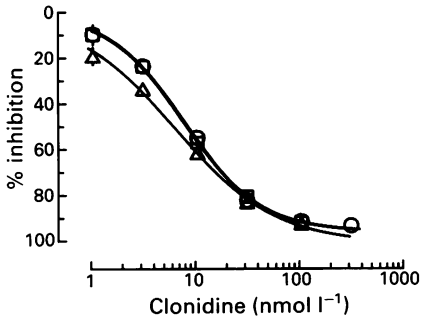

Figure 5 Interaction between clonidine and AR-C 239 on the evoked overflow of tritium. Brain slices were stimulated four times with 4 pulses/100 Hz (S_1–S_4). AR-C 239, when used, was present throughout superfusion at a fixed concentration. Clonidine was added before S_2, S_3 and S_4 at increasing concentrations. Abscissa scale, concentration of clonidine. Ordinate scale, percent inhibition caused by clonidine, calculated from S_n/S_1 overflow ratios. Clonidine alone (○); in the presence of AR-C 239 100 nM (△); 1 μM (□). (○) and (□) groups are hard to distinguish because values were almost identical. Means of 4–15 (clonidine alone 7–17) brain slices; vertical lines show s.e.mean. pA_2 for AR-C 239: <6.

detectable in the presence of prazosin 1 μM (Figure 4) and AR-C 239 0.1 and 1 μM (Figure 5).

An evaluation of b_n/b_1 ratios indicated that the basal outflow of tritium was not changed when clonidine was added before S_2, S_3 and S_4 (not shown).

Discussion

The pulse trains used in the present experiments are too short (30 ms train duration) for the development of autoinhibition and, hence, permit the determination of undistorted agonist EC_{50} and antagonist K_D (pA_2) values (see Starke, 1987; Singer, 1988). The lack of any release-enhancing effect of yohimbine in the present study (Table 1) confirms the absence of α_2-adrenoceptor-mediated autoinhibition.

Prazosin, in contrast to yohimbine, increased the evoked overflow of tritium (Table 1), an increase that has been observed previously in rabbit brain cortex slices (Reichenbacher et al., 1982). Since there was no autoinhibition, the effect of prazosin was not due to antagonism at autoreceptors. The observation emphasizes that caution is necessary when one interprets effects of prazosin on the release of noradrenaline. Prazosin possesses reserpine-like properties (Commarato et al., 1978; Hedler et al., 1981) which sometimes become manifest at low concentrations (10 nM; Bradley & Doggrell, 1983). A reserpine-like mechanism probably also underlies the acceleration of basal tritium efflux observed in the present experiments. It may be due to this property that prazosin enhances the electrically evoked release of noradrenaline (Cubeddu & Weiner, 1975), rather than to any blockade of prazosin-sensitive α_{2B}- (or α_1-) adrenoceptors. We do not know why BRL 44408 and BRL 41992 reduced the evoked overflow of tritium. One possibility is a partial agonist action at α_2-autoreceptors as has been observed for the presumed α-adrenoceptor antagonists phentolamine and idazoxan in several tissues (Limberger & Starke, 1983; Angus & Lew, 1984; Ennis & Lattimer, 1984) including rabbit brain cortex (Heepe & Starke, 1985).

Besides freedom from autoinhibition, our experimental model fulfils other prerequisites for the determination of K_D values (Limberger et al., 1989): the baseline conditions are stable (see also Table 2) and the clonidine exposure time suffices for steady-state. Since the antagonism by yohimbine and BRL 44408 was surmountable (Figures 1 and 2) and the Q parameter (Waud, 1975), which corresponds to the slope of the Schild plot, close to unity, our K_D values of these two drugs are correct estimates of the antagonist-autoreceptor dissociation constant. The K_D for BRL 41992, of which only one effective concentration could be tested, is less reliable. Pronounced increases in the basal efflux of tritium made higher concentrations of prazosin, AR-C 239 and BRL 41992 unsuitable for autoreceptor characterization.

In this and our previous study (Limberger et al., 1989) we have measured the following antagonist K_D values at rabbit brain cortex α_2-autoreceptors, with clonidine as agonist (in nM): rauwolscine 5, yohimbine 11 (mean of previous and present study), BRL 44408 15, idazoxan 51, BRL 41992 630, prazosin = AR-C 239 > 1,000. This order of affinities agrees with the order found at α_{2A} but not at α_{2B} binding sites (Bylund et al., 1988; for the BRL compounds, Young et al., 1989). For instance, at the autoreceptors, as at α_{2A} binding sites, prazosin and AR-C 239 possess much lower affinity than yohimbine and idazoxan, whereas at α_{2B} sites the affinity of prazosin is only 5 times lower than that of yohimbine and as high as that of idazoxan, and the affinity of AR-C 239 even surpasses that of idazoxan (Bylund et al., 1988). Furthermore, at the autoreceptors the affinity ratio BRL 44408/BRL 41992 is 42 and at α_{2A} binding sites it is 61, whereas at α_{2B} sites it is 0.008 (Young et al., 1989). Clearly, in terms of the proposed $\alpha_{2A/B}$ dichotomy, the presynaptic autoreceptors of rabbit brain cortex are α_{2A}. In this they agree with the α_{2A}-autoreceptors of rat brain cortex (Gobbi et al., 1990), despite species differences between rabbit and rat α_2-autoreceptors which are shown by other antagonists (see Introduction for references).

Additional α_2 binding sites have been suggested recently. For instance, in the rat submaxillary gland, [^{3}H]-rauwolscine labelled a site distinct from α_{2A} and α_{2B} (Michel et al., 1989). However, rauwolscine had 3 times less affinity for this site than idazoxan, whereas for the rabbit brain cortex autoreceptors it had a 10 times higher affinity than idazoxan. Both the 'α_{2C}' site in an opossum kidney-derived cell line (Bylund, 1988) and the human 'α_2C2' site recently cloned (Lomasney et al., 1990) are too sensitive to prazosin to correspond to the autoreceptors. The most likely binding equivalent of the autoreceptors is, in fact, the α_{2A} site.

There were several reasons for choosing clonidine as the agonist. First, in previous experiments we found no difference in the K_D values of antagonists when tested against clonidine, noradrenaline and α-methylnoradrenaline (Limberger et al., 1989). Secondly, noradrenaline and other catecholamines displace [^{3}H]-noradrenaline from the tissue despite the presence of desipramine, when high concentrations have to be tested against antagonists. Thirdly, clonidine has about equal α_{2A} and α_{2B} binding affinity or is slightly α_{2B}-selective (see Bylund et al., 1988; Michel et al., 1989) whereas 5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline (UK 14304), which is more selective than clonidine for α_2-receptors in general (as opposed to α_1), prefers the α_{2A} subtype. We were particularly interested in finding an α_{2B}-adrenoceptor.

There may be presynaptic α_{2B}-autoreceptors at the noradrenaline-releasing neurones of other tissues such as rat submandibular gland (see Bylund, 1988) and rat atria (Connaughton & Docherty, 1990). It should be noted, however, that rat atria were previously postulated to possess a mixture of presynaptic α_2- and α_1-autoreceptors (see Docherty, 1989), and the relationship between the two suggestions ($\alpha_2 + \alpha_1$ versus α_{2B}) has not been clarified.

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Haemodynamic effects of human α-calcitonin gene-related peptide following administration of endothelin-1 or N^G-nitro-L-arginine methyl ester in conscious rats

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1 We investigated the peripheral haemodynamic effects of human α-calcitonin gene-related peptide (CGRP) following administration of endothelin-1 or N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide production, in conscious, chronically-instrumented, Long Evans rats.

2 Infusion of endothelin-1 (3 nmol $kg^{-1} h^{-1}$) caused hypertension, bradycardia and renal, mesenteric and hindquarters vasoconstrictions. Co-infusion of human α-CGRP (1.5 nmol $kg^{-1} h^{-1}$) reduced the hypertension and abolished the hindquarters vasoconstriction caused by endothelin-1 but the renal and mesenteric vasoconstrictor actions of endothelin-1 were not affected.

3 Infusion of human α-CGRP (15 nmol $kg^{-1} h^{-1}$) in the presence of endothelin-1 caused hypotension and hyperaemic vasodilatation in the hindquarters; the mesenteric vasoconstrictor effects of endothelin-1 were diminished, but there was only a transient reversal of the renal vasoconstrictor effects of endothelin-1.

4 Pretreatment with the non-peptide angiotensin II receptor antagonist, DuP 753 (10 mg kg^{-1}), caused slight hypotension associated with renal, mesenteric and hindquarters vasodilatations, but DuP 753 did not affect responses to endothelin-1 infusion. However, under these conditions co-infusion of human α-CGRP (15 nmol $kg^{-1} h^{-1}$) caused a sustained reversal of the renal vasoconstrictor effects of endothelin-1.

5 These results indicate that the failure of human α-CGRP to cause sustained reversal of the renal vasoconstrictor effects of endothelin-1 in the absence of DuP 753 was due to activation of the renin-angiotensin system (possibly as a consequence of the hypotension).

6 In the second experiment, L-NAME (10 mg kg^{-1}) caused renal, mesenteric and hindquarters vasoconstrictions similar to those seen in the presence of endothelin-1. However, the renal vasoconstrictor effects of L-NAME were reversed completely by human α-CGRP (15 nmol $kg^{-1} h^{-1}$), even though the latter caused hypotension comparable to that seen in the presence of endothelin-1. These results are consistent with a lack of functional activation of the renin-angiotensin system by human α-CGRP in the presence of L-NAME.

7 The vasoconstrictor effects of L-NAME on the hindquarters were completely reversed by infusion of human α-CGRP, but hindquarters flow and vascular conductance did not rise above baseline levels. Hence these results indicate the hindquarters hyperaemic vasodilator effects of human α-CGRP seen in the presence of endothelin-1 were contributed to by nitric oxide-mediated mechanisms.

Keywords: Human α-CGRP; endothelin-1; N^G-nitro-L-arginine methyl ester (L-NAME); peripheral blood flow

Introduction

In a recent study (Gardiner *et al.*, 1990d) it was found that infusion of endothelin-1 caused marked constriction of the internal carotid vascular bed in conscious rats, and that this effect was antagonized by co-infusion of nimodipine or human α-calcitonin gene-related peptide (CGRP). In the case of human α-CGRP it was suggested that the antagonism of the vasoconstrictor effects of endothelin-1 might show regional variations, since the internal carotid vasoconstriction was reversed at a time when systemic arterial blood pressure was still elevated, indicating persistent vasoconstriction in other vascular beds. Thus, the first aim of the present study was to investigate the functional antagonism between the vasoconstrictor effects of endothelin-1 and the influence of human α-CGRP in the renal, mesenteric and hindquarters vascular beds.

The results indicated that the hindquarters vasoconstrictor response to endothelin-1 was readily reversed by co-infusion of human α-CGRP, whereas the renal vasoconstrictor effects of endothelin-1 were resistant to reversal by human α-CGRP (see Results). Previously we (Bennett *et al.*, 1989; Gardiner *et al.*, 1990a) had found that the renal vasodilator effects of rat α-CGRP were enhanced by the angiotensin converting enzyme inhibitor, captopril, and suggested that activation of the renin-angiotensin system either directly (Kurtz *et al.*, 1988) and/or indirectly might oppose the vasodilator action of the rat α-CGRP. However, we could not exclude the possibility that captopril inhibited the catabolism of the peptide and thereby enhanced its effects.

Therefore, the second aim of the present study was to determine if inhibition of the cardiovascular actions of angiotensin II influenced the ability of human α-CGRP to oppose the renal vasoconstrictor effects of endothelin-1. In order to avoid the putative problems with the use of captopril in this protocol (see above), and the complications arising from the partial agonistic effects of the peptide analogues of angiotensin II (e.g. Tomlinson *et al.*, 1990), we used the non-peptide angiotensin II receptor antagonist, DuP 753 (Wong *et al.*, 1990; Batin *et al.*, 1991), which is extremely potent but totally lacking in agonistic effects.

In vivo, endothelin-1 is produced by endothelial cells and its release is inhibited by nitric oxide (Boulanger & Lüscher, 1990), the major endothelium-derived relaxing factor (see Moncada & Higgs, 1990). In conscious rats, administration of N^G-nitro-L-arginine methyl ester (L-NAME; Moore *et al.*, 1990) causes hypertension associated with marked regional vasoconstrictions (Gardiner *et al.*, 1990f). L-NAME inhibits production of nitric oxide from L-arginine (Ishii *et al.*, 1990; Mülsch & Busse, 1990), hence its regional haemodynamic effects could be due to loss of nitric oxide-mediated vasodilator tone and/or disinhibition of endothelin-1 release. If the

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latter were the case the haemodynamic profile of L-NAME and endothelin-1 might be similar. The question of whether or not human α-CGRP opposes the vasoconstrictor effects of L-NAME and endothelin-1 in a comparable fashion depends on the extent to which the vasodilator action of human α-CGRP is dependent on release of nitric oxide from endothelial cells; this is contentious (Gray & Marshall, 1990) and could vary in different vascular beds. Therefore, the third aim of the present work was to compare the regional haemodynamic profiles of endothelin-1 and L-NAME and to assess the degree of reversal of these effects by human α-CGRP.

Methods

All experiments were carried out on male, Long Evans rats (350–450 g). Under sodium methohexitone anaesthesia (60 mg kg^{-1}, i.p., supplemented as required) miniaturized, pulsed Doppler probes (Haywood *et al.*, 1981) were implanted to monitor renal, superior mesenteric and hindquarters blood flows in conscious animals. All the techniques have been described in detail elsewhere (Gardiner *et al.*, 1989a,b; 1990d,f).

Continuous recordings of the Doppler shift signals were made by use of a Crystal Biotech VF-1 mainframe (Crystal Biotech, Holliston, USA) modified to operate with a pulse repetition frequency of 125 kHz and fitted with modified HVPD-20 modules, to avoid problems with signal aliasing (Gardiner *et al.*, 1990c). Mean arterial blood pressure and mean Doppler shift signals were used to calculate percentage changes in regional vascular conductances (Gardiner *et al.*, 1990c). The following protocols were run:

Effects of endothelin-1

Conscious, Long Evans rats (n = 8) received an i.v. infusion of endothelin-1 (3 nmol kg^{-1} h^{-1} at 0.3 ml h^{-1}) over a period of 20 min.

Effects of human α-CGRP during infusion of endothelin-1

Ten min after the onset of endothelin-1 infusion (as above), conscious Long Evans rats (n = 8) were given a concurrent infusion of human α-CGRP (1.5 nmol kg^{-1} h^{-1} at 0.3 ml h^{-1}) and the two peptides were given together for the following 10 min.

The protocol as above was also performed but with a 10 fold higher dose of human α-CGRP (i.e. 15 nmol kg^{-1} h^{-1} at 0.3 ml h^{-1}).

The same animals (Group 1 in Table 1) were used in protocols 1 and 2; the protocols were randomized and spread over 2 days. At least 90 min separated each experimental run and all variables were back to baseline before the next intervention was begun.

Effects of human α-CGRP during infusion of endothelin-1 in the presence of DuP 753

In a separate group (Group 2 in Table 1) of conscious, Long Evans rats (n = 9) human α-CGRP (15 nmol kg^{-1} h^{-1}; 0.3 ml h^{-1}) was administered during the last 10 min of a 20 min infusion of endothelin-1 (as above) in animals that had received DuP 753 (10 mg kg^{-1} bolus in 100 μl; Wong *et al.*, 1990; Batin *et al.*, 1991), 10 min before the onset of the endothelin infusion.

Effects of human α-CGRP after administration of L-NAME

Elsewhere (Gardiner *et al.*, 1990f) we have shown that an i.v. bolus dose of L-NAME (10 mg kg^{-1}) causes haemodynamic effects that are established within 10 min of administration and are maintained for the following 60 min at least. Therefore, an additional group (Group 3 in Table 1) (n = 8) of Long Evans rats was given an i.v. bolus injection of L-NAME (10 mg kg^{-1} in 100 μl) and 10 min later an infusion of human α-CGRP (15 nmol kg^{-1}, h^{-1}, 0.3 ml h^{-1}) was begun and continued for the following 10 min.

The resting, baseline values for all cardiovascular variables in the 3 separate groups of animals used in the present study are shown in Table 1. There were no significant differences between the groups (Kruskal-Wallis test).

Drugs and peptides

Human α-CGRP (Celltech Ltd) and endothelin-1 (Peptide Institute) were dissolved in isotonic saline containing 1% bovine serum albumin (Sigma). 2-n-butyl-4-chloro-5-hydroxymethyl-1-[2-(1H-tetrazole-5-yl)bi-phenyl-4-yl-methyl]imidazole, potassium salt (DuP 753, Du Pont de Nemours, USA; gift from Dr R. Smith) and L-NAME hydrochloride (Sigma) were dissolved in isotonic saline.

Statistics

Within-group comparisons were carried out with Friedman's test (Theodorsson-Norheim, 1987) and between-group comparisons with Wilcoxon's rank sums test or the Kruskal-Wallis test, as appropriate.

Results

Effects of endothelin-1

Infusion of endothelin-1 at 3 nmol kg^{-1} h^{-1} for 20 min caused a progressive rise in mean arterial blood pressure and a bradycardia (Figure 1), accompanied by reductions in blood flows and conductances in the renal, mesenteric and hindquarters vascular beds (Figures 1 and 2). The profiles of change were similar to those described previously (Gardiner *et al.*, 1990b).

Table 1 Resting, baseline cardiovascular variables in the three separate groups of Long Evans rats studied

Group	*1* (n = 8)	*2* (n = 9)	*3* (n = 8)
Heart rate (beats min^{-1})	325 ± 15	318 ± 16	319 ± 10
Mean arterial blood pressure (mmHg)	104 ± 3	105 ± 1	102 ± 4
Doppler shift (kHz)			
Renal	8.5 ± 1.4	11.0 ± 0.9	11.4 ± 1.5
Mesenteric	6.3 ± 0.9	5.3 ± 0.4	7.9 ± 0.5
Hindquarters	4.0 ± 0.5	4.4 ± 0.3	4.0 ± 0.4
Vascular conductance ([kHz mmHg^{-1}]10^3)			
Renal	83 ± 14	104 ± 8	111 ± 14
Mesenteric	61 ± 9	51 ± 4	77 ± 5
Hindquarters	39 ± 5	42 ± 2	40 ± 5

Values are mean ± s.e.mean.

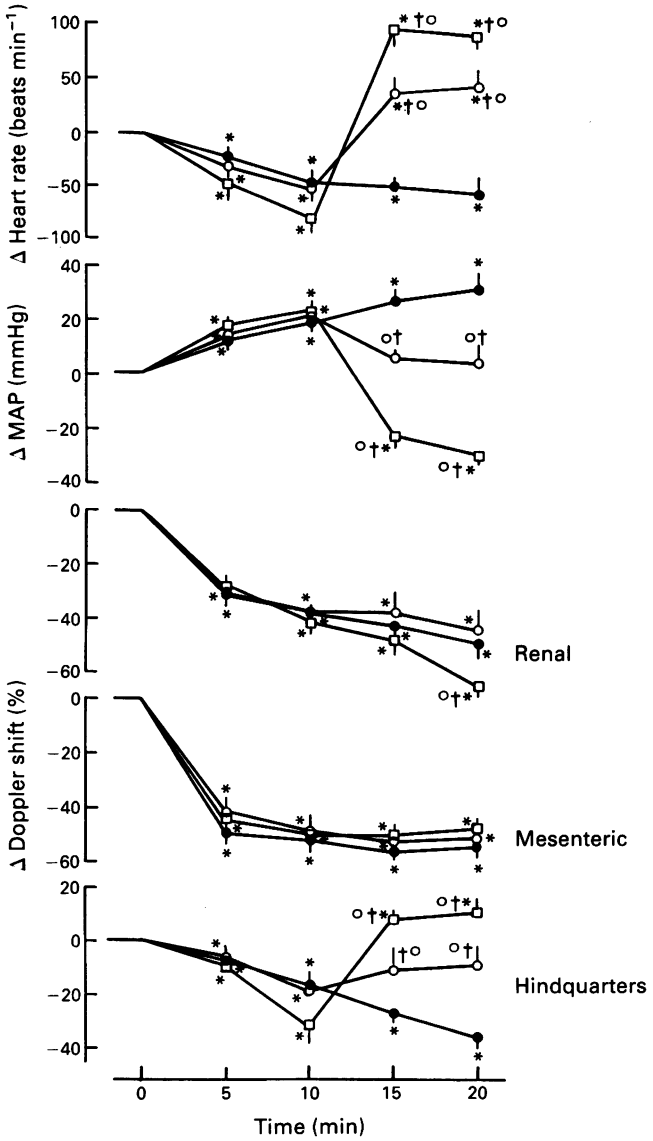

Figure 1 Changes in heart rate, mean arterial blood pressure (MAP) and regional blood flows (Doppler shift) in the same Long Evans rats ($n = 8$) during infusion of endothelin-1 alone (3 nmol kg^{-1} h^{-1}) for 20 min (●), during infusion of endothelin-1 alone for 10 min followed by co-infusion of endothelin-1 and human α-calcitonin gene-related peptide (α-CGRP) (1.5 nmol kg^{-1} h^{-1}) for the subsequent 10 min (○) or during infusion of endothelin-1 and human α-CGRP (15 nmol kg^{-1} h^{-1}) for the subsequent 10 min (□). Values are mean and bars show s.e.means. * $P < 0.05$ versus baseline. † $P < 0.05$ versus 10 min value, ° $P < 0.05$ versus corresponding value in the presence of endothelin-1 alone.

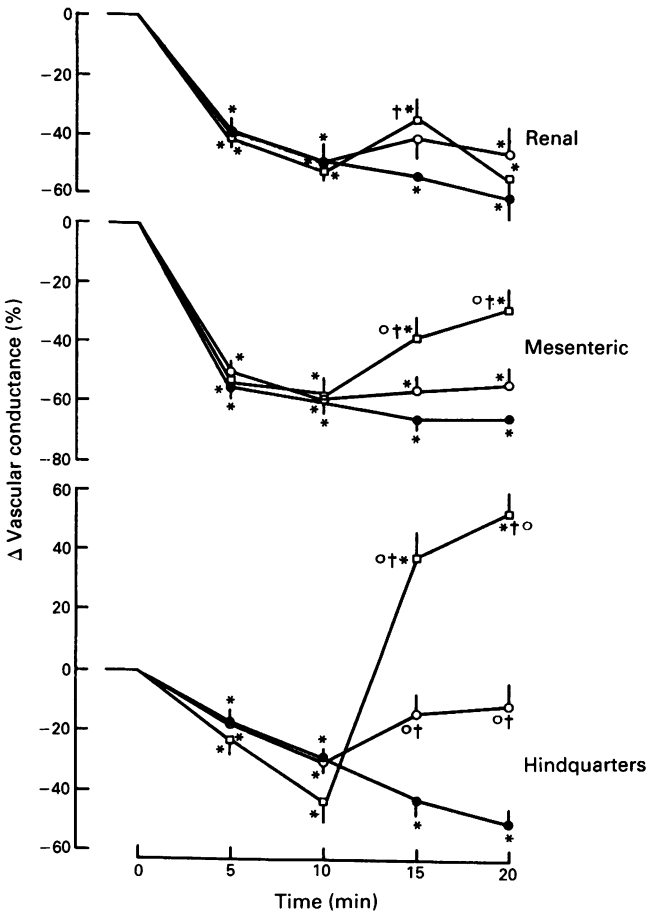

Figure 2 Changes in regional vascular conductances in the same Long Evans rats ($n = 8$) as in Figure 1 during infusion of endothelin-1 alone (3 nmol kg^{-1} h^{-1}) for 20 min (●), during infusion of endothelin-1 alone for 10 min followed by co-infusion of endothelin-1 and human α-calcitonin gene-related peptide (α-CGRP) (1.5 nmol kg^{-1} h^{-1}) for the subsequent 10 min (○), or during infusion of endothelin-1 alone for 10 min followed by co-infusion of endothelin-1 and human α-CGRP (15 nmol kg^{-1} h^{-1}) for the subsequent 10 min (□). Values are mean and bars show s.e.means. * $P < 0.05$ versus baseline, † $P < 0.05$ versus 10 min values, ° $P < 0.05$ versus corresponding value in the presence of endothelin-1 alone.

Effects of human α-CGRP during infusion of endothelin-1

While endothelin-1 was being infused alone during the first 10 min the cardiovascular effects were indistinguishable from those described above. Co-infusion of human α-CGRP at 1.5 nmol kg^{-1} h^{-1} caused a significant ($P < 0.05$) reduction in the endothelin-1-induced hypertension, accompanied by a pronounced tachycardia (Figure 1). Although the reduction in hindquarters blood flow caused by endothelin-1 was abolished by human α-CGRP, the latter did not significantly affect renal or mesenteric blood flows (Figure 1). Thus, the hindquarters vasoconstrictor effect of endothelin-1 was reversed by human α-CGRP at a time when the renal and mesenteric vasoconstrictor effects were not significantly changed (Figure 2).

Infusion of endothelin-1 alone in the third protocol caused effects very similar to the responses evoked by the peptide in the first protocol (Figures 1 and 2). Co-infusion of human α-CGRP at 15 nmol kg^{-1} h^{-1} caused hypotension and tachycardia (Figure 1). The reduction in renal blood flow was enhanced, whereas the reduction in hindquarters blood flow was converted to a significant increase above baseline during infusion of endothelin-1 and human α-CGRP; mesenteric blood flow showed no significant change (Figure 1). Hence there was a significant increase in hindquarters vascular conductance above baseline levels; mesenteric vasoconstriction was diminished, but there was only a transient rise in renal vascular conductance, which always remains below baseline (Figure 2).

Effects of human α-CGRP during infusion of endothelin-1 in the presence of DuP 753

Injection of DuP 753 (10 mg kg^{-1}) caused a slight, but sustained fall in mean arterial blood pressure (maximum at 10 min, -9 ± 2 mmHg, $P < 0.05$), accompanied by a tachycardia (40 ± 9 beats min^{-1}, $P < 0.05$). There were increases in flow in renal ($9 \pm 2\%$, $P < 0.05$), mesenteric ($23 \pm 5\%$, $P < 0.05$) and hindquarters ($14 \pm 4\%$, $P < 0.05$) vascular beds, together with rises in vascular conductance (renal, $19 \pm 2\%$; mesenteric, $35 \pm 6\%$; hindquarters, $23 \pm 5\%$, all $P < 0.05$). In the presence of DuP 753 the haemodynamic effects of endothelin-1 infusion were not different from those seen with endothelin-1 alone. However, the subsequent administration of human α-CGRP elicited a sustained renal vasodilatation in

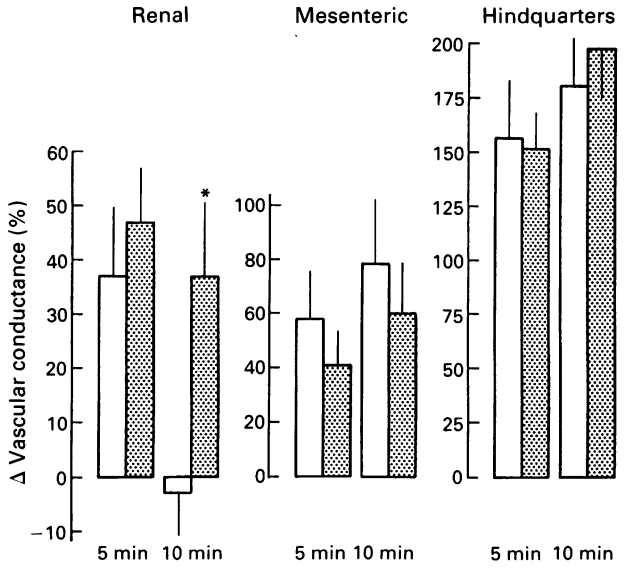

Figure 3 Changes in regional vascular conductances 5 and 10 min after infusion of human α-calcitonin gene-related peptide (α-CGRP) (15 nmol kg^{-1} h^{-1}) in the presence of an endothelin-1 infusion (3 nmol kg^{-1} h^{-1} beginning 10 min before human α-CGRP) (open columns, data from Figure 2) or in the presence of an endothelin-1 infusion beginning 10 min before human α-CGRP and 10 min after administration of DuP 753 (10 mg kg^{-1}) (stippled columns) in a separate group of animals ($n = 9$). Values are mean and bars show s.e.means. The changes are expressed relative to the value before human α-CGRP was administered. * $P < 0.05$ versus value in the presence of endothelin-1 alone.

contrast to the transient effect seen in the absence of DuP 753 (Figure 3). The mesenteric and hindquarters vasodilator responses to human α-CGRP in the presence of endothelin-1 were unaffected by DuP 753 (Figure 3).

Effects of human α-CGRP following administration of L-NAME

Injection of L-NAME caused hypertension and bradycardia (Figure 4) accompanied by reductions in renal, mesenteric and hindquarters blood flows and conductances (Figures 4 and 5). The changes in vascular conductances following administration of L-NAME were very similar to those seen during infusion of endothelin-1 (Figure 5), although the changes in mean arterial blood pressure, heart rate and renal blood flow were different (Figure 4). In the presence of L-NAME, human α-CGRP caused tachycardia and hypotension similar to those seen in the presence of endothelin-1 (Figure 4). However, there were differences in the changes in renal and hindquarters blood flows indicating that human α-CGRP caused more marked renal vasodilatation and less marked hindquarters vasodilatation in the presence of L-NAME than in the presence of endothelin-1 (Figures 4 and 5). The mesenteric vasodilator response to human α-CGRP was similar under the two conditions (Figure 5).

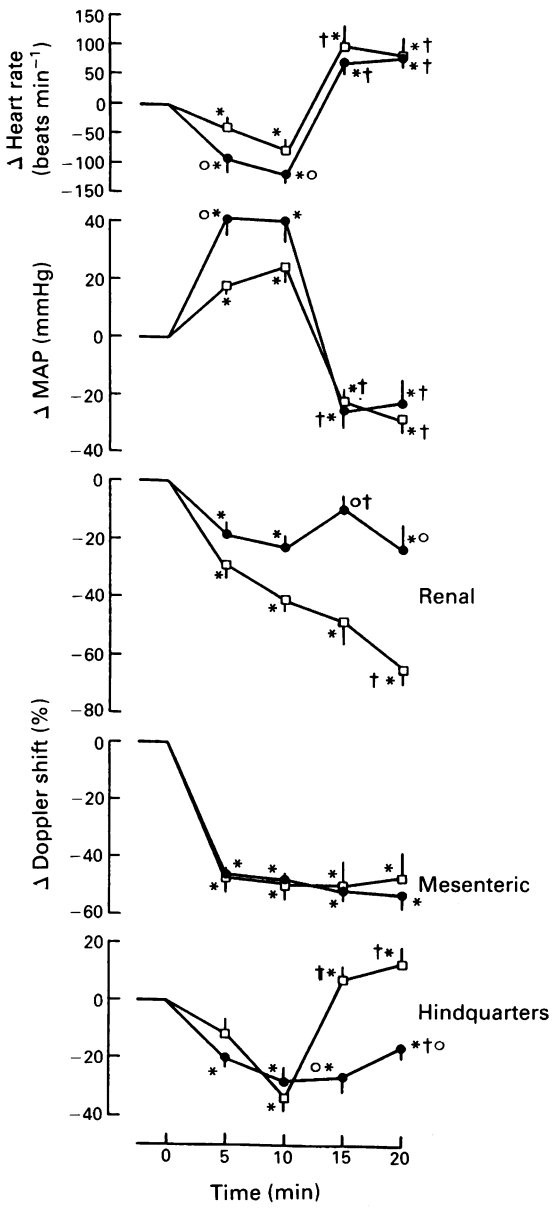

Figure 4 Changes in heart rate, mean arterial blood pressure (MAP) or regional blood flows (Doppler shift) following infusion of endothelin-1 alone (3 nmol kg^{-1} h^{-1}) for 10 min and co-infusion of endothelin-1 and human α-calcitonin gene-related peptide (α-CGRP) (15 nmol kg^{-1} h^{-1}) for the subsequent 10 min (□), (data from Figure 1): (●) indicates changes in cardiovascular variables after injection of N^{G}-nitro-L-arginine methyl ester (10 mg kg^{-1} bolus) followed 10 min later by infusion of human α-CGRP (15 nmol kg^{-1} h^{-1}) for the subsequent 10 min in a separate group of Long Evans rats ($n = 8$). Values are mean and bars show s.e.means. * $P < 0.05$ versus baseline, † $P < 0.05$ versus 10 min value. ° $P < 0.05$ versus corresponding value in the presence of endothelin-1.

Discussion

The present work produced two main findings: (1) the ability of human α-CGRP to oppose the vasoconstrictor effects of endothelin-1 varies in different vascular beds; (2) the haemodynamic changes following administration of endothelin-1 or L-NAME and the ability of human α-CGRP to reverse these changes are not identical.

In a previous study (Gardiner *et al.*, 1990d) we suggested that the functional antagonism between the vasoconstrictor effects of endothelin-1 and the vasodilator effects of human α-CGRP might vary in different vascular beds. This was demonstrated to be so in the present work since the hindquarters vasoconstrictor effects of endothelin-1 were abolished by infusion of human α-CGRP at a rate that had no effect on renal or mesenteric vascular conductances. Furthermore, in the presence of endothelin-1, infusion of human α-CGRP at a higher rate, sufficient to cause hypotension, tachycardia and marked elevations in hindquarters blood flow and vascular conductance above baseline levels, caused only a modest reduction in the mesenteric vasoconstriction and a transient reversal in renal vasoconstriction. In the latter vascular bed the ability of human α-CGRP to reverse endothelin-1-induced vasoconstriction was augmented in the presence of DuP 753, a non-peptide, angiotensin II antagonist (Wong *et al.*, 1990). These results are consistent with activation of the renin-

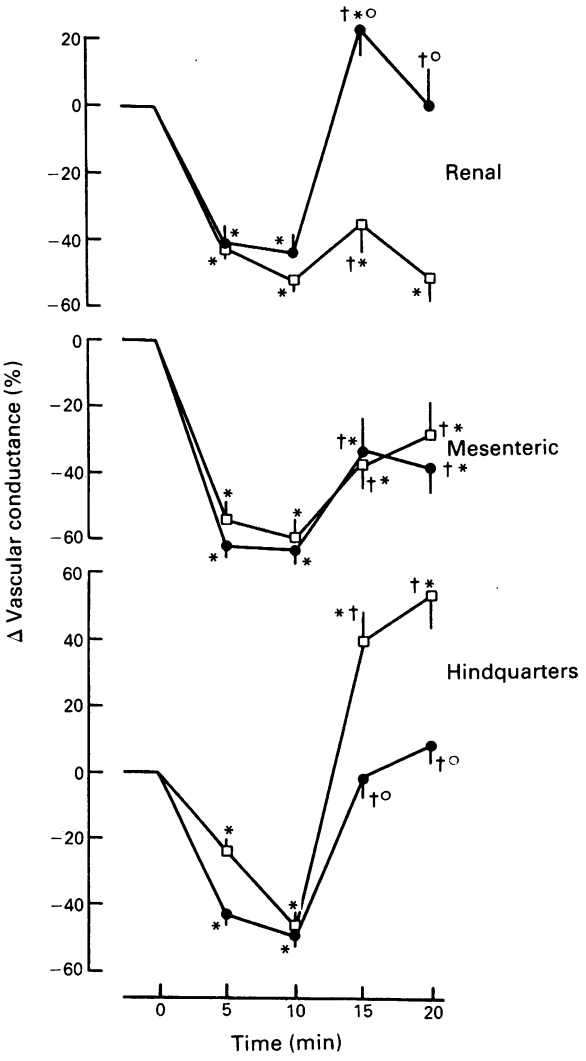

Figure 5 Changes in regional vascular conductances following infusion of endothelin-1 (3 nmol kg^{-1} h^{-1}) for 10 min and co-infusion of endothelin-1 and human α-calcitonin gene-related peptide (α-CGRP) (15 nmol kg^{-1} h^{-1}) for the subsequent 10 min (□), (data from Figure 2); (●) indicates changes in cardiovascular variables after injection of N^G-nitro-L-arginine methyl ester (10 mg kg^{-1} bolus) followed 10 min later by infusion of human α-CGRP (15 nmol kg^{-1} h^{-1}) for the subsequent 10 min in a separate group of Long Evans rats ($n = 8$). Values are mean and bars show s.e.means. * $P < 0.05$ versus baseline; † $P < 0.05$ versus 10 min value; ° $P < 0.05$ versus corresponding value in the presence of endothelin-1.

angiotensin system in the presence of human α-CGRP offsetting its renal vasodilator effects. Such findings corroborate previous observations showing that captopril causes marked renal vasodilatation in the presence of rat α-CGRP (Bennett *et al.*, 1989; Gardiner *et al.*, 1990a), and indicate that those effects were due to antagonism of angiotensin II-mediated vasoconstriction rather than to some other action of captopril augmenting the effects of rat α-CGRP.

It is possible that activation of the renin-angiotensin system by human α-CGRP was a direct (Kurtz *et al.*, 1988), and/or an indirect effect, consequent upon the fall in mean arterial blood pressure. Whatever the explanation, it is notable that this occurred in the presence of endothelin-1 which has been shown to inhibit renin release *in vitro* (Rakugi *et al.*, 1988; Takagi *et al.*, 1988, 1989; Matsumura *et al.*, 1989b). However, *in vivo*, any inhibitory effects of endothelin-1 on renin release may be obscured by stimulatory effects secondary to changes in renal function (e.g. Goetz *et al.*, 1988; Miller *et al.*, 1989; Otsuka *et al.*, 1989; Matsumura *et al.*, 1989a; Tsuchiya *et al.*, 1990). Moreover, it is feasible that the stimulatory effect of human α-CGRP on renin release is greater than endothelin's inhibitory effect. However, the relative potencies of endothelin-1 to inhibit, and human α-CGRP to stimulate, renin release have not been assessed.

It is notable that in the presence of endothelin-1, human α-CGRP acted to cause mesenteric vasodilatation, albeit to a modest extent. In previous studies in conscious rats, administration of human α-CGRP alone was found to cause a fall in mesenteric vascular conductance, and we argued that this might have been due to reflex vasoconstrictor effects (Gardiner *et al.*, 1989a). The present results are consistent with *in vitro* studies showing mesenteric vasodilator responses to human α-CGRP, since those experiments were carried out in preconstricted preparations (Marshall *et al.*, 1986). The lack of a marked mesenteric vasodilator response to human α-CGRP in the present work *in vivo*, even when the mesenteric vasculature was preconstricted with endothelin-1, could have been due to activation of baroreflex mechanisms consequent upon the hypotension caused by human α-CGRP. In this context it is interesting that the mesenteric vasodilator effects (unlike the renal effects) of human α-CGRP were not augmented in the presence of DuP 753. A similar picture was seen in the hindquarters, consistent with a relative lack of vasoconstrictor effect of angiotensin II in that vascular bed (Gardiner *et al.*, 1988). However, angiotensin II can exert substantial mesenteric vasoconstrictor effects (Gardiner *et al.*, 1988), as was clear from the marked vasodilator response to DuP 753. Hence, the apparent lack of involvement of the renin-angiotensin system in opposing the mesenteric vasodilator effects of human α-CGRP in the presence of endothelin-1 was probably due to the hypotension eliciting baroreflex-mediated vasoconstrictor effects that had a more extensive influence in this vascular bed than in the renal circulation.

As reported elsewhere, administration of L-NAME caused hypertension associated with marked renal, mesenteric and hindquarters vasoconstrictions, consistent with a substantial involvement of nitric oxide-mediated mechanisms in the maintenance of resting regional vascular conductances (Gardiner *et al.*, 1990e,f). In the present work we compared the haemodynamic effects of endothelin-1 and L-NAME since there is a possibility that disinhibition of endothelin-1 release contributes to the vasoconstrictor effects seen following suppression of nitric oxide production (Boulanger & Lüscher, 1990) with L-NAME. Although we were able to match very closely the renal, the mesenteric and the hindquarters vasoconstrictor effects of endothelin-1 and L-NAME, the changes in mean arterial blood pressure, heart rate and renal blood flow were different following administration of the two substances. At the doses used, L-NAME produced a greater rise in mean arterial blood pressure than did endothelin-1 even though their vasoconstrictor effects were the same. Hence it is likely that endothelin-1 produced a more marked decrease in cardiac output than did L-NAME. However, it does not follow that release of endogenous endothelin-1 would cause the same haemodynamic effects as administering exogenous endothelin-1. Therefore, direct assessment of the possible contribution of endogenous endothelin-1 to the haemodynamic actions of L-NAME will have to await the availability of a selective antagonist of the cardiovascular actions of endothelin-1.

In the presence of L-NAME, human α-CGRP caused a complete reversal of the renal vasoconstriction in spite of mean arterial blood pressure falling to a level similar to that seen in the presence of endothelin-1 (under which circumstances there was activation of the renin-angiotensin system sufficient to oppose the renal vasodilator effects of human α-CGRP; see above). These results indicate that in the presence of L-NAME, human α-CGRP may have caused little stimulation of the renin-angiotensin system, but there are no studies available regarding the effects of L-NAME on renin release. The fact that the initial overshoot in renal vascular conductance following administration of human α-CGRP in

the presence of L-NAME was transient (see Figure 5) might indicate activation of the renin-angiotensin system at that stage.

The occurrence of renal and mesenteric vasodilator responses to human α-CGRP in the presence of L-NAME that were greater (renal) or similar (mesenteric) to those in the presence of endothelin-1 might indicate that endothelium-derived nitric oxide was not involved in these responses, consistent with other findings (Grace *et al.*, 1987). However, *in vivo*, even classical, endothelium-dependent vasodilators can elicit substantial responses in the presence of L-NAME (Gardiner *et al.*, 1990g).

The picture in the hindquarters was more clear-cut in as much as the overshoot in blood flow and vascular conductance seen following administration of human α-CGRP in the presence of endothelin-1 was absent when human α-CGRP was given after L-NAME, although the hindquarters vasoconstrictor effect of the latter was still reversed by human α-CGRP. These findings indicate that about 50% of the hindquarters vasodilator response to human α-CGRP in the present experiments could have been mediated by nitric oxide-mediated mechanisms sensitive to L-NAME. Differential involvement of such processes in different vascular beds could account for the regional differences in sensitivity to the vasodilator effects of human α-CGRP and the variations in the ability of this peptide to reverse endothelin-1-induced regional vasoconstrictions (see above). In this connection it is of interest that, although the carotid vascular bed is more sensitive than the hindquarters to the vasodilator effects of human α-CGRP under normal conditions (Gardiner *et al.*, 1989a), in the presence of endothelin-1 the carotid vasodilator effects of human α-CGRP (Gardiner *et al.*, 1990d) were less than those in the hindquarters (this study). The augmented hindquarters vasodilator effect of human α-CGRP was abolished in the presence of L-NAME, whereas the latter has no effect on the carotid vasodilator effects of human α-CGRP (unpublished observations). Collectively these results indicate a substantial nitric oxide-mediated vasodilator effect of human α-CGRP localized to the hindquarters vascular bed.

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Specific neurokinin receptors mediate plasma extravasation in the rat knee joint

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1 Plasma extravasation in the rat knee joint was induced by intra-articular injection of neurokinins and specific neurokinin receptor agonists.

2 Pronounced plasma extravasation was produced by substance P (SP, 4–185 μM) and to a lesser extent by neurokinin-B (NKB, 83–413 μM), whereas neurokinin-A (NKA, 88–440 μM) and calcitonin gene-related peptide (CGRP, 26–130 μM) had no significant effect.

3 The specific neurokinin$_1$ receptor agonist [Sar^9, Met(O$_2$)^{11}]-substance P (NK$_1$ agonist) in doses of 0.4–70 μM appeared to be more potent than SP in eliciting plasma extravasation. The neurokinin$_2$ receptor agonist [Nle^{10}]-neurokinin A$_{4-10}$ (NK$_2$ agonist) was not effective at 70 μM but produced a small and significant effect at 330 μM, whereas the neurokinin$_3$ receptor agonist [MePhe^7]-neurokinin B (NK$_3$ agonist) was without effect at 40 μM or 400 μM.

4 Injections of SP or NKA into the synovial cavity of the rat knee were equally effective in producing marked plasma extravasation in remote sites such as the forelimb and hindlimb paws.

5 Co-administration experiments showed that the effects of SP were synergistic with NKA or the NK$_1$ receptor agonist, but not with CGRP or the NK$_2$ receptor agonist.

6 The rank order of potency was NK$_1$ agonist $\geq$ SP > NKB > NK$_2$ agonist suggesting that NK$_1$ receptors mediate plasma extravasation in the rat knee joint.

Keywords: Neuropeptides; neurokinins; substance P; tachykinins; joint inflammation; calcitonin gene-related peptide (CGRP); plasma extravasation; neurokinin A; neurokinin B; neurogenic inflammation

Introduction

Substance P (SP), a neuropeptide contained in sensory (C) fibres (Hokfelt *et al.*, 1975), has long been implicated as the mediator of neurogenic inflammation (Jancso *et al.*, 1967; Lembeck & Holzer, 1979; Gamse *et al.*, 1980; Lembeck *et al.*, 1982). A family of peptides structurally related to SP, known as the tachykinins, have now been characterized. More appropriate nomenclature for these peptides is the neurokinin family, since these are synthesized and stored in nervous structures and act as neurotransmitters. The neurokinins include the physalaemin-like compound, neurokinin A (NKA, also called neuromedin L or substance K) and neurokinin B (NKB, also called neuromedin K) (Kimura *et al.*, 1983; Kangawa *et al.*, 1983; Minamino *et al.*, 1984). The occurrence of several neurokinins in mammalian tissues suggests the existence of different types of neurokinin receptors. Based on the rank order of potencies of the neurokinins in both pharmacological (Regoli *et al.*, 1987a,b) and biochemical (Buck *et al.*, 1984; Beaujouan *et al.*, 1984) studies, three distinct neurokinin receptor populations have been postulated: (1) the NK$_1$ (or SP-P) receptor at which SP is the agonist; (2) the NK$_2$ (or SP-E) receptor at which NKA is the agonist; and (3) the NK$_3$ (or SP-N) receptor at which NKB is the agonist.

It is now recognised that sensory afferents commonly contain more than one type of neuropeptide, with SP and calcitonin gene-related peptide (CGRP) being often co-localised in many types of nociceptive afferent fibres (Fischer *et al.*, 1985). It is also possible that other neurokinins such as NKA and NKB may be co-localised with SP in nerve fibres. SP has been shown to induce plasma extravasation when injected into the synovial cavity of the knee (Lam & Ferrell 1989a,b). This suggests that NK$_1$ receptors are present in articular tissues as SP is the preferential endogenous ligand for this receptor. However, endogenous neurokinins tend to show cross-reactivity with other neurokinin receptors (Drapeau *et al.*, 1987). The recent description of selective agonists for NK$_1$, NK$_2$ and NK$_3$ receptors (Drapeau *et al.*, 1987) offers the opportunity to assess the types of neurokinin receptors present in the knee joint. Thus, the present study is an attempt to identify the neurokinin receptor types mediating plasma extravasation by comparing the rank order of potency of the various neuropeptides (including CGRP) in the rat knee joint. Interactions between the different agents with SP were also assessed.

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Methods

Experiments were performed on male Wistar rats (~300 g) deeply anaesthetized by intraperitoneal injection of urethane (1.13 g kg^{-1}) and diazepam (2.5 mg kg^{-1}). Evans blue (75 mg kg^{-1}) was injected into the external jugular vein. The experimental procedure consisted of injection of 0.2 ml of the naturally-occurring neuropeptides or the specific neurokinin receptor agonists into the synovial cavity of one knee, the other being injected with 0.9% saline to provide an internal control. These were left in the joint for 4 h after which the animals were injected with Euthatal and exsanguinated. A 4 h period was chosen as the time course of neurokinin-induced plasma extravasation was not known and it also allowed comparisons with results of previous investigations (Lam & Ferrell, 1989a,b). The anterior and posterior portions of the knee joint capsule on both sides were dissected free from each rat. The amount of tissue obtained from each animal was small, necessitating pooling of samples from five rats. These samples were weighed and Evans blue extracted by a modified dye extraction technique (Harada *et al.*, 1971), details of which have been given previously (Lam & Ferrell, 1989a). The amount of dye recovered was calculated by comparing the absorbance of the fluid obtained at 620 nm (LKB Ultrospec II) with that of a standard curve prepared with known concentrations of Evans blue solution. As Evans blue binds to plasma proteins normally restricted to the vascular compartment, its presence in the capsule provides an index of altered vascular permeability.

In a group of rats, the effects of co-administration of SP with other naturally occurring neuropeptides and with the specific NK_1 receptor agonists were investigated. The procedures were the same as above with the combined volume of drugs co-administered remaining at 0.2 ml. In another group of rats, the effects of intra-articular injection of NKA and NKB on plasma extravasation in the rat hind paw were also assessed. Measurements of Evans blue content in rat paws were performed individually as the amount of tissues obtained from each paw are much greater than those obtained from the joint capsules. For each dose of the neuropeptides, 5 rat paws were used. Experiments involving measurements of Evans blue content from joint capsules were obtained from 3–6 groups of five rats. Data are presented as the mean difference (± s.e.mean) in Evans blue content between the control and the test knee in each group. The figures represent means ± s.e.mean and differences were considered significant if the P values were 5% or less (unpaired t test). Human α-CGRP was kindly donated by Celltech Ltd (Berkshire). All other drugs were purchased from Cambridge Biochemicals Ltd. In all cases physiological saline solution was used as the solvent.

Results

Plasma extravasation in the knee joint induced by naturally-occurring neuropeptides

Substance P (SP) injected into the synovial cavity produced plasma extravasation in a dose-dependent manner (Figure 1). The threshold for this response was 1 μg in 0.2 ml volume, representing a dose of 3.7 μM, with 50 μg (180 μM) giving a maximal response. CGRP and NKA in concentrations up to 132 μM and 440 μM respectively, were ineffective in eliciting plasma extravasation. These results are illustrated in Figure 1, which shows that plasma extravasation induced by CGRP and NKA did not differ significantly from the control situation when saline was injected into both knees. Significant plasma extravasation was produced with 80 μM NKB, but increasing the dose of NKB to 410 μM failed to produce further significant increase in the plasma extravasation (Figure 1). The maximum plasma extravasation produced by NKB was less than half of that produced by SP.

Plasma extravasation in the knee joint induced by neurokinin receptor agonists

The effects of intra-articular injection of different neurokinin receptor agonists were compared with the effects of SP. [Sar^9, $Met(O_2)^{11}$]-substance P was the chosen specific NK_1 receptor agonist in the present studies. [Nle^{10}]-neurokinin A_{4-10} and [$MePhe^7$]-neurokinin B were the chosen specific NK_2 and NK_3 receptor agonists, respectively. The NK_1 receptor agonist produced dose-dependent inflammatory responses which closely resembled those produced by SP (Figure 2). Although the NK_1 agonist appeared to be more potent than SP, their dose-response curves did not differ significantly. The NK_2 receptor agonist produced a small degree of plasma extravasation at 70 μM which was not significantly different from that produced by injection of saline alone. At a higher concentration of 330 μM, the NK_2 receptor agonist produced a significant plasma extravasation which was about 40% of that produced by SP (Figure 2). Two concentrations of the NK_3 receptor agonist were tested, at both 40 μM and 400 μM, but no significant plasma extravasation was observed (Figure 2).

Plasma extravasation in the rat paw induced by intra-articular administration of neurokinins

During the course of the experiments it was observed that although intra-articular injection of NKA produced no significant plasma extravasation in the knee joint, Evans blue extravasation was prominent elsewhere, especially in the skin of the forelimb and hindlimb paws. It was decided therefore to determine the extent of Evans blue extravasation in the hind-

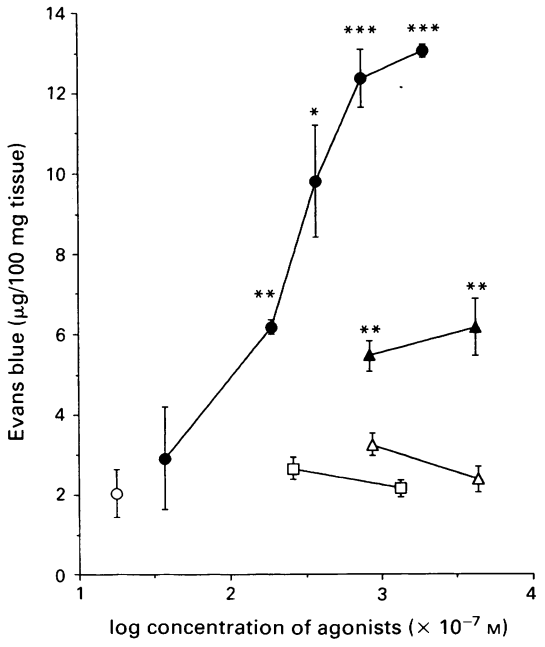

Figure 1 Effects of increasing doses of substance P (●), neurokinin A (△), neurokinin B (▲), and calcitonin gene-related peptide (□) on plasma extravasation into the knee joint capsule. Evans blue content represents the difference between the test (neuropeptide-injected) and the control (saline-injected) knee for each group of five animals. Mean of $n = 3$–4; vertical bars show s.e.mean. Significant difference from saline injection in both knees (○): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

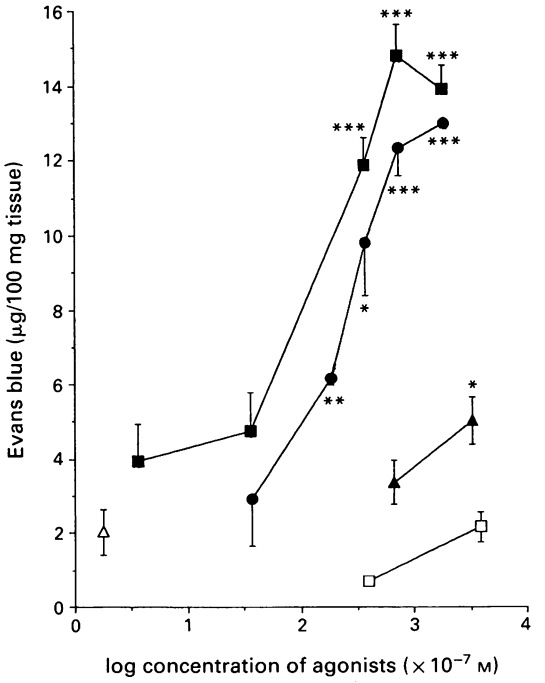

Figure 2 Effects of increasing doses of substance P (●), and neurokinin$_1$ (NK_1) (■), NK_2 (▲), and NK_3 (□) receptor agonists on plasma extravasation into the knee joint capsule. Evans blue content represents the difference between the test (neurokinin-injected) and the control (saline-injected) knee for each group of five animals. Mean of $n = 3$–4; vertical bars show s.e.mean. Significant difference from saline injection in both knees (△): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

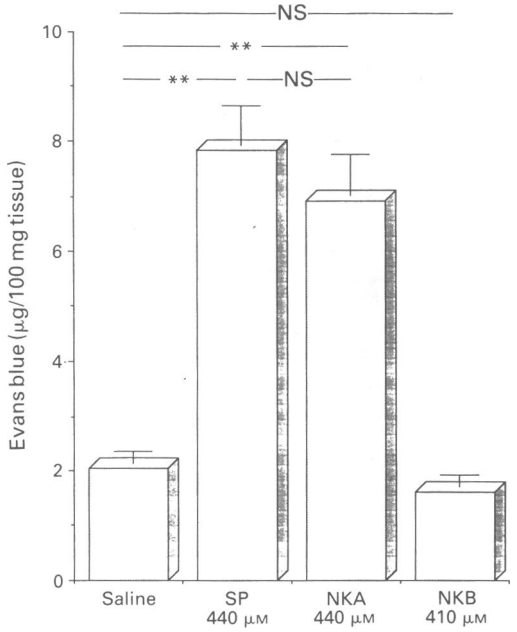

Figure 3 Effects of intra-articular injections of substance P (SP), neurokinin A (NKA), and neurokinin B (NKB) on plasma extravasation into the contralateral hindlimb paw. Evans blue content represents plasma extravasation from five individual rat paws (Mean with s.e.mean shown by vertical bars). NS = no significant difference from saline control. Significant difference from saline control: * $P < 0.05$, ** $P < 0.01$.

limb paw along with that occurring in the knee joint capsules following intra-articular administration of the neurokinins. As illustrated in Figure 3, NKA at 440 μM, a concentration that had no effect in the knee joint, produced marked plasma extravasation in the hindlimb paw contralateral to the neuropeptide-injected knee. In contrast, NKB, which produced significant plasma extravasation in the knee joint capsules at 410 μM, had no effect on the paw at this dose (Figure 3). SP at the same concentration as NKA (440 μM) produced similar plasma extravasation in the paw (Figure 3).

Plasma extravasation in the knee joint induced by co-administration of substance P with naturally-occurring neuropeptides and neurokinin receptor agonist

The effect of a submaximal dose of SP (18 μM) on plasma extravasation was investigated with co-administration of CGRP, NKA, NKB, and with the specific NK_1 receptor agonist. CGRP (130 μM) had no significant effect on plasma extravasation on its own. When co-adminstered with SP, no alteration on the SP-induced plasma extravasation was observed (Figure 4a). As no effect was observed with this, the highest dose, lower doses were not used. Co-administration of SP with 400 μM NKA, which by itself was ineffective in producing a response, resulted in a small but significant increase on the SP-induced plasma extravasation (Figure 4b). Co-administration of SP with 400 μM NKB on the other hand did not affect the SP-induced plasma extravasation although NKB at this concentration was effective in producing plasma extravasation on its own (Figure 4c). The specific NK_1 receptor agonist which itself is a potent inflammatory agent, when co-administered with SP resulted in summation of the individual responses (Figure 4d).

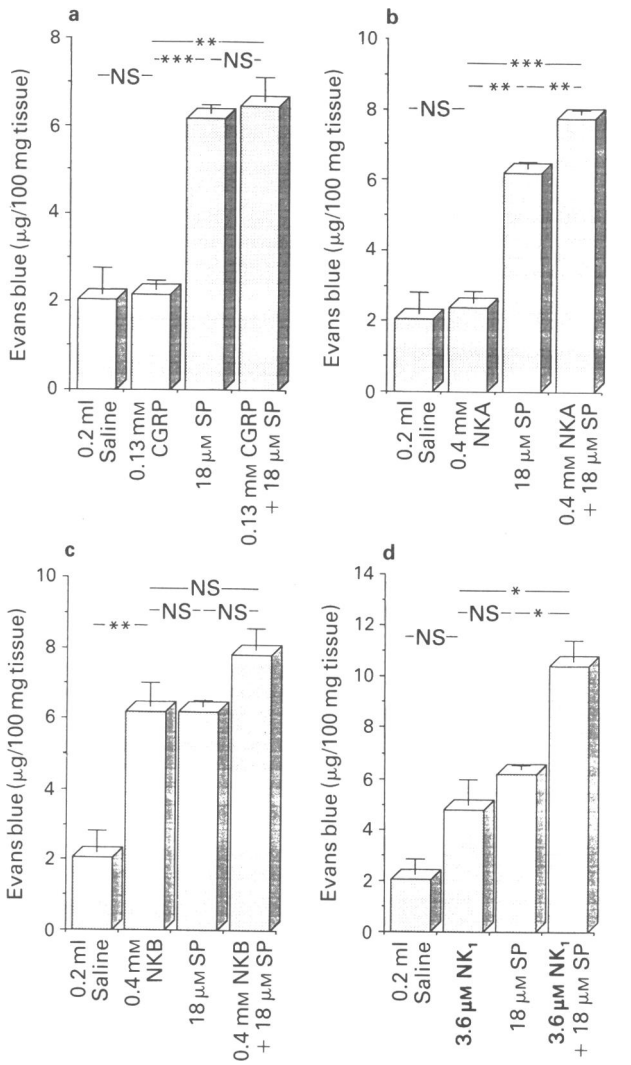

Figure 4 Effects of co-administration of substance P (SP) with (a) calcitonin gene-related peptide (CGRP), (b) neurokinin A (NKA), (c) neurokinin B (NKB), and (d) specific neurokinin$_1$ receptor agonist (NK_1) on plasma extravasation into the knee joint capsule. Evans blue represents the difference between the test (neuropeptide-injected) and the control (saline-injected) knee for each group of five animals. Mean with s.e.mean shown by vertical bars. $n = 3$. NS = no significant difference. Significant difference: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Discussion

Plasma extravasation in rat knee, induced by intra-articular injection of pro-inflammatory agents has been shown to have a significant neurogenic component (Lam & Ferrell, 1989a). The neuropeptide SP present in articular C-afferent fibres has been confirmed as an important mediator of the inflammatory process (Lam & Ferrell, 1989a,b). Marked plasma extravasation can be elicited by direct administration of SP into the synovial cavity of rat knee (Lam & Ferrell, 1989a,b) which can be inhibited by pretreatment with substance P antagonist D-Pro^4, D-Trp^{7,9,10} SP(4–11) (Lam & Ferrell, 1989a). The plasma extravasation induced by SP is thought to be mediated by interactions from the carboxyl terminus of the neuropeptide with specific vascular receptors (Foreman *et al.*, 1983). An N-terminal analogue of SP as verified in human skin (Foreman *et al.*, 1983) failed to induce plasma extravasation. The common C-terminal of the tachykinins suggest that they should all induce plasma extravasation as was found to be the case in studies on rat skin (Brain & Williams, 1989; Andrews *et al.*, 1989). The present studies on the rat knee have shown that this is not always the case, as among the neuropeptides tested, only SP and to a lesser extent NKB, were effective in producing plasma extravasation in the rat knee but not NKA or CGRP.

Antagonists of histamine and 5-hydroxytryptamine have been shown to inhibit partially SP-induced plasma extrava-

sation in rat knee (Lam & Ferrell, 1990) and skin (Brain & Williams, 1989). Inflammatory responses induced by NKA and NKB on rat skin were not affected by these antagonists (Brain & Williams, 1989). This suggests that SP-induced plasma extravasation but not that of the other neurokinins is partially dependent on stimulation of mast cell amine release, which is mediated via the N-terminal amino acids (Foreman *et al.*, 1983). In the rat knee, the importance of this N-terminus contribution is possibly greater and hence the difference in potency of SP from the other neurokinins (which share the same C-terminus and not the N-terminus) in eliciting plasma extravasation is even greater.

As SP is the preferential agonist on NK_1 receptors (Lee *et al.*, 1986), this suggests that inflammatory processes in the rat knee are probably mediated by NK_1 receptors. The lack of effect of NKA which is the preferential agonist on NK_2 receptors (Lee *et al.*, 1986) at a concentration as high as 440 μM suggest that the NK_2 receptor is not important in this response. NKB which is the preferential agonist on NK_3 receptors (Lee *et al.*, 1986) elicited plasma extravasation which was less pronounced than that produced by SP. This suggests that perhaps NK_3 receptors are also involved in this response, but as high concentrations of NKB (83–413 μM) were required, the effects could well be due to cross-reactivity of NKB on NK_1 receptors. Furthermore, the concentrations of NKB in the spinal cord and dorsal root ganglia are 35–40 times less than SP (Ogawa *et al.*, 1985), and the presence of NKB has not yet been demonstrated in the peripheral nerve fibres. These observations taken together do not favour the involvement of NK_3 receptors in the present inflammatory model.

Andrew and co-workers (1989) in their studies on plasma extravasation using a vacuum-induced blister model on rat footpad skin, reached a similar conclusion to ours and considered the NK_1 receptor to be the mediator in the inflammatory response. Plasma extravasation in the rat paw was also investigated in the present study which showed that at a high concentration (440 μM), NKA although not effective on the rat knee, was able to elicit a marked plasma extravasation in the rat paw. However, NKB (410 μM) which was effective at the knee joint was without effect on the rat paw. This could suggest that plasma extravasation at the two sites may be mediated by different types of neurokinin receptors. However, closer examination of the results suggests that both effects are more likely to be mediated by NK_1 receptors, as (i) the effect of NKA on the rat paw is apparent only at high concentrations which would possibly cross-react with NK_1 receptors, and (ii) SP which is potent at the knee joint also produced the most marked plasma extravasation in the rat paw. The reason for NKB being effective on the rat knee but not in the rat paw may be due to less of the neuropeptide being absorbed into the circulation or due to NKB being more susceptible to inactivation by peptidase present in the circulation, hence resulting in an insufficient amount reaching distant sites to produce a response.

In the present study, the receptor type mediating plasma extravasation in the rat knee joint was investigated further with specific neurokinin receptor agonists. The results showed that the specific NK_1 receptor agonist [Sar^9, $Met(O_2)^{11}$]-substance P was as, if not more, potent than SP as an inflammatory agent in the joint, hence confirming that the plasma extravasation is mediated principally by NK_1 receptors. The involvement of NK_2 receptors is unlikely as the endogenous NK_2 receptor agonist, NKA was without effect, and also the specific NK_2 receptor agonist [Nle^{10}]-neurokinin A_{4-10} showed only small responses even at a high concentration (330 μM). Thus, the plasma extravasation induced by the NK_2 agonist is more likely to be due to cross-reactivity of the NK_2 agonist on NK_1 receptors. The present studies on the specific NK_3 receptor agonist [$MePhe^7$]-neurokinin B have shown that it is ineffective as an inflammatory agent in the rat knee. Taken together these findings and earlier discussions on the endogenous NK_3 receptor agonist, NKB, lead to the conclusion that the NK_3 receptor plays no part in the present inflammatory model.

CGRP and SP when co-administered have been shown to be synergistic in their effects on plasma extravasation in rat skin (Brain & Williams, 1985; 1989). CGRP is a potent vasodilator and it is therefore not surprising to find that as a consequence of this action, CGRP can potentiate SP-induced plasma extravasation. However, the present study has shown no potentiation on the SP-induced plasma extravasation when CGRP was co-administered with SP. Thus, there are differences between the two different inflammatory models despite evidence to suggest that the NK_1 receptor is the common mediator in both cases. The differences could well be due to the facts that much higher concentrations of SP (μM instead of pM) and much longer duration of action (4 h instead of 30 min) of the drug at the site of injection were required to establish measurable inflammatory responses in the present model, which may have resulted in short-lived interactions being missed. It is known that both in rat (Brain & Williams, 1989) and in human skin (Brain & Williams, 1988), the vasodilator effects of CGRP can be inhibited when SP is present. This is thought to result from SP-stimulated mast cells releasing proteases which degrade CGRP and thus terminate its vasodilator activity (Brain & Williams, 1989). The fact that CGRP potentiated SP-induced plasma extravasation in the skin model (Brain & Williams, 1989) irrespective of whether the CGRP lost its activity prematurely due to proteolysis, suggests that CGRP is active as a vasodilator over the short period that SP is increasing microvascular permeability. In the present inflammatory model, as a consequence of the larger dose and longer duration of SP in the joint, the effect of proteases released by SP stimulation on mast cells may be much greater than in the skin model. Thus, degradation of CGRP would be greater in the present model resulting in no potentiation of the SP-induced plasma extravasation by CGRP.

It is interesting to note that CGRP was without effect on plasma extravasation in the rat knee joint despite its potent vasodilator effect which has been demonstrated by laser Doppler flowmetry in the rat knee (Grice *et al.*, 1990). This finding suggests that plasma extravasation is more dependent on changes in vascular permeability than changes in vascular tone in this model.

Co-administration of NKA with SP showed a slight additive effect on the SP-induced plasma extravasation, whereas no summation was observed when NKB was co-administered with SP. The magnitude of summation of NKA on the SP response was of the order of 1.56 μg Evans blue/100 mg tissue which was in fact less than the variability from injecting saline alone (2.04 μg Evans blue/100 mg tissue). Although this summation was shown to be significant statistically, the importance of this is doubtful as the present studies have shown that NKA is not an effective agent in inducing plasma extravasation in the rat knee. Moreover, NKB is a better agent than NKA in the rat knee and yet it did not alter the SP-induced response. Laser Doppler flowmetry studies (Grice, 1990) have also shown that the NK_2 receptor agonist which acts on the same receptors as NKA, is not as potent a vasodilator when compared to SP or the NK_3 receptor agonist which acts on the same receptors as NKB.

It was expected that when the NK_1 receptor agonist was co-administered with SP, the plasma extravasation induced by SP would be enhanced as the two agents were each effective in their own right. The present results confirm this view, thus providing further evidence that the effects of the NK_1 receptor agonist and SP resulted from their actions on NK_1 receptors in the rat knee.

In conclusion, the rank order of potency determined in the present inflammatory model was NK_1 receptor agonist $\geqslant$ SP > NKB > NK_2 receptor agonist, which is in keeping with that proposed for the NK_1 receptor (Regoli *et al.*, 1987a). Taken together the present studies indicate that the inflammatory effects of neurokinins in the rat knee joint are probably mediated by NK_1 receptors.

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Effect of capsaicin on PAF-induced bronchial hyperresponsiveness and pulmonary cell accumulation in the rabbit

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1 Platelet activating factor (PAF), but not the carrier molecule bovine serum albumin (BSA) induced bronchoconstriction in the anaesthetized rabbit. This bronchoconstriction was not altered by prior treatment with capsaicin.

2 Rabbits demonstrated increased airways responsiveness to histamine 24 h after exposure to PAF but not to BSA. PAF failed to increase airways responsiveness to histamine in animals pretreated with capsaicin (80 mg kg^{-1}).

3 A significant increase in inflammatory cells was obtained in bronchoalveolar lavage (BAL) 24 h after PAF exposure in vehicle-treated rabbits. This was associated with an increase in the numbers of neutrophils and eosinophils. Capsaicin treatment inhibited the PAF-induced influx of inflammatory cells found in BAL, although this was not associated with an inhibition of PAF-induced pulmonary eosinophilia.

4 Capsaicin-induced motor effects were modest in epithelium-intact rabbit bronchial preparations, but were significantly enhanced in epithelium-denuded preparations in the presence of thiorphan. The contractile response to capsaicin was significantly inhibited in tissues exposed to a consecutive dose of capsaicin. Furthermore, ruthenium red abolished capsaicin-induced contraction in epithelium-denuded preparations.

5 Tissue content of calcitonin gene-related peptide-like immunoreactivity and substance P-like immunoreactivity was not reduced in bronchus and iris obtained from capsaicin-treated rabbits, although capsaicin-induced contractile responses in rabbit bronchus obtained from animals previously treated with capsaicin were significantly reduced. Furthermore, airway responses to histamine, methacholine and electrical field stimulation *in vitro*, were not altered by pretreatment of rabbits *in vivo* for 3 days with capsaicin.

6 In conclusion, PAF-induced airways responsiveness and pulmonary cell accumulation is inhibited by *in vivo* capsaicin pretreatment in the rabbit, via a mechanism that may not involve depletion of sensory neuropeptides.

Keywords PAF; bronchial hyperresponsiveness; capsaicin; sensory neuropeptides

Introduction

It is becoming increasingly apparent that platelet activating factor (PAF) may be an important mediator in the pathogenesis of asthma (Page, 1988) and it has been shown recently, that PAF induces an increase in airways responsiveness and recruitment of inflammatory cells in a number of species including the guinea-pig (Coyle *et al.*, 1988), rabbit (Coyle *et al.*, 1990a) and man (Cuss *et al.*, 1986). Neuropeptides including substance P, neurokinin A and calcitonin gene-related peptide (CGRP) may be localized to sensory nerves in the lung of a number of animal species including man (Lundberg *et al.*, 1984; Hua *et al.*, 1985; Palmer *et al.*, 1987; Hislop *et al.*, 1990) where they may play a physiological role in non-adrenergic non-cholinergic (NANC) transmission (Barnes, 1986). Furthermore, it has been suggested that stimulation of afferent sensory nerves by inflammatory mediators participate in an axon reflex involved in the pathogenesis of bronchial asthma (Barnes, 1986). Nonetheless, few studies have attempted to investigate formally the role of sensory neuropeptides in the pathogenesis of bronchial hyperresponsiveness and airways inflammation that characterize asthma.

Capsaicin, a chemical well recognized for its ability both to release acutely and deplete chronically the content of sensory neuropeptides of a subset of primary afferent nerves in guinea-pigs and rats, has been widely used as a tool in these species to investigate the role of sensory neuropeptides in various biological processes (Maggi & Meli, 1988). Capsaicin has been shown to produce no protection (Ingenito *et al.*, 1989), partial protection (Saria *et al.*, 1983) or complete protection (Manzini *et al.*, 1987) against antigen-induced bronchospasm in guinea-pigs. Furthermore, antigen-induced bronchial hyperresponsiveness but not the pulmonary accumulation of eosinophils was inhibited in ovalbumin-sensitized guinea-pigs treated with capsaicin (Ladenius & Briggs, 1989). Similarly, toluene-diisocyanate (TDI)-induced bronchial hyperresponsiveness in guinea-pigs was inhibited by capsaicin pretreatment, although in this study no affect was seen on the pulmonary accumulation of polymorphonuclear leukocytes (Thompson *et al.*, 1987). Airways responsiveness to ovalbumin and 5-hydroxytryptamine was also reduced in ovalbumin-sensitized rats treated with capsaicin after but not before sensitization (Alving *et al.*, 1987).

Both rabbit and man are similar in the relatively sparse innervation by sensory nerves of their lungs (Laitinen *et al.*, 1983). Furthermore, capsaicin-induced contractile responses are modest in human bronchi *in vitro* (Lundberg *et al.*, 1983), a similar finding to that in the rabbit bronchus. Therefore, in the present study we have sought to investigate the contribution of sensory C-fibres in modulating PAF-induced bronchial

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hyperresponsiveness and cellular recruitment in the rabbit by investigating the effect of capsaicin pretreatment on these processes.

Methods

Capsaicin treatment

Rabbits were treated either with capsaicin (total dose of 80 mg kg^{-1}, s.c.) administered over a period of 3 days (5 mg kg^{-1} on day 1, 50 mg kg^{-1} on day 2, 25 mg kg^{-1} on day 3) or with vehicle (ethanol 10%, Tween 10%, distilled water 80% s.c.). Both vehicle and capsaicin-treated rabbits received at intervals of 2 h, theophylline 2 mg kg^{-1}, atropine 1.2 mg kg^{-1}, diphenhydramine 2.5 mg kg^{-1}, and chlordiazepoxide 1.2 mg kg^{-1} (i.p.). Vehicle or capsaicin was then injected 15 min after premedication. Lung function was assessed in rabbits 2 days after the last vehicle and capsaicin injection. In another series of experiments in which no lung function was performed in rabbits, *in vitro* experiments were performed 4 days after the last vehicle or capsaicin injection.

In vivo *experiments*

New Zealand White rabbits (3–3.5 kg) were anaesthetized with diazepam (5 mg ml^{-1}; 2.5 mg kg^{-1}, i.p.) and fentanyl citrate (0.315 mg ml^{-1}; 0.4 ml kg^{-1}, i.m.) and placed in a supine position. Rabbits were intubated with a 3.0 mm endotracheal tube which was connected to a pneumotachograph (Bore 00) and the flow determined by a Validyne pressure transducer. Pleural pressure was estimated by placing an oesophageal balloon in the lower third of the oesphagus to obtain the maximum end expiratory pressure. Thoracic pressure was measured by a sidehole catheter on the distal end of the tracheal tube. Transpulmonary pressure, the difference between thoracic and pleural pressure was measured using a differential pressure transducer (Validyne). The flow was integrated to obtain a continuous recording of tidal volume. Measurements of total lung resistance (RL) were calculated by a pulmonary mechanical analyser (Buxco, Model 6, Sharon, CT, U.S.A.) as described previously (Giles *et al.*, 1974). Anaesthesia was maintained throughout the course of the experiment by administration of 0.2–0.3 ml of fentanyl citrate intramuscularly every 30–40 min according to the protocol described by Flecknall (1987).

Assessment of airway responsiveness to histamine in vivo

Airway responsiveness to histamine was determined by exposing rabbits to increasing concentrations of histamine (1.25–80 mg ml^{-1}) administered to the lungs directly via an endotracheal tube. Aerosols were generated by a Devilbiss ultrasonic nebuliser which has previously been demonstrated to generate particles of which 80% are less than 0.5 μm in diameter.

On day 1, after measurement of baseline lung function, animals were exposed to an aerosol of sterile saline for 2 min immediately followed by lung function measurements. Increasing concentrations of histamine were administered, with each dose being given for 2 min until there was at least a doubling in RL. The concentration of histamine which produced a 50% increase in RL (PC_{50}) was determined for each animal and used as an index of airway responsiveness.

On day 2, vehicle and capsaicin-treated rabbits were reanaesthetized and exposed to an aerosol of 0.25% bovine serum albumin (BSA) for 2 min, followed by aerosolised PAF (80 μg ml^{-1}) administered over a 1 h period as described by Coyle *et al.* (1990a). A group of vehicle-treated rabbits received an aerosol of BSA followed by exposure to an aerosol of BSA containing the same concentration of ethanol as received by the PAF-treated animals (approx. 1%). In some animals, lung function was monitored for a 1 h period after exposure to BSA or PAF.

On day 3, increasing concentrations of histamine were administered to the anaesthetized rabbits and the PC_{50} for histamine determined as on day 1.

Bronchoalveolar lavage

On completion of the concentration-effect curve to histamine on day 1 (pre) and day 3 (post), a bronchoalveolar lavage (BAL) was performed. Rabbits were lavaged by passing a cannula into the airways via the endotracheal tube until it was wedged at the first bifurcation. A volume of 3 ml of 10% normal rabbit serum in 0.9% sterile saline was injected and immediately aspirated from the lungs. Total cell counts were determined under light microscopy with an improved Neubauer haemocytometer. Cytospins were prepared, fixed and stained with Lendrum's stain. A total of 200 cells were counted differentially and classified as neutrophils, eosinophils or mononuclear cells based on standard morphological criteria.

In vitro *experiments*

Following the completion of a BAL on day 3 (described above), the rabbits were killed by exsanguination whilst under anaesthesia. The lungs were quickly removed and placed in cold oxygenated Krebs-Henseleit solution. Rabbit intrapulmonary bronchi (1–3 mm, i.d.) were dissected free of parenchymal tissue and visible blood vessels. Bronchial rings were suspended under 1 g tension in organ baths containing Krebs-Henseleit solution aerated with 95% O_2, 5% CO_2 and maintained at 37°C. Changes in isometric tension were measured with a Grass force-displacement transducer (FTO3C) and recorded on a Lectromed chart recorder. All bronchial preparations were allowed to equilibrate for 45 min and decreases in resting tension which may have occurred during this period were compensated for by readjustment of tension to 1 g. The bathing solution was changed every 15 min before the addition of pharmacological agonists.

Following equilibration in Krebs-Henseleit solution, bronchial preparations were chosen randomly and exposed to either methacholine (0.1–100 μM), histamine (1–100 μM), capsaicin (10–100 μM) or electrical field stimulation (EFS; 100 V, 0.5 ms, 0.1–30 Hz; 30 s duration). Furthermore, in all bronchial preparations exposed to capsaicin *in vitro*, a second application of capsaicin after 45 min was given to assess the effects of capsaicin-induced desensitization. In the case of the EFS studies, all bronchial preparations were preincubated for 30 min with indomethacin (5 μM) and propranolol (5 μM).

In other experiments, bronchi were obtained from animals in which no lung function was performed. New Zealand White rabbits (1.5–2.0 kg) were treated with vehicle or capsaicin as described above. Four days later, animals were killed with an overdose of ketamine (25 mg kg^{-1}, 0.5 ml kg^{-1}, i.v.). Main bronchi were dissected free from adhering lung tissue and bronchial rings were mounted on stainless steel hooks and suspended under 500 mg tension in a 5 ml organ bath containing Krebs-Henseleit solution as described above. In some preparations, the epithelium was removed by gentle rubbing of the luminal surface with a cotton wool applicator. The efficiency of this removal was confirmed histologically. Changes in isometric tension were measured with a Grass force-displacement transducer (FTO3C) and recorded on a Basile Unirecord 7050 polygraph. Each preparation was allowed to equilibrate for at least 30 min before the addition of pharmacological agonists. Indomethacin (5 μM) was present throughout the experiment. Thiorphan (10 μM) was added to the organ bath at least 10 min before administration of capsaicin

(100 μM), neurokinin A (NKA, 0.1 μM), substance P (1 μM) and calcitonin gene-related peptide (1 μM).

CGRP-LI and SP-LI radioimmunoassay

Calcitonin gene related peptide-like immunoreactivity (CGRP-LI) was assayed as previously described (Manzini et al., 1989). Briefly, experimental samples or samples containing known concentrations of rat-CGRP standard were incubated for 48 h at 4°C with anti-human CGRP rabbit anti-serum (Peninsula CA, U.S.A.) that demonstrates 100% cross-reactivity toward both α and β rat-CGRP. Then [^{125}I]-human-CGRP (Amersham U.K.) was added and incubated for a further 48 h at 4°C. Separation of bound and free antigen was by double antibody precipitation. The lower detection limit was 2.5 fmol/tube.

Substance P-like immunoreactivity (SP-LI) was assayed as previously described (Geppetti et al., 1987). Briefly, SP-standard or samples were incubated overnight at 4°C with rabbit anti-SP anti-serum and [^{125}I]-Bolton and Hunter conjugate SP (Amersham, U.K.). Bound and free antigen were separated by double antibody precipitation. The sensitivity of the assay was 1.1 fmol/tube. The anti-serum cross-reacts to 1% with NKA, 0.5% NKB and less than 0.1% with phylsalaemin and eledoisin.

Analysis of results

Results from all studies were expressed as mean ± s.e.mean. Differences in the bronchoconstrictor responses to PAF were analysed with an unpaired Student's t test, and considered significant if $P < 0.05$. *In vivo* histamine potency derived from measurements of resistance (PC_{50}) was expressed as the derived PC_{50} (antilog PC_{50}) together with the lower and upper limits of the s.e.mean. The pre-post test differences for each group were analyzed with an analysis of variance (ANOVA) and the location of this difference was performed with an unpaired Student's t test (two-tailed) with a Bonferroni correction (Wallenstein et al., 1980) and considered significant if $P < 0.05$.

The logarithm of the concentration of agonist or frequency of EFS which elicited a 50% maximal response was designated as $-\log_{10}$ EC_{50} (pD_2) or $-\log_{10}$ EF_{50} respectively. In the case of the EFS data, responses were expressed as a % of the maximum response to methacholine. Statistical analysis of agonist potency and EFS data was performed by ANOVA. In other experiments, motor responses were expressed as absolute increases in tension (mg) and as a % of the maximum response to methacholine or carbachol. The significance of differences between mean values was determined by the Wilcoxon test, Mann-Whitney U test or non-paired Student's t test, difference being considered significant if $P < 0.05$.

The variability of the results for the cell data was confirmed with a Barlett's test for homogeneity of variance, and a logarithmic scale was found to be more appropriate. For the differential cells, analysis was performed on log $10(x + 0.005)$ because of recorded zero counts. Pre cell data were analysed by ANOVA to examine whether capsaicin treatment alone had an affect on cells in BAL. The pre-post test differences for each group were analyzed by ANOVA and the difference in means analyzed by a non-paired Student's t test with a Bonferroni correction and considered signficant if $P < 0.05$.

Drugs

Calcitonin gene-related peptide, diphenhydramine hydrochloride, histamine dihydrochloride, histamine diphosphate, indomethacin, methacholine hydrochloride, neurokinin A, propranolol hydrochloride, ruthenium red, theophylline, thiorphan, Tween 80 (polyoxyethylenesorbitanmonooleate), capsaicin (8-methyl-N-vanillyl-6-nonenamide), bovine serum albumin Grade V (endotoxin free) and normal rabbit serum were all obtained from Sigma (Poole, Dorset); PAF was purchased from Novabiochem (Nottingham, UK); chlordiazepoxide hydrochloride, diazepam (Roche, U.K.); fentanyl citrate (Janssen Pharmaceutical Ltd, U.K.). Composition of the Krebs-Henseleit solution was (mM): NaCl 117.6, $NaHCO_3$ 25, D-glucose 11.1, KCl 5.4, $MgSO_4$ 0.57, KH_2PO_4 1.03 and $CaCl_2$ 2.5. Thiorphan was dissolved in 5% Na_2CO_3, capsaicin in ethanol and all other drugs in Krebs-Henseleit solution.

Results

Effect of capsaicin treatment on PAF-induced bronchoconstriction

PAF (80 μg ml^{-1}) induced acute bronchoconstriction in vehicle-treated animals, with RL increasing by 100 ± 15% ($n = 4$). The PAF-induced bronchoconstriction was not altered in capsaicin-treated animals, as assessed by changes in RL (135 ± 25%, $n = 4$; $P > 0.05$). Vehicle-treated animals exposed to 0.25% BSA demonstrated a small but significant bronchoconstriction (RL 16 ± 2%, $n = 3$; $P < 0.05$).

Baseline RL measurements in vehicle and capsaicin-treated animals were 15.2 ± 1.7 cmH_2O l^{-1} s^{-1} ($n = 9$) and 14.7 ± 2.2 cmH_2O l^{-1} s^{-1} ($n = 6$) respectively.

Effect of capsaicin treatment on PAF-induced bronchial hyperresponsiveness

Histamine induced a concentration-dependent increase in airways resistance in vehicle- and capsaicin-treated rabbits. Capsaicin treatment alone failed to alter significantly airway responsiveness to histamine (pre) ($P > 0.05$). A significant treatment effect was observed between post and prehistamine PC_{50} values for the 3 groups ($P < 0.005$). In vehicle-treated rabbits, the values of PC_{50} for histamine before and 24 h after exposure to aerosolized BSA were 20 mg ml^{-1} (12–32) and 17 mg ml^{-1} (13–23) respectively, ($n = 5$, Figure 1). However, there was a significant increase in airways responsiveness to histamine 24 h after PAF exposure compared with BSA exposure in vehicle-treated rabbits (pre PC_{50} 44.7 mg ml^{-1} (40–50); post PC_{50} 13.8 mg ml^{-1} (9–20), $n = 7$, Figure 1; $0.002 < P < 0.01$). However, in capsaicin-treated rabbits, PAF

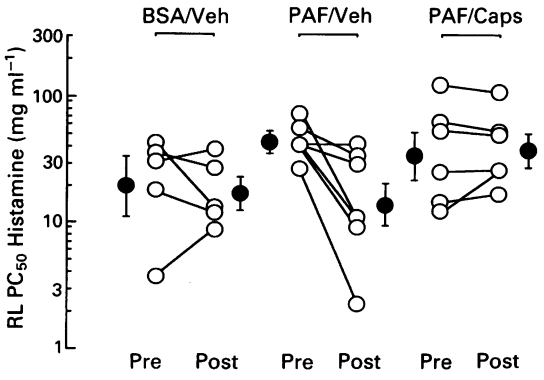

Figure 1 Individual histamine lung resistance (RL) PC_{50} values before (pre) and 24 h after (post) aerosol exposure to bovine serum albumin (BSA) or platelet activating factor (PAF) in vehicle-treated (Veh) or capsaicin-treated (Caps) rabbits. Mean values are also shown and the vertical bars represent the lower and upper limits of the s.e.mean.

failed to increase significantly airways responsiveness to histamine compared with PAF exposure in vehicle-treated rabbits (pre PC_{50} 35.1 mg ml^{-1} (24–50); post PC_{50} 39.4 mg ml^{-1} (30–51), $n = 6$, Figure 1; $0.01 < P < 0.025$).

Effect of capsaicin treatment on cell counts in bronchoalveolar lavage

Both total and differential cell counts were enumerated from bronchoalveolar lavage (BAL; Table 1). The percentage of neutrophils, eosinophils, and mononuclear cells recovered from BAL (pre) in vehicle-treated rabbits were $7 \pm 4\%$, $1 \pm 0.6\%$ and $92 \pm 10\%$, respectively ($n = 11$). Similarly, the percentages of neutrophils, eosinophils and monocytes recovered from BAL (pre) in capsaicin-treated rabbits were $21 \pm 18\%$, $1.2 \pm 0.9\%$ and $78 \pm 22\%$, respectively ($n = 5$). Capsaicin treatment alone failed to increase significantly the total number of cells recovered in BAL ($P > 0.05$), although a significant increase in the number of neutrophils was observed ($0.02 < P < 0.05$). A significant treatment effect was observed between post and pre total cell counts for the 3 groups ($P < 0.005$). Total cell counts were significantly elevated in BAL, 24 h after exposure to PAF compared with BSA in vehicle-treated rabbits ($P < 0.005$; Table 1). This was reflected by a significant increase in the number of neutrophils ($0.02 < P < 0.05$) and eosinophils ($P < 0.005$) but not monocytes ($P > 0.05$) in BAL.

The PAF-induced influx of total cells was significantly less in capsaicin-treated than in vehicle-treated rabbits ($0.01 < P < 0.025$), Table 1). Similarly, the PAF-induced influx of neutrophils was not observed in capsaicin-treated rabbits ($P > 0.05$; pre vs post). In contrast, there was no significant difference in the PAF-induced eosinophil influx in capsaicin-treated compared with vehicle-treated rabbits ($P > 0.05$).

Effect of capsaicin on in vitro *smooth muscle function*

In bronchi obtained from animals in which lung function was performed, methacholine, histamine and EFS produced a concentration/frequency-dependent contraction in rabbit intrapulmonary bronchi. The contractile potencies (pD_2) of histamine, methacholine and $-\log EF_{50}$ values for electrical field stimulation (EFS) were not significantly different in preparations taken from vehicle or capsaicin-treated rabbits exposed to either BSA or PAF ($P > 0.05$, Table 2).

Table 2 Contractile potency (pD_2) of histamine, methacholine and $-\log EF_{50}$ values for elecrical field stimulation (EFS) in vehicle or capsaicin-treated rabbits exposed to either 0.25% bovine serum albumin (BSA) or platelet activating factor (PAF, 80 μg ml^{-1})

	BSA/ Vehicle	*PAF/ Vehicle*	*PAF/ Capsaicin*
Methacholine	5.40 ± 0.09 (4, 17)	5.64 ± 0.18 (6, 23)	5.56 ± 0.08 (6, 29)
Histamine	4.67 ± 0.25 (3, 11)	4.73 ± 0.24 (6, 23)	4.70 ± 0.06 (5, 23)
EFS	0.484 ± 0.117 (4, 7)	0.514 ± 0.119 (7, 12)	0.774 ± 0.079 (7, 13)

Results expressed as mean ± s.e.mean. Numbers in parentheses indicate the number of rabbits followed by the total number of bronchial preparations used.
pD_2 values for histamine and methacholine and $-\log EF_{50}$ values for EFS were not significantly different between groups ($P > 0.05$, ANOVA).

Capsaicin (10–100 μM) produced only modest contractions of bronchi from vehicle-treated rabbits ($12 \pm 3\%$ methacholine E_{max}; $n = 23$ out of 41 bronchial preparations from 10 rabbits). In contrast, the response to capsaicin was significantly reduced when repeated a second time in these same preparations ($3.5 \pm 0.8\%$, $n = 4$ out of 33 preparations; $P < 0.01$, Wilcoxon rank test). Furthermore, capsaicin also produced contractions in bronchi from capsaicin-treated rabbits ($3.7 \pm 1.4\%$, $n = 6$ out of 21 bronchial preparations from 5 rabbits), although these were significantly smaller than in those from vehicle-treated rabbits ($P = 0.025$, Mann-Whitney U test).

In epithelium-intact bronchi obtained from animals in which no lung function had been performed, capsaicin (100 μM) produced a modest contractile response ($13 \pm 6\%$ carbachol E_{max} in 4 out of 6 bronchial preparations) even in the presence of thiorphan (10 μM). In contrast, in epithelium-denuded thiorphan-pretreated preparations, capsaicin (100 μM) produced significantly greater contractions ($29 \pm 4\%$, $n = 6$, $P < 0.05$). Furthermore, capsaicin-induced contractile responses were significantly reduced in preparations from capsaicin-treated rabbits compared with those from vehicle-treated rabbits ($14.0 \pm 3.9\%$, $n = 6$ vs $30.5 \pm 2.9\%$, $n = 11$;

Table 1 Number of cells recovered from bronchoalveolar lavage in vehicle-treated or capsaicin-treated rabbits before (pre) and 24 h after (post) aerosol exposure to bovine serum albumin (BSA, 0.25%) or platelet activating factor (PAF 80 μg ml^{-1}.)

	$\times 10^5$ cells/ml			
	Total	*Neutrophil*	*Eosinophil*	*Monocyte*
BSA/Vehicle				
Pre	2.66 ± 0.40	0.30 ± 0.21	0.062 ± 0.028	2.30 ± 0.45
Post	2.54 ± 0.50	0.33 ± 0.26	0.006 ± 0.004	2.18 ± 0.60
PAF/Vehicle				
Pre	1.98 ± 0.28	0.055 ± 0.032	0.003 ± 0.003	1.94 ± 0.26
Post	11.8 ± 2.99*	6.71 ± 3.83~	0.565 ± 0.250*	4.56 ± 1.70
PAF/Capsaicin				
Pre	5.92 ± 2.31	1.25 ± 1.04#	0.074 ± 0.056	4.62 ± 1.28
Post	5.64 ± 0.89†	1.43 ± 0.61NS	0.502 ± 0.314NS	3.76 ± 0.72

Results expressed as mean ± s.e.mean. Number of animals in each group are as follows: BSA/Vehicle, $n = 5$; PAF/Vehicle, $n = 6$; PAF/Capsaicin, $n = 5$. Capsaicin treatment significantly increased the number of neutrophils (pre) in BAL cf. vehicle treatment #$0.02 < P < 0.05$; non-paired t test). Analysis of the pre-post test differences revealed a significant difference in the 3 groups for total cells and eosinophils ($P < 0.005$).
PAF significantly increased the total number of cells, neutrophils and eosinophils recovered in BAL cf. BSA (*$P < 0.005$, ~$0.02 < P < 0.05$ and *$P < 0.005$ respectively, non-paired t test).
PAF-induced influx of total cells in BAL was significantly inhibited in capsaicin-treated cf vehicle-treated rabbits (†$0.01 < P < 0.02$, non-paired t test) but not eosinophils (NS$P > 0.05$, non-paired t test). No significant increase in BAL neurophils by PAF in capsaicin-treated rabbits (NS$P > 0.05$, pre cf post; paired t test).

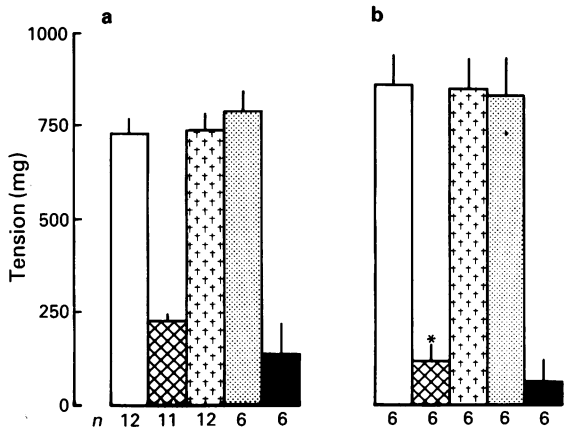

Figure 2 Tension generated in epithelium-denuded bronchi obtained from vehicle (a) or capsaicin-treated (b) rabbits to carbachol (10 μM, open columns), capsaicin (100 μM, cross-hatched column), neurokinin A (0.1 μM, columns with crosses), substance P (1 μM, stippled column) and calcitonin gene-related peptide (1 μM, solid column) all in the presence of thiorphan (10 μM) and indomethacin (5 μM). n indicates the number of preparations tested. Response to capsaicin significantly reduced in bronchi from capsaicin-treated rabbits cf vehicle-treated rabbits; $^*P < 0.01$, non-paired t test

$P < 0.01$, Figure 2). Capsaicin pretreatment did not affect the responses to carbachol, substance P, CGRP or NKA (Figure 2).

The contractile responses of epithelium-denuded bronchial preparations to capsaicin were significantly inhibited by the presence of ruthenium red (10 μM) (30 $\pm$ 6% $n = 5$ vs 2 $\pm$ 2% $n = 6$, $P < 0.05$), whereas those to NKA (0.1 μM) were not significantly affected by such treatment (114 $\pm$ 3% vs 99 $\pm$ 8%, $n = 6$, $P > 0.05$).

There were no significant differences in the tissue levels of CGRP-LI and SP-LI in bronchi and SP-LI in the iris from capsaicin-treated rabbits compared with the levels obtained in vehicle-treated rabbits ($P > 0.05$, non-paired t test, Table 3). In contrast, tissue levels of CGRP-LI in the iris from capsaicin-treated rabbits were significantly greater than in those from vehicle-treated rabbits ($P < 0.05$, Table 3).

Discussion

It has been demonstrated previously that in rabbit airways PAF can induce at least three effects: (a) platelet-dependent (Coyle *et al.*, 1990a), histamine-mediated (Halonen *et al.*, 1985) bronchoconstriction; (b) non-specific increase in airways responsiveness to spasmogens (Nieminen *et al.*, 1989; Coyle *et al.*, 1990a) and (c) an influx of inflammatory cells in the lung as assessed by BAL (Coyle *et al.*, 1990a). Our present data confirm these results, and in addition indicate that *in vivo* capsaicin treatment significantly inhibits PAF-induced bronchial hyperresponsiveness and the recruitment of total inflammatory cells, while not significantly affecting PAF-induced bronchoconstriction.

Capsaicin has been used extensively in rats and guinea-pigs as a tool to invesigate the function of primary afferent nerves and to reduce the levels of tissue sensory neuropeptides (Maggi & Meli, 1988). Its use has led to the suggestion that sensory neuropeptides may be important in a wide range of biological activities, including certain aspects of pulmonary pathophysiology. In the present study, we have demonstrated that bronchi removed from rabbits treated for 3 days with capsaicin, in contrast to those from vehicle-treated animals fail to contract *in vitro* to further stimulation by exogenously applied capsaicin. Furthermore, capsaicin-induced contractions were inhibited by pretreatment with ruthenium red, which has been proposed as a selective inhibitor of capsaicin-induced stimulation of sensory nerve endings (Maggi & Meli, 1988). Nevertheless, capsaicin pretreatent did not result in significant depletion of tissue neuropeptide levels in the rabbit (this study; personal communication J.-A. Karlsson).

A number of possibilities may explain the apparent dissociation between capsaicin-induced loss of function without loss in neuropeptide content. It is possible that part of the SP-LI and CGRP-LI detected in rabbit bronchi may be present in capsaicin-insensitive neuropeptide containing nerves, as has been demonstrated in the gut (Holzer *et al.*, 1980; Hua *et al.*, 1985), or that the majority of neuropeptides in sensory nerves exist in pools which are not sensitive to *in vivo* capsaicin treatment (Hakanson *et al.*, 1987), or that another neuropeptide may be involved. Alternatively, an impairment of sensory nerve function, without a reduction in neuropeptide content within the sensory nerve may occur (Lembeck & Donnerer, 1981; Maggi *et al.*, 1987; 1990a, b; Chahl, 1989). Intracameral injection of capsaicin into the eye of the rabbit induces miosis and hyperaemia which are not observed in rabbits treated neonatally or as adults with capsaicin (Butler & Hammond, 1980; Tervo, 1981). However, according to Tervo (1981), this loss of function in the eye is not associated with a loss in neuropeptide content, a finding confirmed in the present study. In contrast, capsaicin applied to rabbit saphenous nerve was accompanied by a reduction in neuropeptide content 9–12 days later, with a corresponding loss in function (Lynn & Shakhanbeh, 1988). Whether this is a result of the much higher local concentrations of capsaicin around the nerve compared with that achieved within the eye or lungs following subcutaneous injection of capsaicin is not known. However, it does indicate that rabbit tissues have the potential to demonstrate a quantifiable loss in neuropeptide content when treated with capsaicin *in vitro* (Hakanson *et al.*, 1987; Lynn & Shakhanbeh, 1988). However, a partial resistance of rabbit tissues to the excitatory actions of capsaicin has already been presented with regard to its effects on conduction in cutaneous nerves (Baranowski *et al.*, 1986), its inability to induce degeneration of primary afferents (Lynn & Shakhanbeh, 1988) and its poor efficacy in producing desensitization (Amann & Lembeck, 1990). These studies indicate that, unlike rats and guinea-pigs, the rabbit is particularly resistant to the effects of capsaicin. However, whether this can account for the discrepancy between loss in function with no apparent loss in neuropeptide content is speculative. Lastly, capsaicin may produce an as yet unidentified non-specific action, although a non-specific effect of capsaicin on airway smooth muscle or nerves can be ruled out as contractile responses of bronchi induced by exogenously applied spasmogens or EFS were unchanged in tissues obtained from animals previously treated with capsaicin.

Table 3 Tissue levels of calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) and substance P-like immunoreactivity (SP-LI) in bronchial and iridial tissue from rabbits pretreated with vehicle or capsaicin (80 mg kg^{-1})

	Bronchus	*Iris*	
	(pmol g^{-1} wt weight)		n
CGRP-LI			
Vehicle-treated	11.2 $\pm$ 1.5	35.8 $\pm$ 6.2	6
Capsaicin-treated	13.8 $\pm$ 2.1	49.9 $\pm$ 12.7*	6
SP-LI			
Vehicle-treated	0.96 $\pm$ 0.15	6.5 $\pm$ 1.1	6
Capsaicin-treated	1.01 $\pm$ 0.10	10.9 $\pm$ 1.3	6

Results expressed as mean $\pm$ s.e.mean. n represents the number of tissues tested.
Significant increase in CGRP-LI immunoreactivity in iridial tissue from capsaicin-treated compared with vehicle-treated rabbits: $^*P < 0.05$, non-paired t test.

Capsaicin treatment alone did not alter airway responses to histamine *in vivo*. However, while it has been reported that responses to histamine are reduced in capsaicin-treated guinea-pigs (Biggs & Ladenius, 1990), it has also been reported that responses to muscarinic agonists are not affected (Thompson *et al.*, 1987; Biggs & Ladenius, 1990). Our data indicate that capsaicin does not mediate a non-specific effect on airway smooth muscle, nor does histamine induce bronchoconstriction by activation of sensory nerves. Against this background therefore, it is of interest that PAF-induced bronchial hyperresponsiveness to histamine was inhibited in rabbits treated for 3 days with capsaicin. Our results support previous observations that have demonstrated inhibition of TDI and antigen-induced bronchial hyperresponsiveness by capsaicin treatment of guinea-pigs (Thompson *et al.*, 1987; Ladenius & Briggs, 1989), although these authors concluded that sensory neuropeptides may be involved in the expression of bronchial hyperresponsiveness. The demonstration of a loss of function *in vitro*, as assessed by the lack of effect of capsaicin in treated animals, without a detectable loss in neuropeptide content, questions the involvement of the release of sensory neuropeptides in the PAF-induced increase in airways responsiveness observed *in vivo*.

We have also demonstrated in the present study that PAF induces an influx of inflammatory cells (both neutrophils and eosinophils) in the lung as assessed by BAL. We have also shown that this influx is inhibited in rabbits treated with capsaicin, an effect associated primarily with an inhibition of neutrophil accumulation. Whether this inhibition of neutrophil accumulation is responsible for the inhibition of the PAF-induced bronchial hyperresponsiveness by capsaicin remains to be established. Our previous work has suggested that both PAF (Coyle *et al.*, 1990a) and allergen-induced (Coyle *et al.*, 1990b) bronchial hyperresponsiveness in the rabbit are dependent upon platelet activation and associated with the eosinophil rather than neutrophil accumulation. Furthermore, PAF and allergen-induced eosinophil infiltration are platelet-dependent, thus making it unlikely that the effects of capsaicin are mediated by alterations in platelet function, as this would have also resulted in a reduction in eosinophil infiltration, a finding not consistent with our present data with capsaicin-treated rabbits. Similarly, platelet depletion (Lellouch-Tubiana *et al.*, 1988), but not capsaicin-treatment (Ladenius & Biggs, 1989), inhibited antigen-induced pulmonary eosinophilia in allergic guinea-pigs.

The finding that capsaicin treatment failed to inhibit either allergen-induced influx of eosinophils in guinea-pig lung (Ladenius & Briggs, 1989), or PAF-induced pulmonary eosinophil accumulation in the rabbit (present study) suggest that eosinophil recruitment *per se* may not be responsible for the expression of bronchial hyperresponsiveness. This is supportive of data obtained in other species (Aoki *et al.*, 1988; Sanjar *et al.*, 1990). However, the activation status of the eosinophils in this study was not evaluated, and thus we cannot rule out the possibility that as we (Coyle *et al.*, 1990a, b) and others (Frigas & Gleich, 1986) have previously proposed, the eosinophil may still be important for the expression of PAF-induced bronchial hyperresponsiveness in the rabbit via the release of cytotoxic mediators. Nonetheless, the apparent dissociation between eosinophil accumulation and bronchial hyperresponsiveness suggested by our present data is also supported by other recent experimental results showing that in guinea-pigs, cytokines can cause pulmonary eosinophilia without bronchial hyperresponsiveness (Chapman *et al.*, 1990) and in man, chronic eosinophilic bronchitis is not associated with hyperresponsiveness (Gibson *et al.*, 1989).

In summary, the present results suggest that PAF-induced bronchial hyperresponsiveness and the infiltration of inflammatory cells (in particular neutrophils) may be inhibited by capsaicin pretreatment, but that these inhibitory effects may be independent of depletion of sensory neuropeptides.

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Stereoselective effects of the enantiomers of bupivacaine on the electrophysiological properties of the guinea-pig papillary muscle

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1 Direct myocardial effects of the S(−)- and R(+)-enantiomers of bupivacaine were compared in the guinea-pig isolated papillary muscle by recording transmembrane action potentials with the standard microelectrode technique.

2 In 5.4 mM K^+, at a stimulation rate of 1 Hz, the maximal rate of depolarization ($\dot{V}_{max}$) was reduced to 59.9 ± 1.4% ($n = 10$) of control (mean ± s.e.mean) in the presence of 10 μM R(+)-bupivacaine, and to 76.7 ± 1.2% ($n = 14$) in the presence of the same concentration of S(−)-bupivacaine. This was mainly due to a difference in time constant at which block dissipated during the diastolic period. Recovery was slower in the presence of R(+)-bupivacaine. The slower recovery in the presence of R(+)-bupivacaine resulted also in a more pronounced frequency-dependent block of $\dot{V}_{max}$.

3 Time constants for recovery from use-dependent block became significantly faster for both enantiomers on hyperpolarization, while no significant change was observed at depolarization. At all membrane potentials recovery was slower in the presence of R(+)-bupivacaine.

4 The action potential duration (APD) was shortened to a greater extent in the presence of R(+)-bupivacaine over a large range of stimulation frequencies.

5 We conclude that S(−)-bupivacaine affects $\dot{V}_{max}$ and APD in the guinea-pig papillary muscle less than the R(+)-enantiomer at different rates of stimulation and resting membrane potentials.

Keywords: Bupivacaine; enantiomers; stereoselectivity; heart; guinea-pig papillary muscle; standard microelectrode technique; maximal rate of depolarization; $\dot{V}_{max}$

Introduction

Bupivacaine (Marcaine) is a commonly used drug for regional anaesthesia. During the last decade, several reports described cases of severe, even fatal, cardiotoxicity (sudden cardiovascular collapse) after presumed accidental intravascular injection of bupivacaine (Albright, 1979), or after administration of large overdoses of bupivacaine (Covino, 1986).

The mechanism of the cardiotoxicity of bupivacaine is complex. In several studies the observed cardiotoxicity was not due to a direct effect on the heart, but indirectly to a blockade of the sympathetic cardiac innervation (Hotvedt *et al.*, 1983), or to a central nervous system-mediated mechanism with potentially fatal arrhythmias (Thomas *et al.*, 1986). In many other studies *in vivo* (by injection *in situ*; intracoronary, intraatrial) and *in vitro*, a direct cardiotoxicity was shown with conductance disturbances and severe negative inotropic effects. In several studies, animals died most often of ventricular arrhythmias or ventricular fibrillation preceded by a progressive widening of the QRS interval (Kotelko *et al.*, 1984; Hotvedt *et al.*, 1985; Rosen *et al.*, 1985; Nath *et al.*, 1986). But in some other studies, animals died in complete cardiodepression without rhythm disturbances (Liu *et al.*, 1982).

Numerous papers have shown that bupivacaine is much more cardiotoxic than lignocaine. The ratio of the potencies to depress cardiac contractility by bupivacaine and lignocaine is analogous to the anaesthetic potency ratio (*in vivo* 4–5 : 1) (Nath *et al.*, 1986; Buffington, 1989) and this ratio is too small to explain the observed large differences in cardiotoxicity. The much larger potency ratio required to depress cardiac conduction (16 : 1 to affect seriously QRS or PR conduction times and cause severe dysrhythmias like ventricular fibrillation) seems to be the primary mechanism (Komai & Rusy, 1981; Nath *et al.*, 1986). Bupivacaine is much more arrhythmogenic than lignocaine. Bupivacaine has been shown to cause Purkinje fibre-ventricular muscle conduction block in rabbit heart preparations *in vitro* at a concentration of 10–15 μM (Moller & Covino, 1988).

Clinically used bupivacaine is a racemic mixture of S(−)-bupivacaine and R(+)-bupivacaine. Previous studies (Luduena *et al.*, 1972; Aberg, 1972; Aps & Reynolds, 1978) demonstrated that the potency and duration of the anaesthesia *in vivo* were equal or in some cases larger for S(+)-bupivacaine than for the R(+)-enantiomer. Furthermore S(−)-bupivacaine was less toxic than R(+)-bupivacaine: the LD_{50} was about 30 to 40% lower for R(+)-bupivacaine when administered intravenously in mice, rat, and rabbit or subcutaneously in mice and rat (Luduena *et al.*, 1972; Aberg, 1972). The mechanism of the observed difference in the *in vivo* toxicity of the enantiomers of bupivacaine has never been examined. As stereoselective effects of class I antiarrhythmics, which are also local anaesthetics, have been reported (Carmeliet *et al.*, 1986; Block *et al.*, 1988; Hill *et al.*, 1988), we have explored whether the observed difference in the *in vivo* toxicity between the two enantiomers could be attributed to a different potency on the heart. Since the cardiotoxicity of bupivacaine seems to be mainly of electrophysiological origin, we have studied the effects of its enantiomers on the electrophysiological properties of guinea-pig isolated papillary muscle at different membrane potentials and stimulation frequencies.

Methods

Guinea-pigs (300–500 g) of either sex were killed by a blow on the head, the hearts were quickly removed and transferred to Tyrode solution of the following composition (mM): NaCl 127, $NaHCO_3$ 23.8, KCl 5.4, $MgCl_2$ 0.5, $CaCl_2$ 1.8 and glucose 5. The solution was gassed with 95% O_2 and 5% CO_2 and had a pH of 7.4. Papillary muscles of the right ventricle were dissected and fixed to the bottom of a perfusion bath by thin

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cotton threads. The bath (volume 0.25 ml) was perfused with Tyrode solution at a constant rate (2.7 ml min^{-1}). The preparation was allowed to recover for 2 h during which stimuli (0.3 ms in duration; intensity: 1.5 times threshold) were applied at a rate of 1 Hz via bipolar Ag–AgCl electrodes by a programmable stimulator. Transmembrane potentials were recorded with an intracellular and extracellular microelectrode (filled with 3 M KCl, resistance 1.0–1.5 10^7 Ohm), and displayed on a Tektronix 5441 storage oscilloscope. The maximal rate of depolarization ($\dot{V}_{max}$) was measured by electronic differentiation with a bandwidth from 0 to 10 kHz. The action potential duration (APD) was measured directly from the oscilloscope by extrapolating the terminal slope of the action potential to the resting membrane potential. APD_{90} and APD_{50} are the APD after 90 and 50% repolarization, respectively.

In all experiments the preparation was stimulated at 1 Hz and perfused with normal Tyrode solution, containing 5.4 mM external K^+ unless otherwise stipulated. The experiments were only evaluated when the microelectrode impalement was stable during the whole duration of the experiment and when $\dot{V}_{max}$ did not change with the stimulation intensity or duration.

The enantiomers of bupivacaine HCl were kindly provided by Astra Belgium. Concentrated stock solutions were made in twice distilled water and kept in a freezer at −20°C. Drug solutions were made as needed by appropriate dilution.

Concentration-effect relationships were recorded by increasing the drug concentration stepwise from 0.3 μM to 80 μM. Each concentration was applied for 15 min and the diverse action potential parameters were measured at the end of these periods.

Tonic block was defined as the percentage decrease of the first $\dot{V}_{max}$ after a long period of rest (5 min) in presence of the drug, as compared with the control in the same conditions. Phasic block was defined as the further decrease in $\dot{V}_{max}$ as stimulation was continued at 1 Hz, calculated relative to the first $\dot{V}_{max}$ in control conditions.

To record the recovery process of $\dot{V}_{max}$, the preparation was stimulated at a rate of 60 min^{-1}. Rest periods of increasing duration were applied, after which the preparation was again stimulated at 1 Hz until steady state. The $\dot{V}_{max}$ of the first action potential after each rest period was noted as a function of the time of rest. The rest period was gradually increased until no further increase of the first $\dot{V}_{max}$ after the rest period could be obtained. For recovery periods shorter than 1 s a method with extrapulses was used. Every 15th pulse an extrastimulus was applied, first as close as possible to the preceding action potential, then the coupling interval gradually was increased. The $\dot{V}_{max}$ of the extra evoked action potential was noted as a function of the coupling interval.

Inactivation curves were obtained by increasing stepwise the external K^+ concentration from 2.7 mM to 21 mM with exactly 5 min interval and noting $\dot{V}_{max}$ as a function of the resting membrane potential.

Results were fitted by non linear regression: using the sum of 2 exponentials for recovery experiments, and the Boltzmann equation for inactivation curves. Results in tables and figures are indicated by mean ± standard error of mean (s.e.mean). Statistics were calculated by the two tailed Student's t test; $P < 0.05$ was considered as statistically significant. When more than two means had to be compared we made use of analysis of variance (ANOVA). ANOVA was followed by multiple pairwise comparisons, based on Bonferroni's t test where the significance level is set at $P < 0.05/m$ (where m = the number of simultaneous comparisons).

Results

General description of the effects on the action potential

Typical action potential recordings in the presence of 10 μM S(−)-bupivacaine (a) and R(+)-bupivacaine (b) are shown in Figure 1. Both enantiomers caused a reduction of the maximal rate of rise ($\dot{V}_{max}$), action potential amplitude (APA) and action potential duration (APD). No effect on the resting membrane potential was observed. The reduction of $\dot{V}_{max}$ as well as APD was more pronounced with R(+)-bupivacaine than with S(−)-bupivacaine.

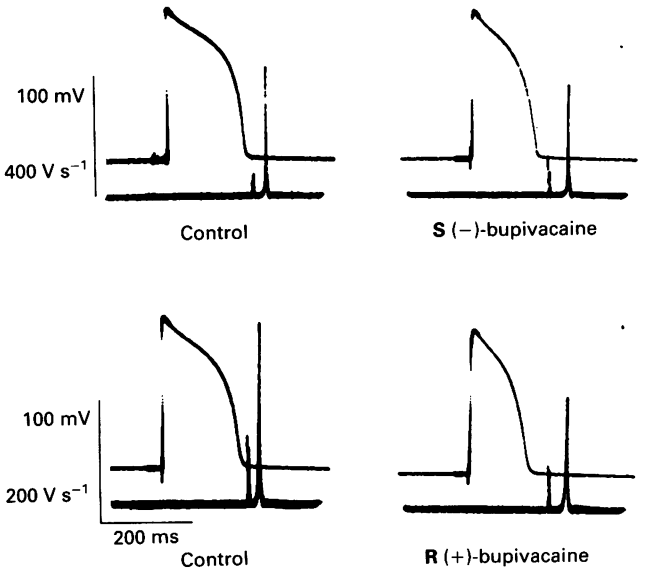

Figure 1 Action potential and $\dot{V}_{max}$ in control conditions and 45 min after superfusion with the 2 enantiomers of bupivacaine 10 μM. The guinea-pig papillary muscle was stimulated at 1 Hz. External K^+ was 5.4 mM. Both enantiomers reduced $\dot{V}_{max}$ and shortened the action potential. The maximal rate of depolarization was clearly more reduced by the R(+)-enantiomer of bupivacaine.

Concentration-effect relationships Concentration-effect curves for the different parameters were made at a stimulation frequency of 1 Hz. Although the number of experiments was restricted, the concentration-effect curve for $\dot{V}_{max}$ (Figure 2) showed statistically significant differences between the two enantiomers at all concentrations; R(+)-bupivacaine caused a more marked reduction of $\dot{V}_{max}$ than S(−)-bupivacaine. The concentration-effect curve for R(+)-bupivacaine had a mean K_d value of 16.1 ± 0.2 μM and a Hill coefficient of 0.86 ± 0.06 ($n = 3$) while for S(−)-bupivacaine we obtained a K_d value of 38.7 ± 2.7 μM and a Hill coefficient of 1.04 ± 0.05 ($n = 4$).

The effect of the drug on APD was less pronounced. At a concentration of 80 μM, the APD was reduced to about 70% for both enantiomers. The concentration-effect curve for the APD showed no statistically significant differences between the two enantiomers. For the rest of the experiments a concentration of 10 μM was chosen.

Steady state effects of 10 μM bupivacaine To obtain steady state values a wash-in period of 45 min was needed. Table 1 shows the mean steady state values for the different action potential parameters in control and in the presence of either enantiomer.

At a concentration of 10 μM and a stimulation frequency of 1 Hz, $\dot{V}_{max}$ was about 77% of control in the presence of S(−)-bupivacaine while $\dot{V}_{max}$ amounted to about 60% in the presence of R(+)-bupivacaine. The difference in potency was statistically significant.

We also observed statistically significant differences in effect on the action potential amplitude (APA), which was also more depressed in the presence of the dextrorotatory isomer of bupivacaine.

In contrast to the results obtained after 15 min perfusion (concentration-effect curves, Figure 2b), the APD and APD_{90} after 45 min perfusion were significantly more shortened with R(+)-bupivacaine than in the presence of S(−)-bupivacaine (two-tailed Student's unpaired t test). In the presence of R(+)-

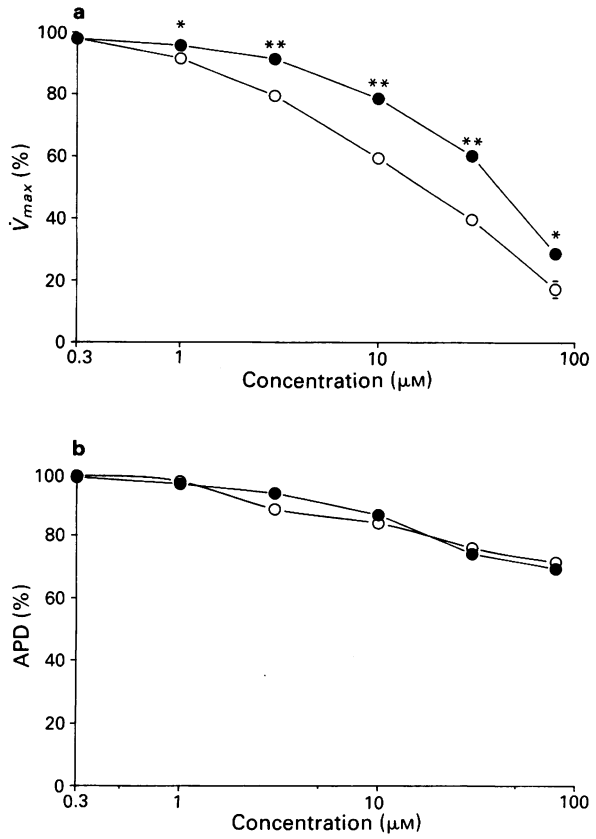

Figure 2 $\dot{V}_{max}$ (a) and APD (b) relative to the control as a function of the concentration of R(+)-bupivacaine (○, $n = 3$) and S(−)-bupivacaine (●, $n = 4$) in guinea-pig papillary muscles. The preparation was stimulated at 1 Hz in 5.4 mM K^+. The control $\dot{V}_{max}$ and APD were $345 \pm 17\,V\,s^{-1}$ and 169 ± 8 ms for R(+)-bupivacaine, and $330 \pm 1\,V\,s^{-1}$ and 171 ± 5 ms for the S(−)-enantiomer. S.e.mean are indicated by vertical bars when they are larger than the symbols. Statistical significance of the difference between the 2 enantiomers is indicated by asterisks: $*P < 0.05$; $**P < 0.01$.

bupivacaine APD was reduced to $76.5 \pm 2.5\%$ ($n = 11$) of control and to $83.4 \pm 1.5\%$ ($n = 14$) in the presence of S(−)-bupivacaine.

Tonic and phasic block of $\dot{V}_{max}$ At a stimulation frequency of 1 Hz and an external potassium concentration of 5.4 mM, the total block of $\dot{V}_{max}$ consisted of tonic and use-dependent block. In these conditions phasic block was more pronounced than tonic block. Tonic block was $7.7 \pm 0.8\%$ ($n = 5$) for S(−)-bupivacaine and $10.7 \pm 1.9\%$ ($n = 5$) for the R(+)-enantiomer, results that were not statistically significantly different. Tonic block disappeared with hyperpolarization (2.7 mM K^+) and increased with depolarization (8.1 mM K^+). In 2.7 mM K^+, tonic block was $-1.9 \pm 1.6\%$ ($n = 4$) for S(−)-bupivacaine and $2.8 \pm 2.8\%$ ($n = 2$) for R(+)-bupivacaine; in 8.1 mM K^+, tonic block increased to $34 \pm 3\%$ ($n = 3$) and $47 \pm 2\%$ ($n = 2$), respectively.

In 5.4 mM K^+ phasic block was larger for R(+)-bupivacaine [$30.3 \pm 1.6\%$ ($n = 5$)] than for S(−)-bupivacaine [$16.5 \pm 1.3\%$ ($n = 5$)], which was statistically significant.

In order to examine further the difference in potency, time-dependent and potential-dependent characteristics of both drugs were studied.

Frequency and time-dependent effects

$\dot{V}_{max}$*-stimulation interval relationship* Figure 3(a) shows the relationship between $\dot{V}_{max}$ and the stimulation interval. In control conditions, diminishing the stimulation interval from 10 s to 0.25 s caused a 10% decrease of $\dot{V}_{max}$, presumably due to a slow component in the inactivation of the sodium current. Bupivacaine reduced the $\dot{V}_{max}$ at all stimulation intervals. At an interval of 10 s, $\dot{V}_{max}$ was on average $85.4 \pm 1.7\%$ ($n = 5$) for S(−)-bupivacaine and $82.6 \pm 1.4\%$ ($n = 5$) for R(+)-bupivacaine when compared with the control. The reduction of $\dot{V}_{max}$ was quite constant at stimulation intervals between 2 to 10 s in the presence of S(−)-bupivacaine and between 4 to 10 s in the presence of R(+)-bupivacaine. Only when the stimulation interval was diminished from 2 to 0.25 s did the effect on $\dot{V}_{max}$ become more pronounced. At an interval of 0.25 s, $\dot{V}_{max}$ was decreased to about $29.0 \pm 0.9\%$ ($n = 5$) for S(−)-bupivacaine and to about $16.9 \pm 1.8\%$ ($n = 5$) for R(+)-bupivacaine compared to the control at a stimulation interval of 10 s. The difference in block between the two enantiomers became relatively larger as the stimulation interval was shortened: at an interval of 0.25 s, $\dot{V}_{max}$ in the presence of R(+)-bupivacaine was 1/5 of the $\dot{V}_{max}$ at an interval of 10 s, while in presence of the S(−)-enantiomer this ratio was only 1/3.

The difference in effect between the two enantiomers was statistically significant for stimulation intervals between 2 s and 0.25 s (frequencies between 30 min^{-1} and 240 min^{-1}). At longer stimulation intervals this difference was not statistically significant. These experiments confirm that in 5.4 mM K^+ the difference in effect of the 2 enantiomers on $\dot{V}_{max}$ is due to a

Table 1 Steady state effects of bupivacaine (10 μM)

	$\dot{V}_{max}$ ($V\,s^{-1}$) mean ± s.e.mean	(n)	*APA* (mV) mean ± s.e.mean	(n)	*Em* (mV) mean ± s.e.mean	(n)
Control	329 ± 12	(14)	127.5 ± 0.3	(14)	−88.7 ± 0.7	(14)
S(−)-bupivacaine	253 ± 12	(14)	124.8 ± 0.3	(14)	−88.6 ± 0.7	(14)
%	76.7 ± 1.2**	(14)	97.9 ± 0.2*	(14)		
Control	320 ± 15	(11)	126.4 ± 0.5	(11)	−88.7 ± 0.8	(11)
R(+)-bupivacaine	193 ± 9	(10)	122.7 ± 0.6	(11)	−88.6 ± 0.8	(11)
%	59.9 ± 1.4**	(10)	97.1 ± 0.3*	(11)		
	APD (ms) mean ± s.e.mean	(n)	APD_{90} (ms) mean ± s.e.mean	(n)	APD_{50}(ms) mean ± s.e.mean	(n)
Control	180 ± 4	(14)	173 ± 4	(14)	150 ± 4	(14)
S(−)-bupivacaine	150 ± 4	(14)	142 ± 5	(14)	115 ± 4	(14)
%	83.4 ± 1.5*	(14)	82.0 ± 1.6*	(14)	76.2 ± 1.7	(14)
Control	180 ± 5	(11)	173 ± 5	(11)	148 ± 4	(11)
R(+)-bupivacaine	137 ± 4	(11)	129 ± 4	(11)	104 ± 4	(11)
%	76.5 ± 2.5*	(11)	75.1 ± 2.5*	(11)	70.9 ± 2.8	(11)

Stimulation frequency: 60 min^{-1}. $[K^+]_o = 5.4$ mM
Student's unpaired t test between S(−)- and R(+)-bupivacaine: $*P < 0.05$; $**P < 0.001$.

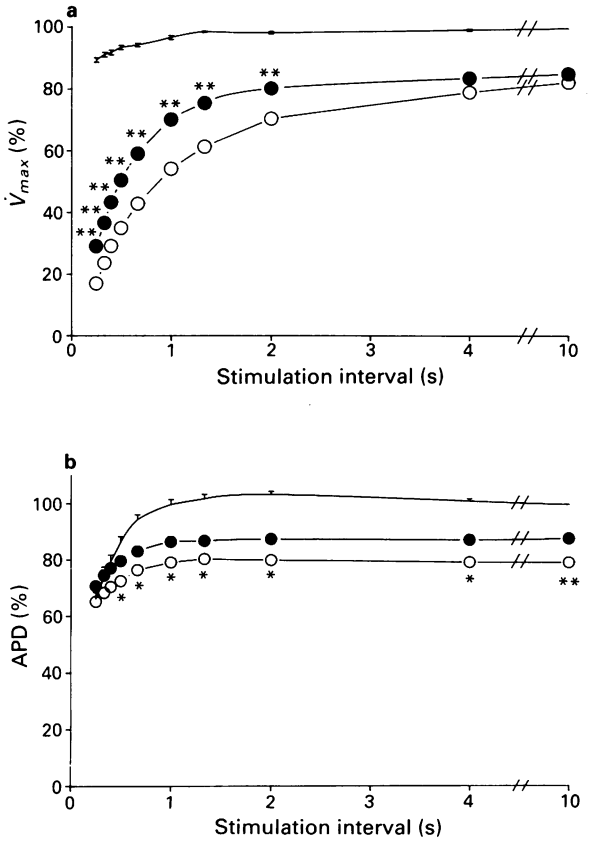

Figure 3 Effect of stimulation interval on $\dot{V}_{max}$ (a) and APD (b) in guinea-pig papillary muscles. Relative change in $\dot{V}_{max}$ and APD as a function of the stimulation interval in control conditions (curve without symbols), and in the presence of 10 μM **R**(+)-bupivacaine (○) and 10 μM **S**(−)-bupivacaine (●). The preparation was bathed in 5.4 mM K^+. The control value at a stimulation interval of 10 s was taken as 100% : 313 ± 6 $V s^{-1}$ and 178 ± 6 ms for **R**(+)-bupivacaine, 322 ± 18 $V s^{-1}$ and 176 ± 7 ms for **S**(−)-bupivacaine. The number of experiments was 5 for both enantiomers for the effect on $\dot{V}_{max}$ and 4 for the effect on the APD. S.e.mean are indicated by vertical bars when they are larger than the symbols. Repeated measures ANOVA revealed significant differences between the two enantiomers both for $\dot{V}_{max}$ and the APD. Statistical significance of the difference between the two enantiomers is indicated with asterisks; $*P < 0.05$; $**P < 0.01$ (Student's *t* test).

difference in use-dependent block rather than a difference in tonic block.

APD-stimulation interval relationship Figure 3(b) shows the effect of the two enantiomers of bupivacaine on the action potential duration at different stimulation intervals. A stepwise decrease in the interval from 10 s to 0.25 s in control conditions caused a gradual shortening of the APD to about 66% of its value at 10 s. At a stimulation interval of 10 s, APD was 87.8 ± 1.3% ($n = 4$) of the control value in the presence of **S**(−)-bupivacaine and 79.3 ± 1.0% ($n = 4$) in the presence of **R**(+)-bupivacaine. At simulation intervals from 10 to 0.4 s, both enantiomers decreased APD. At intervals of 0.3 and 0.25 s, the APD in the presence of **S**(−)-bupivacaine and **R**(+)-bupivacaine was not significantly different from the one obtained in control conditions (two tailed Student's paired *t* test). At a stimulation interval of 0.25 s, APD was 103.5 ± 1.0% ($n = 3$) of the control for **S**(−)-bupivacaine and 96.7 ± 5.4% ($n = 3$) for **R**(+)-bupivacaine. The action potential duration appeared to be less affected at short stimulation intervals than at long stimulation intervals.

At all intervals the action potential duration was less in the presence of **R**(+)-bupivacaine than in the presence of **S**(−)-bupivacaine but the difference was only statistically significant at stimulation intervals greater than 0.4 s.

Onset and recovery kinetics of/from use-dependent block of $\dot{V}_{max}$

Onset kinetics: When the preparation was stimulated at a frequency of 1 Hz in 5.4 and 8.1 mM K^+ after a long period of rest (5 min), the $\dot{V}_{max}$ of the first action potential was smaller in presence of the drug than in control conditions (cf. Figure 4). For the second action potential, $\dot{V}_{max}$ was much lower than the first for both enantiomers of bupivacaine and continued stimulation did not cause a significant further decrease of $\dot{V}_{max}$. Therefore the rate at which the use-dependent block developed after a rest period was too fast to be fitted. We did not record onset at higher rates of stimulation.

Recovery kinetics: The kinetics at which $\dot{V}_{max}$ reprimed during a rest period could be fitted as a sum of two exponentials; a fast one representing the recovery of the drug free channels, and a slower one representing the recovery of the drug occupied channels. Figure 4 (right panels) shows typical recovery patterns for **S**(−)-bupivacaine and **R**(+)-bupivacaine at different external K^+ concentrations. Table 2 summarizes the fast and slow recovery time constants at different external

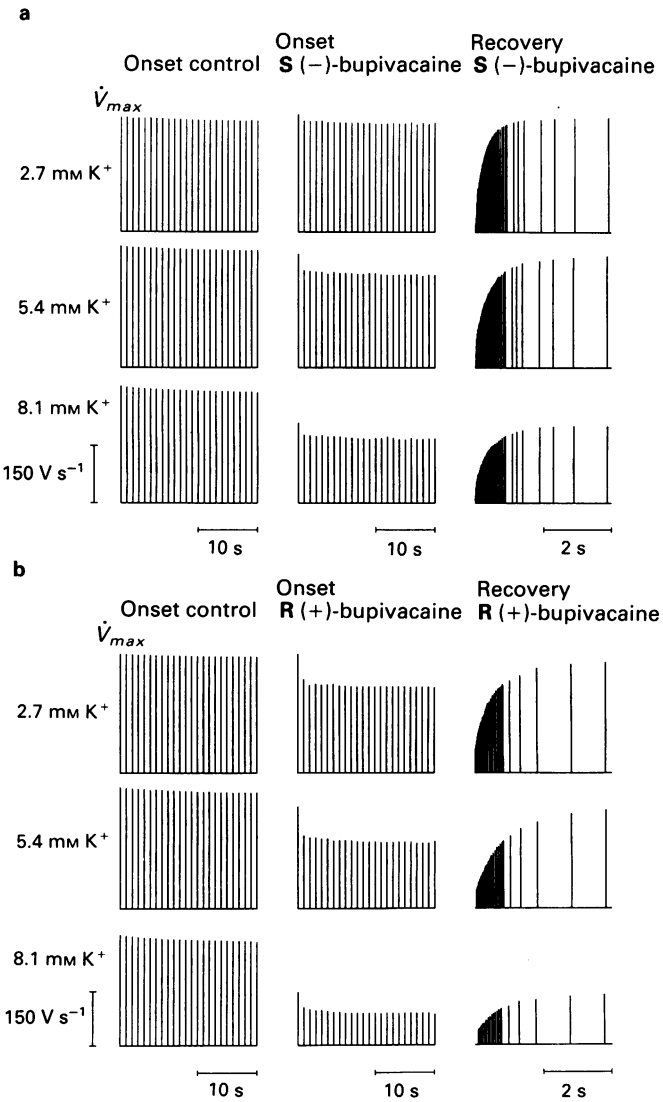

Figure 4 Onset of rate-dependent block of $\dot{V}_{max}$ in control conditions (left), and onset and recovery (middle and right panel) in presence of 10 μM **S**(−)-bupivacaine (a) and **R**(+)-bupivacaine (b) in different external K^+ concentrations for a typical experiment on guinea-pig papillary muscles. For onset, $\dot{V}_{max}$ is plotted as a function of time when the preparation is stimulated after 5 min of rest. For recovery, rest periods of variable duration were applied and the $\dot{V}_{max}$ of the first action potential after each period of rest is plotted as a function of the time of rest. Basic frequency was 1 Hz. Tonic block was larger and recovery slower for the **R**(+)-enantiomer. Tonic block increased with depolarization for both enantiomers.

Table 2 Recovery time constants of $\dot{V}_{max}$ at different K^+-concentrations

	K^+ 2.7 mM		K^+ 5.4 mM		K^+ 8.1 mM	
	mean ± s.e.mean	(*n*)	mean ± s.e.mean	(*n*)	mean ± s.e.mean	(*n*)
S(−)-bupivacaine						
fast (ms)	30 ± 11	(3)	19 ± 5	(5)	25 ± 2	(4)
slow (ms)	365 ± 19*	(5)	560 ± 28**	(8)	533 ± 20**	(5)
R(+)-bupivacaine						
fast (ms)	8	(1)	14 ± 2	(4)	30	(1)
slow (ms)	585 ± 83*	(3)	981 ± 86**	(7)	842 ± 42**	(4)

Concentration bupivacaine: 10 μM. Basic frequency: 60 min^{-1}.
Repeated measures ANOVA on time constants obtained in experiments where effects of differing K^+ concentration were measured in the same preparation, revealed significant differences between enantiomers and significant effects of changing K^+ concentration. Comparison between S(−)- and R(+)-bupivacaine: *$P < 0.05$; **$P < 0.001$ (Student's *t* test). Multiple pairwise comparisons between time constants in the different K^+ concentrations revealed that recovery in 2.7 mM K^+ was faster than in 5.4 mM K^+ for each enantiomer (Bonferroni *t* test).

K^+ concentrations. At all K^+ levels, recovery of $\dot{V}_{max}$ was significantly slower in the presence of R(+)-bupivacaine than in the presence of S(−)-bupivacaine. In 5.4 mM K^+, the slow phase had a time constant of about 981 ms in the presence of R(+)-bupivacaine against about 560 ms for S(−)-bupivacaine.

When hyperpolarizing the membrane by superfusion with 2.7 mM $[K^+]_o$, the recovery became significantly faster (τ = 585 ms for R(+)-bupivacaine and 365 ms for S(−)-bupivacaine) while no significant change was observed during depolarization with 8.1 mM $[K^+]_o$ (see Figure 4).

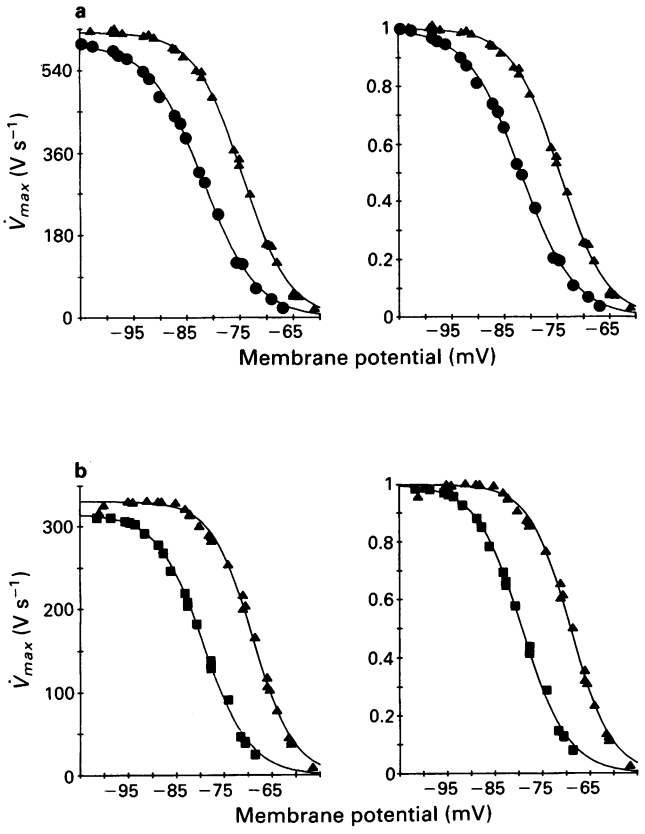

Figure 5 Inactivation curve of $\dot{V}_{max}$ in guinea-pig papillary muscle. Typical inactivation curves at a frequency of 0.8 min^{-1} in control conditions (▲), in the presence of 10 μM S(−)-bupivacaine (a, ●) and in the presence of 10 μM R(+)-bupivacaine (b, ■). In the right part of the figure, inactivation curves are normalized. In presence of the drug, $\dot{V}_{max}$ was depressed at all resting membrane potentials except at very negative potentials, and the inactivation curve was shifted to more negative membrane potentials. Inactivation curves were more shifted by the R(+)-enantiomer.

Voltage dependence of the effects on $\dot{V}_{max}$

Figure 4 showed an increase of tonic block with depolarization, suggesting voltage-dependence for both enantiomers. The voltage-dependence of the effect on $\dot{V}_{max}$ was investigated by recording inactivation curves.

To avoid time-dependent effects, a very low stimulation frequency (0.8 min^{-1}) was applied. Figure 5 shows typical inactivation curves. At all potentials the $\dot{V}_{max}$ was depressed except at very negative membrane potentials; in 2.7 mM K^+, $\dot{V}_{max}$ was 98.6 ± 1.9% (n = 4) of the control for S(−)-bupivacaine and 99.2 ± 1.8% (n = 3) for R(+)-bupivacaine.

Both enantiomers shifted the inactivation curves to more negative potentials. R(+)-bupivacaine shifted the half maximal potential with −11.1 ± 0.7 mV (n = 3) to more negative potentials, while S(−)-bupivacaine caused a shift of −8.1 ± 0.5 mV (n = 4). The difference between the two enantiomers was statistically significant. There was no statistically significant difference between the two enantiomers in the slope of the inactivation curves.

Discussion

The present study provides strong evidence that the interaction of bupivacaine with cardiac cell membranes is dependent upon the stereochemical conformation of the drug. R(+)-bupivacaine causes a stronger reduction of $\dot{V}_{max}$ at different stimulation rates and resting membrane potentials. This could be at the basis of the difference in lethal toxicity between R(+)-bupivacaine and the S(+)-enantiomer, previously reported (Luduena *et al.*, 1972; Aberg, 1972).

A mean apparent dissociation constant, K_d of 16 μM was found for the reduction of $\dot{V}_{max}$ at 1 Hz by R(+)-bupivacaine versus a K_d of 39 μM for S(−)-bupivacaine. These K_d values are very similar to the dissociation constants calculated by Leeson *et al.* (1989) for the inhibition of the veratridine effect on frog sciatic nerve and the reduction of I_{Na} in nerves (12 μM for R(+)-bupivacaine and 42 μM for S(−)-bupivacaine). Conversely, the anaesthetic potency *in vivo* of S(−)-bupivacaine seems to be larger or equal (Luduena *et al.*, 1972). Another study on isolated frog nerves (Aberg, 1972), reported no difference in nerve-blocking effect between the two enantiomers.

In 5.4 mM K^+, both enantiomers caused tonic block and a marked use-dependent block developed at high stimulation frequencies. Since hyperpolarization completely relieved tonic block in the presence of 10 μM of either enantiomer of bupivacaine, both enantiomers must have a small affinity for channels in the rested state. Since depolarization strongly increased tonic block, we conclude that the observed tonic block was due to binding to the inactivated state (I-block). The marked shift of the inactivation curves at a frequency where all use-

dependent effects are absent, also reveals the I-block. This is in agreement with the results of Clarkson & Hondeghem (1985) who showed in voltage-clamped guinea-pig papillary muscle that bupivacaine is mainly an inactivated state blocker with low affinity for rested and activated sodium channels.

The affinity of the inactivated channel appears to be larger for R(+)-bupivacaine than for S(−)-bupivacaine since the shift of the inactivation curves was more pronounced in the presence of R(+)-bupivacaine. The difference in shift was less striking than the one we obtained with the enantiomers of tocainide (Carmeliet *et al.*, 1986).

At 1 Hz and 5.4 mM K^+, an important amount of use-dependent block was present. Use-dependent block of $\dot{V}_{max}$ results from block of Na^+ channels during the action potential and incomplete unblocking during the diastolic period. R(+)-bupivacaine unblocked significantly slower than did S(−)-bupivacaine at all tested membrane potentials. In 5.4 mM K^+, the time constant for recovery from use-dependent block was 560 ms for S(−)-bupivacaine and 981 ms for R(+)-bupivacaine. This is in agreement with the absence of use-dependent block of $\dot{V}_{max}$ at stimulation intervals larger than 2 or 4 s. The slower unblocking of R(+)-bupivacaine blocked channels explains the larger reduction of $\dot{V}_{max}$ at stimulation intervals lower or equal to 1.3 s.

When cells were hyperpolarized, recovery from use-dependent block became significantly faster, as is also the case with lignocaine. No increase of recovery time was seen upon depolarization. Faster recovery of $\dot{V}_{max}$ upon hyperpolarization could be due to unblocking via a faster transition pathway: from the drug occupied inactivated state, over the drug occupied rested state, to the drug free rested state (ID → RD → R) as suggested by Bean *et al.* (1983) for lignocaine.

In summary, R(+)-bupivacaine has a larger effect on $\dot{V}_{max}$ at different resting membrane potentials and stimulation rates than does S(−)-bupivacaine. Very recently the larger block of the Na^+ current by R(+)-bupivacaine in guinea-pig isolated cardiac myocytes was explained by Valenzuela *et al.* (1990) as being due to differences in the degree of activation unblocking and the magnitude of the voltage shift in gating kinetics of drug associated channels. Activation unblocking would be lower and voltage shift would be larger for R(+)-bupivacaine.

As far as the action potential duration is concerned, we did not observe any significant difference in effect of the two enantiomers after 15 min; however after 45 min, when complete steady state conditions are achieved, R(+)-bupivacaine caused a more pronounced shortening of the action potential than its enantiomer. Shortening of the action potential is presumably due to a similar mechanism as described by Carmeliet & Saikawa (1982) for lignocaine, namely inhibition of the slowly inactivating Na^+ current. As R(+)-bupivacaine has a larger blocking effect on $\dot{V}_{max}$, which is a measure for the fast Na^+ current, one can speculate that it has presumably also a larger effect on the slowly inactivating Na^+ current and, as a consequence, the effect on APD would be more pronounced.

In contrast to the effect on $\dot{V}_{max}$, both enantiomers reduced the APD more at lower stimulation rates than at higher stimulation rates; at the highest frequencies the APD was not statistically different from the control APD. This finding is analogous to that of Varro *et al.* (1986) for different class I antiarrhythmic drugs. The exact reason for this phenomenon is not known. One possible explanation is that since the amplitude of the slow Na^+ current is less pronounced at higher frequencies, the slow Na^+ current contributes less to APD at higher frequencies and so drugs blocking this current would have less influence on APD at higher frequencies. However, block of i_K (delayed potassium current) could also play a role.

The fact that R(+)-bupivacaine has a greater effect on $\dot{V}_{max}$ and APD is possibly the explanation for the observed higher *in vivo* toxicity. Reduction of $\dot{V}_{max}$ slows conduction. Too much slowing of conduction and shortening of the APD predisposes for reentrant phenomena. Since S(−)-bupivacaine has less influence on the heart *in vitro* (present results) and has a higher LD_{50} value, but shows a comparable anaesthetic potency *in vivo*, clinical use of only the S(−)-enantiomer of bupivacaine in analogy to ropivacaine (congener of bupivacaine and optically pure S-enantiomer) could improve the margin of safety of this widely used drug. Of course, other parameters such as pharmacokinetics and overall pharmacodynamic activity also have to be considered. Protein binding, tissue binding, clearance, uptake in heart and brain, convulsive toxicity, negative inotropic effect should all be considered for both enantiomers.

Our results with the enantiomers of bupivacaine, having identical physicochemical properties, favour the hypothesis that a specific receptor site at the sodium channel must be involved in local anaesthetic action rather than a non specific effect of disturbing the membrane.

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Announcement

Sixth Southeast Asian/Western Pacific Regional
Meeting of Pharmacologists

30th June–4th July 1991
Hong Kong

Organised by The Hong Kong Pharmacology Society

Pharmacology for Better Therapeutics

Topics: Anti-inflammatory agents, autacoids, cardiovascular pharmacology, chemotherapy, clinical pharmacology, drug metabolism, endocrine pharmacology, gastrointestinal pharmacology, haematoergical pharmacology, herbal medicine, hormones, immunopharmacology, neuropharmacology, pharmacokinetics, receptors, renal pharmacology, signal transduction, toxicology, vasoactive agents.

Venue: Hong Kong Convention and Exhibition Centre, Wanchai, Hong Kong

Conference Secretariat: The Travel Advisers Ltd, Room 1006, Silvercord Tower I, 30 Canton Road, Kowloon, Hong Kong. Tel: (852) 369 8321, Fax: (852) 369 1078

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   —to include Key words (10 maximum) at end of summary.
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