British Journal of Pharmacology

June 1991

Volume 103 Number 2 pages 1283–1620

Br. J. Pharmacol. (1991), **103**, 1285–1287

SPECIAL REPORT

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Decreased $GABA_A$ receptor subunit mRNA concentrations following chronic lorazepam administration

Inwha Kang & [1]Lawrence G. Miller

Division of Clinical Pharmacology, Depts. of Psychiatry and Pharmacology, Tufts Univ. School of Medicine and New England Medical Center, Boston, MA, U.S.A.

Chronic benzodiazepine administration has been associated with alterations in binding and function at the $GABA_A$ receptor. To evaluate effects of chronic benzodiazepine exposure on messenger RNA (mRNA) concentrations for several $GABA_A$ receptor subunits, we treated mice with lorazepam, 2 mg kg^{-1} daily for 1–28 days and evaluated mRNAs for the alpha1 and gamma2 subunits by Northern hybridization. In cerebral cortex, concentrations of mRNA for the alpha1 and gamma2 subunits were unchanged from vehicle or control after 1–10 days of lorazepam. However, after 14 days of treatment mRNA concentrations for both subunits decreased to approximately 50% of control values and remained decreased at 28 days. In contrast, no significant alterations were observed for either subunit mRNA in hippocampus or cerebellum over the same time course. Alterations in mRNAs in cortex occur after the development of tolerance and receptor downregulation in this model.

Keywords: $GABA_A$ receptor; alpha1 subunit; gamma2 subunit; benzodiazepine; chloride

Introduction Both behavioural and neurochemical alterations have been observed during chronic benzodiazepine administration in animals. For most effects of benzodiazepines, tolerance occurs and may limit the clinical utility of these compounds (Greenblatt & Shader, 1978). Although differing neurochemical alterations have been reported by several investigators, several recent studies indicate decreases in benzodiazepine receptor binding and $GABA_A$ receptor function associated with chronic benzodiazepine administration (Tietz *et al.*, 1986; Miller *et al.*, 1988; 1989; Marley & Gallager, 1989).

Benzodiazepines exert their effects in the central nervous system binding to a specific site located on the $GABA_A$ receptor complex (Olsen & Tobin, 1990). Several subunits of the $GABA_A$ receptor have been cloned and sequenced in the past several years (e.g., Schofield *et al.*, 1987; Pritchett *et al.*, 1989). Although the receptor structure remains uncertain, and indeed may vary among brain regions, it is likely to be a heteroligomeric protein including alpha, beta, gamma, and possibly delta polypeptide subunits (Olsen & Tobin, 1990).

In view of the alterations in receptor binding and function observed during chronic benzodiazepine administration, we evaluated mRNA concentrations for the most abundant subunits, alpha1 and gamma2, in three brain regions to assess their relation to receptor alterations.

Methods *Drug administration* Lorazepam (2 mg kg^{-1} daily) was dissolved in PEG400, placed in Alzet 2001/2002 pumps, and implanted subcutaneously in mice under brief ether anaesthesia. This regimen has been shown to produce constant drug concentrations in plasma and brain, behavioural tolerance, and receptor downregulation (Miller *et al.*, 1988). Control mice received vehicle only.

RNA isolation and Northern hybridization Total RNA was isolated as previously described (Auffray & Rougeon, 1980). RNA was electrophoresed on agarose (1.2%) gels containing 2.2 M formaldehyde, then transferred to nylon membranes. After prehybridization for 1 h, membranes were hybridized at 42°C overnight with ^{32}P-labelled DNA probes (5–10 × 10^6 c.p.m., approximately 3 ng ml^{-1}). After washing at 42°C, membranes were exposed to X-ray film at −80°C for 1–3 days. To test for possible interlane variability and nonspecific effects, membranes were subsequently washed and reprobed with ^{32}P-labelled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA. Based on 18S and 28S ribosomal RNA, alpha1 hydribization occurred at 4.5 kb, gamma2 at 4.4 kb, and GAPDH at 1.1 kb. Results were quantitated by densitometry (LKB Ultroscan) with linearity assessed by ^{32}P standards.

Probes and probe preparation Oligonucleotide probes were synthesized based on the first 45 transcribed bases of the reported alpha1 and gamma2 sequences (Schofield *et al.*, 1987; Pritchett *et al.*, 1989). For GAPDH, a riboprobe was generated from a cDNA human clone provided by Dr D. Chikaraishi. Oligonucleotide probes were 5′ end-labelled with ^{32}P by use of T4 polynucleotide kinase.

Data analysis Results are presented as relative units. For each blot, control RNA from an untreated animal was included. Units were determined as subunit density corrected for GAPDH density (subunit/GAPDH), in turn divided by control density (control/GAPDH). Data were analyzed by analysis of variance with Dunnett's test.

Results Treatment of mice for 1, 2, 4, 7, and 10 days with lorazepam did not alter concentrations of alpha1 subunit mRNA in cortex (Figure 1a, b). However, after 14 days of exposure, mRNA concentrations were decreased to approximately 50% of vehicle or shorter treatment levels. This decrement persisted in animals treated with lorazepam for 28 days. Similar results were observed for the gamma2 subunit in cortex (Figure 1a, b). Concentrations of mRNA were not altered from 1 to 10 days, but were decreased by about 50% after 14 days of lorazepam exposure. This decrement also persisted at 28 days of treatment. Neither alpha1 nor gamma2 subunit mRNAs were altered by vehicle exposure.

In hippocampus, concentrations of mRNA for both the alpha1 and gamma2 subunits were not significantly altered at 14 and 28 days compared to 1 and 7 days of lorazepam administration (Figure 2). In cerebellum, concentrations of mRNA for both alpha1 and gamma2 subunits remained unchanged from 1 to 28 days (Figure 2). In both hippocampus and cerebellum, vehicle administration did not alter either alpha1 or gamma2 mRNA.

Discussion These data indicate that mRNAs for two $GABA_A$ receptor subunits, alpha1 and gamma2, are decreased in

[1] Author for correspondence at: Box 1007, New England Medical Center, 750 Washington St., Boston, MA 02111, U.S.A.

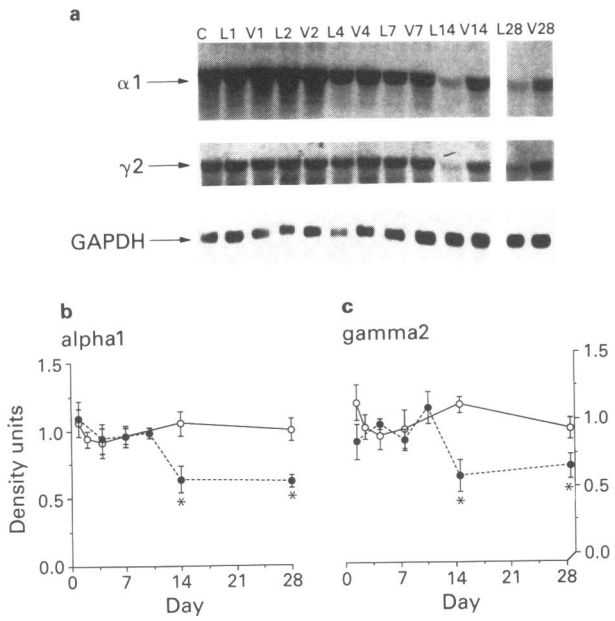

Figure 1 Effects of lorazepam and vehicle administration on alpha1 and gamma2 subunit mRNA concentrations in cortex. Mice were treated with lorazepam (2 mg kg^{-1} daily) or vehicle as described, and mRNA concentrations determined by Northern hybridization. (a) Northern hybridization. Results are from a representative hybridization repeated 2–3 times, from an experiment repeated three times. C = control, L1 = day 1 lorazepam, V1 = day 1 vehicle, etc. (b) Relative mRNA concentrations. The left panel is alpha1 mRNA and the right panel is gamma2 mRNA. Autoradiographs were analyzed by densitometry in arbitrary absorbance units. (○) Vehicle treatment; (●) lorazepam treatment. Results are mean, s.e.mean shown by vertical bars, n = 2-3 determinations each from 3 separate experiments. Units are sample absorbance/GAPDH absorbance. *$P < 0.05$ vs. vehicle. There are no significant differences among vehicle treatments.

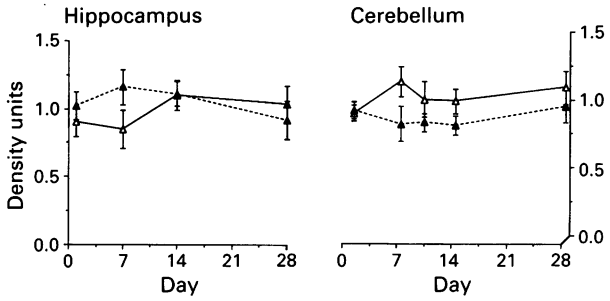

Figure 2 Effects of lorazepam administration on alpha1 and gamma2 subunit mRNA concentrations in hippocampus and cerebellum. Mice were treated with lorazepam (2 mg kg^{-1}, daily) and mRNA concentrations determined by Northern hybridization: (△) alpha1 mRNA; (▲) gamma2 mRNA. Results are mean, s.e.mean shown by vertical bars, n = 2–3 determinations each from 2 separate experiments. Units are sample absorbance/GAPDH absorbance. There are no significant differences among mRNA concentrations.

cortex by chronic lorazepam administration. This effect appears to be due to benzodiazepine exposure: treatment with vehicle only does not produce any changes in mRNA concentrations. In addition, a control mRNA coding for GAPDH is unaffected by lorazepam treatment. Additional evidence for the specificity of this effect derives from regional studies. Concentrations of mRNA for these subunits are decreased in cortex but not hippocampus or cerebellum. It is also notable that mRNA concentrations for the alpha1 and gamma2 subunits are altered in parallel.

For lorazepam at the dose used in this study, receptor binding and function in cortex are decreased after 7 days (Miller *et al.*, 1988), but the mRNA concentrations in cortex remain unchanged at 7 days; decrements do not occur until 14 days. In addition, the regional specificity of changes in binding and receptor function differed from alterations in mRNAs. Benzodiazepine binding and GABA-dependent chloride uptake were decreased in hippocampus and unchanged in cerebellum during chronic lorazepam administration (Miller *et al.*, 1988). Thus, the later onset for changes in mRNA compared to receptor effects may indicate that mRNA alterations do not account for initial receptor downregulation. It is possible that reductions in mRNA concentrations in cortex help to sustain receptor downregulation observed at 14 days. Receptor alterations in the absence of mRNA changes in hippocampus also suggest that genetic and receptor effects are unrelated. An alternative hypothesis is that both binding and mRNA alterations in regions such as hippocampus may be heterogeneous and thus not always evident in regional tissue samples.

One preliminary report has assessed alpha1 mRNA during chronic benzodiazepine administration (Heninger *et al.*, 1989). In this study, rats received diazepam by intermittent injection for 3 weeks. Decreases in alpha subunit mRNA, apparently alpha1, were observed in cortex at this time. Thus, results in a similar but not identical system corroborate the alterations observed in the present study. Our results extend the prior study to the gamma2 subunit, to a detailed time course, and to several brain regions.

A number of neurotransmitter receptors and ion channels exist as multiple subtypes, apparently by the use of distinct genes to encode receptor subunits polypeptides (Olsen & Tobin, 1990). Such heterogeneity may contribute to increased information-processing ability among neurones. Differential regulation of expression of these subunits might also modulate responses to drugs or other stimuli. Our results indicate that several $GABA_A$ receptor subunit mRNAs are affected by chronic administration of a ligand which binds to this receptor. This alteration may help to modulate chronic effects of xenobiotics.

Supported in part by grants DA-05258 and MH-34223 from the U.S. Public Health service. L.G.M. is the recipient of a Faculty Development Award in Clinical Pharmacology from the Pharmaceutical Manufacturers Association Foundation.

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(Received January 30, 1991
Accepted February 13, 1991)

Br. J. Pharmacol. (1991), **103**, 1288–1294 © Macmillan Press Ltd, 1991

Cyclic AMP counteracts mitogen-induced inositol phosphate generation and increases in intracellular Ca^{2+} concentrations in human lymphocytes

*Lambertus J.H. van Tits, *†Martin C. Michel, †Harvey J. Motulsky, **Alan S. Maisel & [1,]*Otto-Erich Brodde

* Biochemical Research Labs, Dept. Medicine, Div. Renal and Hypertensive Diseases, University of Essen, D-4300 Essen, F.R.G. †Dept. of Pharmacology, M-036, University of California, San Diego, La Jolla, CA 92093 and **Cardiology Section 111–A, Veterans Administration Medical Center, San Diego, CA 92161, U.S.A.

1 The effects of increases in intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) on mitogen-induced generation of inositol phosphates and increases in intracellular Ca^{2+} concentration were investigated in human peripheral blood mononuclear leukocytes (MNL).

2 The mitogens concanavalin A (Con A), pokeweed mitogen (PWM) and phytohaemagglutinin (PHA) concentration-dependently stimulated generation of inositol phosphates. Catecholamines inhibited this process with an order of potency: isoprenaline > adrenaline > noradrenaline indicating involvement of β_2-adrenoceptors. This order of potency was also consistent with the catecholamine potencies for stimulating the generation of cyclic AMP.

3 In addition to catecholamines, the cyclic AMP formation-stimulating agents prostaglandin E_1 (PGE_1) and forskolin concentration-dependently inhibited mitogen-induced inositol phosphate generation, too. Moreover, the inhibitory effect of isoprenaline was potentiated by co-incubation with the phosphodiesterase inhibitor isobutylmethylxanthine demonstrating that these inhibitory effects were mediated by cyclic AMP.

4 Con A and PHA concentration-dependently increased the intracellular Ca^{2+} concentration in human MNL (assessed by the fluorescent indicator dye Fura-2). This increase was almost completely blocked by chelation of extracellular Ca^{2+}, demonstrating influx rather than mobilization from intracellular stores.

5 The elevation of intracellular Ca^{2+} was not blocked by pretreatment with pertussis toxin, 100 ng ml^{-1}, for 16 h.

6 Isoprenaline, PGE_1, and forskolin, however, inhibited the mitogen-stimulated elevation of intracellular Ca^{2+}. This inhibition was enhanced by the phosphodiesterase inhibitors isobutylmethylxanthine and Ro 20-1724, demonstrating mediation by cyclic AMP.

7 We conclude that catecholamines and other cyclic AMP increasing agents can inhibit mitogen-stimulated generation of inositol phosphates and elevation of intracellular Ca^{2+} in resting human MNL. Since generation of inositol phosphates and increases in intracellular Ca^{2+} are very early events in activation of MNL, attenuation of these events might well contribute to the inhibitory effect of cyclic AMP on MNL function.

Keywords: Mononuclear leukocytes; cyclic AMP; inositol phosphates; calcium; human lymphocytes; immunoregulation

Introduction

The function of the immune system is regulated by various neurotransmitters and hormones. Of special interest are those agents which elevate lymphocyte adenosine 3':5'-cyclic monophosphate (cyclic AMP) concentrations because they can inhibit lymphocyte function in multiple ways *in vitro* (for reviews see Bourne *et al.*, 1974; Felten *et al.*, 1987; Kammer, 1988). For example, it has been demonstrated that cyclic AMP increasing hormones can inhibit mitogen-stimulated lymphocyte proliferation, antibody formation by B-cells, and cytolytic activity of T-cells. These inhibitory effects of cyclic AMP formation stimulating hormones are mediated by cyclic AMP because they can also be elicited by receptor-independent stimulation of cyclic AMP formation (e.g. by the diterpene forskolin) and by compounds mimicking cyclic AMP (e.g. 8-Br-cyclic AMP). Moreover, some data suggest that cyclic AMP increasing agents can also impair immune function *in vivo*. For example it has been shown that prolonged exposure to β-adrenoceptor agonists can decrease the number of thymocytes in mice (Durant, 1986) and of circulating lymphocytes in man (Maisel *et al.*, 1990).

The activation of resting lymphocytes is a multistep process involving early biochemical alterations such as formation of inositol phosphates and increases in intracellular Ca^{2+} leading to activation of a protein kinase C, which occur during the first seconds and minutes of lymphocyte activation (Tsien *et al.*, 1982; Wolff & Akerman, 1982; Taylor *et al.*, 1984; Sugiura & Waku, 1984; Chen *et al.*, 1986; Cambier *et al.*, 1987; Hadden, 1988). These early alterations are followed within a couple of hours by later events such as secretion of interleukin-2 (IL-2), and expression of IL-2 receptors and certain oncogenes. This second wave of biochemical events can also be inhibited by elevations of intracellular cyclic AMP (Feldman *et al.*, 1987; Mary *et al.*, 1987; Johnson *et al.*, 1988; Kammer, 1988). As secretion of IL-2 and expression of IL-2 receptors depend on an elevation of intracellular Ca^{2+} and an activation of a protein kinase C (Cambier *et al.*, 1987; Hadden, 1988), it is possible that cyclic AMP formation stimulating agents inhibit lymphocyte activation by attenuating the generation of these early biochemical signals. Therefore, we have investigated the effects of various cyclic AMP formation stimulating agents on the mitogen-stimulated generation of inositol phosphates and increases of intracellular Ca^{2+} in resting human mononuclear leukocytes (MNL,

[1] Author for correspondence at: Biochem. Forschungslabor, Med. Klinik & Poliklinik, Abtlg. Nieren- und Hochdruckkrankheiten Universitätsklinikum, Hufelandstr. 55, D-4300 Essen 1, Fed. Rep. Germany.

mostly lymphocytes), with special emphasis on the effects of β-adrenoceptor agonists.

Methods

Mononuclear leukocyte preparation

Mononuclear leukocytes (MNL), containing >95% lymphocytes, were prepared from the venous blood of healthy young volunteers according to the method of Böyum (1968). In some experiments, MNL were cultured in RPMI medium supplemented with 20% foetal calf serum, 2 mM glutamine and 25 $\mu g\,ml^{-1}$ gentamycin in the absence or presence of 100 $ng\,ml^{-1}$ pertussis toxin for 16–20 h prior to the Ca^{2+} measurements. These cells were washed twice in buffer 2 h before the Ca^{2+} measurements in order to remove the pertussis toxin.

Cyclic AMP measurements

Accumulation of cyclic AMP was measured in intact MNL according to previously published techniques (Brodde *et al.*, 1985). Briefly, MNL were suspended in phosphate buffered saline containing 0.1 mM theophylline (in order to inhibit cyclic AMP degradation by phosphodiesterases) and 0.025% bovine serum albumin. Aliquots of 300 μl (2×10^6 cells) were incubated for 15 min at 37°C in the presence or absence of indicated stimuli, in a total volume of 330 μl. Incubation was terminated by boiling for 5 min. After cooling, samples were centrifuged at 12,000 *g* for 10 min, and cyclic AMP content was determined in 50 μl aliquots of the supernatant by radioimmunoassay.

In some experiments MNL were preincubated with mitogens for 15 min at 37°C before theophylline (0.1 mM final concentration) and isoprenaline were added. Incubations were continued for another 15 min, and then stopped and samples processed as described above.

Inositol phosphate generation

Generation of inositol phosphates was determined according to published techniques (Minneman & Johnson, 1984) with minor modifications. MNL were suspended in Krebs-Henseleit solution containing (mM): NaCl 118, KCl 4.7, $CaCl_2$ 1.3, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24.9, glucose. H_2O 11, EDTA 1 μM and 0.1% bovine serum albumin, equilibrated with 95% O_2/5% CO_2 at 37°C. Cells were incubated with [^{3}H]-myo-inositol (10 $\mu Ci\,ml^{-1}$ with 3×10^7 cells ml^{-1}) for 2 h at 37°C. Thereafter, the cells were washed and resuspended in Krebs-Henseleit solution supplemented with 10 mM LiCl (to block inositol phosphate breakdown). Aliquots of this cell suspension (450 μl containing 5×10^6 cells) were preincubated for 15 min under 95% O_2/5% CO_2; thereafter, 50 μl of drug solution (mitogens in the presence or absence of cyclic AMP formation stimulating agents) was added. Samples were incubated for another 30 min at 37°C, and the reaction was stopped by addition of 1.5 ml chloroform-methanol (1:2, v:v). Each sample was mixed thoroughly 4 times in a period of 1 h, 0.5 ml of chloroform and 0.5 ml of water added sequentially, mixed again twice and centrifuged at 3000 *g* for 12 min to separate the phases. Columns containing 1 ml of a 1:1 slurry of Dowex AG 1-X8 anion exchange resin (200–400 mesh) in the formate form, previously stored in 0.1 M formic acid, were washed twice with 10 ml of 10 mM Tris-formate (pH 7.4) and once with 7 ml water. Aliquots of 1.5 ml of the upper phase of each sample were filled up to 5 ml with water and poured on the columns. After washing the columns with 5 ml of water and twice with 5 ml of 25 mM ammonium formate successively (to elute remaining [^{3}H]-inositol), the [^{3}H]-inositol phosphates retained on the columns were eluted with 1 ml of 2 M ammonium formate dissolved in 0.1 M formic acid; thus, the eluate contained a mixture of inositolmonophosphate, -bisphosphate and -trisphosphate in which inositolmonophosphate is the major constituent (accumulating from breakdown of the other phosphates in the presence of LiCl). Scintillation fluid (9 ml) was added to the eluent and the samples were counted in a Beckman LS 9000 liquid scintillation counter (Beckman Inst., Fullerton, CA) at an efficiency of about 42%. Following each experiment, the columns were regenerated with 2 ml of 2 M ammonium formate dissolved in 0.1 M formic acid and 2 ml of 1 M formic acid, washed with 10 ml of 10 mM Tris-formate (pH 7.4) and stored in 0.1 M formic acid. With this regeneration procedure, the columns could be used for up to 2 months without any loss of capacity.

Ca^{2+} measurements

Intracellular Ca^{2+} concentrations were measured with the fluorescent dye Fura-2 according to standard procedures previously described in detail (Motulsky & Michel, 1988). Briefly, loading of the cells with Fura-2 and the subsequent fluorescence measurements were performed at room temperature in buffer containing (mM): NaCl 120, KH_2PO_4 5, $Mg(CH_3COO)_2$ 1, $CaCl_2$ 1, Hepes 20 and glucose 1 $mg\,ml^{-1}$ at pH 7.4. Our calculations to convert raw fluorescence data into intracellular Ca^{2+} concentrations included correction factors for dilution of cells by addition of drugs (1–2%), and for the presence of extracellular Fura-2 (accounting for 10–25% of the total fluorescence), and (in some experiments) the presence of EGTA. Experiments at 37°C yielded qualitatively similar but more variable results as more dye leaked out of the cells; this dye leakage was attenuated by addition of 2.5 mM probenecid but even under those conditions it accounted for at least 50% of total fluorescence after an incubation period of more than 10 min (data not shown).

Chemicals

Myo-[2-^{3}H(N)]-inositol (specific activity 20 $Ci\,mmol^{-1}$) and a radioimmunoassay for cyclic AMP were purchased from New England Nuclear (Dreieich, FRG) and Amersham (Braunschweig, FRG), respectively. ICI 118,551 (erythro-(±)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol) was a gift from ICI-Pharma (Plankstadt, FRG), CGP 20712A (1-[2-((3-carbamoyl-4-hydroxy)phenoxy)ethylamino]-3-[4-(1-methyl-4 - trifluoromethyl - 2 - imidazolyl)phenoxy] - 2 - propanol methanesulphonate) from Ciba-Geigy (Basel, Switzerland), Ro 20-1724 (4-(3,4-dibutoxybenzyl)-2-imidazolidinone) from Hoffman-La Roche (Basel, Switzerland), and nifedipine from Pfizer (New York, N.Y., U.S.A.). Dowex AG 1-X8 anion exchange resin (200–400 mesh, formate form) was obtained from Bio-Rad Laboratories (Richmond, VA, U.S.A.), Fura-2 acetoxymethoxyester was obtained from Molecular Probes (Eugene, OR, U.S.A.), forskolin was from Calbiochem (La Jolla, CA, U.S.A.), and pertussis toxin from List (Campbell, CA, U.S.A.). Phytohaemagglutinin (PHA) for the experiments on cyclic AMP and inositol phosphate generation was from Wellcome (Beckenham, U.K.), and the PHA for the Ca^{2+} experiments was from Sigma (St. Louis, MO, U.S.A.); these two preparations differ with respect to their purity grades and, therefore, their potencies. Pokeweed mitogen (PWM) was from Boehringer (Mannheim, FRG), concanavalin A (Con A) and all other chemicals were from Sigma and of the highest commercially available purity grade.

Data analysis

Maximum and the half-maximal effective concentrations of agonists were calculated from concentration-response curves by fitting the experimental data to a sigmoid curve by computer-assisted non-linear regression analysis. To obtain optimal estimates for the maximal effect and the concentration of agonist causing 50% of the maximal effect (EC_{50}), the Hill-slope and the bottom of the curve were fixed at 1 and the measured basal value, respectively, during the curve fitting

procedure. Data are shown as mean ± s.e.mean of n experiments. Differences between groups were compared with two-tailed t tests as appropriate, with $P < 0.05$ considered statistically significant.

Results

Cyclic AMP accumulation

Isoprenaline, adrenaline, and noradrenaline concentration-dependently increased the intracellular cyclic AMP content in human MNL (Table 1). Although all three catecholamines elicited a similar maximal cyclic AMP accumulation, their potencies differed considerably with a rank order of isoprenaline > adrenaline > noradrenaline. Prostaglandin E_1 (PGE_1) and forskolin also stimulated cyclic AMP accumulation in MNL with maximal stimulatory effects 4–5 and 0.66 times as large as those in response to isoprenaline, respectively (Table 1).

As other investigators have reported enhanced hormone-stimulated cyclic AMP accumulation in the presence of mitogenic lectins (Fredholm *et al.*, 1987; Dailey *et al.*, 1988), we also determined isoprenaline-stimulated cyclic AMP accumulation following exposure to mitogens for 30 min. This protocol was chosen to yield a mitogen exposure of the MNL comparable to that during the inositol phosphate accumulation experiments. However, neither PWM (2.4 $\mu g\,ml^{-1}$) nor PHA (1.8 $\mu g\,ml^{-1}$) significantly altered the potency or maximal effects of isoprenaline on MNL cyclic AMP accumulation (Figure 1).

Inositol phosphate accumulation

Incubation of MNL, prelabelled with [^{3}H]-myo-inositol, with various concentrations of PHA (0.2–14.4 $\mu g\,ml^{-1}$), Con A (0.14–36 $\mu g\,ml^{-1}$) and PWM (0.15–4.8 $\mu g\,ml^{-1}$) for 30 min at 37°C concentration-dependently increased the formation of [^{3}H]-inositol phosphates (Figure 2); the maximal effect was highest for Con A and PHA and lowest for PWM. All further experiments were performed using 1.8 $\mu g\,ml^{-1}$ PHA, 18 $\mu g\,ml^{-1}$ Con A, and 2.4 $\mu g\,ml^{-1}$ PWM.

β-Adrenoceptor agonists decreased mitogen-stimulated inositol phosphate generation by 30–50% depending on the mitogen used (Figure 3a–c). The maximal inhibitory effect was similar for all three catecholamines but their potency differed, the rank order being: isoprenaline > adrenaline > noradrenaline (Figure 3). The catecholamine-induced inhibition of the inositol phosphate generation was completely blocked by 100 nM of the β_2-adrenoceptor selective antagonist ICI 118,551 (Bilski *et al.*, 1983) but was not affected by 500 nM of the β_1-adrenoceptor selective antagonist CGP 20712A (Dooley *et al.*, 1986; Figure 4a–c).

In order to investigate whether the inhibitory effects of the catecholamines are related to their stimulation of cyclic AMP generation, we studied the effects of PGE_1 and forskolin (which stimulate cyclic AMP generation independently of β-adrenoceptor activation) and of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX). Forskolin, a poor stimulator of cyclic AMP generation in resting human MNL (see Table 1) weakly reduced mitogen-stimulated inositol phosphate generation (20–35% inhibition; Figure 5a–c). In contrast, PGE_1 (which stimulates cyclic AMP generation considerably more than the catecholamines, see Table 1) inhibited mitogen-stimulated inositol phosphate accumulation to a much larger extent than the catecholamines (Figure 5a–c). Moreover, IBMX (0.1 mM) considerably enhanced the inhibition of mitogen-stimulated inositol phosphate generation by isoprenaline (Figure 6). Thus, inhibition of inositol phosphate generation by these various cyclic AMP promoting agents was strongly related to the increase in the intracellular level of cyclic AMP.

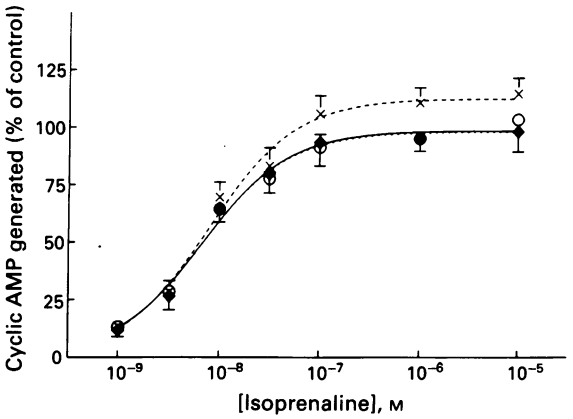

Figure 1 Isoprenaline-induced cyclic AMP generation in the absence and presence of pokeweed mitogen (PWM) and phytohaemagglutinin (PHA). Lymphocytes were preincubated for 15 min without (control) or with PHA (1.8 $\mu g\,ml^{-1}$) or PWM (2.4 $\mu g\,ml^{-1}$) prior to addition of isoprenaline. Values are mean of 4 experiments for control (○; max. cyclic AMP generation 45.3 ± 7.8 pmol/10^6 cells in 15 min), PHA (◆; 50.4 ± 5.9 pmol/10^6 cells in 15 min) and PWM (×; 47.6 ± 4.9 pmol/10^6 cells in 15 min); vertical bars show s.e.mean. Mean data were fitted to a sigmoid curve.

Table 1 Concentration-response relationships for catecholamine-, prostaglandin E_1- and forskolin-stimulated adenosine 3′:5′-cyclic monophosphate (cyclic AMP) generation in human lymphocytes

	EC_{50} (nM)	*Maximal cyclic AMP accumulation* (pmol/10^6 cells in 15 min)
Isoprenaline	7.8 ± 1.4	47.6 ± 10.6
Adrenaline	48 ± 12	49.4 ± 8.6
Noradrenaline	5100 ± 2900	47.5 ± 12.3
Forskolin	31000 ± 7000	31.4 ± 10.5
Prostaglandin E_1	1560 ± 800	249 ± 60

Lymphocytes were incubated for 15 min at 37°C with 5–7 concentrations of the indicated agonists in the presence of 0.1 mM theophylline, and the cyclic AMP content was determined as described in methods. The agonist concentration causing a half-maximal increase in cyclic AMP content, the maximal increase and the Hill slope were calculated for each experiment by fitting the experimental data to a sigmoid curve using computer assisted non-linear regression analysis. The Hill slopes did not differ significantly from 1.0. Each value is the mean ± s.e.mean of 4 experiments.

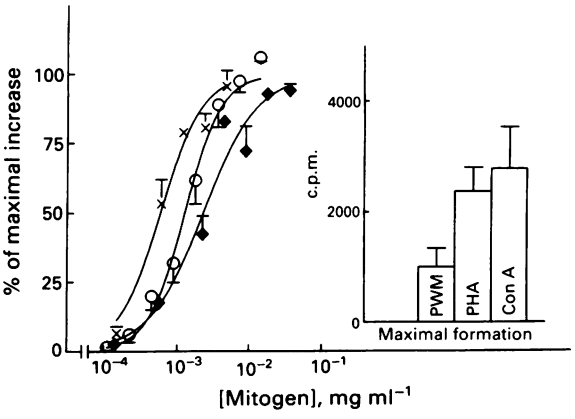

Figure 2 Concentration-response curves for mitogen-induced accumulation of inositol phosphates in human lymphocytes. The EC_{50} values for pokeweed mitogen (PWM, ×), phytohaemagglutinin (PHA, ○) and concanavalin A (Con A, ◆) were 0.6 $\mu g\,ml^{-1}$, 1.3 $\mu g\,ml^{-1}$, and 2.3 $\mu g\,ml^{-1}$, respectively. Inset: Maximal inositol phosphate generation by mitogens expressed as c.p.m. Data are mean of 3–5 experiments; vertical bars show s.e.mean.

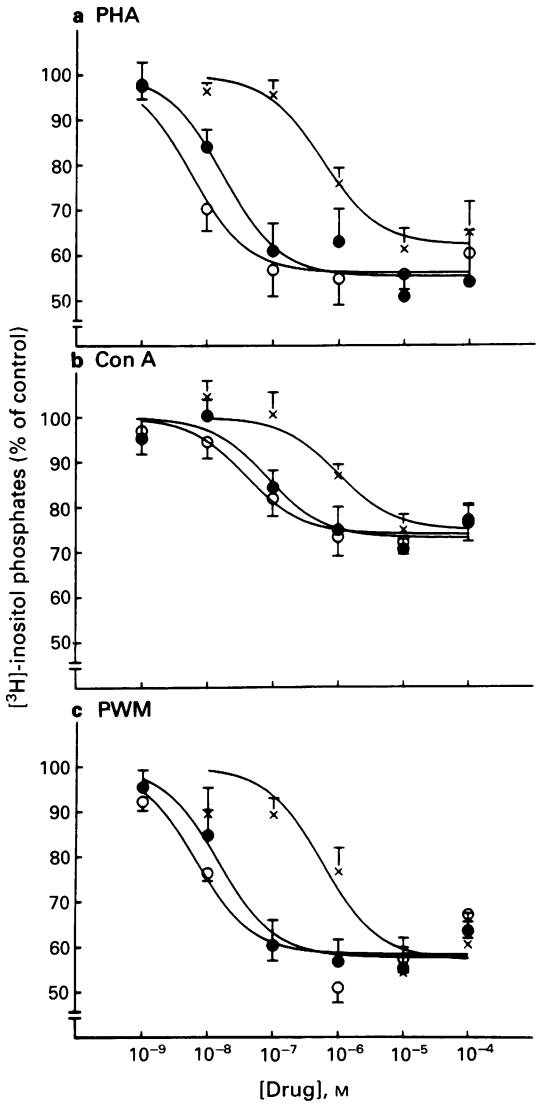

Figure 3 Inhibition of mitogen-induced inositol phosphate generation by isoprenaline (○), adrenaline (●) and noradrenaline (×) in human lymphocytes: (a) phytohaemagglutinin (PHA); (b) concanavalin A (Con A) and (c) pokeweed mitogen (PWM). Mean data were fitted to a competition curve.

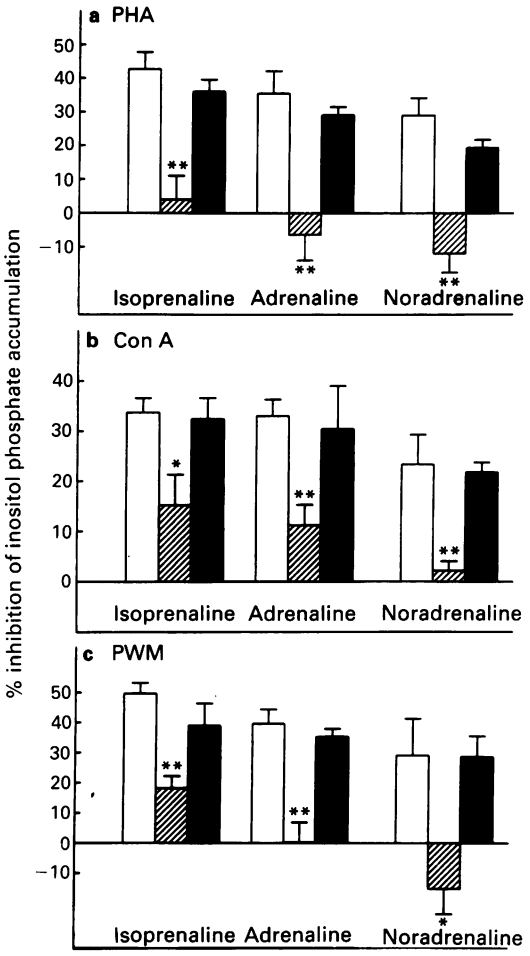

Figure 4 Effects of the β_1-adrenoceptor selective antagonist CGP 20712 A (500 nM, filled columns) and the β_2-adrenoceptor selective antagonist ICI 118,551 (100 nM, hatched columns) on catecholamine-evoked inhibition (open columns) of mitogen-induced inositol phosphate generation in human lymphocytes: (a) phytohaemagglutinin (PHA); (b) concanavalin A (Con A) and (c) pokeweed mitogen (PWM). Data are expressed as % inhibition of the formation of [^{3}H]-inositol phosphates caused by the mitogens in the respective experiments. Values are mean of 3–6 experiments; vertical bars show s.e.mean. * $P < 0.05$; ** $P < 0.01$ vs. control.

Increases of intracellular Ca^{2+} concentrations

The basal intracellular Ca^{2+} concentration was 62 ± 5 nM ($n = 12$ with 8–22 replicates in each experiment). Con A and PHA concentration-dependently elevated intracellular Ca^{2+} to a peak level within 1–3 min; as expected, the Ca^{2+} concentration later declined from the peak to a plateau above the basal level which was maintained for more than 30 min (data not shown). From the peak Ca^{2+} concentration elicited by 4 mitogen concentrations we constructed concentration-response curves (Figure 7). PWM elicited only very small and inconsistent increases of intracellular Ca^{2+} (maximally 25 nM at 96 $\mu g\,ml^{-1}$) regardless of whether experiments were performed at 25°C or 37°C (data not shown). Chelation of extracellular Ca^{2+} by addition of 5 mM EGTA immediately prior to the mitogens completely abolished the Ca^{2+} increases stimulated by Con A and PHA ($n = 4$; data not shown). Thus, the mitogen-induced increases in intracellular Ca^{2+} were predominantly due to influx rather than intracellular mobilization in accordance with recently reported data in human and murine resting lymphocytes (Alcover *et al.*, 1987; Linch *et al.*, 1987; Gelfand *et al.*, 1987).

Overnight pretreatment of MNL cells with pertussis toxin (100 $ng\,ml^{-1}$) ADP-ribosylated more than 90% of the pertussis toxin substrates of these cells, but did not inhibit the mitogen-stimulated Ca^{2+} increases (data not shown). Such a lack of effect of pertussis toxin on the inhibition of Ca^{2+} increases has previously also been reported in human and murine-T-lymphocytes (Macintyre *et al.*, 1988; Aussel *et al.*, 1988; Gray *et al.*, 1989; Stewart *et al.*, 1989).

The cyclic AMP generation-stimulating agents PGE_1 (10 μM), forskolin (20 μM) and isoprenaline (10 μM) did not significantly alter the intracellular Ca^{2+} concentration of resting MNL (2 ± 2 nM for PGE_1, 1 ± 0 nM for forskolin, and 1 ± 1 nM for isoprenaline, $n = 4$–8 each). However, all three agents inhibited the PHA- and Con A-stimulated Ca^{2+} elevations (Figure 8). Inhibition of the Ca^{2+} elevation was associated with a prolonged time-to-peak (data not shown) which was not quantified in detail. Inhibition of PWM-stimulated Ca^{2+} increases was not tested because these increases were too small and inconsistent to begin with (see above). Blocking the degradation of cyclic AMP with the phosphodiesterase inhibitors IBMX (0.1 mM) and Ro 20-1724 (0.25 mM) enhanced the inhibitory effects of PGE_1, forskolin, and isoprenaline (Figure 9), demonstrating that the inhibition is mediated by cyclic AMP.

Discussion

The activation of T-lymphocytes is a multistep process. Two of the first signals which can be observed biochemically are an

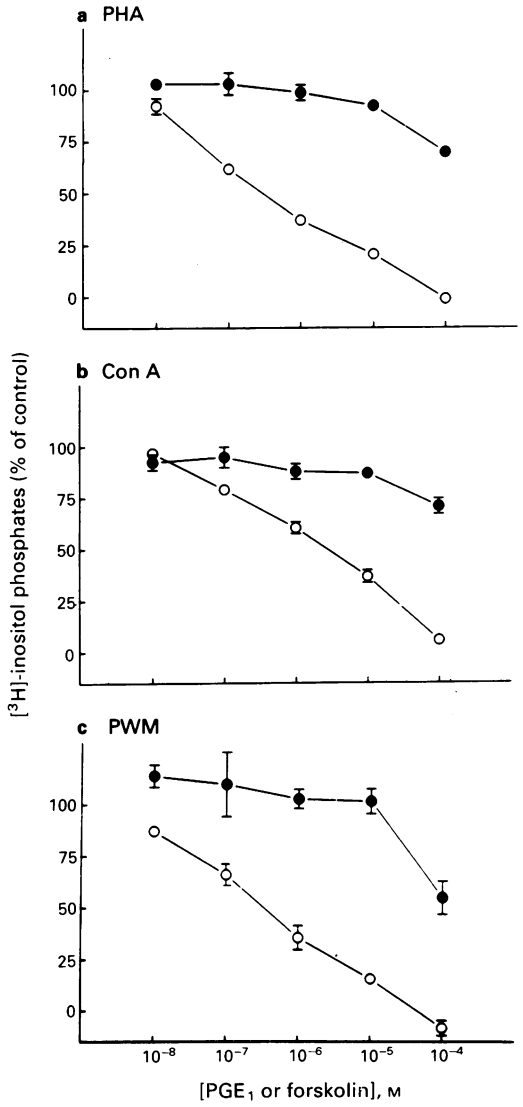

Figure 5 Inhibition of mitogen-induced inositol phosphate generation in human lymphocytes by prostaglandin E_1 (○) and forskolin (●): (a) phytohaemagglutinin (PHA); (b) concanavalin A (Con A) and (c) pokeweed mitogen (PWM). Values are mean of 3–5 experiments; vertical bars show s.e.mean.

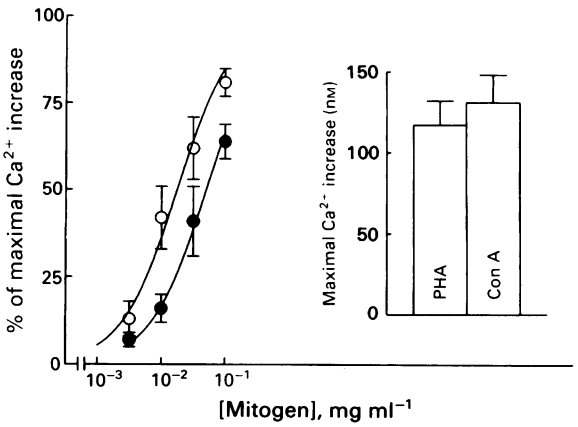

Figure 7 Mitogen-stimulated increases of intracellular Ca^{2+} in human lymphocytes. The EC_{50} values for phytohaemagglutinin (PHA, ○) and concanavalin A (Con A, ●) were 22.8 $\mu g\,ml^{-1}$ and 65.5 $\mu g\,ml^{-1}$, respectively. Inset: Maximal Ca^{2+} increase expressed in nM. Data are mean of 7 experiments; vertical bars show s.e.mean.

elevation of the free intracellular Ca^{2+} concentration and the hydrolysis of phosphatidyl-inositol-bisphosphate into inositol-(1,4,5)-trisphosphate (IP_3) and diacylglycerol (DAG) leading to activation of a protein kinase C (Chen *et al.*, 1986; Cambier *et al.*, 1987; Linch *et al.*, 1987; Gelfand *et al.*, 1987; Hadden, 1988; Gupta, 1989). Although generation of IP_3 is the major stimulus to elevate intracellular Ca^{2+} in many model systems (Berridge & Irvine, 1989) it is still a matter of debate whether Ca^{2+} increases in lymphocytes by mitogenic lectins are caused by IP_3 or not (King, 1988). Our study did not directly address this question, but the observation that PWM was approximately half as effective as Con A in generating inositol phosphates but a very poor stimulus for Ca^{2+} elevations support the idea that Ca^{2+} influx in lymphocytes may not necessarily be predominantly caused by inositol phosphate generation.

In our study, we have used the T-lymphocyte specific mitogenic lectins PHA and Con A and the non-specific B- and T-lymphocyte mitogen PWM to mimic the activation of resting lymphocytes by antigens. The characteristics of inositol phosphate generation and elevation of intracellular Ca^{2+} in human MNL as stimulated by PHA, Con A and PWM in our and other studies (i.e. concentration-dependency; dependency of Ca^{2+} increase on influx; lack of pertussis toxin sensitivity) are very similar to those described previously for other polyclonal T-lymphocyte activators such as monoclonal anti-CD3 antibodies (for review see Chen *et al.*, 1986; Cambier *et al.*,

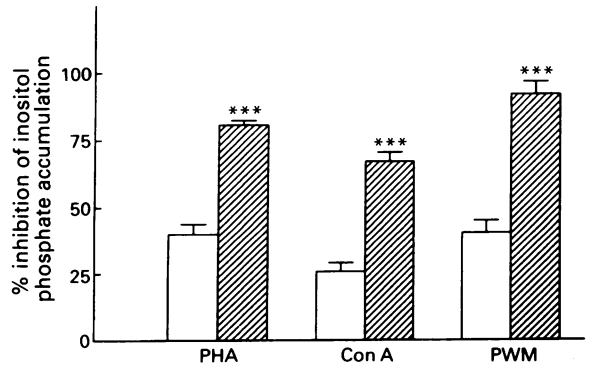

Figure 6 Effects of the phosphodiesterase inhibitor isobutylmethylxanthine (0.1 mM, hatched columns) on isoprenaline-evoked inhibition of mitogen-induced inositol phosphate generation (open columns) in human lymphocytes. Mitogens: phytohaemagglutinin (PHA); concanavalin A (Con A) and pokeweed mitogen (PWM). Data are expressed as % inhibition of formation of [^{3}H]-inositol phosphates caused by the mitogens in the respective experiments. Values are mean of 5 experiments; vertical bars show s.e.mean. *** $P < 0.001$ vs. control in the absence of isobutylmethylxanthine.

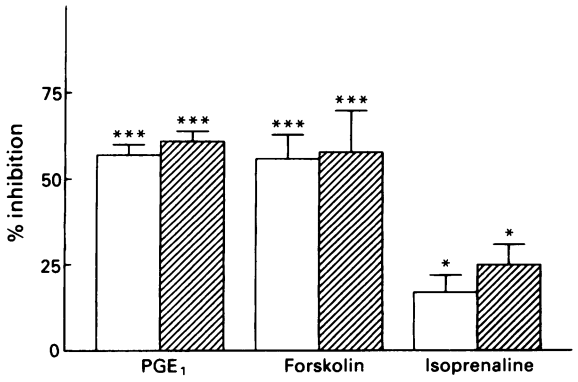

Figure 8 Inhibition of phytohaemagglutinin (PHA) (open columns) and concanavalin A (Con A) (hatched columns)-induced Ca^{2+} increases in human lymphocytes by prostaglandin E_1 (PGE_1, 10 μM), forskolin (20 μM) or isoprenaline (10 μM). Values are mean of 4–7 experiments; vertical bars show s.e.mean. The mitogen-induced rise in intracellular Ca^{2+} in these experiments was 119 ± 17 nM for PHA and 94 ± 8 nM for Con A. * $P < 0.05$, *** $P < 0.001$ vs. control in the absence of cyclic AMP increasing agents.

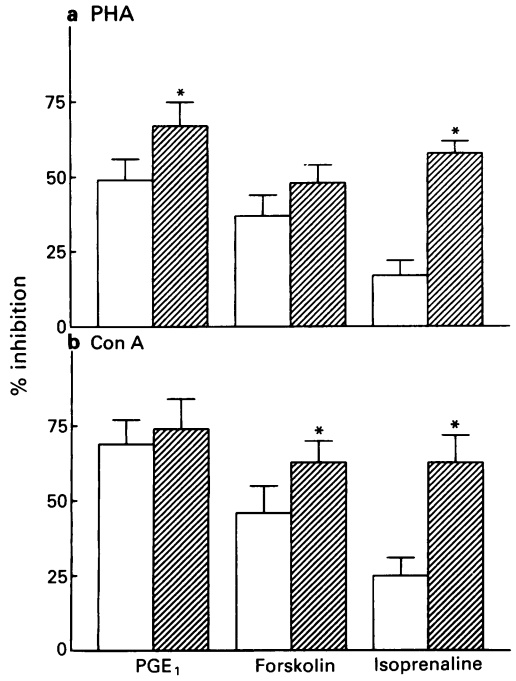

Figure 9 Effect of phosphodiesterase inhibition by isobutylmethylxanthine and Ro 20-1724 (100 and 250 μM, respectively) on mitogen-stimulated Ca^{2+} increases in human lymphocytes. Either the phosphodiesterase inhibitors (hatched columns) or vehicle (open columns) were added to the cells, followed 20–30 s later by prostaglandin E_1 (1 μM), forskolin (2 μM), or isoprenaline (10 μM), and 20–30 s later by phytohaemagglutinin (PHA, 100 $\mu g\,ml^{-1}$, a) or concanavalin A (Con A, 100 $\mu g\,ml^{-1}$, b). Data shown are mean of 4–6 experiments; vertical bars show s.e.mean. * $P < 0.05$ vs. inhibition of Ca^{2+} increase in the absence of phosphodiesterase inhibitors.

1987; Hadden, 1988). Therefore, this paradigm is likely to give reliable estimates of the biochemical alterations occurring in resting lymphocytes undergoing activation by antigens. On the other hand, it should be noted that the mitogenic lectins used in this study may utilize, at least partially, different pathways for lymphocyte activation.

Various authors have shown that cyclic AMP can play an inhibitory role in the activation of resting T-lymphocytes. Thus, increases in the intracellular level of cyclic AMP inhibit mitogen- or antigen-induced T-cell proliferation, cytotoxic T-lymphocyte function, lymphokine secretion, and production and effects of IL-2 (Bourne *et al.*, 1974; Felten *et al.*, 1987; Feldman *et al.*, 1987; Mary *et al.*, 1987; Kammer, 1988; Johnson *et al.*, 1988). Whether this inhibitory effect of cyclic AMP, however, is due to interaction with the phospholipase C/IP_3/DAG pathway, is still a matter of debate. Imboden *et al.* (1986) and Sommermeyer & Resch (1990) found in the human T cell tumour line Jurkat that cholera toxin (which via ADP-ribosylation activates the adenylate cyclase coupled G_s-protein thereby stimulating cyclic AMP formation) inhibits antigen-induced IP_3-formation and increases in intracellular Ca^{2+}, but this effect could not be mimicked by other cyclic AMP increasing agents and/or cyclic AMP analogues. On the other hand, Lerner *et al.* (1988) in mouse T-lymphocytes and Windebank *et al.* (1988) in cloned human NK cell lines could demonstrate that increases in the intracellular level of cyclic AMP, regardless of whether it was receptor-dependent (PGE_1) or -independent (forskolin), evoked inhibition of IP_3 formation and increases in intracellular Ca^{2+}.

These data prompted us to investigate in mixed human lymphocytes the effects of various agents known to stimulate the formation of cyclic AMP on the mitogen-induced generation of inositol phosphates (as a parameter of phosphatidylinositol-bisphosphate hydrolysis) and increases in intracellular Ca^{2+}. The results clearly demonstrate that all agents used in this study that elevate cyclic AMP levels (see Table 1) also inhibit mitogen-induced generation of inositol phosphates and increases in the intracellular Ca^{2+} concentration.

The fact, that (i) inhibition of the break-down of cyclic AMP by phosphodiesterase inhibitors potentiated the inhibition of inositol phosphate generation and of intracellular Ca^{2+} rises and (ii) the amount of inhibition of inositol phosphate generation was strongly related to the magnitude of cyclic AMP elevations is in favour of the idea that cyclic AMP is responsible for inducing these inhibitory effects. Thus, the present data obtained in human MNL confirm those of Lerner *et al.* (1988) obtained in mouse T-lymphocytes and of Windebank *et al.* (1988) obtained in cloned human NK cells, that cyclic AMP-induced inhibition of cell proliferation and cytotoxicity, respectively, is accompanied (and/or induced?) by inhibition of increases in the intracellular Ca^{2+} concentrations and generation of inositol phosphates.

In the present study, β-adrenoceptor agonists increased the intracellular cyclic AMP content and inhibited the mitogen-induced generation of inositol phosphates with an order of potency: isoprenaline > adrenaline > noradrenaline. This order of potency (Lands *et al.*, 1967) and the fact that catecholamine-induced inhibition of inositol phosphate generation could be abolished by the β_2-adrenoceptor selective antagonist ICI 118,551 (Bilski *et al.*, 1983) but not affected by the β_1-adrenoceptor selective antagonist CGP 20712 A (Dooley *et al.*, 1986) strongly support the view that this effect is mediated via β_2-adrenoceptor stimulation, as is to be expected since human MNL contain exclusively β_2-adrenoceptors (for review see Brodde *et al.*, 1987).

It should be noted that cyclic AMP (a) inhibited inositol phosphate generation and Ca^{2+} increases, although both responses may occur independently; (b) inhibited the second messenger generation by all three mitogens, although they may stimulate second messenger generation via at least partially different pathways (see above); and (c) inhibited second messenger generation whether near maximally effective (Con A and PWM) or low (PHA) mitogen concentrations were used. Thus, our data suggest a prominent role of cyclic AMP-increasing agents in the regulation of the generation of second messengers which are important in the activation of resting lymphocytes.

Taken together our data demonstrate that catecholamines can attenuate the generation of two second messengers which are important in the activation of resting human lymphocytes. This appears to be mediated via β_2-adrenoceptors by elevating intracellular cyclic AMP. Thus, attenuation of early second messenger responses involved in the activation of resting lymphocytes may represent (or at least participate in) the molecular basis for the immunomodulatory effects of the sympathetic nervous system.

This work was supported by the Gesellschaft zur Erforschung und Bekämpfung des hohen Blutdrucks (Essen, FRG) and the Deutsche Forschungsgemeinschaft (DFG Ph 43/1-1). M.C.M. was a recipient of a fellowship of the Deutsche Forschungsgemeinschaft. The skilful technical assistance of Mrs Martina Michel is gratefully acknowledged.

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(Received June 25, 1990
Revised January 10, 1991
Accepted January 15, 1991)

Br. J. Pharmacol. (1991), **103**, 1295–1302 © Macmillan Press Ltd, 1991

Alkaline buffers release EDRF from bovine cultured aortic endothelial cells

[1]Jane A. Mitchell, [2]Gilberto de Nucci, [3]Timothy D. Warner & John R. Vane

William Harvey Research Institute, Saint Bartholomew's Hospital Medical College, Charterhouse Square, London EC1M 6BQ

1 Release of endothelium-derived relaxing factor (EDRF) and prostacyclin (PGI_2) from bovine cultured aortic endothelial cells (EC) was measured by bioassay and radioimmunoassay, respectively.

2 Bradykinin (BK, 3–30 pmol), adenosine diphosphate (ADP, 2–6 nmol) or the sodium ionophore monensin (40–100 nmol) injected through a column of EC released EDRF. L-Arginine free base (FB; 10–20 μmol) or D-arginine FB (10–20 μmol) injected through the column of EC released similar amounts of EDRF and also caused an increase in pH of the Krebs solution perfusing the EC from 7.5–8.0 to 8.6–9.5. Sodium carbonate (Na_2CO_3) an alkaline buffer which caused the same changes in the pH of the Krebs solution also induced the same release of EDRF. The hydrochloride salts of L- or D-arginine did not cause either release of EDRF when injected through the column of EC or increases in the pH of the Krebs solution.

3 Inhibitors of either diacylglycerol lipase (RHC 80267) or kinase (R59022) inhibited the release of EDRF induced by BK or ADP but potentiated the release induced by L-arginine FB, monensin (40–100 nmol) or alkaline buffer (Na_2CO_3). R59022 and RHC 80267 infused through the EC increased the basal release of EDRF.

4 When calcium chloride was omitted from the Krebs solution the release of EDRF induced by alkaline buffer (Na_2CO_3; pH 8.6–9.5) or L-arginine FB (10–20 μmol) was selectively inhibited when compared to that induced by BK (3–30 pmol) or ADP (2–6 nmol). This inhibition was reversed when calcium (2.5 mM) was restored.

5 N^G-monomethyl-L-arginine (NMMA; 30 μM) inhibited release of EDRF induced by BK (10–30 pmol) or alkaline buffers (Na_2CO_3 or D-arginine FB; pH 8.6–9.5). This inhibition was partially reversed by L- but not D-arginine FB or HCl (30–100 μM).

6 Prostacyclin was released when BK (10 pmol), ADP (2 nmol) or arachidonic acid (30 nmol) were injected through the column of EC. However, monensin (40 nmol) or alkaline buffers (pH 8.6–9.5) did not release detectable amounts of PGI_2 as measured by radioimmunoassay for 6-oxo-prostaglandin $F_{1\alpha}$.

7 Thus alkalinisation of the external bathing solution can release EDRF from cultured EC by a mechanism which does not involve receptor activation and which depends on the presence of extracellular calcium.

Keywords: Prostacyclin; N^G-monomethyl-L-arginine; monensin; calcium; diacylglycerol kinase inhibitor; diacylglycerol lipase inhibitor; bradykinin; adenosine diphosphate; pH

Introduction

Macrophages can synthesize nitrates and nitrites from the guanidino group of L-arginine (Iyengar *et al.*, 1987; Hibbs *et al.*, 1987), a process which is inhibited by N^G-monomethyl-L-arginine (L-NMMA; Hibbs *et al.*, 1987). After the demonstration that nitric oxide (NO) accounted for the biological activity of endothelium-derived relaxing factor (EDRF, Palmer *et al.*, 1987), several groups showed that NO was formed in macrophages and that this pathway was dependent on the metabolism of L-arginine (Marletta *et al.*, 1988; Hibbs *et al.*, 1988; Stuehr *et al.*, 1989). L-Arginine is also a precursor of EDRF in the endothelial cell (EC; Palmer *et al.*, 1988a) and EDRF release is reversibly inhibited by L-NMMA (Palmer *et al.*, 1988b). Although L-arginine does not cause or augment the release of EDRF from porcine (Palmer *et al.*, 1988a) or bovine (this paper) cultured EC, the inhibition by L-NMMA of EDRF release is reversed by L-arginine (Palmer *et al.*, 1988b).

We have shown that the receptor-mediated release of EDRF and prostacyclin (PGI_2) is coupled (de Nucci *et al.*, 1988a) although other agonists which do not act on receptors such as the calcium ionophore A23187 (Gryglewski *et al.*, 1986a) or thimerosal (Förstermann *et al.*, 1986; Mülsch *et al.*, 1987) can also release EDRF and prostacyclin (PGI_2) from EC. Here we demonstrate that L-arginine or D-arginine free base (FB) but not their hydrochloride (HCl) salts induce release of EDRF from EC without coupled release of PGI_2 and that this EDRF release is not due to receptor activation. This novel mechanism of EDRF release is based on alkalinization of the Krebs solution and depends on the presence of extracellular calcium. Some of this work has been presented to the British Pharmacological Society (de Nucci *et al.*, 1988b).

Methods

Endothelial cell culture

Endothelial cells were isolated by treatment of bovine aortae with 0.02% (wt/vol) trypsin, and seeded onto Cytodex 3 microcarrier beads (Pharmacia). The beads were stirred for 3–4 days until they were covered with a confluent layer of EC, as previously described (de Nucci *et al.*, 1988a).

Detection of EDRF by bioassay

The EC on beads (2–3 ml carrying 1–2 × 10^7 cells) were packed into a jacketed column and perfused (5 ml min^{-1} at

[1] Author for correspondence.
[1,3] Present address: Abbott Laboratories, D47S AP9A, Abbott Park, Il 60064-2204, U.S.A.
[2] Present address: Departamento de Farmacologia, Faculdade de Ciencias Medicas – UNICAMP, PO Box 6111, 13081 – Campinas – SP, Brazil.

37°C) with gassed (95% O_2:5% CO_2) Krebs solution, which contained superoxide dismutase (SOD, 10 u ml^{-1}; Gryglewski *et al.*, 1986b) and indomethacin (5 μM). The effluent from the column superfused a cascade (Vane, 1964) of four spirally cut strips of rabbit aortae (RbA) that were denuded of endothelium. The effluent from the column reached the consecutive RbA after 1, 4, 7 and 10 s. Drugs were given over the assay tissues (OT) as a control or through the column of endothelial cells (TC).

The assay tissues were superfused with Krebs solution containing either U46619 (30 nM) or noradrenaline (100–300 nM) until a stable contraction was achieved. Glyceryl trinitrate (GTN) was then used to calibrate the relaxations of the RbA. The recording of the RbA was electrically adjusted so that they showed a similar relaxation with a given dose of GTN.

Measurement of prostacyclin release

In experiments to measure PGI_2 release, indomethacin was omitted from the Krebs solution perfusing the EC but added as an infusion over the bioassay tissues. The column effluent was collected and analysed by radioimmunoassay (RIA) for 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$) as a measure of PGI_2 release by the EC (Salmon, 1978).

Removal of phosphate from the Krebs solution

When L- or D-arginine FB or sodium carbonate (Na_2CO_3) buffer were used, the column of EC was perfused with Krebs solution without KH_2PO_4 to avoid precipitation of phosphates. An appropriate solution of KH_2PO_4 was infused over the RbA in order to complete the salt requirement of the Krebs solution.

Measurement of pH

The pH was measured with a Corning pH 105 meter.

Statistics

During OT infusions of the test drugs (R59022, RHC 80267, calcium chloride or L-NMMA) control injections of EDRF-releasing agents were made through the column of EC. The resulting relaxations of the 4 RbAs were each measured in mm and added together to express the control release. Test drugs were then infused TC and the EDRF-releasing agents were retested. The relaxations of the 4 RbAs were again measured and added together and the result expressed as a % of the control release. Any changes in the sensitivity of the RbAs occurring during the course of the experiment were assessed by changes in relaxations induced by a given dose of GTN. Thus, if the GTN response was increased or decreased by x fold with respect to control it was assumed that this represented a similar change in the sensitivities of the tissues to EDRF. The measured responses to EDRF at the same time point were therefore multiplied by the factor x before being compared with control. Unpaired Student's t test was used and a P value of less than 0.05 taken to be significant.

Materials

The Krebs solution (pH 7.5–8) had the following composition (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.17, $CaCl_2 \cdot 6H_2O$ 2.5, $NaHCO_3$ 25 and glucose 5.6. Bradykinin acetate (BK), adenosine diphosphate di(monocyclohexylammonium) salt (ADP), arachidonic acid sodium salt (AA), L-arginine free base (FB), L-arginine hydrochloride (HCl), D-arginine FB, D-arginine HCl, monensin sodium salt and indomethacin were supplied by Sigma (Poole, Dorset). Glyceryl trinitrate (GTN) was obtained from Lipha (Middlesex). Trypsin was obtained from Flow Laboratories (Hertfordshire). Superoxide dismutase from bovine erythrocytes was a gift from Grunenthal (Stolberg, W. Germany). Sodium carbonate (Na_2CO_3; anhydrous, Analar, BDH Chemicals Ltd, Poole, Dorset) buffer was freshly prepared each day (100 mg ml^{-1}) in distilled water. This was then diluted with distilled water to produce a solution of the same buffering capacity in Krebs solution as the equivalent volume of L- or D-arginine FB (1 M stock) such that 10–20 μl of either injected into 5 ml of Krebs solution increased the pH from 7.5–8.0 to 8.6–9.5.

[^{3}H]-6-oxo-$PGF_{1\alpha}$ was purchased from New England Nuclear; 6-oxo-$PGF_{1\alpha}$ and the antiserum for the RIA were gifts from Dr J.A. Salmon (Wellcome Research Laboratories, Beckenham). U46619, the code name for 9,11-dideoxy-9α,11α-methanoepoxy-prostaglandin $F_{2\alpha}$ was a gift from Dr J. Pike (Upjohn Co., Kalamazoo, U.S.A.). R59022, the code name for 6-(2)-4((4-fluorophenyl)phenylmethylene)-1-piperidinyl)-7-methyl-5H-thiazolo-(3,2-a)pyrimidin-5-one, was supplied by Janssen Pharmaceutical (Oxon). RHC 80267 (1,6-di(o-(carbamoyl) cyclohexanone oxime)hexane) was a gift from Dr J. Sutherland (Revlon Health Care Group, U.S.A.). N^G-monomethyl-L-arginine citrate (L-NMMA) was obtained from Ultrafine Chemicals (Manchester).

Results

Release of EDRF by extracellular alkalinization

L- or D-arginine FB (10 or 20 μmol) injected TC released equal amounts of EDRF with a concomitant increase in the pH of the Krebs solution from 7.5–8.0 to 8.6–9.5 (D-arginine FB caused the release of EDRF which was 101 $\pm$ 4% of that induced by the same concentration of L-arginine FB; $n = 4$). L- or D-arginine HCl (10 or 20 μmol) did not release EDRF or increase the pH of the Krebs solution. When Na_2CO_3 solution was added to L- or D-arginine HCl in order to increase the pH, injections TC resulted in EDRF release which was of a similar magnitude to that induced by Na_2CO_3 solution given alone ($n = 4$). Injections of Na_2CO_3 which caused the same increase in the pH of the Krebs solution as L-arginine FB resulted in the release of EDRF which was 99 $\pm$ 16% ($n = 10$) of that induced by L-arginine FB. Figure 1 shows the pH-dependent release of EDRF induced by L-arginine FB (20 μmol) or L arginine HCl (20 μmol; with the addition of Na_2CO_3).

The effect of RHC 80267 on the release of EDRF

Bradykinin (3–30 pmol), ADP (2–6 nmol), L-arginine FB (10–20 μmol) or monensin (40–100 nmol) injected TC induced release of EDRF in the presence of the diacylglycerol lipase inhibitor RHC 80267 (10 μM; Sutherland & Amin, 1982) infused OT. When RHC 80267 was infused TC there was a relaxation of the RbAs which diminished down the cascade, indicating an increase in the basal release of EDRF (45 $\pm$ 12%, $n = 4$). The stimulated release of EDRF induced by BK or ADP was inhibited in the presence of RHC 80267 ($n = 5$) whereas that induced by L-arginine FB ($n = 5$) or monensin ($n = 3$) was potentiated when compared to the control release of EDRF ($P < 0.05$). Figure 2a shows the effects of RHC 80267 (30 μM) on EDRF release induced by BK (10 pmol), ADP (2 nmol), L-arginine FB (20 μmol) and monensin (40 pmol). Figure 2b shows the release of EDRF as the % $\pm$ the s.e.mean of control for 3–5 experiments.

The effect of R59022 on the release of EDRF

Similar effects to those obtained with RHC 80267 were observed when the diacylglycerol kinase inhibitor R59022 (10–30 μM; de Chaffoy de Courcelles *et al.*, 1985) was infused through the column of EC. The maintained basal release of EDRF was increased (37 $\pm$ 9%) in 5 out of 8 experiments and

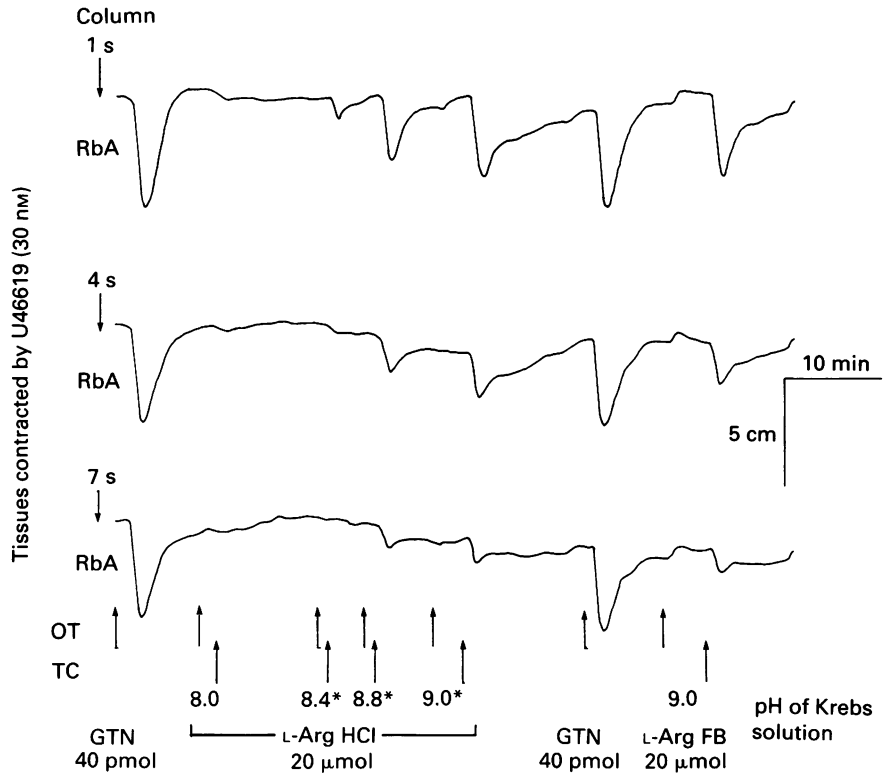

Figure 1 The effect of increasing the extracellular pH of endothelial cells on endothelium-derived relaxing factor (EDRF) release. Effluent from a column of endothelial cells superfused a cascade of four rabbit aortic strips (RbA) precontracted by an infusion of U46619 (30 μM), three of which are shown. Glyceryl trinitrate (GTN; 40 pmol) injected over the tissues (OT) caused a similar relaxation of the three RbA. L-Arginine free base (L-Arg FB; 20 μmol) injected OT caused an increase in the pH of the Krebs solution to pH 9 with no effect on the assay tissues. When injected through the column of endothelial cells (TC) it caused the release of EDRF characterized by relaxation of the RbAs which diminished down the cascade. L-Arginine HCl (L-Arg HCl; 20 μmol) neither increased the pH of the Krebs solution nor released EDRF when given TC. Increasing amounts of a solution of Na_2CO_3, denoted in the figure by *, which increased the pH of the Krebs solution to pH 8.4, 8.8 or 9.0 induced pH-dependent release of EDRF when added to the solution of L-Arg HCl. Similar results were obtained in three other experiments.

transiently increased in the other 3; the EDRF release induced by BK (10–30 pmol; $n = 6$) or ADP (2–6 nmol; $n = 8$) was inhibited; and the release induced by L-arginine FB (10–20 μmol; $n = 6$) or alkaline buffer (Na_2CO_3; pH 8.5–9.0; $n = 3$) was potentiated when compared to the control release of EDRF ($P < 0.05$). Figure 3a shows the inhibition of EDRF release induced by ADP (2 nmol) in the presence of R59022 (30 μM) TC and potentiation of release induced by L-arginine FB (20 μmol). Figure 3b shows the release of EDRF as the % of control release for 3–6 experiments.

The removal of extracellular calcium on the release of EDRF

Endothelial cells were perfused either with normal Krebs solution containing 2.5 mM Ca^{2+} or with a low Ca^{2+} buffer which consisted of Krebs solution without $CaCl_2$, thus leaving a small concentration of Ca^{2+} due to contamination from the other salts. At all times the RbA were perfused with Krebs solution containing the full complement of Ca^{2+}. In conditions of normal Ca^{2+} EDRF was released from the EC by BK (3–30 pmol), ADP (2–6 nmol), L-arginine FB (10–20 μmol) or alkaline buffer (Na_2CO_3 buffer; pH 8.6–9.5). In low Ca^{2+} Krebs solution the release induced by L-arginine FB or alkaline buffer was inhibited to a greater extent than the release induced by BK or ADP. When normal Ca^{2+} was restored to the buffer the inhibition of EDRF release was reversed ($n = 3$–4). Figure 4a shows the selective inhibition of EDRF release induced by L-arginine FB (20 μmol) when extracellular Ca^{2+} (2.5 mM) was removed TC. Figure 4b shows the release of EDRF as the % of control release for 3–4 experiments.

L-Arginine (at concentrations which do not alter pH) on the release of EDRF

L-Arginine FB or HCl (100–300 μM), infused through the column of EC, did not affect the pH of the Krebs solution, the basal release of EDRF or potentiate the release of EDRF induced by other agonists (results not shown; $n > 10$).

N^G-monomethyl-L-arginine (L-NMMA) on the release of EDRF

N^G-monomethyl-L-arginine (10–30 μM) had no direct effect on the RbAs or on the relaxant effects of GTN but when infused TC caused an increase in tone of the RbAs which diminished down the cascade, indicating a decrease of the basal release of EDRF. L-NMMA, dose-dependently inhibited the release of EDRF induced by BK (10–30 pmol; $n = 3$) or by alkaline buffer (D-arginine FB; 10–20 μmol; $n = 4$). This inhibition and increase in tone of the RbA was lessened by L-arginine FB or HCl (30–100 μM; $n = 4$) but not by D-arginine FB or HCl (30–100 μM; $n = 4$; data not shown). Figure 5a shows the inhibition of EDRF release induced by D-arginine FB (20 μmol) with L-NMMA (30 μM) infused TC and the partial reversal in the presence of L-arginine HCl (100 μM) TC. Figure 5b shows the release of EDRF as the % of control release for 3–5 experiments.

Release of prostacyclin from endothelial cells

BK (30 pmol; $n = 26$), ADP (2 nmol; $n = 11$) or arachidonic acid (30 nmol; $n = 7$) given TC induced the release of pros-

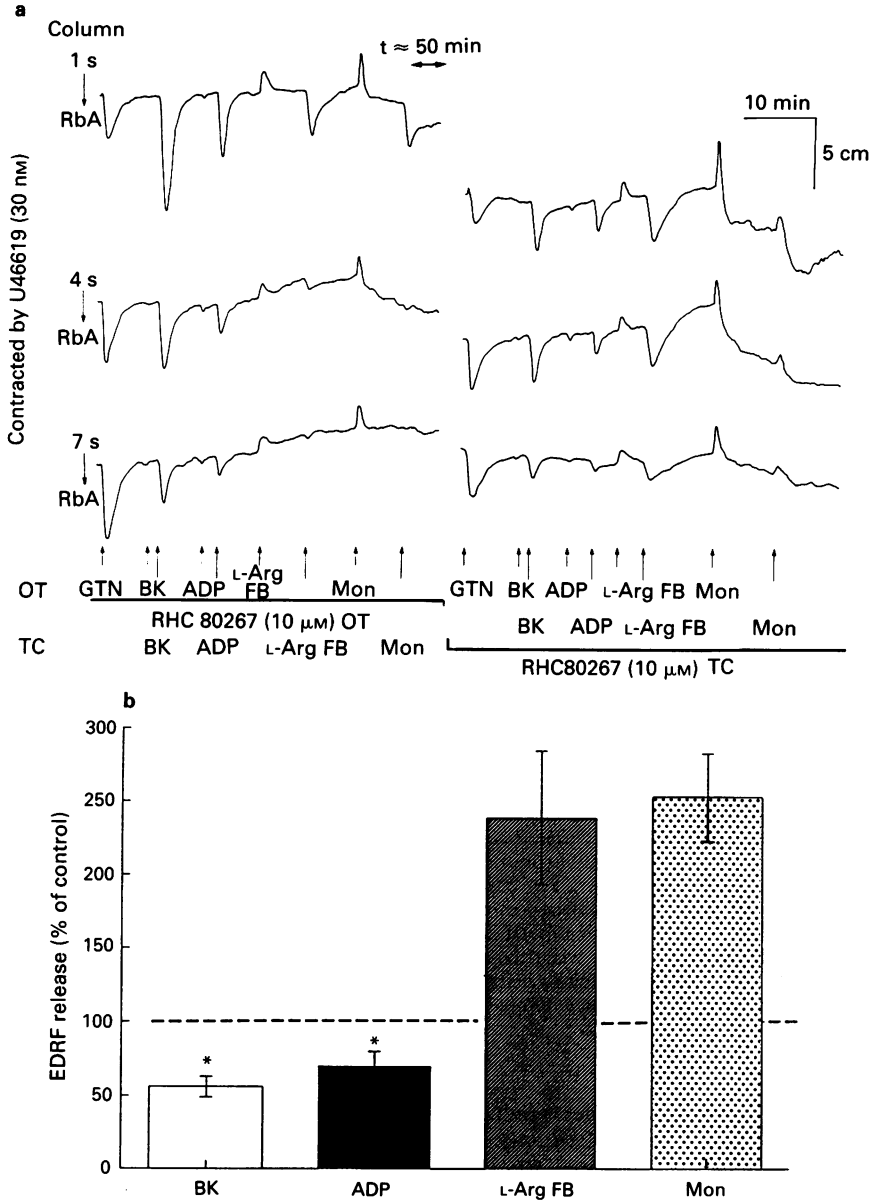

Figure 2 (a) Effect of diacylglycerol lipase inhibitor (RHC 80267) on endothelium-derived relaxing factor (EDRF) release. Experimental details as in Figure 1. When RHC 80267 (10 μM) was infused over the assay tissues (OT) glyceryl trinitrate (GTN; 40 pmol, OT) induced similar relaxations of all three RbA. Bradykinin (BK; 10 pmol) or ADP (2 nmol) given through the column of endothelial cells (TC) released more EDRF than L-arginine free base (L-Arg FB; 20 μmol) TC as shown by the larger relaxations of the assay tissues. Monensin (Mon; 40 nmol) TC also caused a release of EDRF. When RHC 80267 was infused TC there was a clear increase in the basal release of EDRF seen as a graded loss of tone of the assay tissues. However, the stimulated release of EDRF induced by BK or ADP was smaller while that induced by L-Arg FB or Mon was larger, such that they released more EDRF than when RHC 80267 was infused OT. Similar results were obtained in three other experiments. (b) The effect of RHC 80267 (10 μM) on the release of EDRF induced by BK (3–30 pmol; $n = 5$; open column), ADP (2–6 nmol; $n = 5$; solid column), L-Arg FB (10–20 μmol; $n = 5$; hatched column) or Mon (40–100 nmol; $n = 3$; stippled column) as % of control (dotted line); s.e.mean shown by vertical bars. RHC 80267 decreased the release of EDRF in response to BK or ADP, but increased that in response to L-Arg FB or Mon. Thus, in the presence of RHC 80267 the release of EDRF induced by either BK or ADP was significantly different from that induced by either L-Arg FB or Mon (indicated by * $P < 0.05$ for both comparisons).

tacyclin into the Krebs solution perfusing the column of EC as measured by RIA for 6-oxo-$PGF_{1\alpha}$. However, L-arginine FB (20 μmol; $n = 10$), alkaline buffer (Na_2CO_3; pH 8.6–9.5; $n = 4$) or monensin (40 nmol; $n = 3$) given TC did not induce the release of prostacyclin from EC above the limit of detection of the assay (0.5 ng ml^{-1}) (Table 1).

Discussion

Our results demonstrate that L-arginine FB and D-arginine FB, but not the hydrochloride salts can release EDRF from bovine cultured aortic EC. The recent proposal that L-arginine is the precursor of EDRF (Schmidt *et al.*, 1988; Palmer *et al.*, 1988a) could indicate that the selective release of EDRF by L-arginine FB occurs because of an increased synthesis of EDRF. However the findings that: (i) L-arginine HCl neither released EDRF nor potentiated EDRF release when given to the EC at the same concentrations; (ii) D-arginine FB released similar amounts of EDRF as L-arginine FB with similar increases in pH; (iii) a buffer, such as Na_2CO_3, which caused a similar increase in pH of the Krebs solution as L- or D-arginine FB, also released similar amounts of EDRF, all point to the conclusion that an increase in extracellular pH leads to the release of EDRF. The failure of R59022 to inhibit the release of EDRF induced by L- or D-arginine FB, Na_2CO_3

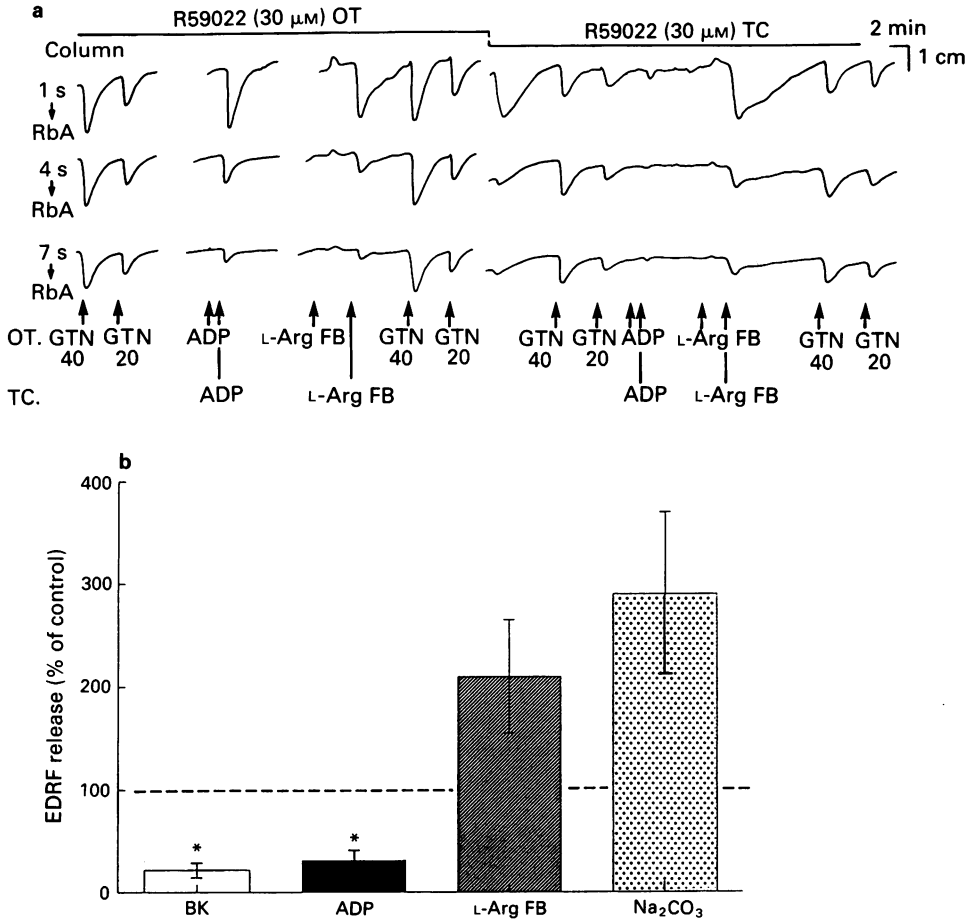

Figure 3 (a) Effect of diacylglycerol kinase inhibitor (R59022) on endothelium-derived relaxing factor (EDRF) release. Experimental details as in Figure 1. When R59022 (30 μM) was infused over the assay tissues (OT) glyceryl trinitrate, 20 or 40 pmol (GTN; 20 or 40) OT induced similar relaxations of all three rabbit aortic strips (RbA). ADP (2 nmol) or L-arginine free base (L-Arg FB; 20 μmol) injected through the column (TC) induced release of EDRF that caused relaxations of the top RbA equivalent to that induced by GTN 40. Infusions of R59022 TC caused a transient release of EDRF and inhibited the release of EDRF induced by ADP (smaller on the top RbA than GTN, 40). However, the EDRF release induced by L-Arg FB was potentiated (larger on the top RbA than GTN 40). Similar results were obtained in three other experiments. (b) The effect of R59022 (10–30 μM) on the release of EDRF induced by BK (10–30 pmol; $n = 6$; open column), ADP (2–6 nmol; $n = 8$; solid column), L-Arg FB (10–20 μmol; $n = 6$; hatched column) or alkaline buffer (Na_2CO_3; pH 8.6–9.5; $n = 3$; stippled column) as % of control release (dotted line); s.e.mean shown by vertical bars. R59022 decreased the release of EDRF in response to BK or ADP, but increased that in response to L-Arg FB or Na_2CO_3. Thus, in the presence of R59022 the release of EDRF induced by either BK or ADP was significantly different from that induced by either L-Arg FB or Na_2CO_3 (indicated by * $P < 0.05$ for both comparisons).

or monensin contrasts with its inhibitory action on the release of EDRF induced by other agonists (de Nucci *et al.*, 1988a). These results provide evidence that the release induced by L- or D-arginine FB, monensin or Na_2CO_3 is not receptor-mediated and shares a common mechanism for the activation of endothelial cells.

The novel finding that the diacylglycerol lipase inhibitor RHC 80267 (Sutherland & Amin, 1982) inhibits the release of EDRF induced by BK or ADP confirms our previous observation that the mechanism of EDRF release by these agonists involves activation of a phospholipase C (de Nucci *et al.*, 1988a). RHC 80267 should increase the concentrations of diacylglycerol in the cells leading to activation of protein kinase C and phosphorylation of G-proteins (Yamanishi *et al.*, 1983). This would act as a negative feedback system as previously reported for neutrophils (Smith *et al.*, 1987) and endothelial cells (de Nucci *et al.*, 1988a). The finding that the release of EDRF induced by L- or D-arginine FB, Na_2CO_3 or monensin

Table 1 The release of prostacyclin induced by different agonists

	6-oxo-$PGF_{1\alpha}$ (ng ml^{-1})				
Time (min)	0	1	2	3	*n*
BK (10 pmol)	<0.5	1.3 ± 0.3	2.5 ± 0.4	1.7 ± 0.3	26
ADP (2 nmol)	<0.5	1.2 ± 0.4	1.3 ± 0.3	<0.5	11
AA (30 nmol)	<0.5	4.8 ± 1.0	6.4 ± 1.2	4.0 ± 1.5	7
L-Arg FB (20 μmol)	<0.5	<0.5	<0.5	<0.5	10
Na_2CO_3 (pH 8.6–9.5)	<0.5	<0.5	<0.5	<0.5	4
Mon (40 nmol)	<0.5	<0.5	<0.5	<0.5	3

Prostacyclin release induced by bradykinin (BK), ADP, arachidonic acid (AA), L-arginine free base (L-Arg FB), alkaline buffer (Na_2CO_3) and monensin (Mon) was estimated by radioimmunoassay of 6-oxo-$PGF_{1\alpha}$ at times 0, 1, 2 and 3 min after doses of the above agents. The concentrations of 6-oxo-$PGF_{1\alpha}$ are given as the mean ± s.e.mean of *n* experiments.

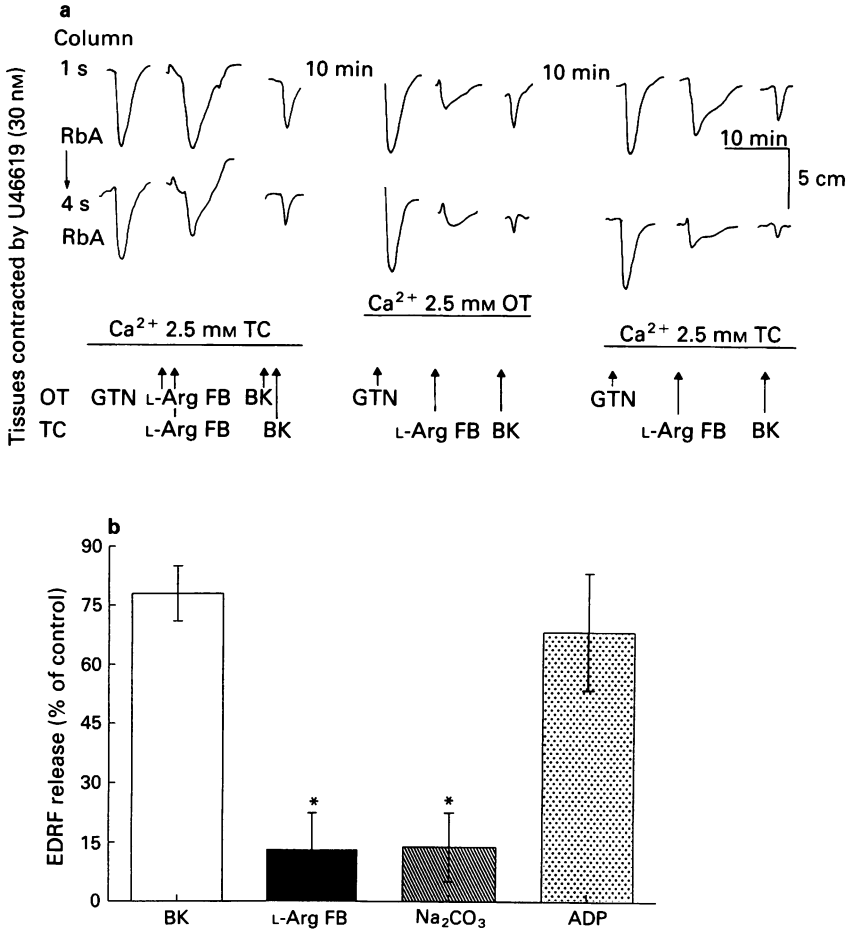

Figure 4 (a) Effect of lowering the extracellular calcium on endothelium-derived relaxing factor (EDRF) release. Experimental details as in Figure 1 but only two of four RbA are shown. Glyceryl trinitrate (GTN; 40 pmol) given over the assay tissues (OT) caused relaxations which were similar down the cascade. When calcium (Ca^{2+}; 2.5 mM) was infused through the column of endothelial cells (TC) EDRF release induced by L-arginine free base (L-Arg FB; 20 μmol) was greater than that induced by bradykinin (BK; 3 pmol). When the infusion of Ca^{2+} was changed from TC to OT and the endothelial cells were perfused with low Ca^{2+} Krebs solution, the release of EDRF induced by L-Arg FB was similar in magnitude to that induced by BK. When Ca^{2+} was returned TC, the release of EDRF induced by L-Arg FB was again greater than that induced by BK. Similar results were obtained in three other experiments. (b) The effect of removal of extracellular calcium on the release of EDRF induced by BK (3–30 pmol; $n = 4$; open column), L-Arg FB (10–20 μmol; $n = 4$; solid column), alkaline buffer (Na_2CO_3; pH 8.6–9.5; $n = 3$; hatched column) or ADP (2–6 nmol; $n = 3$; stippled column) as % of control; s.e.mean shown by vertical bars. Removal of the extracellular calcium significantly decreased the release of EDRF induced by L-Arg FB or Na_2CO_3 with respect to control and with respect to BK or ADP under the same conditions (indicated by * $P < 0.05$ all ways).

is potentiated by RHC 80267 reinforces the view that these agents are releasing EDRF by a mechanism different from that of BK or ADP.

It is interesting that the sodium ionophore, monensin causes a similar pattern of release of EDRF and PGI_2 to alkaline pH in that it releases EDRF with no simultaneous release of PGI_2. This could indicate that the release of EDRF in response to an increased extracellular pH is mediated by a similar mechanism to that of monensin. Most eukaryotic cells have several pH regulating mechanisms and the interaction between them is complicated. There is general agreement that all cells possess an electro-neutral Na^+/H^+ antiporter and that this antiporter has a pH regulating function after acid loads (Deitmer & Ellis, 1980; Krulwich, 1983). In addition most cells have a pH regulating anion antiporter, through which they can normalize pH after acid or alkali loads (Russel & Boron, 1976; Madshus, 1988). Adams *et al.* (1989) recently showed that monensin could elevate intracellular calcium in bovine pulmonary arterial cells and associated this action with the presence of a $Na^+:Ca^{2+}$ exchanger. Whether an increase in extracellular pH can activate a similar ion exchanger in the EC which leads to EDRF release remains to be investigated. A possible mechanism could be that after an increase in the Na^+ content of the EC, due to an alkali load, the EC exchanges Na^+ for Ca^{2+} and the resulting increase in intracellular Ca^{2+} causes EDRF release. This hypothesis is supported by the finding that extracellular Ca^{2+} has a more important function in the release of EDRF induced by alkaline pH than for that induced by BK or ADP where mobilisation of intracellular Ca^{2+} is probably involved (Lambert *et al.*, 1986; Derian & Moskowitz, 1986). The release of EDRF with little or no PGI_2 release by alkaline buffers and its dependence on extracellular Ca^{2+} fits well with the suggestion that a mobilisation of intracellular Ca^{2+} is vital for PGI_2 release (Luckoff, 1988).

The finding that L-NMMA inhibits the release of EDRF induced by an increase in pH as well as by receptor activation and that L-arginine lessens this inhibition suggests that L-NMMA acts at a fundamental point in the EDRF-generating system. The finding that L-arginine does not increase basal or stimulated release of EDRF in our system confirms previous observations using porcine aortic cultured EC (Palmer *et al.*, 1988a) or fresh vascular preparations (Thomas *et al.*, 1989). The intracellular concentration of L-arginine in cultured EC is approximately 100 μM and EC have the ability to maintain this arginine concentration during pro-

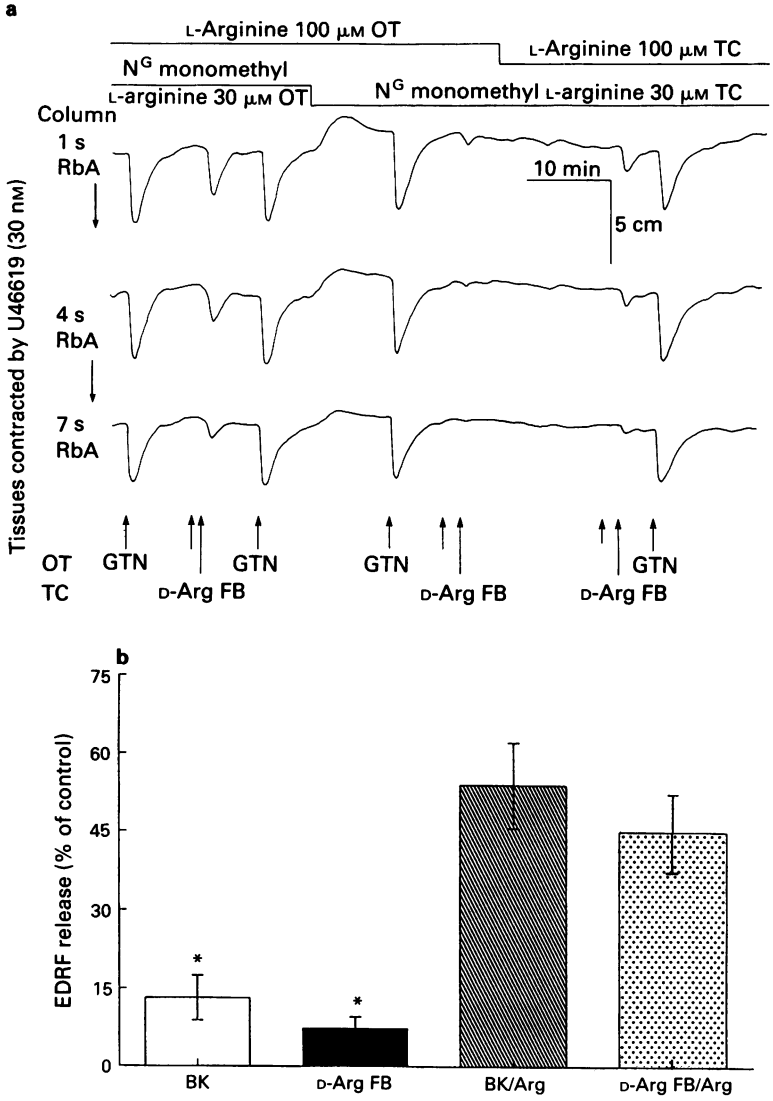

Figure 5 (a) The effect of N^G-monomethyl-L-arginine (L-NMMA) on endothelium-derived relaxing factor (EDRF) release induced by D-arginine free base (D-Arg FB). Experimental details as in Figure 1. Glyceryl trinitrate (GTN; 40 pmol) given over the RbA (OT) induced relaxations which were similar on all the RbAs. Infusion of L-NMMA (30 μM) OT had no direct effect on the tone of the RbA nor on the detection of EDRF release induced by D-Arg FB (20 μmol). Infusion of L-NMMA through the column of endothelial cells (TC) caused contraction of the RbAs and an inhibition of the release of EDRF induced by D-Arg FB. Additional infusions of L-Arg HCl (100 μM) partially reversed the inhibition, but did not fully restore the release of EDRF induced by D-Arg FB. Similar results were obtained in three other experiments. (b) The effect of L-NMMA (30 μM) on EDRF release induced by BK (10–30 pmol; $n = 4$; open column) or alkaline buffer (D-Arg FB; 10–20 μmol; $n = 4$; solid column) and the effect of the addition of L-Arg (100 μM) for BK ($n = 4$; hatched column) or D-Arg FB ($n = 4$; dotted column) as % of control release; s.e.mean shown by vertical bars. In the presence of L-NMMA the release of EDRF induced by BK or D-Arg FB was significantly different from control and from the release in the presence of a co-infusion of L-Arg (both indicated by, * $P < 0.05$).

longed EDRF release (Mitchell *et al.*, 1990a) by generating L-arginine from an intracellular store (Mitchell *et al.*, 1990b). This facility of EC could explain why the application of L-arginine does not affect the synthesis of EDRF.

The authors wish to thank Dr Renia Botting for her editorial help and Miss Elizabeth Wood for culturing the endothelial cells. The William Harvey Research Institute is supported by a grant from Glaxo Group Research Limited.

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(Received May 12, 1990
Revised November 29, 1990
Accepted January 7, 1991)

Br. J. Pharmacol. (1991), **103**, 1303–1308 © Macmillan Press Ltd, 1991

CGP 35348, a new $GABA_B$ antagonist, prevents antinociception and muscle-relaxant effect induced by baclofen

M. Malcangio, C. Ghelardini, A. Giotti, P. Malmberg-Aiello & [1]A. Bartolini

Department of Preclinical and Clinical Pharmacology, University of Florence, Viale G.B. Morgagni 65, I-50134 Firenze, Italy

1 CGP 35348, a new $GABA_B$ antagonist, was examined on antinociception induced by (±)-baclofen by use of the hot plate and writhing tests in mice and the paw pressure test in rats. CGP 35348 was also studied in mice on (±)-baclofen-induced impairment of rota-rod performance.

2 CGP 35348, injected either i.p. (60–100 mg kg^{-1} in mouse) or intracerebroventricularly (i.c.v.) (0.5–2.5 μg per mouse; 25 μg per rat) prevented (±)-baclofen-induced antinociception.

3 CGP 35348 did not modify oxotremorine- and morphine-induced antinociception in mice and rats.

4 CGP 35348 (2.5 μg i.c.v. per mouse) also prevented (±)-baclofen-induced impairment of the rota-rod test.

5 Two other $GABA_B$ antagonists, phaclofen (50 μg i.c.v. per mouse) and 2-OH-saclofen (2.5 μg–10 μg i.c.v. per mouse) did not modify (±)-baclofen-induced antinociception.

7 These results suggest that, at present, CGP 35348 is the only compound able to antagonize (±)-baclofen-induced antinociception.

Keywords: Analgesia; $GABA_B$-antagonism; 2-OH-saclofen; CGP 35348; baclofen; muscle relaxation

Introduction

The antinociceptive effect of systemically administered baclofen has been well documented in rodents (Cutting & Jordan, 1975; Levy & Proudfit, 1977; Bartolini *et al.*, 1981; Hill *et al.*, 1981; Sawynok & La Bella 1982; Vaught *et al.*, 1985) and has also been observed in man (Corli *et al.*, 1984). Baclofen exerts an antinociceptive effect which is not reduced by $GABA_A$ (Bartolini *et al.*, 1981; Sawynok & La Bella, 1982), opioid (Levy & Proudfit, 1979; Bartolini *et al.*, 1981) or muscarinic receptor antagonists (Bartolini *et al.*, 1981). It has been suggested that baclofen antinociception has both supraspinal and spinal components (reviewed by Sawynok, 1987) and catecholamines and substance P may play some role in the antinociceptive effect (Sawynok 1983; 1989; Sawynok *et al.*, 1984; Sawynok & Dickson, 1985b; Hwang & Wilcox, 1989).

Although some compounds, namely (+)-baclofen, δ-aminovaleric acid and phaclofen have been reported to prevent (±)-baclofen-induced antinociception, their lack of brain penetration and low potency have hampered *in vivo* studies (Bowery, 1989). In fact, (+)-baclofen and δ-aminovaleric acid only inhibit the antinociception if injected intrathecally at doses at least 20 times higher than those of (−)-baclofen (Sawynok & Dickson, 1985a; Sawynok, 1986).

Similarly, phaclofen was only able to antagonize the antinociception when administered intracerebroventricularly (Giuliani *et al.*, 1988).

It appeared, therefore, worthwhile to investigate whether CGP 35348 (3-aminopropyl-diethoxymethyl-phosphinic acid) and 2-hydroxysaclofen (3-amino-2-(4-chlorphenyl)-2-hydroxy-propylsulfonic acid), two new $GABA_B$ antagonists described respectively by Olpe *et al.* (1990) and by Kerr *et al.* (1988), were more effective antagonists of baclofen antinociception than phaclofen.

The effect of CGP 35348 on baclofen-induced muscle relaxant activity (Levy & Proudfit, 1977; 1979; Wilson & Yash, 1978; Sawynok & La Bella, 1982; Hammond & Drower, 1984) has also been investigated.

[1] Author for correspondence.

Methods

Male Swiss-Webster mice (25–30 g) and Wistar rats (120–150 g) were used. Fifteen mice or five rats were housed per cage. The cages were brought into the experimental room 24 h before the experiment for acclimatization. The animals were fed a standard laboratory diet and tap water *ad libitum*.

Intracerebroventricular (i.c.v.) injection was performed during short ether anaesthesia, isotonic saline being used as solvent according to the method described by Haley & McCormick (1957). Briefly, during anaesthesia, mice or rats were grasped firmly by the loose skin behind the head. A 0.4 mm external diameter, hypodermic needle attached to a 10 μl syringe was inserted perpendicularly through the skull and no more than 2 mm into the brain of mouse and 4 mm into the brain of rat, where 5 μl (for mouse) or 10 μl (for rat) of solution were injected. The injection site was 1 mm (for mouse) or 2 mm (for rat) to the right or left of the midline along a line drawn through the anterior base of the ears.

To ascertain the exact point into which drugs were administered, some mice or rats were injected i.c.v. with 5 μl or 10 μl of diluted 1:10 Indian ink and their brains were examined macroscopically after sectioning.

Hot plate

Mice were placed inside a stainless steel container thermostatically set at 52.5 ± 0.1°C in a precision water-bath from KW Mechanical Workshop, Siena Italy. The reaction time(s) was measured with a stop-watch before and at various times after treatment. The endpoint used was the licking of the fore or hind paws. Those mice which scored below 12 and over 18 s in the pretest were rejected. An arbitrary cut-off time of 45 s was adopted.

Paw pressure (Randall-Selitto)

The nociceptive threshold was determined with an analgesimeter (Ugo Basile, Varese, Italy) in rats. The instrument exerts

a force, expressed in g, which is applied at a constant rate with a cone-shaped pusher on the upper surface of the normal rat hind paw. The force was continuously monitored by a pointer moving along a linear scale. The pain threshold was given by the force which induced initial struggling of the rat. Those rats which scored below 60 g or over 120 g during pretests were rejected. An arbitrary cut-off value of 300 g was adopted.

Writhing

Mice were injected i.p. with 0.6% aqueous solution of acetic acid (10 ml kg^{-1}). The number of stretching movements was counted for 10 min, starting 5 min after acetic acid injection.

Rota-rod

Mice were tested on a rota-rod treadmill (Ugo Basile, Varese, Italy). The apparatus consisted of a base platform and a rotating rod of 3 cm diameter with a non-slippery surface. This rod was placed at a height of 15 cm from the base. The rod, 30 cm in length, was divided into 5 equal sections by 6 disks. Thus up to 5 mice were tested simultaneously on the apparatus, with a rod-rotating speed of 16 r.p.m. The integrity of motor coordination was assessed on the basis of the endurance time of the animals on the rotating rod. One day before the test, the animals were trained twice. On the day of the test only mice able to stay balanced on the rotating rod between 70 and 120 s (cut-off time) were selected. The performance time was measured before and at various times after treatment.

Drugs and reagents

The following drugs were used: (±)-baclofen (β-*p*-chlorophenyl GABA) and CGP 35348 (3-aminopropyl-diethoxymethyl-phosphinic acid) (CIBA-GEIGY), phaclofen (β-*p*-chlorophenyl-3-aminopropylphosphonic acid) and 2-hydroxysaclofen (3-amino-2-(4-chlorphenyl)-2-hydroxypropylsulphonic acid) (Tocris), oxotremorine (Sigma), morphine hydrochloride (Carlo Erba). All drugs were dissolved in isotonic (NaCl 0.9%) saline solution immediately before use. Drug concentrations were prepared in such a way that the necessary dose could be administered in a volume of 10 ml kg^{-1} by either s.c. or i.p. injection.

Statistical analysis

Significant differences were determined by analysis of variance (ANOVA), after Cochran's and Bartlett's tests had shown homogeneity of variances. Multiple comparisons with appropriate controls were made by ANOVA followed by the multiple range test for least significant differences (LSD). Means with 95% confidence intervals that did not overlap were considered significantly different (for statistical methods used, see Snedecor & Cochran, 1973). All data were analyzed by the programme STATGRAPHICS (1986, STSC Inc. USA).

Results

Effect of CGP 35348, 2-hydroxysaclofen and phaclofen on (±)-baclofen-induced antinociception in mice and rats

Pretreatment of mice by systemically administered CGP 35348 inhibited the increase in paw licking latency induced by (±)-baclofen in the hot plate test. Intraperitoneally injected CGP 35348 was ineffective at 30 mg kg^{-1} while at 60 mg kg^{-1} it significantly reduced (±)-baclofen antinociception which was abolished at the dose of 100 mg kg^{-1} (Table 1). All three doses of CGP 35348 alone did not modify paw licking latency of mice with respect to controls (Table 1).

Similarly, i.c.v. injection of CGP 35348 in mice caused a dose-dependent antagonism of (±)-baclofen-induced antinociception in the hot plate test (Table 2). CGP 35348 at the lowest dose of 0.1 μg i.c.v., failed to modify significantly the antinociceptive effect of (±)-baclofen whereas 0.5 μg and 2.5 μg reduced or abolished, respectively, the response to (±)-baclofen.

Mice treated with CGP 35348 2.5 μg i.c.v. appeared excited and their motility was significantly increased compared to controls (data not shown). The excitability was accompanied by a decreased paw licking latency in the hot plate test (Table 2). Higher doses of CGP 35348 were not tested in the hot plate assay since alterations in the behaviour of the mice were even more evident. A dose of 10 μg per mouse induced hypermotility and scratching while 25 μg produced seizures.

The effect of 2-hydroxy-saclofen was examined at doses of 2.5, 10 and 17.5 μg i.c.v. per mouse. However only 2.5 and 10 μg were used in the hot plate test in mice since 17.5 μg induced salivation and passivity. In contrast to CGP 35348, neither 2.5 nor 10 μg 2-hydroxy-saclofen antagonized the effect of baclofen on licking latency, but rather it was increased (Table 2). Similarly, phaclofen (50 μg per mouse i.c.v.) failed to antagonize the effect of baclofen (Table 2). This dose of phaclofen was the highest possible to be injected without impairing the overall behaviour of the mouse.

CGP 35348, injected both i.c.v. and systemically, did not modify oxotremorine- or morphine-induced increases in paw

Table 1 Effect of systemically injected CGP 35348 on (±)-baclofen activity on paw licking latency in the hot plate test (52.5°C)

Pretreatment (mg kg^{-1} i.p.)	*Treatment* (mg kg^{-1} s.c.)	n	*Licking latency in mice* (s) *before pretreatment*	*after treatment* (min) 30	45
Saline 10 ml	Saline 10 ml	20	14.1 ± 0.4	14.9 ± 0.7	14.6 ± 0.6
CGP 35348 30	Saline 10 ml	10	15.7 ± 1.1	14.2 ± 1.2	14.4 ± 0.7
CGP 35348 60	Saline 10 ml	10	14.8 ± 0.7	14.8 ± 1.8	15.6 ± 1.2
CGP 35348 100	Saline 10 ml	10	16.1 ± 0.6	14.5 ± 1.3	15.5 ± 1.4
Saline 10 ml	Baclofen 4	25	14.4 ± 0.8	30.9 ± 2.4*	33.0 ± 2.1*
CGP 35348 30	Baclofen 4	10	14.5 ± 0.8	26.7 ± 3.3*	32.9 ± 2.7*
CGP 35348 60	Baclofen 4	8	13.0 ± 1.7	19.0 ± 1.5*	25.9 ± 3.6*
CGP 35348 100	Baclofen 4	10	13.8 ± 0.9	12.9 ± 1.6	17.2 ± 1.4

Pretreatment injection was made 5 min before the 'treatment' drug injection. Significant differences were determined by ANOVA followed by LSD multiple range test: * $P < 0.05$ versus S/S controls.

Table 2 Effect of CGP 35348, phaclofen and 2-OH-saclofen i.c.v. injected, on (±)-baclofen activity on paw licking latency in the hot plate test (52.5°C)

Pretreatment μg i.c.v.	*Treatment* mg kg^{-1} s.c.	n	*Licking latency in mice* (s) *before pretreatment*	*after treatment* (min) 30	45
Saline 5 μl	Saline 10 ml	18	14.5 ± 0.6	14.6 ± 1.6	15.6 ± 1.6
CGP 35348 0.1	Saline 10 ml	10	14.2 ± 1.2	15.5 ± 1.1	14.0 ± 1.5
CGP 35348 0.5	Saline 10 ml	10	14.7 ± 0.8	13.5 ± 0.9	14.6 ± 0.9
CGP 35348 2.5	Saline 10 ml	15	14.5 ± 0.6	12.0 ± 0.5*	12.0 ± 0.9*
2-OHsaclofen 2.5	Saline 10 ml	10	13.5 ± 0.9	13.9 ± 1.6	14.2 ± 1.7
2-OHsaclofen 10	Saline 10 ml	10	14.3 ± 0.7	17.2 ± 1.5	18.4 ± 2.8
Phaclofen 50	Saline 10 ml	19	13.4 ± 0.4	16.4 ± 1.6	14.7 ± 2.0
Saline 5 μl	Baclofen 4	20	13.4 ± 0.9	30.8 ± 2.6*	32.3 ± 2.1*
CGP 35348 0.1	Baclofen 4	10	14.6 ± 0.9	25.6 ± 3.1*	27.9 ± 3.1*
CGP 35348 0.5	Baclofen 4	10	14.4 ± 0.8	17.4 ± 1.8	23.3 ± 2.7*
CGP 35348 2.5	Baclofen 4	20	12.6 ± 1.1	13.0 ± 1.7	14.6 ± 1.5
2-OHsaclofen 2.5	Baclofen 4	10	14.0 ± 0.8	41.3 ± 1.9*†	36.7 ± 2.6*†
2-OHsaclofen 10	Baclofen 4	10	13.8 ± 0.6	40.9 ± 2.1*†	41.2 ± 1.9*†
Phaclofen 50	Baclofen 4	46	14.3 ± 0.4	29.1 ± 1.9*	32.4 ± 1.9*

All pretreatment drugs were injected 5 min prior to the 'treatment' injection. Significant differences were determined by ANOVA followed by LSD multiple range test. *$P < 0.05$ versus S/S controls. †$P < 0.05$ versus S/baclofen group.

licking latency detected 15, 30 and 45 min after injection (Table 3).

CGP 35348, administered i.c.v., was tested against (±)-baclofen in two other classical models of antinociception. Figure 1 shows results obtained in the acetic acid-induced writhing test, in which CGP 35348 (2.5 μg per mouse) antagonized the (±)-baclofen-induced decrease in the number of stretches while 2-hydroxy-saclofen (2.5 μg i.c.v. per mouse)

Table 3 Lack of antagonism by CGP 35348 of effect of oxotremorine and morphine on paw licking latency in the hot plate test (52.5°C)

Pretreatment	*Treatment* (mg kg^{-1} s.c.)	n	*Licking latency in mice* (s) *before pretreatment*	*after treatment* (min) 30
Saline i.p.	Saline s.c.	20	14.1 ± 0.4	14.9 ± 0.7
Saline i.p.	Morphine 10	10	12.9 ± 0.7	32.9 ± 2.9*
CGP 35348 100 mg kg^{-1} i.p.	Morphine 10	10	13.1 ± 0.7	33.3 ± 1.9*
Saline i.p.	Oxotremorine 0.1	10	13.2 ± 0.6	39.4 ± 0.6*
CGP 35348 100 mg kg^{-1} i.p.	Oxotremorine 0.1	10	13.5 ± 0.6	32.2 ± 1.9*
Saline i.c.v.	Saline s.c.	18	14.5 ± 0.6	14.6 ± 1.6
Saline i.c.v.	Morphine 10	10	13.6 ± 1.1	40.0 ± 4.8*
CGP 35348 2.5 μg i.c.v.	Morphine 10	10	13.3 ± 0.8	39.2 ± 2.3*
Saline i.c.v.	Oxotremorine 0.1	10	14.2 ± 0.7	34.2 ± 2.3*
CGP 35348 2.5 μg	Oxotremorine 0.1	10	13.3 ± 0.6	40.6 ± 1.9*

All pretreatment drugs were injected 5 min prior to the 'treatment' injection. The licking latency scores were obtained at the time of maximum effect for both antinociceptive substances. Significant differences were determined by ANOVA followed by LSD multiple range test.

*$P < 0.05$ versus S/S controls.

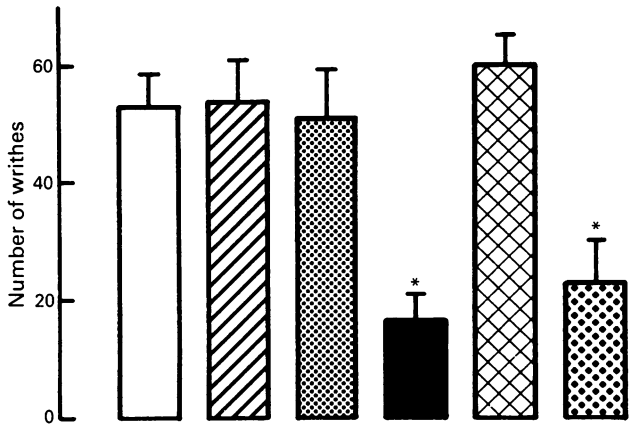

Figure 1 Effect of CGP 35348 (2.5 μg, i.c.v.) and 2-hydroxysaclofen (2.5 μg, i.c.v.) on (±)-baclofen (2 mg kg^{-1}, s.c.)-induced inhibition of mice writhing test. Groups were (pretreatment/treatment): saline/saline (open column); CGP 35348/saline (hatched column); 2-hydroxysaclofen/saline (finely stippled column); saline/(±)-baclofen (solid column); CGP 35348/(±)-baclofen (cross-hatched column); 2-hydroxysaclofen/(±)-baclofen (large stippled column). Pretreatment was performed 5 min before treatment and the writhing test was performed 45 min after treatment. At least 10 mice were used for each group. Bars represent s.e.mean. * $P < 0.05$ versus S/S controls, ANOVA followed by LSD range test.

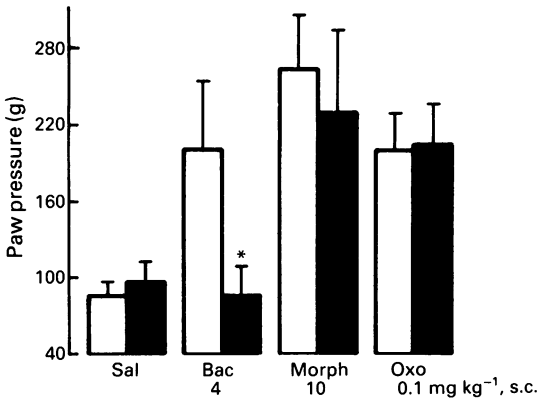

Figure 2 Effect of CGP 35348 (25 μg, i.c.v.) on (±)-baclofen-(Bac)-, morphine (Morph)- and oxotremorine (Oxo)-induced capacity to withstand increased paw pressure in the rat. I.c.v. pretreatment with saline (open columns) and CGP 35348 (solid columns), was given 5 min before treatment. Values shown were obtained 30 min after saline (Sal)-, baclofen- and oxotremorine-treatment and 15 min after morphine-treatment. At least 6 rats were used for each group. Bars represent s.e.mean. * $P < 0.05$ ANOVA followed by LSD range test.

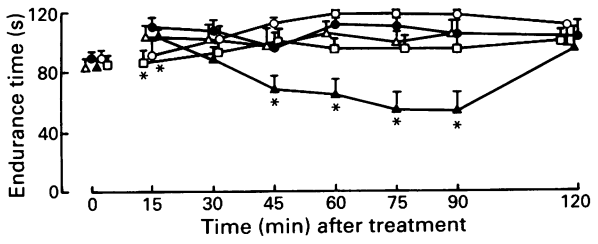

Figure 3 Effect of (±)-baclofen and CGP 35348 on rota-rod test performance in mice. Groups were treated (i.c.v./s.c.) as follows: saline/saline (●); saline/baclofen 4 mg kg^{-1} (△); saline/baclofen 6 mg kg^{-1} (▲); CGP 35348 2.5 μg/saline (○); CGP 35348 2.5 μg/baclofen 6 mg kg^{-1} (□). All pretreatment drugs were injected 5 min prior to the 'treatment' injection. Endurance time of mice on the treadmill was measured before treatment and then starting 15 min after treatment up to 120 min. At least ten mice were used for each group. * $P < 0.05$ versus saline controls by ANOVA followed by LSD range test. Bars represent s.e.mean.

failed to alter the frequency. The stretch frequency was not modified by treatment with CGP 35348 or 2-hydroxy-saclofen alone. Antagonism of the effect of (±)-baclofen (2 mg kg^{-1}, s.c.) by systemically injected CGP 35348 (60 mg kg^{-1}, i.p., 5 min before baclofen) was also confirmed in this test performed 30 min after baclofen (pretreatment/treatment: number of writhes mean ± s.e.mean; saline i.p./saline s.c.: 51 ± 6.8; CGP 35348 i.p./saline s.c.: 51.1 ± 10.8; CGP 35348 i.p./baclofen s.c.: 55.9 ± 7.5; saline i.p./baclofen s.c.: 25.5* ± 11.9; $n = 10$; * $P < 0.05$ versus S/S controls).

Figure 2 shows results obtained with the rat in the paw-pressure test. (±)-Baclofen (4 mg kg^{-1}, s.c.) elevated the ability of the rat to withstand paw pressure and this effect was antagonized by CGP 35348 (25 μg per rat) pretreatment. However, CGP 35348 did not change the morphine- or oxotremorine-induced capacity to withstand increased paw pressure.

Effect of CGP 35348 on (±)-baclofen-induced muscle-relaxation in mice

In addition to its antinociceptive activity, (±)-baclofen is an effective muscle-relaxant and this property could contribute to the results obtained in the antinociception models. However, in the antinociception experiments, the maximum doses of (±)-baclofen and CGP 35348 were 4 mg kg^{-1} and 2.5 μg i.c.v. per mouse respectively. Data shown in Figure 3 show that the endurance time on the rota-rod for mice injected with an antinociceptive dose of (±)-baclofen (4 mg kg^{-1}) was not altered with respect to controls for up to 120 min. Similarly, CGP 35348 injected i.c.v. at a dose of 2.5 μg, did not alter the rota-rod endurance time from 30 to 120 min after injection (Figure 3).

A decrease in the mouse endurance time on the rota-rod was induced by (±)-baclofen at a dose of 6 mg kg^{-1} which started 45 min after subcutaneous injection and persisted unchanged for 90 min but disappeared after 120 min (Figure 3).

CGP 35348 (2.5 μg i.c.v. per mouse) injected 5 min before (±)-baclofen (6 mg kg^{-1}) prevented this decrease in the rota-rod endurance time induced by (±)-baclofen which was detected for up to 120 min (Figure 3).

Discussion

The present results demonstrate that the GABA-analogue CGP 35348, antagonized (±)-baclofen-induced antinociception in mice and in rats as well as the (±)-baclofen muscle relaxant effect in mice. The effect of CGP 35348 appears to be specific to $GABA_B$ receptors since the antagonist was not able to modify morphine- or oxotremorine-induced antinociception.

Two other putative $GABA_B$ antagonists, phaclofen and 2-hydroxysaclofen, which were used for comparative purposes, failed to prevent the antinociceptive effect of (±)-baclofen. Antagonism of (±)-baclofen antinociception by CGP 35348 was obtained by both systemic and i.c.v. administration.

CGP 35348 efficacy after i.c.v. administration supports the contention that (±)-baclofen-induced antinociception is centrally mediated (Liebman & Pastor, 1980; Sawynok, 1987). CGP 35348 antagonism of (±)-baclofen antinociception occurred even after i.p. administration revealed the ability of this molecule to cross the blood-brain barrier although high doses (60–100 mg kg^{-1}) were necessary. These results are in agreement with Bittiger *et al.* (1990) who reported that CGP 35348 at doses of 30 mg kg^{-1} i.v. and 100 mg kg^{-1} i.p. was able to inhibit the depressant effect of cortically applied baclofen and to reverse the shift in the EEG induced by baclofen in freely moving rats.

The ability of CGP 35348 to cross the blood-brain barrier is a major advance by comparison with other putative $GABA_B$ antagonists. (+)-Baclofen and δ-aminovaleric acid have been

reported to antagonize partially the effects of (−)-baclofen, when injected intrathecally at high doses (10 μg) (Sawynok & Dickson, 1985a; Sawynok 1986). Similarly phaclofen was described as an antagonist of (±)-baclofen antinociception when injected i.c.v. at high doses (12.5–50 μg per mouse), being without effect after intraperitoneal administration (Giuliani *et al.*, 1988). These *in vivo* doses of phaclofen are almost 20 times higher than those of CGP 35348 used in the present work. Interestingly, a similar ratio of potency between phaclofen and CGP 35348 in antagonizing the (−)-baclofen effect has been reported *in vitro* in the spinal cord and the hippocampus (Olpe *et al.*, 1990).

However, in our study, phaclofen injected intracerebroventricularly was unable to prevent (±)-baclofen-induced antinociception. Discrepancies in the activity of phaclofen as a $GABA_B$ receptor antagonist have already been noted by others since earlier results showing phaclofen as a $GABA_B$ antagonist (Kerr *et al.*, 1987; Dutar & Nicoll, 1988) have not been substained (Robinson *et al.*, 1989; Stirling *et al.*, 1989; Wang & Dun, 1990). However the low potency of phaclofen may be the main cause of the contrasting results present in the literature (Bowery, 1989).

2-Hydroxysaclofen, although reported as more potent than phaclofen (Kerr *et al.*, 1988) was unable to prevent (±)-baclofen-induced antinociception in our experimental conditions even when injected directly into the cerebral ventricles. In the hot plate test, 2-hydroxysaclofen augmented the antinociceptive action of baclofen.

Among the three $GABA_B$ antagonists tested, CGP 35348 was the only one able to antagonize (±)-baclofen antinociception. CGP 35348 given i.c.v. but not i.p., induced a decrease in paw licking latency in the hot plate test. This effect of CGP 35348 does not seem to be due to an hyperalgesic action since neither in the mouse writhing test nor in the rat paw pressure test did CGP 35348 have any effect by itself. It seems likely that the decreased licking latency in the hot plate test may depend on an increased excitability of mice treated with CGP 35348 i.c.v. injected.

It is well known that (±)-baclofen has a muscle-relaxant effect in addition to its antinociceptive effect (Levy & Proudfit, 1977; 1979; Wilson & Yash, 1978; Sawynok & La Bella, 1982; Hammond & Drower, 1984). These two effects are not related since antinociception occurs at smaller doses of (±)-baclofen but CGP 35348, injected directly into cerebral ventricles, was also able to antagonize (±)-baclofen-induced motor incoordination. These results not only confirm CGP 35348 antagonism of muscle-relaxation induced by (±)-baclofen (already shown by administering CGP 35348 i.p. by Olpe *et al.* (1990)), but also demonstrate that this effect, like antinociception, is centrally mediated.

A comparison of the activities of phaclofen, 2-hydroxysaclofen and CGP 35348, has demonstrated that CGP 35348 is the most active $GABA_B$ antagonist *in vivo*, and thus will be a valuable tool for the investigation of the physiological role of $GABA_B$ receptors.

The authors wish to thank Prof. N.G. Bowery for helpful comments and suggestions in the preparation of the manuscript. Thanks to CIBA-GEIGY (Basel, CH) for the gift of CGP 35348 and to Mrs Mary Forrest for revision of the manuscript. This study was supported by grants from Ministero dell'Università e della Ricerca Scientifica e Technologica and CNR (progetto speciale di Comitato).

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(Received November 2, 1990
Revised January 15, 1991
Accepted January 21, 1991)

Br. J. Pharmacol. (1991), **103**, 1309–1312 © Macmillan Press Ltd, 1991

Effect of *pertussis* pretreatment on plasma glucose and insulin responses to lithium in rats

O. García Hermida, [1]T. Fontela, *M. Ghiglione & L.O. Uttenthal

Centro de Investigaciones Biológicas, C.S.I.C., Velázquez 144, 28006 Madrid, Spain, and *Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad de Salamanca, Espejo 2, 37007 Salamanca, Spain

1 Administration of lithium to rats causes a rise in plasma glucose and suppresses glucose-stimulated insulin secretion. These effects are blocked by the α_2-adrenoceptor antagonist, yohimbine.

2 Pretreatment of rats with *Bordetella pertussis* toxin resulted in a reversal of the usual plasma glucose and insulin responses to intravenously administered lithium (4 mEq kg^{-1}). There was a slow fall in plasma glucose, while plasma insulin rose to 267 ± 42% (±s.e.mean) of control values at 30 min. The effect of lithium on glucose-stimulated insulin secretion was also reversed; there was a marked increase in the insulin response which contrasted with the suppression seen in normal controls.

3 In perifused islets of Langerhans isolated from *pertussis* pretreated rats, the previously described inhibition by lithium of the second phase of glucose-stimulated insulin secretion from normal islets was almost completely abolished.

4 The results are consistent with the hypothesis that these effects of lithium are mediated by the influence of catecholamines on the islets. When the inhibitory effect of α_2-adrenoceptors is abolished by *pertussis* treatment, which blocks the action of the inhibitory guanine nucleotide-binding protein G_i, effects of β-adrenoceptor stimulation predominate, leading to an increased secretion of insulin.

Keywords: Lithium; *pertussis* toxin; adrenoceptors; glucose; insulin; islets of Langerhans

Introduction

Administration of lithium to rats leads to a rise in plasma glucose, while the rise in plasma insulin that would normally accompany this is abolished. Correspondingly it has been shown that glucose-stimulated insulin release is inhibited by lithium, both *in vivo* and *in vitro* (Anderson & Blackard, 1978; Shah & Pishdad, 1980). These effects are blocked by pretreatment of rats with the non-selective α-adrenoceptor antagonist, dihydroergotamine (Fontela *et al.*, 1986; 1987) and the selective α_2-adrenoceptor antagonist, yohimbine (Fontela *et al.*, 1990). It has therefore been suggested that this action of lithium is mediated via catecholaminergic stimulation, acting predominantly on the α_2-adrenoceptors of the pancreatic β cell. Activation of these receptors is associated with an inhibition of adenylate cyclase, brought about by the action of the inhibitory guanine nucleotide-binding protein G_i (Yamazaki et al., 1982). *Pertussis* toxin (islet activating protein) blocks the action of G_i by catalysing its ADP-ribosylation, which prevents its interaction with the receptor (Okajima *et al.*, 1985). The toxin is highly specific, in that the stimulatory protein G_s, which interacts with the β-adrenoceptors, is not affected (Murayama & Ui, 1983). Indirect confirmation of the involvement of catecholamines in the effects of lithium on insulin secretion can thus be obtained by the use of *pertussis* toxin to abolish the inhibitory action of the α_2-adrenoceptors while leaving the stimulatory effects of β-receptors intact. If the actions of lithium are mediated by catecholamines acting predominantly via α_2-adrenoceptors in the normal rat, they should be reversed in *pertussis*-treated animals, in which β-adrenoceptor effects predominate. We have therefore studied the effects of lithium on plasma glucose and insulin, and on glucose-stimulated insulin secretion *in vivo* and *in vitro*, in rats previously exposed to *pertussis* toxin.

Methods

Animals

Male Wistar rats (250–300 g) were kept under standard conditions previously described (Fontela *et al.*, 1990). They were injected i.p. with 0.125 ml of a killed liquid culture of *Bordetella pertussis* (8×10^{11} organisms ml^{-1}), containing active *pertussis* toxin (Swiss Serum and Vaccine Institute, Berne, Switzerland), or with an equal volume of vehicle. Experiments were carried out 5 days later. Food was withdrawn the night before each experiment, but free access to water was maintained.

In vivo *experiments*

Rats were anaesthetized with pentobarbitone sodium 20 mg kg^{-1} i.p., and the previously described experimental protocol was followed (Fontela *et al.*, 1990). Venous blood samples were collected before and at 5, 30, 60, 90 and 120 min after rapid (30 s) i.v. infusion of 0.5 M LiCl or control 0.5 M NaCl (4 mEq kg^{-1}), or after the further i.v. infusion (5 min later) of 0.55 M glucose (0.5 g kg^{-1}) for studies of glucose-induced insulin secretion. Plasma glucose was measured immediately by the glucose oxidase method and the remaining plasma was frozen at −18°C for determination of immunoreactive insulin (IRI) by radioimmunoassay (Amersham, UK).

Islet experiments

Pancreatic islets were isolated from control and *pertussis* pretreated rats by the collagenase digestion technique (Lacy & Kostianovsky, 1967). The islets were perifused at 0.9–1.0 ml min^{-1} in four parallel Millipore chambers, one pair containing 50 islets each from control rats, and the other pair containing the same number of islets from *pertussis* pretreated rats, as previously described (Fontela *et al.*, 1990). Two perifusion media were employed: a standard medium of normal ionic composition (Na^+ 139, K^+ 5, Ca^{2+} 2, Mg^{2+} 2, Cl^- 124 and HCO_3^- 24 mEq l^{-1}, pH 7.4) used for the first 30 min period and throughout the experimental time-period in the control chamber of each pair; and an otherwise identical lithium medium containing Na^+ 134 and Li^+ 5 mEq l^{-1}, used for the last 60 min time period in the experimental chamber of each pair. Both media were supplemented with bovine serum albumin 5 mg ml^{-1} and with glucose 3.2 mmol l^{-1} for the first 30 min and 16.7 mmol l^{-1} for the final 60 min of perifusion. The media were gassed with 95% O_2 and 5% CO_2, and were collected in graduated tubes at 1 or 5 min intervals. The volumes collected were recorded, and aliquots were removed

[1] Author for correspondence.

and frozen at −18°C for subsequent radioimmunoassay of IRI.

Analysis of data

The magnitude of the *in vivo* insulin response (total integrated response) was calculated as the area between straight lines joining adjacent points and the baseline, from time 0 to 120 min. For isolated islets the total insulin secreted into the medium per islet during 60 min of perifusion with glucose 16.7 mmol l^{-1} was calculated. The 'K value' (rate of fall of plasma glucose concentration after i.v. glucose administration) was calculated as the percentage fall in plasma glucose per minute during the period 5 to 30 min after the injection of glucose. The normal 'K value' is around 1.7 and lower values are indicative of glucose intolerance. Results are expressed as means ± s.e.mean, and differences between means of experimental and contemporaneous control values were analysed by the two-tailed Student's *t* test for unpaired data.

Results

In vivo *studies*

Effects of lithium on plasma glucose and insulin concentrations in control and pertussis *pretreated rats* Pretreatment of rats with *pertussis* toxin reversed the effects of lithium seen in control rats (Figure 1). Whereas acute lithium administration caused a rise in plasma glucose without a corresponding rise in plasma insulin in controls (Figure 1a and c), in the *pertussis*-treated animals there was a marked rise in plasma insulin to 267 ± 42% ($P < 0.005$) of control values at 30 min (Figure 1b), and a slow fall in plasma glucose to 79 ± 2% ($P < 0.001$) of control values at 120 min (Figure 1d).

Effects of lithium on glucose tolerance and glucose-induced insulin release in control and pertussis *pretreated rats* A similar reversal of lithium effects was seen in studies of i.v. glucose tolerance (Figure 2). Whereas lithium administration reduced glucose-stimulated insulin release in control rats (the total integrated response was 4799 ± 473 min · μu ml^{-1} as compared with 6426 ± 350 min · μu ml^{-1} in saline-injected rats, $P < 0.05$) and lowered the 'K value' of glucose disappearance from 1.85 ± 0.05% to 1.57 ± 0.02% ($P < 0.001$), in *pertussis*-treated rats the integrated insulin response rose from saline control values of 5950 ± 190 min · μu ml^{-1} to 8850 ± 280 min · μu ml^{-1} ($P < 0.001$) and the 'K value' from 1.64 ± 0.11% to 2.16 ± 0.08% ($P < 0.005$).

In vitro *studies*

Effects of lithium on glucose-induced insulin release from perifused islets of control and pertussis *pretreated rats* As shown in Figure 3, 16.7 mmol l^{-1} glucose elicits a biphasic release of IRI. The concurrent administration of Li^+ 5 mmol l^{-1} inhibits the second phase of IRI release, reducing total release from 143 ± 9 to 78 ± 9 μu per islet ($P < 0.005$), equivalent to a fall of 45 ± 6%. By contrast, in islets from *pertussis* pretreated rats, the inhibition of second-phase IRI release by lithium was greatly diminished, total IRI release falling from 140 ± 7 to 123 ± 8 μu per islet, a reduction of only 12 ± 6% that was not statistically significant ($P > 0.1$). It will be noted that *pertussis* pretreatment by itself did not significantly alter glucose-stimulated IRI-release in the absence of lithium.

Discussion

The extensive studies of Ui and his colleages (Ui, 1984) have established that *pertussis* toxin catalyses an ADP-ribosylation of the inhibitory guanine nucleotide-binding protein G_i that interacts with α-adrenoceptors, muscarinic ACh and certain

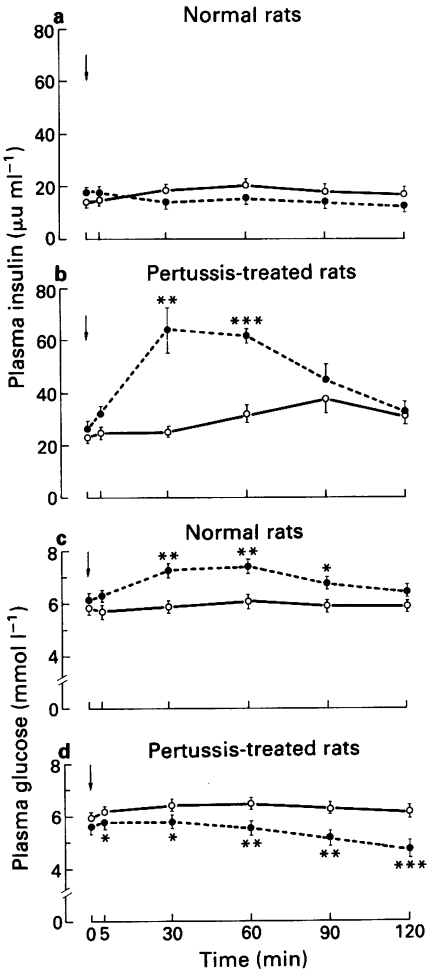

Figure 1 Effect of *pertussis* pretreatment on responses of plasma glucose and insulin to i.v. LiCl 4 mEq kg^{-1} in rats. (○) Results for NaCl-injected animals; (●) results for LiCl-injected animals. Time of LiCl or NaCl injection is marked by an arrow. Upper panels (a, b) show plasma insulin concentrations, and lower panels (c, d) plasma glucose concentrations in (a, c) untreated controls, and (b, d) *pertussis* pretreated rats. Each result represents the mean of five to eight rats and vertical bars show s.e.mean. Asterisks indicate the statistical significance of differences from the corresponding saline control values: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

other receptors, and is responsible for the negative signal transduction that leads to an inhibition of adenylate cyclase. ADP-ribosylation of G_i prevents its interaction with the receptor (Okajima *et al.*, 1985) and thus releases adenylate cyclase from G_i-mediated inhibition. At the same time, adenosine 3′ : 5′-cyclic monophosphate (cyclic AMP) production resulting from ligand-activation of stimulatory receptors is enhanced in the intact cell (Katada *et al.*, 1982), indicating a functional association between inhibitory and stimulatory guanine nucleotide-binding (G_s) proteins in the normal signal transduction from these receptors (Murayama & Ui, 1983; Asano *et al.*, 1984).

Previous studies (Fontela *et al.*, 1986; 1987; 1990) have suggested that the effect of lithium in raising plasma glucose levels and inhibiting glucose-stimulated insulin secretion is mediated by catecholaminergic influences on the pancreatic islets, acting principally via α_2-adrenoceptors. In the normal rat pancreas, α-adrenoceptors predominate over β-adrenoceptors, so that the overall effect of catecholamines is to inhibit insulin secretion (Katada & Ui, 1977). Treatment with *pertussis* toxin blocks the effects of α-adrenoceptor activation and thus exposes the unopposed effects of β-adrenoceptor activation. If lithium acts via a catecholamine related mechanism, its effects on insulin secretion should

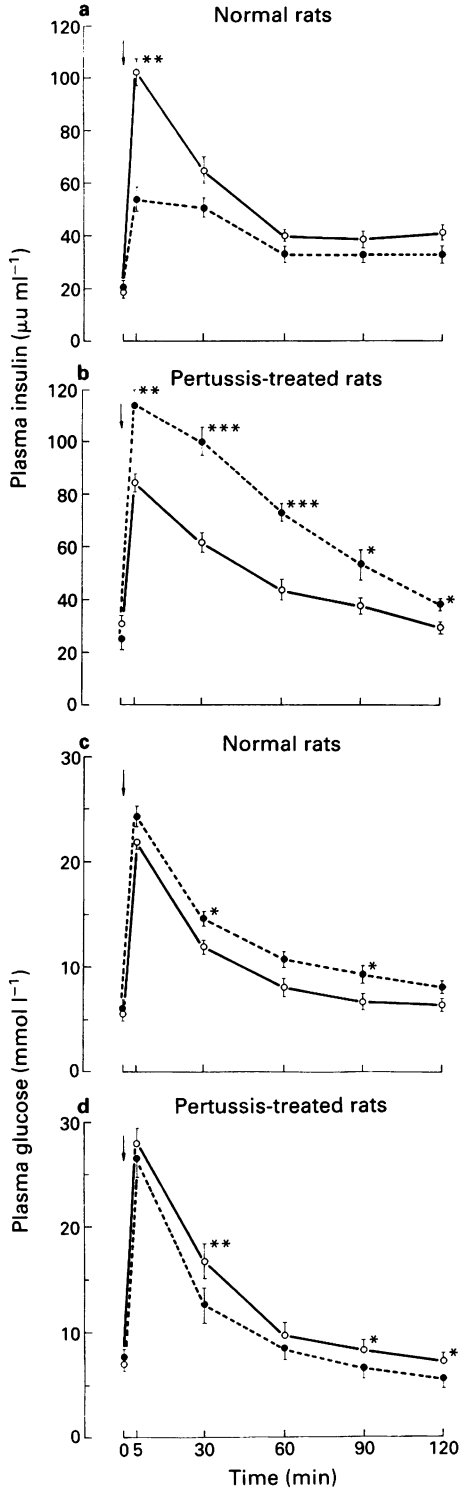

Figure 2 Effect of *pertussis* pretreatment on lithium-modified responses of plasma glucose and insulin after i.v. glucose 0.5 g kg^{-1} in rats. Results are shown for animals injected with NaCl (○) or LiCl 4 mEq kg^{-1} (●) 5 min before the injection of glucose, marked by an arrow. Upper panels (a, b) show plasma insulin concentrations, and lower panels (c, d) plasma glucose concentrations in (a, c) untreated controls, and (b, d) *pertussis* pretreated rats. Each result represents the mean of four to seven rats and vertical bars show s.e.mean. Asterisks indicate the statistical significance of differences from the corresponding saline control values: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

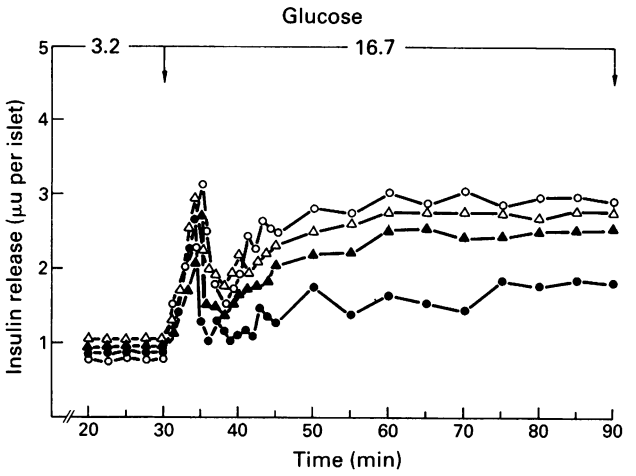

Figure 3 Effect of *pertussis* pretreatment on the inhibition by lithium of glucose-stimulated insulin release from perifused isolated islets. After isolation, islets were perifused for 30 min with 3.2 mmol l^{-1} glucose, and then exposed for a further 60 min to 16.7 mmol l^{-1} glucose either in a medium of normal ionic composition (control islets (○) and islets from *pertussis* pretreated rats (△)) or in a medium containing Li^+ 5 mmol l^{-1} (control islets (●) and islets from *pertussis* pretreated rats (▲)). Results are shown from one of seven similar experiments.

therefore, like those of adrenaline, be reversed in *pertussis*-treated animals. The results of the present study show that this reversal does in fact take place, so that lithium raises plasma insulin levels after an overnight fast, and causes a dramatic enhancement of glucose-stimulated insulin secretion in the intact animal. Thus far, the results are consistent with the pothesis that lithium is acting on the islets via catecholamine effects.

The effect of lithium in reducing plasma glucose levels in the fasted, *pertussis*-treated animal is susceptible of two explanations: first, it may clearly be attributed to the rise in plasma insulin, but secondly, there may be an additive effect from the action of lithium potentiating peripheral glucose uptake (Clausen, 1968). The latter effect is normally outweighed by the hyperglycaemic action of the catecholaminergic response in untreated animals, but may apply in the therapeutic situation where diabetic patients whose insulin secretion is already impaired have shown an improved glucose tolerance on lithium treatment (Jones *et al.*, 1983).

In the perifused rat islet preparation, the fact that pretreatment of the rats with non-selective α- or selective α_2-adrenoceptor antagonists almost fully blocks the inhibitory action of lithium on the second phase of glucose-stimulated insulin release (Fontela et al., 1987; 1990) suggests that this effect of lithium depends on the release within the isolated islets of noradrenaline from nerve terminals that persist around and within the islets. Other, more complex hypotheses to explain the effect, such as interactions of lithium with the unblocked receptor and signal transduction mechanisms, are also possible, but would presuppose actions for which there is no direct evidence. The finding that *pertussis* pretreatment partially reverses the inhibitory effect of lithium is consistent with the prevention of negative signal transduction from the α_2-adrenoceptors, but the absence in these circumstances of a stimulation of insulin secretion via β-adrenoceptors contrasts with the results *in vivo*. The *in vivo* stimulation of insulin secretion by lithium in *pertussis*-treated rats clearly depends on nervous and humoral mechanisms that cannot act on the isolated islets. These mechanisms would include a greater intensity of noradrenergic effect from the intact sympathetic system, the effect of circulating adrenaline, as inferred from the partial blockade of lithium action by bilateral adrenalectomy (Fontela *et al.*, 1986), and the effects of a number of neuropeptides and circulating peptide hormones that may stimulate insulin secretion via G_s-linked receptors. The significance of these *in vivo* effects that cannot act on the isolated islets is clearly demonstrated by the fact that the inhibition of glucose-stimulated insulin secretion by lithium is more profound *in vivo* than in the isolated islets (Fontela *et al.*, 1990). The fact

that the present and earlier results point to a major role for catecholamines in mediating the effect of lithium on insulin secretion does not exclude the possibility of significant co-operative effects mediated by other substances, such as neuropeptides and peptide hormones.

We are grateful to the Swiss Serum and Vaccine Institute and Laboratorios Berna, Madrid, Spain, for their gift of *B. pertussis* suspension. This work was supported by Grant PM-89-0005 from the Dirección General de Investigación Científica y Técnica, and is dedicated to the memory of Dr J. Gómez-Acebo, who died after its inception.

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(Received October 2, 1990
Revised January 22, 1991
Accepted January 23, 1991)

Br. J. Pharmacol. (1991), **103**, 1313–1320 © Macmillan Press Ltd, 1991

A calcium channel antagonist stereoselectively decreases ethanol withdrawal hyperexcitability but not that due to bicuculline, in hippocampal slices

M.A. Whittington & H.J. Little

Pharmacology Department, The Medical School, University Walk, Bristol, BS8 1TD

1 Extracellular recordings were made from CA1 area of isolated hippocampal slices of the mouse after chronic ethanol administration *in vivo*, with orthodromic stimulation of the Schaffer collateral/commissural fibres.

2 The (+)-isomer of the calcium channel antagonist PN 200-110 (isradipine) significantly decreased all the recorded signs of hyperexcitability in the slices during ethanol withdrawal. These included increased paired pulse potentiation and decreases in the thresholds for elicitation of single and multiple population spikes.

3 The (−)-isomer of PN 200-100 did not affect ethanol withdrawal hyperexcitability in the slices.

4 Neither isomer of PN 200-110 affected the field potentials in slices from control animals.

5 The γ-aminobutyric acid (GABA) antagonist, bicuculline, lowered thresholds for eliciting population spikes in hippocampal slices from untreated animals. The active, (+)-isomer of PN 200-110 did not affect this action of bicuculline in hippocampal slices from untreated animals.

6 The stereoisomerism of the action of PN 200-110 on ethanol withdrawal hyperexcitability in the hippocampal slice was therefore the same as that seen in blockade of calcium channels. The results suggested that ethanol withdrawal hyperexcitability recorded in the isolated hippocampal slice involved increased activity of voltage-sensitive calcium channels.

Keywords: Calcium channel antagonist; ethanol withdrawal; hippocampus; bicuculline

Introduction

Cessation of long-term ethanol intake results in a withdrawal syndrome, consisting, in rodents, of tremor, hyperreactivity and convulsions (Goldstein & Pal, 1971). The occurrence of this syndrome is an integral part of ethanol dependence and in man it is one of the causes of continued addiction to alcohol. The underlying neurophysiological changes that cause the syndrome are not yet understood. Acutely, ethanol has many neuronal effects, including decreases in the actions of the excitatory amino acid, N-methyl-D-aspartate (NMDA) (Lovinger *et al.* 1989), potentiation of the chloride flux induced by γ-aminobutyric acid (GABA) agonists (Ticku *et al.*, 1986) and blockade of calcium uptake (Harris & Hood, 1980).

It is not clear whether the acute effect of ethanol is selective for any particular calcium channel subtype. It has been suggested that many alcohols, including ethanol, have a selective action in blocking the 'T'-subtype of calcium channels (Llinas, 1988). Of a range from methanol to decanol, octanol was the most effective, and ethanol the least so, in blocking this low threshold calcium current in brainstem slices (Llinas & Yarom, 1986), while the high threshold 'calcium spike' was unaltered. Takahashi *et al.* (1989), however, did not find that octanol was selective for any particular subtype of calcium channel.

There is growing evidence that neuronal calcium channels may be important in ethanol-dependence. Administration of dihydropyridine calcium channel antagonists decreased the behavioural manifestations of the ethanol withdrawal syndrome (Dolin *et al.*, 1987; Littleton *et al.*, 1990). The number of dihydropyridine-sensitive binding sites in the CNS, thought to represent voltage-sensitive calcium channels, was increased by chronic ethanol administration (Dolin *et al.*, 1987; 1988a; Dolin & Little, 1989). This was a functional increase, as the actions of dihydropyridine compounds on phosphatidyl inositol turnover and on neurotransmitter release were also increased (Dolin *et al.*, 1987). The increase in dihydropyridine-sensitive sites with long-term ethanol intake is likely to be an adaptive response to its acute effects in decreasing calcium influx.

Whilst many changes in neuronal function have been demonstrated to occur after chronic ethanol treatment, it has been difficult to show whether or not they are causally related to tolerance and dependence. In the case of changes in neuronal calcium channels, additional evidence has been found, in that prevention of the increase in dihydropyridine-sensitive binding sites also prevented the development of ethanol tolerance and withdrawal. Chronic administration of calcium channel antagonists, during the ethanol intake, prevented the appearance of tolerance to the acute actions of ethanol (Dolin *et al.*, 1987; Wu *et al.*, 1987; Dolin & Little, 1989), the withdrawal syndrome (Whittington & Little, 1988) and the increase in the number of dihydropyridine-sensitive binding sites (Dolin *et al.*, 1988a; Dolin & Little, 1989).

We are currently investigating the neuronal basis of the ethanol withdrawal hyperexcitability and the protective action of the dihydropyridines. We have examined in detail the time course and pattern of hyperexcitability in isolated hippocampal slices after chronic ethanol administration in the mouse (Whittington & Little, 1990). Recordings were made from the CA1 area, with stimulation of the Schaffer collateral/commissural pathway. After the ethanol treatment there was an initial, transient, increase in paired pulse potentiation and a decrease in paired pulse inhibition, during the first 4 h of the recording period. Thresholds for eliciting single and multiple population spikes were decreased by the chronic ethanol schedule, for both orthodromic and antidromic stimulation. The threshold changes were apparent from approximately 3 h from the start of recording and reached a maximum at about 7 h. The input/output curves for population spike area and population excitatory postsynaptic potential (e.p.s.p.) slope showed a shift to the left for the slices from ethanol-treated animals, but no change in the maximal response. We suggested that several different mechanisms may underlie these changes, including a decrease in GABA-mediated inhibition, increased calcium influx and decreases in potassium currents.

The present study examined the actions of the isomers of the dihydropyridine calcium channel antagonist, PN 200-110 (isradipine) on the changes recorded from the isolated hippocampal slice after chronic ethanol treatment *in vivo*. The

ethanol treatment schedule was the same as we have used in our previous electrophysiological studies (Whittington & Little, 1990), and in behavioural and receptor binding experiments (Whittington & Little, 1988; Dolin *et al.*, 1988a). On withdrawal, it produced an increase in convulsive behaviour on handling, from approximately 4 to 12 h from withdrawal, but no spontaneous convulsions (Whittington & Little, 1988). The isomers of PN 200-110 have been found to show stereospecificity in their effects on calcium channels in rabbit aortic tissue (Hof *et al.*, 1986). The (+)-isomer was a potent calcium channel antagonist, while the (−)-isomer was without activity. For comparison, we have compared the effects of PN 200-110 on ethanol withdrawal hyperexcitability with its effect on hyperexcitability induced in hippocampal slices, from naive animals, by the GABA antagonist, bicuculline. The results provide further evidence for the importance of neuronal calcium channels in ethanol-dependence.

Methods

Chronic ethanol treatment

Male C57 mice were given either ethanol 24% v/v in tap water as sole fluid, or tap water for between 16 and 19 weeks. Ethanol intake was measured every four days, giving a mean ($\pm$s.e.mean) value of $12.1 \pm 0.9\,g\,kg^{-1}\,day^{-1}$. The mice were weighed at regular intervals during the treatment and there were no significant differences between the weights of the chronic ethanol group and the controls. The weights were 25–29 g at the start of the treatment, the mice being between 6 and 8 weeks old.

Addition of drugs

Stock solutions of the isomers of PN 200-110 were made up in pure dimethylsulphoxide (DMSO) and kept frozen. These were diluted each day in the perfusion medium to give final concentrations of 2 μM PN 200-110 and 0.02% DMSO. The PN 200-110 isomers were present in the perfusion medium throughout the entire recording period, from the beginning of the experiment. This experimental schedule was necessary because our previous studies (Whittington & Little, 1990) showed that the patterns of ethanol withdrawal hyperexcitability changed during the 7 h recording period and we wished to study the effects of PN 200-110 on all the manifestations of ethanol withdrawal. DMSO, at 0.02%, was added to the perfusion medium when no PN 200-110 was present.

A stock solution of bicuculline was made up in 0.1 M HCl, titrated to pH 3 with NaOH, and kept frozen. Dilutions were made in perfusion medium when required; the pH of the perfusion medium was unchanged by the addition of the bicuculline solution. Slices from untreated animals, were perfused with increasing concentrations of bicuculline, 100 nM to 1 μM, after the slices had been allowed to settle for 1 h, and recordings had been made in control conditions for 1 h. Each concentration of bicuculline was applied for 40 min, except for the highest concentration that was studied for 60 min. The effects of the (−)-isomer of PN 200-110 were studied by including this compound in the perfusion medium from the beginning of each experiment, in order to provide a direct comparison with its effects on ethanol withdrawal hyperexcitablility.

Preparation of isolated hippocampal slices

On the day of study each animal was killed by cervical dislocation between 09 h 00 min and 09 h 30 min. The ethanol drinking fluid was not removed from the cages prior to this. In the bicuculline studies, slices were prepared from untreated animals. The brain was dissected out in HEPES buffer (see below) and the hippocampus removed and cut transversely into 400 μm thick slices. The slices were placed in the recording chamber supported by lens tissue, on a nylon mesh over which the perfusion medium was passed at a flow rate of $1.7\,ml\,min^{-1}$. The perfusion fluid was a standard Krebs solution (composition given below) and was maintained at 30 ± 0.5°C throughout the experiments. Extracellular field potentials were studied by use of recording electrodes filled with 2 M KCl, with resistances between 5 and 15 MΩ. Square wave, paired pulse stimulation, using constant current, was given through stimulating electrodes consisting of two silver insulated wires, 0.2 mm in diameter, passed through 1 mm diameter silicon tubing. Stimulation parameters were 50 μs duration and 0.1 Hz.

Slice integrity was validated on the basis of three parameters: threshold for single population spike elicitation less than 100 μA, maximum spike height over 4 mV and no multiple spikes at stimulus strengths up to 1.25 × threshold. These criteria were applied at the beginning of the recording period; no slice was rejected from either chronic treatment group.

Certain precautions were taken to ensure that the comparisons between hippocampal slices from different animals were valid. The positioning of the electrodes was normalised between slices by use of anatomical markers. The ends of the arc of granular cells in the dentate gyrus were used as a reference point along the slice to indicate how far across the CA1 region to position the recording electrode. In all cases the tip of the recording electrode was between 50 μ and 150 μ from the surface of the slices. The apex of the CA3 region above the fimbria was chosen for the position of the stimulating electrode. Two recording chambers were used concurrently; slices from the different treatment groups were alternated between the two.

Field potential recordings

Orthodromic stimulation was applied through the Shaffer collateral/commissural fibres and recordings were made from the CA1 pyramidal cell layer. Measurements were made of the thresholds for elicitation of single population spikes and multiple population spikes. The field excitatory postsynaptic potentials (e.p.s.ps) recorded from the cell body layer provided an indication of the synaptic activity and the resultant hyperexcitability of the pyramidal cell dendrites. The threshold for multiple population spikes was defined as the minimum stimulus current required to produce a second population spike event superimposed on the same field e.p.s.p. as the first. The latter usually occurred in the second response to the paired pulse stimuli, at stimulus strengths supramaximal for the elicitation of single spikes.

Paired pulse potentiation was measured as the area of the second evoked population spike divided by the area of the first evoked spike. Pulses were applied at 70 ms intervals with a stimulation level of 1.25 times the threshold for eliciting single population spikes (see Whittington & Little, 1990). Stimulus levels for paired pulse measurements were adjusted to correct for the changes in threshold throughout the recording period, i.e. the stimulation was kept at 1.25 × threshold throughout.

In the studies on the ethanol withdrawal hyperexcitability, the slices were incubated for 30 min before the start of recording. The first recordings were therefore taken 45 min after withdrawal of the animal from ethanol. Readings of first and multiple spike thresholds, paired pulse potentiation at 70 ms separation, and the field e.p.s.p. slope for the first response, were taken every 15 min from the start of the recording. Full input/output relationships were established at 1, 3, 5 and 7 h after ethanol withdrawal (i.e. from the time of removal of brain). The stimulation levels used for these were from threshold to maximal population spike area, with nine intermediate stimulation settings.

In the bicuculline experiments, the measurements of the thresholds and paired pulse potentiation were made every 10 min and full input/output curves obtained at the end of each 40 min perfusion period (60 min for the control period and for the highest bicuculline concentration).

Expression and analysis of results

The field potentials were analysed on line by Acorn computer. The areas of the population spikes of the field potentials were measured, in $nV\,s^{-1}$ (nVs). Barnes (1979) found that use of the areas of the population spikes minimised variability between slices. In every case a minimum of four responses were averaged for each stimulus. The slope of the field e.p.s.p., for the first of the paired responses, was measured, by computer, as the gradient between two cursors placed at 20% and 80% of the maximum field e.p.s.p. deflection between the end of the fibre volley and the start of the population spike event.

Five tissues, each from a different animal, were studied for each treatment group, with the exception of the control group for the bicuculline experiments, in which six slices were used. Thresholds for eliciting single and multiple population spikes were expressed as mean ± s.e.mean. Paired pulse potentiation was expressed as mean ± s.e.mean, for the area of the second population spikes divided by the area of the first spike, in each pair.

The times given are all from removal of tissues. For the chronic ethanol treatment group, this corresponded with the withdrawal of ethanol as the ethanol remained in the drinking fluid up to this time. As ethanol was not included in the perfusion medium it would have washed out of the slices during the dissection and recording period.

The input/output data were fitted by nonlinear regression analysis to sigmoidal curves. The stimulation levels required to produce half maximal responses, the gradients and the amplitudes of the maximal responses were calculated from these curves, for the population spike areas and for the field e.p.s.p. slopes. The minimum field e.p.s.p. slopes for the induction of population spikes were also calculated. To determine the relationship between the population spike size and the field e.p.s.p. slopes, the individual measurements of the population spike areas (i.e. at all stimulation levels used) were plotted against the corresponding field e.p.s.p. slopes and the gradients of these lines calculated.

Comparisons were made between results from the different treatment groups by analysis of variance, for the thresholds and for the paired pulse potentiation. In view of the transient nature of the changes in paired pulse potentiation, comparisons were made for this measurement between 1 h and 4 h only. All other measurements were compared between 1 h and 7 h. In the bicuculline experiments, analysis of variance was used to compare the results obtained in the presence and absence of the (+)-isomer of PN 200-110, and the results obtained before and after addition of bicuculline to the bathing medium. In both sets of studies, the results from the calculations on the input/output data were compared by Student's nonpaired *t* test, at each time point.

Composition of solutions

The composition of the perfusion fluid was (mM): NaCl 124, KCl 3.25, KH_2PO_4 1.25, $NaHCO_3$ 20.0, $MgSO_4$ 2.0, $CaCl_2$ 2.0, and D-glucose 10.0. The pH was 7.2 at 30°C. The composition of the HEPES buffered dissection medium was (mM): NaCl 120, KCl 2.0, KH_2PO_4 1.25, $NaHCO_3$ 20.0, $MgSO_4$ 2.0, $CaCl_2$ 2.0, D-glucose 10.0, HEPES acid 7.0 and NaHEPES 2.6. The pH was 7.2 at 23°C.

Results

Changes in field potentials after chronic ethanol treatment

The effects of chronic ethanol treatment on the field potentials in the hippocampal slices were the same as previously described (Whittington & Little, 1990). There was a transient increase in paired pulse potentiation, between 1 h and 4 h into the recording period, followed by decreases in the thresholds required for elicitation of single and multiple population spikes (Figures 1–3, and Figure 5). The changes were all highly significant, $P < 0.005$ for comparison between controls and chronic ethanol-treatment for each parameter.

The input/output curves for population spike areas showed a shift to the left in the slices from ethanol-treated mice, at the 5 h and 7 h time intervals (see Figure 3a in Whittington & Little, 1990). The stimulus required to produce half-maximal response was significantly lower in slices from ethanol-treated mice at the above times ($P < 0.05$). No consistent change in the gradient or the maximum response was seen throughout the recording period. No differences were seen in the gradients, maximum responses or stimulation required for half-maximal responses for the input/output curves for the field e.p.s.p. slopes ($P > 0.05$).

Effects of PN 200-110 on ethanol withdrawal hyperexcitability

Effects of PN 200-110 on the changes in single population spike thresholds after ethanol withdrawal The (+)-isomer of PN 200-110, at 2 μM, significantly diminished the effect of ethanol

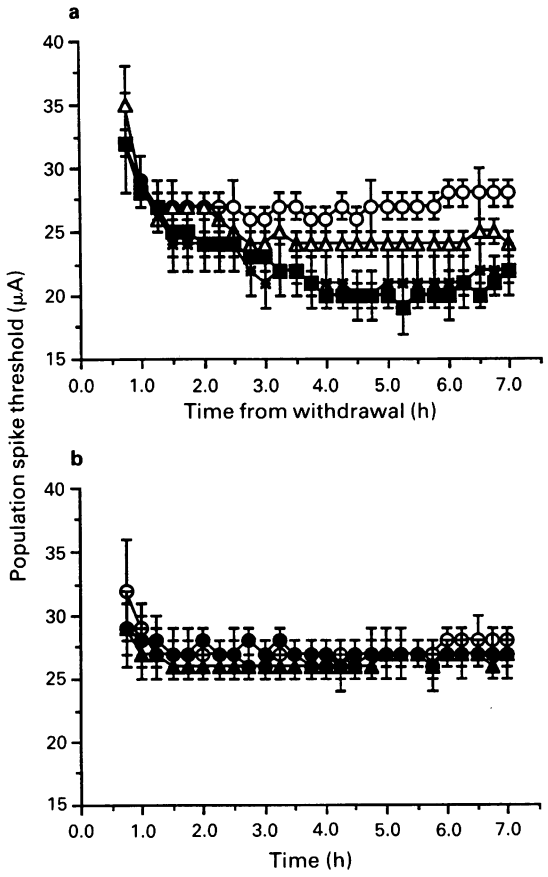

Figure 1 (a) The effects of the isomers of PN 200-110 on the decrease in thresholds for eliciting single population spikes caused by withdrawal from chronic ethanol treatment. PN 200-110, 2 μM, was present in the bathing medium from the beginning of recording. The results when (+)-PN 200-110 was added after ethanol withdrawal were significantly different from those when vehicle was added after ethanol withdrawal ($P < 0.005$) and from controls ($P = 0.03$). The (−)-isomer of PN 200-110 did not affect the lowering of threshold due to the ethanol treatment. Thresholds are in μA, mean with s.e.mean shown by vertical bars, $n = 5$ for each treatment group. Times given are all from preparation of tissues. (○) Controls; (■) chronic ethanol treatment; (△) chronic ethanol treatment and (+)-PN 200-110; (×) chronic ethanol treatment and (−)-PN 200-110. (b) Lack of effect of the isomers of PN 200-110, 2 μM, on single population spike thresholds (mean with s.e.mean shown by vertical bars, $n = 5$ for each treatment group) in tissues from control animals. Neither isomer caused any significant changes in the thresholds ($P > 0.1$). (○) Controls; (▲) (+)-PN 200-110; (●) (−)-PN 200-110.

withdrawal in decreasing the single population spike thresholds (Fig. 1a). Statistical comparison gave $P < 0.005$ for comparison of the effects of (+)-PN 200-110 and vehicle (DMSO) on slices prepared after chronic ethanol-treatment. The effect of the ethanol-treatment was not completely prevented, however, as there was a slight decrease in the single spike threshold in the presence of (+)-PN 200-110, $P < 0.03$ compared with control slices.

(−)-PN 200-110, 2 μM, did not alter the effects of chronic ethanol treatment on the thresholds for single spikes (Figure 1a), $P > 0.1$. Neither isomer of PN 200-110 affected the single spike thresholds in slices prepared from control animals (Figure 1b), $P > 0.1$.

Effects of PN 200-110 on the changes in multiple population spike thresholds after ethanol withdrawal The effects of chronic ethanol treatment in lowering the thresholds for eliciting multiple population spikes were almost compeltely abolished by the addition of 2 μM (+)-PN 200-110 to the bathing medium (Figures 2a and 5); $P < 0.001$ for comparison between vehicle and (+)-PN 200-110 on slices prepared on withdrawal from ethanol treatment. The effect of the ethanol treatment was still just apparent, although greatly reduced ($P < 0.005$, comparison between control slices and ethanol-treatment plus (+)-PN 200-110).

Effects of PN 200-110 on the changes in paired pulse potentiation after ethanol withdrawal The (+)-isomer of PN 200-110 completely prevented the transient increase in paired pulse potentiation produced by ethanol withdrawal (Figure 3a). There were no significant differences ($P > 0.1$) between the results from slices from ethanol-treated animals when (+)-PN 200-110 was added to the bathing medium and the results from control animals that had received no ethanol. The (−)-isomer of PN 200-110 was completely ineffective ($P > 0.1$) in preventing the effects of chronic ethanol treatment (Figure 3a). As shown in Figure 3b, neither isomer affected the degree of paired pulse potentiation in slices from control animals ($P > 0.1$).

Effects of PN 200-110 on the input/output relationships after ethanol withdrawal The decrease in the stimulus intensity required to produce half maximal population spikes, in slices

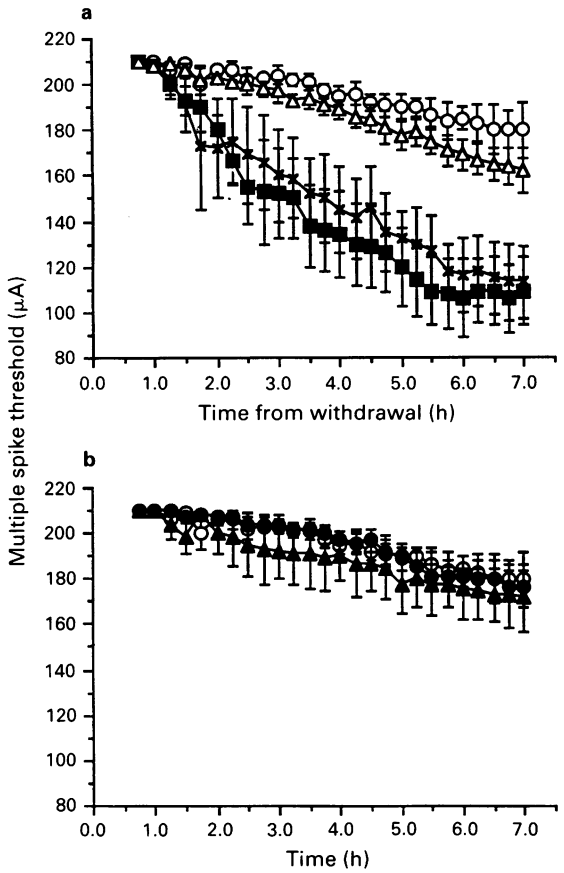

Figure 2 (a) The effects of the isomers of PN 200-110 on the decrease in thresholds for eliciting multiple population spikes caused by withdrawal from chronic ethanol treatment. PN 200-110, 2 μM, was present in the bathing medium from the beginning of recording. (+)-PN 200-110 significantly ($P < 0.001$) decreased the lowering of threshold caused by ethanol withdrawal. The results with (+)-PN 200-110 were significantly different from controls ($P < 0.005$). The (−)-isomer of PN 200-110 did not alter the lowering of threshold due to the ethanol treatment. Thresholds are in μA, mean with s.e.mean shown by vertical bars, $n = 5$ for each treatment group. (○) Controls; (■) chronic ethanol treatment; (△) chronic ethanol treatment and (+)-PN 200-110; (×) chronic ethanol treatment and (−)-PN 200-110. (b) Lack of effect of the isomers of PN 200-110, 2 μM, on multiple population spike thresholds (mean with s.e.mean shown by vertical bars, $n = 5$ for each treatment group) in tissues from control animals. Neither isomer had significant effects on the thresholds ($P > 0.1$). (○) Controls; (▲) (+)-PN 200-110; (●) (−)-PN 200-110.

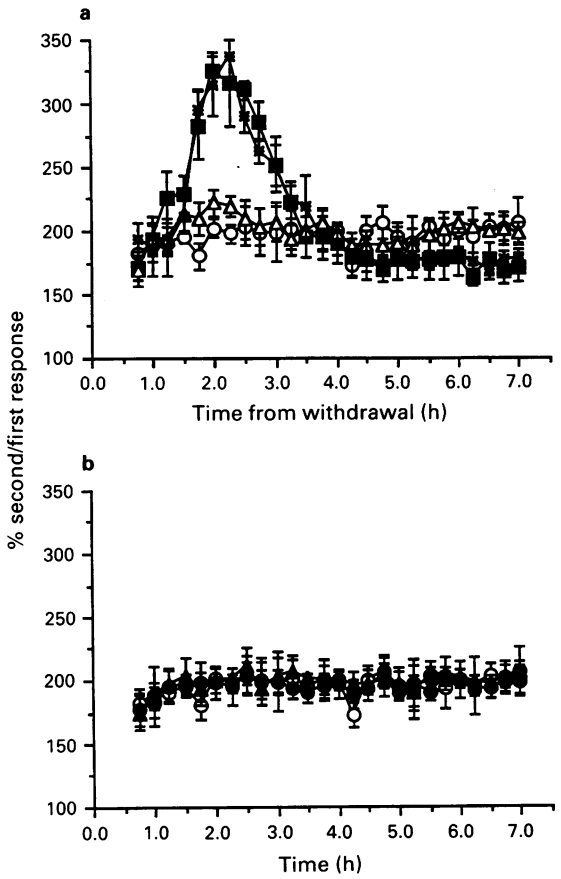

Figure 3 (a) The effects of the isomers of PN 200-110 on the increase in paired pulse potentiation of population spikes, caused by withdrawal from chronic ethanol treatment. PN 200-110, 2 μM, was present in the bathing medium from the beginning of recording. The (+)-isomer of PN 200-110 significantly ($P < 0.001$) diminished the increase in paired pulse potentiation seen after ethanol withdrawal. The results with (+)-PN 200-110 were not significantly different from the control results ($P > 0.1$). The (−)-isomer of PN 200-110 did not alter the effects of ethanol withdrawal on paired pulse potentiation. Results are expressed as area of second response/area of first response, mean with s.e.mean shown by vertical bars, $n = 5$ for each treatment group. Statistical comparisons were made between 1 h and 4 h from preparation of tissues. (○) Controls; (■) chronic ethanol treatment; (△) chronic ethanol treatment and (+)-PN 200-110; (×) chronic ethanol treatment and (−)-PN 200-110. (b) Lack of effect of the isomers of PN 200-110, 2 μM, on paired pulse potentiation in tissues from control animals. Neither isomer caused any significant changes ($P > 0.1$). (○) Controls; (▲)(+)-PN 200-110; (●)(−)-PN 200-100.

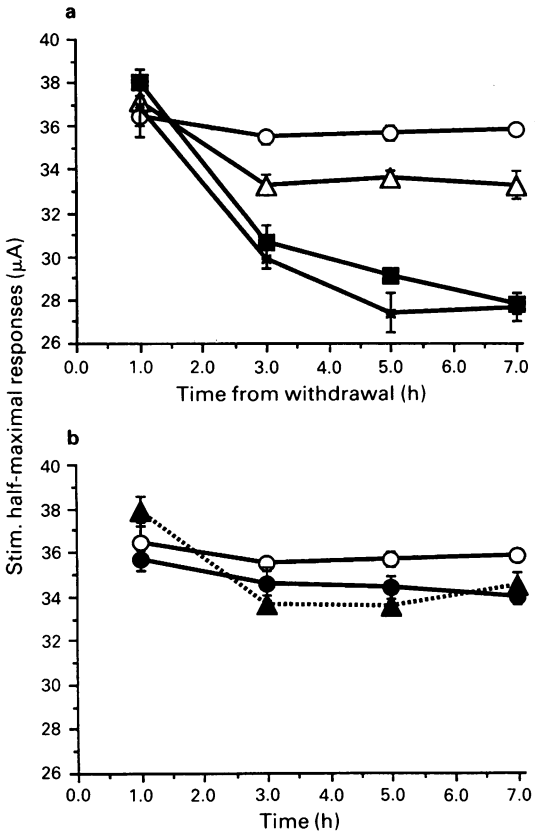

Figure 4 (a) The effects of the isomers of PN 200-110 on the decrease in the stimulation required for half-maximal responses caused by withdrawal from chronic ethanol treatment. PN 200-110, 2 μM, was present in the bathing medium from the beginning of recording. The (+)-isomer of PN 200-110 significantly diminished the decrease in half-maximal stimulation at 3 h, 5 h and 7 h from ethanol withdrawal ($P < 0.05$). The results with (+)-PN 200-110 were not significantly different from the control results ($P > 0.1$). The (−)-isomer of PN 200-110 did not alter the effects of ethanol withdrawal. Results are mean with s.e.mean shown by vertical bars, $n = 5$ for each treatment group. (○) Controls; (■) chronic ethanol treatment; (△) chronic ethanol treatment and (+)-PN 200-110; (×) chronic ethanol treatment and (−)-PN 200-110. (b) Lack of effect of the isomers of PN 200-110, 2 μM, on the stimulation level required for half-maximal responses in tissues from control animals. Neither isomer caused any significant changes ($P > 0.1$). (○) Controls; (▲) (+)-PN 200-110; (●) (−)-PN 200-110.

from ethanol-treated mice, was prevented by (+)-PN 200-110. There were no significant differences ($P > 0.05$) between the results from slices from ethanol-treated mice, recorded when (+)-PN 200-110 was added to the bathing medium, and the results from control animals that had received no ethanol. The (−)-isomer of PN 200-110 was completely ineffective in preventing this effect of withdrawal from chronic ethanol treatment (Figure 4a). No effect of either isomer of PN 200-110 was seen on measurements of the stimulus required to evoke half maximal responses in control slices ($P > 0.1$) (Figure 4b).

Neither isomer of PN 200-110 significantly affected the gradient, maximum response, or stimulus required to evoke half maximal responses, for field e.p.s.ps in slices from control or ethanol-treated mice ($P > 0.05$).

Effects of PN 200-110 on bicuculline-induced hyperexcitability

Comparison between the effects of ethanol withdrawal and those of bicuculline The effects of bicuculline on field potentials in slices from naive animals differed in certain ways from the changes seen after chronic ethanol treatment. Both bicuculline and ethanol withdrawal caused decreases in the thresholds for eliciting single and multiple population spikes, although these effects of the chronic ethanol treatment were seen only in the later part of the recording period. The decrease in the thresholds for single population spikes when bicuculline was added to the bathing medium was significant for bicuculline concentrations of 500 nM and 1 μM, but not at concentrations below 500 nM (Figure 6a). The addition of (+)-PN 200-110, 2 μM, did not alter the effects of bicuculline on the population spike thresholds.

Bicuculline also caused decreases in the thresholds for eliciting multiple population spikes, in preparations from naive animals (Figures 5 and 6b), significant at 500 nM and 1 μM bicuculline, but not at the lower concentrations. The (+)-isomer of PN 200-110, 2 μM, had no effect on this action of bicuculline (Figures 5 and 6b).

Some similarities were therefore seen between the effects of ethanol withdrawal and bicuculline, in that both decreased the thresholds for eliciting single and multiple population spikes, but PN 200-110 affected only those changes caused by the ethanol–treatment. Ethanol withdrawal caused a transient increase in paired pulse potentiation (Figure 3a), but the effects of bicuculline on responses to paired stimuli were difficult to establish. The drug seemed to prevent the phenomenon of paired pulse potentiation, in preparations from naive mice. However, this effect of bicuculline was only seen with concentrations of the drug suffucient to cause long trains of multiple spikes on stimulation, lower concentrations having little effect. Because of this, the response to the second stimulus, normally seen 70 ms after the first response, could not be distinguished from the later population spikes forming part of the response to the first stimulus. It was therefore not possible to measure accurately the effects of bicuculline on paired pulse potentiation.

Effects of PN 200-110 on the action of bicuculline on the input/output relationships The decrease in stimulus intensity required to evoke half maximal population spikes, seen in slices from mice treated with ethanol, was also seen when recording from control slices in the presence of bicuculline. At concentrations between 200 nM and 1 μM, bicuculline significantly reduced this measure of hippocampal excitability ($P < 0.01$). The (+)-isomer of PN 200-110, 2 μM, did not significantly alter the effect of bicuculline on the stimulus levels required to evoke half maximal population spikes ($P > 0.1$). The stimuli required were 64 ± 5 μA (no, (+)-PN 200-110) and 64 ± 4 μA (with PN 200-110 2 μM) for 200 nM bicuculline, and 39 ± 2 μA (no PN 200-110) and 41 ± 1 μA (with (+)-PN 200-110) for 1 μM bicuculline.

Discussion

The stereospecificity of the effects of the isomers of PN 200-110 in preventing ethanol withdrawal hyperexcitability in hippocampal slices was the same as that on peripheral tissues. Hof *et al.* (1986) demonstrated that the (+)-isomer of this compound possessed the cardiovascular actions characteristic of a calcium channel antagonist, while the (−)-isomer had little action. The same stereospecificity has been shown in the action of the isomers of PN 200-110 on inositol phospholipid hydrolysis in CNS tissue (Kendall & Nahorski, 1985). This suggests the action of PN 200-110 in decreasing the ethanol withdrawal hyperexcitability was due to blockade of calcium channels, rather than to any nonspecific mechanism. We have previously demonstrated that other dihydropyridines, such as (±)-nitrendipine, known to block hippocampal calcium channels (Thayer *et al.*, 1986), also protect against withdrawal hyperexcitability in the isolated hippocampal slice (Whittington & Little, 1989). Dihydropyridine calcium channel antagonists protect against the ethanol withdrawal syndrome *in vivo* with PN 200-110 showing the same pattern of stereospecificity (Little *et al.*, 1986; Littleton *et al.*, 1990).

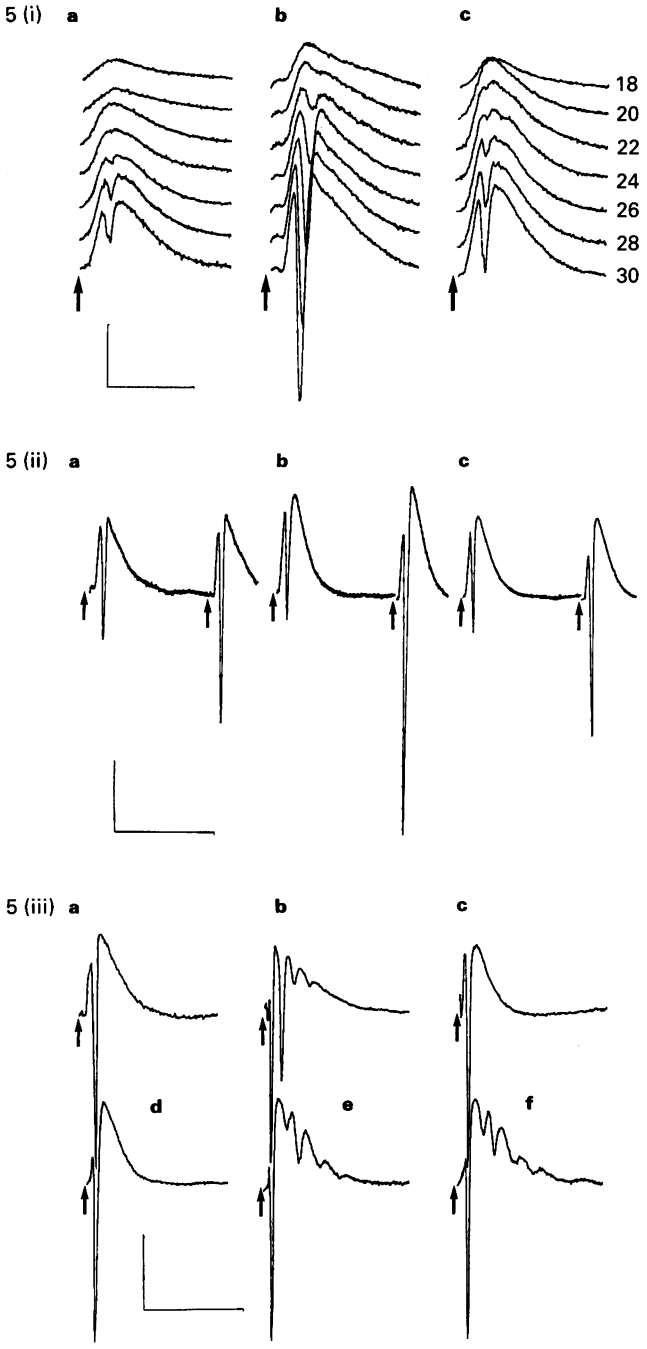

Figure 5 (i) The effects of chronic ethanol treatment, on field potentials, in the absence (b) and presence (c) of 2 μM (+)-PN 200-110. Traces from control slices (a) are included for comparison. These recordings were made 6 h after dissection (i.e. 6 h after ethanol withdrawal where chronic treatment given). Arrows indicate the time of stimulation. The stimulus intensities used for each example are given, in microamps, along the right of the traces. Scale bar = 2 mV/20 ms. (ii) Illustrates the effects of chronic ethanol treatment, on paired pulse potentiation, in the absence (b) and presence (c) of 2 μM (+)-PN 200-110. Traces from control slices (a) are included for comparison. All recordings shown were taken 2 h after dissection (i.e. 2 h after ethanol withdrawal where chronic treatment given). Arrows indicate time of stimulation. The slices were stimulated at 1.25 × the threshold for single population spikes. Scale bar = 4 mV/60 ms. (iii) Comparison of the effects of (+)-PN 200-110, 2 μM, on multiple population spikes caused by withdrawal from ethanol treatment, and on multiple population spikes produced by 500 nM bicuculline added to the bathing medium in control slices. (a) Control; (b) ethanol withdrawal; (c) ethanol withdrawal plus (+)-PN 200-110, 2 μM; (d) control slices; (e) bicuculline, 500 nM; (f) bicuculline, 500 nM plus (+)-PN 200-110, 2 μM. Recordings (a) to (c) were made 6 h after dissection (i.e. 6 h after ethanol withdrawal where chronic treatment given). The effects of bicuculline were studied in control preparations. The arrows indicate the time of stimulation, 150 μA. Scale bar = 4 mV/60 ms.

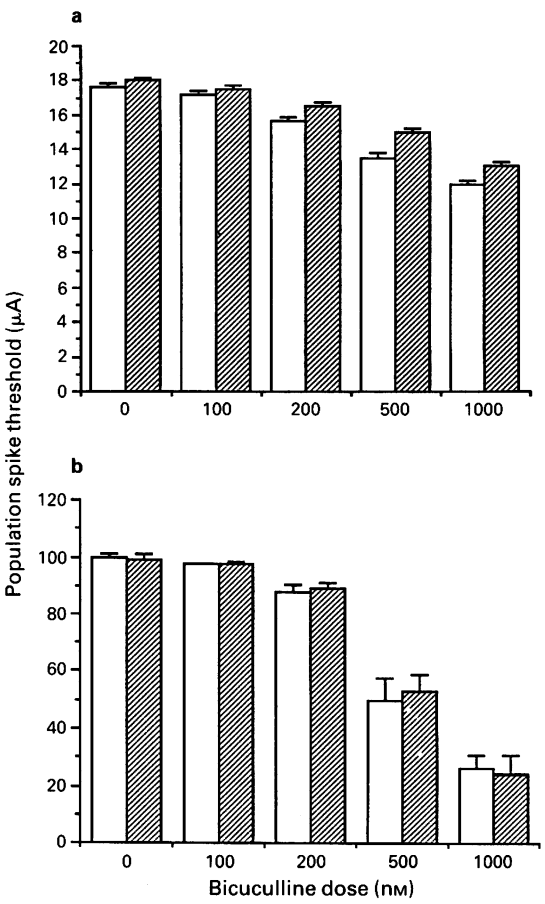

Figure 6 (a) The effects of (+)-PN 200-110 on the decreases in thresholds for eliciting single population spikes caused by a range of doses of bicuculline. The measurements illustrated were those obtained during the last set of measurements at the end of each incubation period. Thresholds are in μA, mean with s.e.mean shown by vertical bars, $n = 6$ for bicuculline alone and $n = 5$ for bicuculline plus (+)-PN 200-110. When the results obtained during the whole of each incubation period were compared, the addition of (+)-PN-110, 2 μM to the perfusion medium, from the beginning of recording, did not significantly alter the thresholds, compared with the effects of bicuculline in the absence of (+)-PN 200-110 ($P > 0.1$). Open columns: bicuculline; hatched columns: bicuculline and (+)-PN 200-110. (b) Lack of effect of (+)-PN 200-110 on the decreases in thresholds for eliciting mutliple population spikes caused by a range of doses of bicuculline. The results are expressed in the same way as those in (a). The measurements when (+)-PN 200-110, 2 μM, was present in the bathing medium from the beginning of recording were not significantly different from those when vehicle was added ($P > 0.1$). Thresholds are in μA, mean with s.e.mean shown by vertical bars, $n = 6$ for bicuculline alone and $n = 5$ for bicuculline plus (+)-PN 200-110. Open columns: bicuculline; hatched columns: bicuculline and (+)-PN 200-110.

Effects of PN 200-110 were seen only in tissues prepared from mice treated chronically with ethanol. This may have been due to the increase in the number of dihydropyridine binding sites reported after chronic ethanol administration (Dolin *et al.*, 1987; 1988a; Dolin & Little, 1989). The actions of dihydropyridine compounds on phosphatidyl inositol turnover and on neurotransmitter release were increased by chronic ethanol administration (Dolin *et al.*, 1987). Although anticonvulsant actions have been reported, these are not seen against all types of convulsions. Seizures due to bicuculline or N-methyl-D-aspartate were unaffected (Dolin *et al.*, 1987; 1988b; 1990).

It is now well established from *in vitro* experiments that higher concentrations of dihydropyridines are required to affect neuronal calcium channels than the corresponding channels in other tissues. Bean (1984) showed that dihydropy-

ridines had higher affinities for inactivated calcium channels other than states. The number of channels in the inactivated state is increased by depolarization and the time the channels spend in the inactivated state varies between different types of tissue. The difference between the binding affinities, that are in the nanomolar range, and the concentrations required for functional effects, is therefore due to the effect of the membrane potential. Binding studies *in vitro* are carried out on homogenized tissue, that are effectively totally depolarized. Greenberg *et al.* (1986) showed that the affinity of (+)-PN 200-110 in intact cultured PC12 cells was increased by depolarization.

Although it was originally thought that dihydropyridine calcium channel antagonists did not affect neuronal calcium channels, actions have recently been reported. Nitrendipine at 1 μM inhibited calcium influx into primary cultures of hippocampal and striatal neurones (Thayer *et al.*, 1986). Docherty & Brown (1986) found that nimodipine at 0.05–10 μM decreased calcium currents in hippocampal CA1 pyramidal cells. Pentylenetetrazol-induced paroxysmal discharges in the CA1 area of hippocampal slices were also reduced by nifedipine at 1 μM (Louvel *et al.*, 1986) and Grover & Teyler (1990) showed that a component of long-term potentiation in the hippocampal slice was prevented by nifedipine at 10 μM. Kendall & Nahorski (1985) found that approximately 1 μM (+)-PN 200-110 was required for maximal effect on depolarization-induced inositol phosphate accumulation. The selectivity of dihydropyridines for the 'L' subtype of channel, originally described in cultured neurones (Nowycky *et al.*, 1985) may not be as clear in normal mammalian neurones (Akaike *et al.*, 1989; Takahashi *et al.*, 1989).

The changes seen in this study in the field potentials of the isolated hippocampal slices after chronic ethanol treatment were the same as those reported in our previous study (Whittington & Little, 1990), both in magnitude and time course. The removal of ethanol from the slices during dissection and perfusion would occur faster than the removal of ethanol from the CNS *in vivo*, so it is not possible to make a direct comparison between the time courses of the *in vivo* and *in vitro* changes.

The (+)-isomer of PN 200-110 significantly decreased all the changes seen in the hippocampal slices after chronic ethanol treatment. It almost compeletely removed the decrease in multiple spike thresholds and the increase in paired pulse potentiation. It was not quite as effective on the action of the ethanol treatment in decreasing the single population spike thresholds as on the other two parameters, but the protective effect was clear. PN 200-110 is very selective in its calcium channel blocking action (Hof *et al.*, 1984). Its stereoselective effects on the ethanol withdrawal hyperexcitability suggest that neuronal calcium channels may be involved in the genesis of ethanol withdrawal hyperexcitability. Dihydropyridine-sensitive channels are not thought to be involved in neurotransmitter release from nerve terminals (Hirning *et al.*, 1988). Dihydropyridine-sensitive sites have been found to be located in the cell bodies and proximal dendrites of hippocampal CA1 pyramidal cells, with particular high densities at the base of major dendrites (Sanna *et al.*, 1986; Westenbroek *et al.*, 1990). This suggests that the hyperexcitability seen after ethanol withdrawal may arise in the basal dendritic regions.

The main changes in field potentials in the CA1 pyramidal cell layer seen after ethanol withdrawal were decreases in thresholds for eliciting single and mutliple population spikes and increases in paired pulse potentiation. The thresholds for multiple population spikes are an indication of the ability of the pyramidal cells to repolarize after firing. Postsynaptic changes that might decrease these thresholds include increased calcium influx during depolarization, prolonging firing, or a decrease in the ability of potassium currents to repolarize the cells. Increased numbers of calcium channels in the base of the dendrites would increase the calcium transients known to occur in that region and therefore increase the transmission of excitation from dendrites to soma. This would result in lowering of the thresholds measured in the present study, as seen following ethanol withdrawal.

One site that has often been suggested as important in ethanol dependence is the GABA receptor-chloride ionophore. It has been suggested that acute ethanol administration potentiates GABA agonists, although the electrophysiological evidence is contradictory (see Little, 1990, for review). Durand & Carlen (1984), using intracellular recording from hippocampal slices, demonstrated decreases in GABA-mediated inhibition after chronic ethanol treatment *in vivo*, but these were seen three weeks after withdrawal from ethanol. In a later paper from this group, however, no changes were found in inhibitory postsynaptic potential (i.p.s.p.) amplitude in CA1 pyramidal cells, 24 h after withdrawal from chronic ethanol treatment (Reynolds *et al.*, 1990). The protective action of PN 200-110 against the hyperexcitability due to ethanol withdrawal, in hippocampal slices in the present study, and *in vivo* in previous work, but not on hyperexcitability due to bicuculline, *in vivo* or *in vitro*, suggests that GABA-mediated inhibition does not play a large part in the ethanol withdrawal syndrome.

We thank the Wellcome Trust and the Brewers' Society for financial assistance in this study and Sandoz AG (Basel) for the gift of the isomers of PN 200-110. H.J.L. is a Wellcome Trust Senior Lecturer.

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(Received November 14, 1990
Revised January 22, 1991
Accepted January 25, 1991)

Br. J. Pharmacol. (1991), **103**, 1321–1326

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The effects of endothelin-1 and N^G-nitro-L-arginine methyl ester on regional haemodynamics in conscious rats with streptozotocin-induced diabetes mellitus

R.J. Kiff, [1]S.M. Gardiner, A.M. Compton & T. Bennett

Department of Physiology & Pharmacology, University of Nottingham Medical School, Queen's Medical School, Nottingham NG7 2UH

1 Resting haemodynamic status and responses to endothelin-1 (0.004, 0.04, 0.4 nmol kg^{-1}) and N^G-nitro-L-arginine methyl ester (L-NAME, 10 mg kg^{-1}) were assessed in conscious, Wistar rats treated with streptozotocin (STZ) to induce diabetes mellitus, and in control animals treated with saline.

2 In the resting state, STZ-treated rats had a bradycardia relative to control animals (291 ± 13 and 337 ± 10 beats min^{-1}, respectively), but mean arterial blood pressures were the same in the two groups (STZ-treated 109 ± 3; control 114 ± 4 mmHg). However, the STZ-treated rats had raised renal (105 ± 9 units) and mesenteric (114 ± 16 units) vascular conductances and reduced hindquarters vascular conductance (26 ± 4 units) relative to control rats (renal, 80 ± 6; mesenteric, 75 ± 7; hindquarters, 37 ± 3 units).

3 Increasing doses of endothelin-1 caused similar, early falls and subsequent rises in mean arterial blood pressures in both groups of rats. Although there were initial hindquarters vasodilatations with endothelin-1 that were not different in STZ-treated and control rats, there were subsequent renal and mesenteric vasoconstrictions that were greater in the former. Hence, the similar rises in mean arterial blood pressures must have been accompanied by a greater reduction in cardiac output in the STZ-treated rats.

4 L-NAME caused similar renal and mesenteric vasoconstrictions in control and STZ-treated rats, but there was a smaller pressor effect and an attenuated hindquarters vasoconstrictor response to L-NAME in STZ-treated rats.

5 Collectively, the results indicate that the resting renal and mesenteric vasodilatations in STZ-treated rats relative to control were probably not due to diminished vascular sensitivity to endogenous endothelin-1 or to enhanced sensitivity to, or production of, nitric oxide in these vascular beds. However, the relative hindquarters vasoconstriction in the resting state and the diminished hindquarters vasoconstrictor response to L-NAME in STZ-treated rats is consistent with diminished nitric oxide production in that vascular bed.

Keywords: Streptozotocin; endothelin-1; N^G-nitro-L-arginine-methyl ester (L-NAME); regional haemodynamics; diabetes mellitus

Introduction

It is known now that endothelial cells make important contributions to cardiovascular regulation through the release of vasoconstrictor and vasodilator factors (Furchgott, 1983). At present, the most potent endothelium-derived vasoconstrictor identified is the peptide, endothelin-1 (Yanagisawa *et al.*, 1988), whereas the major vasodilator produced by endothelial cells is nitric oxide (Palmer *et al.*, 1987; Hutchinson *et al.*, 1987; Moncada & Higgs, 1990). *In vivo* it is likely there is interplay between these factors since there is *in vitro* evidence that endothelin-1 stimulates the release of endothelium-derived relaxing factor (Miller *et al.*, 1989; Warner *et al.*, 1989) whereas the latter inhibits endothelin-1 release, in as much as antagonism of nitric oxide production disinhibits endothelin-1 release (Boulanger & Lüscher, 1990).

Such interactions could contribute to the complex cardiovascular responses to administering exogenous endothelin-1 or manipulating nitric oxide production *in vivo*. For example, in conscious animals i.v. bolus injections of endothelin-1 have been shown to cause a biphasic arterial blood pressure and heart rate response. There is an initial transient fall in blood pressure, which is associated with a tachycardia, followed by a more sustained rise in blood pressure and bradycardia (Gardiner *et al.*, 1989a; Madeddu *et al.*, 1989; Mortensen & Fink, 1990). The initial hypotension is associated with marked vasodilatations in the hindquarters and carotid vascular beds, whereas the subsequent hypertension is associated with vasoconstrictions in renal, mesenteric, hindquarters and carotid vascular beds (Gardiner *et al.*, 1989a).

In conscious rats, bolus injections of N^G-monomethyl-L-arginine (L-NMMA) or N^G-nitro-L-arginine methyl ester (L-NAME) also cause initial hindquarters vasodilatations and, thereafter, marked renal, mesenteric hindquarters and carotid vasoconstrictions, accompanied by hypertension (Gardiner *et al.*, 1990c,d). Since L-NMMA and L-NAME both inhibit nitric oxide production (Palmer *et al.*, 1988; Rees *et al.*, 1990), then it is feasible that the similarity in the haemodynamic effects of endothelin-1, L-NMMA and L-NAME is due in part to the actions of the latter compounds being dependent on disinhibition of the release of endogenous endothelin-1 (Boulanger & Lüscher, 1990).

The production of endothelin-1 and nitric oxide by endothelial cells and the interactions between these factors is likely to be abnormal in conditions in which endothelial cell function is disordered; one such condition is clinical diabetes mellitus (Porta *et al.*, 1987; Stout, 1987; Jensen *et al.*, 1989). Furthermore, studies on tissues taken from animals with experimental diabetes mellitus have indicated abnormalities of vascular function associated with disordered endothelium-mediated responses, although the findings are contradictory (Oyama *et al.*, 1986; Bhardwaj & Moore, 1988; Harris & Macleod, 1988). However, no comparative *in vitro* studies on tissues from normal rats and rats with experimental diabetes mellitus have used appropriately adjusted bathing media to control for the differences in the composition of the extracellular fluid of control and diabetic rats (Hebden *et al.*, 1986), and hence this might be a source of confusion. This problem could

[1] Author for correspondence.

be avoided by carrying out studies *in vivo*, but thus far there have been no investigations of regional haemodynamic status in conscious rats with experimental diabetes mellitus that have considered the possible consequences of endothelial cell dysfunction. So, in the present work we assessed regional haemodynamic status in conscious, unrestrained Wistar rats with diabetes mellitus induced by injection of streptozotocin (STZ), and compared these animals with saline-injected control rats. We found (see Results) that STZ-treated animals had resting renal and mesenteric vasodilatations, but a hindquarters vasoconstriction, relative to control rats. Therefore, we measured regional haemodynamic responses to increasing doses of endothelin-1 in both groups of animals and compared those responses to the effects of administering a maximal dose of L-NAME (Gardiner *et al.*, 1990d).

Methods

Male Wistar rats (aged between 15–20 weeks and weighing 350–500 g) were injected with either STZ (70 mg kg^{-1} in 0.9% NaCl) or isotonic saline (0.9% NaCl) intraperitoneally (i.p.) (Hebden *et al.*, 1986; Tomlinson *et al.*, 1989). Blood glucose levels were determined from a tail vein blood sample, 4 days after the STZ injection, by use of a Reflomat (Boehringer, Mannheim); those rats with blood glucose levels of greater than 12 mmol l^{-1} were considered diabetic and were entered into the experiment.

Fourteen days after STZ or saline injection the rats were anaesthetized (sodium methohexitone 60 mg kg^{-1} i.p., supplemented as required) and miniaturized pulsed Doppler probes (Haywood *et al.*, 1981) were sutured around the left renal and superior mesenteric arteries and the distal abdominal aorta (below the ileocaecal artery to measure hindquarters flow). The wires from the probes were secured to the body wall before being fed subcutaneously to the back of the neck and sutured in place. The animals received an intramuscular injection of ampicillin (Penbritin, Beecham, 7 mg kg^{-1}) and were allowed to recover in their home cages (Gardiner *et al.*, 1990d).

After a further 14 days the animals were re-anaesthetized (sodium methohexitone 40 mg kg^{-1} i.p., supplemented as required). The wires from the probes were soldered to a microconnector (Microtech Inc., Boothwyn, PA, U.S.A.). The right jugular vein was exposed and three venous catheters inserted. An intra-arterial catheter was inserted into the caudal artery and advanced up into the distal abdominal aorta. The catheters were all fed subcutaneously to the back of the neck. The microconnector was fixed to a harness worn by the rat and the catheters were fed through the harness and up a flexible spring, supported by a counter-weight to permit free movement of the animals. The animals were allowed to recover and were housed individually, with unlimited access to food and water (Gardiner *et al.*, 1990d).

The following day the microconnector was linked to a pulsed Doppler flowmeter (VF-1 Crystal Biotech, Massachusetts, U.S.A.) modified to operate with a pulse repetition frequency of 125 kHz (Gardiner *et al.*, 1990b) and the arterial catheter was connected to a pressure transducer. Heart rate, phasic and mean blood pressure and phasic and mean Doppler shift signals were recorded continuously on a Gould ES 1000 recorder. Mean Doppler shift signals were taken as an index of flow (Haywood *et al.*, 1981) and are referred to as such in the text hereafter. Vascular conductances were calculated by dividing the mean Doppler shift values by mean blood pressure (Gardiner *et al.*, 1990d) and are expressed in arbitrary units (i.e. [kHz mmHg^{-1})10^3]).

After a 30 min period of baseline recording each animal received three doses of endothelin-1 (0.004, 0.04 and 0.4 nmol kg^{-1}) in ascending order, with at least 1 h between each dose. Measurements were made before and 10, 14, 36 and 64 s after each endothelin-1 injection. These times were chosen to provide a full profile of the effects of endothelin-1.

At least 2 h after the last dose of endothelin-1, each animal received L-NAME at a dose of 10 mg kg^{-1}. This dose was chosen because it produced maximal effects in other studies (Gardiner *et al.*, 1990d). Measurements were made before L-NAME and at a time when the variables had reached a steady state (approximately 6 min after L-NAME injection).

Blood glucose levels (Reflomat) were determined from a cardiac puncture sample at the end of the experiment. All the STZ-treated rats had blood glucose levels in excess of 20 mmol l^{-1} (the limit of detection of the Reflomat); the blood glucose concentration in the control animals was 5.8 ± 3 mmol l^{-1} (mean ± s.e.mean; $n = 9$).

Data analysis

Data analysis involved the non-parametric, Wilcoxon signed ranks paired test, and the non-parametric Mann-Whitney U test for paired and unpaired data, respectively. $P < 0.05$ was taken as significant.

Drugs

STZ (Sigma) was dissolved in isotonic saline (47 mg ml^{-1}) and used immediately because of its instability. Endothelin-1 (Peptide Institute, Osaka, Japan, obtained through Scientific Marketing Associates, London) was dissolved in isotonic saline containing 1% bovine serum albumin and L-NAME (Sigma) was dissolved in isotonic saline.

Both endothelin-1 and L-NAME were administered in 0.1 ml boluses and the catheter flushed with 0.15 ml isotonic saline.

Results

Resting conditions

Under resting conditions, STZ-treated rats ($n = 11$) had significantly ($P < 0.05$) lower heart rates than control rats ($n = 9$), but there was no difference between the mean blood pressures of the two groups (Table 1). Renal and mesenteric blood flows were not significantly different in the control and diabetic rats, but hindquarters blood flow was significantly ($P < 0.05$) reduced in the latter (Table 1). Calculated vascular conductances showed significant vasodilatation in the renal and mesenteric vascular beds of the diabetic animals ($P < 0.05$) and a significant hindquarters vasoconstriction ($P < 0.05$: Table 1) compared to controls.

Cardiovascular responses to endothelin-1

Blood pressure and heart rate The lowest dose of endothelin-1 (0.004 nmol kg^{-1}) had no significant effect on blood pres-

Table 1 Cardiovascular variables under basal conditions in conscious, unrestrained, control and diabetic Wistar rats

	Control (n = 9)	*Diabetic* ($n = 11$)
Heart rate (beats min^{-1})	337 ± 10	291 ± 13*
Mean arterial blood pressure (mmHg)	114 ± 4	109 ± 3
Doppler shift (kHz)		
Renal	9.2 ± 0.8	11.5 ± 1.0
Mesenteric	8.5 ± 0.7	12.2 ± 1.6
Hindquarters	4.2 ± 0.4	2.9 ± 0.4*
Vascular conductance (arbitrary units)		
Renal	80 ± 6	105 ± 9*
Mesenteric	75 ± 7	114 ± 16*
Hindquarters	37 ± 3	26 ± 4*

Values are mean ± s.e.mean.
* $P < 0.05$ Diabetic versus control, Mann-Whitney U test.

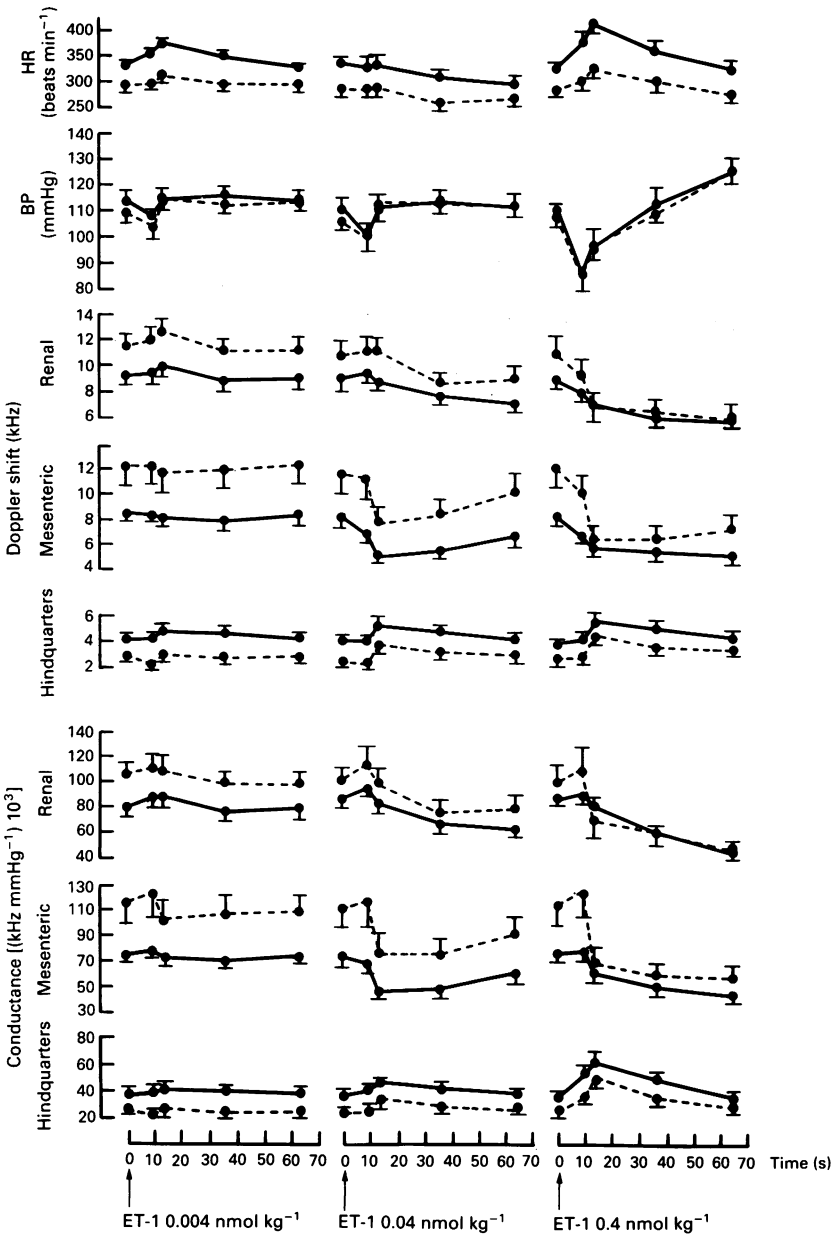

Figure 1 Changes in heart rate (HR), mean arterial blood pressure (BP), mean Doppler shift (blood flow) and vascular conductances of conscious, unrestrained, control (solid line) and streptozotocin (STZ)-treated (dotted line), Wistar rats to three i.v. bolus injections of endothelin-1 (ET-1). Values are the mean, and the bars are the s.e.mean; control $n = 9$, STZ $n = 11$. For clarity all the statistics are given in the text.

sure, and caused only a modest and transient tachycardia in both groups (Figure 1).

The 0.04 nmol kg^{-1} dose of endothelin-1 caused similar significant, but transient falls in blood pressure in both groups (control -12 ± 4 mmHg; STZ -6 ± 2 mmHg, $P < 0.05$: Figure 1); thereafter blood pressure returned to baseline in the control group whereas it rose modestly above baseline in the STZ group ($+8 \pm 2$ mmHg, $P < 0.05$: Figure 1). There were no significant changes in heart rate in either group.

After the highest dose of endothelin-1 (0.4 nmol kg^{-1}) there were marked, transient hypotensive responses in both groups (control -25 mmHg; STZ -24 ± 3 mmHg, $P < 0.05$: Figure 1) followed by rises in blood pressure above baseline (control $+16 \pm 4$ mmHg; STZ $+18 \pm 3$ mmHg, $P < 0.05$: Figure 1). The hypotensive response to endothelin-1 was associated with a greater ($P < 0.05$) increase in heart rate in the control than in the STZ-treated group (Figure 1).

Renal flow and vascular conductance In both groups of rats, endothelin-1 caused dose-related falls in renal flows and vascular conductances (significant for the 0.04 and 0.4 nmol kg^{-1} doses: Figure 1). After the highest dose of endothelin-1 the maximum fall in renal vascular conductance in the STZ-treated group (-52 ± 7 units) was significantly ($P < 0.05$) greater than in the control group (-35 ± 4 units), such that the absolute vascular conductances in the groups were then the same (Figure 1).

Mesenteric flow and vascular conductance There were dose-related falls in mesenteric flow and vascular conductance in both groups which were significant following the 0.04 and 0.4 nmol kg^{-1} doses of endothelin-1 (Figure 1). In the STZ-treated group the maximum fall in mesenteric flow following the 0.4 nmol kg^{-1} dose (-6 ± 1 kHz) and the maximum falls in mesenteric vascular conductance following the 0.04 and 0.4 nmol kg^{-1} doses (-40 ± 8 and -55 ± 9 units, respectively) were greater than the corresponding changes seen in the control group (-3 ± 1 kHz; -25 ± 4 units; -31 ± 6 units, respectively, all $P < 0.05$). Thus, following the 0.4 nmol kg^{-1} dose of endothelin-1 the absolute mesenteric

Table 2 Comparison of the haemodynamic effects of N^G-nitro-L-arginine methyl ester (L-NAME) ($10\,mg\,kg^{-1}$) in conscious, unrestrained, control and diabetic Wistar rats

	Control ($n = 9$)	*Diabetic* ($n = 11$)
Δ Heart rate (beats min^{-1})	-91 ± 13†	-55 ± 4*†
Δ Mean arterial blood pressure (mmHg)	39 ± 3†	31 ± 3*†
Δ Doppler shift (kHz)		
Renal	-2.2 ± 0.7†	-2.7 ± 1.0†
Mesenteric	-4.1 ± 0.5†	-4.6 ± 6†
Hindquarters	-1.1 ± 0.2†	-0.6 ± 0.2†
Δ Vascular conductance (arbitrary units)		
Renal	-34 ± 5†	-39 ± 10†
Mesenteric	-41 ± 6†	-55 ± 8†
Hindquarters	-17 ± 2†	-10 ± 2*†

Values are mean ± s.e.mean.
† $P < 0.05$ change from baseline (Wilcoxon signed rank pairs test).
* $P < 0.05$ for change in STZ group versus change in control group (Mann-Whitney U test).

vascular conductances were the same in control and STZ-treated rats (Figure 1).

Hindquarters flow and vascular conductance The lowest dose of endothelin-1 had no significant effect on hindquarters flow or vascular conductance in either group. Endothelin-1 ($0.04\,nmol\,kg^{-1}$) caused similar rises in hindquarters flow in both groups, but this was associated with a significant ($P < 0.05$) increase in hindquarters conductance in the control group only (Figure 1). The highest dose of endothelin-1 ($0.4\,nmol\,kg^{-1}$) caused similar, significant ($P < 0.05$) increases in hindquarters flow and vascular conductance in both groups (Figure 1).

Cardiovascular responses to N^G-nitro-L-arginine methyl ester

L-NAME ($10\,mg\,kg^{-1}$) caused an increase in blood pressure which was associated with decrease in heart rate and renal, mesenteric and hindquarters flow and vascular conductance in both groups. However, the STZ-treated group demonstrated a significantly smaller increase in blood pressure ($P < 0.05$) and decreases in heart rate ($P < 0.05$) and hindquarters vascular conductance ($P < 0.05$) than the control group (Table 2).

Discussion

Under resting conditions the conscious, STZ-treated Wistar rats in the present study had a bradycardia, with a normal mean blood pressure. Bradycardia following STZ treatment has been reported by several authors (e.g. Stuesse *et al.*, 1982; Carbonell *et al.*, 1987; Hebden *et al.*, 1987a; Tomlinson *et al.*, 1989; 1990). Tomlinson *et al.* (1990) have shown that Brattleboro (i.e. vasopressin-deficient) rats have a bradycardia following treatment with STZ. Thus, elevated circulating vasopressin levels causing coronary vasoconstriction (Boyle & Segel, 1986), or interactions between vasopressin and cardiac neural control (Osborn *et al.*, 1987), are not likely to be responsible for the bradycardia. Clearly, activation of vagal mechanisms (Kofo-Abayomi & Lucas, 1987) and/or inhibition of sympathetic efferent input to the heart, independent of vasopressin, could explain the slow sinus rate following treatment with STZ, but the bradycardia has been found to persist following ganglion blockade (Hebden *et al.*, 1987a; Tomlinson *et al.*, 1990). This observation indicates that myocardial factors (Savarese & Berkowitz, 1979; Dillman, 1982; Ganguly *et al.*, 1983; Sundaresan *et al.*, 1984) may be responsible for the bradycardia in STZ-treated rats. Such factors could also account for the present findings of diminished tachycardia or bradycardia to endothelin-1 and L-NAME, respectively, in STZ-treated rats (although the chronotropic effects of endothelin-1 (Gardiner *et al.*, 1990a) and L-NAME (unpublished observations) are reflex in origin). However, this cannot be the full explanation of the present findings, since cardiac reflex responses to the pressor effects of vasopressin, angiotensin II or methoxamine are not reduced in STZ-treated, Wistar rats (Hebden *et al.*, 1987b).

Several studies have reported on resting arterial blood pressure following STZ treatment; some have shown diabetic rats are hypertensive (Kawashima *et al.*, 1978; Bunag *et al.*, 1982; Funakawa *et al.*, 1983), whereas others have found they are normotensive (Rodgers, 1986; Yamamoto, 1988) or hypotensive (Kohler *et al.*, 1980; Hebden *et al.*, 1987a; Kusaka *et al.*, 1987; Tomlinson *et al.*, 1989; 1990). The difference between the results from this present study and our earlier studies, in which we reported hypotension in diabetic rats (Hebden *et al.*, 1987a,b; Tomlinson *et al.*, 1989; 1990), is that previously both systolic and diastolic blood pressures were recorded, and it was systolic pressure which was consistently reduced (Hebden *et al.*, 1987a; Tomlinson *et al.*, 1989). In the present work we also observed a reduction in systolic blood pressure following STZ treatment (data not shown), but we measured electronically-derived mean blood pressure (in order to calculate vascular conductances) and this was not reduced because in the bradycardic, STZ-treated rats the pressure wave form is broader than in control animals.

Although mean blood pressures were similar in STZ-treated and control rats, their haemodynamic profiles were different, with vasodilatation in the renal and mesenteric vascular beds and vasoconstriction in the hindquarters vascular bed of the diabetic rats, relative to the control animals. There is a marked hypertrophy of the kidney and gastrointestinal tract following treatment with STZ (Caspary, 1973; Christiansen *et al.*, 1981) and it is feasible that associated increases in vascularity contribute to the increases in renal and mesenteric vascular conductances (Lucas & Foy, 1977; Christiansen *et al.*, 1981; Hill & Larkins, 1989). That the mesenteric vasodilatation might be accounted for partly by hypertrophy of the intestine was shown by Hill & Larkins (1989) who found the hyperaemia could be attenuated by underfeeding rats treated with STZ. However, other factors must be involved since Korthuis *et al.* (1987) found that cross-perfusion of jejunum-ileum preparations from control rats with blood from STZ-treated rats caused vasodilatation of a similar degree to that seen in the latter *in vivo*. Subsequent studies showed the intestinal hyperaemia in STZ-treated rats was partly due to hyperglucagonaemia, since it was significantly reduced by a specific antiserum to glucagon (Yrle *et al.*, 1988). However, Yrle *et al.* (1988) suggested other factors, including plasma osmolality and plasma glucose concentration, could have contributed to the intestinal hyperaemia in STZ-treated rats. Factors such as these could have contributed also to the renal vasodilatation observed in the present study. Although renal vasodilatation in STZ-treated rats has been observed previously (e.g. Lucas & Foy, 1977; Jensen *et al.*, 1981) it has not been found in all studies (e.g. Ha & Dunham, 1987; Yrle *et al.*, 1988). However, in many previous investigations measurements were made under anaesthesia (which compromises renal perfusion) so the results cannot be extrapolated to the conscious state.

In the present study, although there were significant elevations in resting conductances in renal and mesenteric vascular beds in STZ-treated, compared to control rats, the former showed more marked vasoconstrictions to endothelin-1, such that following the highest dose of the peptide there were no differences between renal or between mesenteric vascular conductances in the two groups of rats. These results indicate that the resting renal and mesenteric vasodilatations in STZ-treated rats were probably not due to diminished sensitivity to endogenous endothelin-1, but they do not preclude the possibility that endothelial cells in STZ-treated rats produce less

endothelin-1 than normal. However, it has been reported that circulating levels of endothelin-1 are elevated in clinical diabetes mellitus (Takahashi *et al.*, 1989), and that elevated glucose levels enhance endothelin-1 secretion by cultured endothelial cells (Yamauchi *et al.*, 1990).

Following administration of L-NAME the renal and the mesenteric vasoconstrictions were similar in STZ-treated and control rats. Hence, it appears that the resting vasodilatations in the renal and mesenteric vascular beds in the STZ-treated rats were probably not due to increased sensitivity to, and/or increased production of, nitric oxide. In addition, the present results with endothelin-1 and L-NAME indicate that the renal and mesenteric vasodilatations in STZ-treated rats were probably not due to enhanced endothelial nitric oxide production inhibiting release of endogenous endothelin-1 (Boulanger & Lüscher, 1990).

The resting hindquarters vasoconstrictions in the STZ-treated rats in the present study is consistent with the findings of others (Hill & Larkins, 1989) showing reduced skin and skeletal muscle blood flow in diabetic rats. Following STZ-treatment there is atrophy of skeletal muscle (Feng *et al.*, 1990) and hence the reduced hindquarters flow and vascular conductance observed in the present study could have been simply a reflection of reduced vascularity in the wasted muscle. However, the finding that the pressor and hindquarters vasoconstrictor response to L-NAME were attenuated in STZ-treated, compared to control, rats raises the interesting possibility that endothelial cell nitric oxide production, and/or vascular sensitivity to nitric oxide, is diminished specifically in the hindquarters, and this is responsible for the diminished pressor effect of L-NAME. One possible explanation for such a selective endothelial abnormality (i.e. present in the hindquarters but not in the renal or mesenteric vascular beds) is that, following STZ treatment, skeletal muscle glutamine synthesis is enhanced (Feng *et al.*, 1990), and elevated glutamine levels in the environs of the endothelial cells in the hindquarters vascular bed inhibits the generation of nitric oxide from L-arginine (Hecker *et al.*, 1990). If this is the case then it is particularly noteworthy that the hindquarters vasodilator response to endothelin-1 was not diminished in STZ-treated rats, since Whittle *et al.* (1989) have claimed that inhibiting nitric oxide production with L-NMMA antagonizes the vasodilator effects of endothelin-1. However, our proposition that the hindquarters production of nitric oxide could be diminished although the vasodilator response to endothelin-1 was intact is consistent with our previous finding that neither L-NMMA nor L-NAME inhibit the hindquarters vasodilator response to endothelin-1 (Gardiner *et al.*, 1989b; 1990d).

Whatever the explanation of the initial hypotensive response to endothelin-1 it is clear that this effect was similar in STZ-treated and control rats. Furthermore, the subsequent rise in mean arterial blood pressure was the same in both groups. Since the STZ-treated animals showed more marked renal and mesenteric vasoconstrictor responses to endothelin-1 than did control rats, then the accompanying fall in cardiac output (Gardiner *et al.*, 1990e) in the former must have been greater than in the latter. Elsewhere we have shown that the reduction in cardiac output during the hypertension elicited by endothelin-1 is independent of reflex mechanisms (Gardiner *et al.*, 1990a), and hence is likely to be due to the increase in afterload and/or to direct cardiac effects of the peptide. However, Nayler *et al.* (1989) have shown a decreased density of cardiac binding sites for endothelin-1 in STZ-treated rats, and together with the present results, this indicates a greater fall in cardiac output in STZ-treated rats during the hypertensive response to endothelin-1 would be most likely due to the greater rise in afterload. Direct assessment of cardiac haemodynamic status and the cardiac response to L-NAME (Gardiner *et al.*, 1990d) in STZ-treated rats will provide quantitative information on this point.

This work was supported by Wellcome Research Laboratories.

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(Received November 6, 1990
Accepted January 25, 1991)

Br. J. Pharmacol. (1991), **103**, 1327–1332 © Macmillan Press Ltd, 1991

A novel anti-inflammatory peptide from human lipocortin 5

[1]Mauro Perretti, Cristina Becherucci, Kenneth G. Mugridge, Egle Solito, Sergio Silvestri & Luca Parente

Sclavo Research Centre, Laboratory of Pharmacology, Via Fiorentina 1, 53100 Siena, Italy

1 A novel anti-inflammatory peptide (residues 204–212) of human recombinant lipocortin 5 (hrLC5) found on the high similarity region with uteroglobin is described.

2 Peptide 204–212 dose-dependently inhibited the contractions of rat isolated stomach strips elicited by porcine pancreatic phospholipase A_2 (PLA_2). Contractions caused by arachidonic acid (AA), prostaglandin E_2 (PGE_2) and 5-hydroxytryptamine were not affected. No direct enzyme inhibition was observed in a radiochemical assay.

3 PGE_2 release by both human fibroblasts and rat macrophages was reduced by peptide 204–212 in a dose-dependent manner.

4 The development of carrageenin-induced oedema in rats was significantly inhibited by the local administration of peptide 204–212.

5 The pattern and potency of the biological effects of peptide 204–212 are similar to those of antiflammin 2, a lipocortin 1-derived peptide.

6 It is suggested that peptide 204–212 may represent the active site responsible for the anti-inflammatory properties of lipocortin 5.

Keywords: Inflammation; lipocortins; arachidonic acid metabolism; human lipocortin 5; anti-inflammatory peptide

Introduction

The anti-inflammatory activity of glucocorticoids is the overall result of several modulating effects exerted by these drugs on cells and mediators involved in the inflammatory process (recently reviewed by Schleimer *et al.*, 1989). Substantial experimental evidence indicates that one important facet of the steroid action is the inhibition of biosynthesis and release of eicosanoids. It has been proposed that this inhibitory effect is mediated by the induction of lipocortins which are proteins able to inhibit phospholipase A_2 (PLA_2) activity (Flower, 1988). At present, six lipocortins have been identified and sequenced, each of which has both phospholipid and calcium binding properties and shares a common fourfold repeat structure (Pepinsky *et al.*, 1988). The ability of lipocortins to inhibit PLA_2 activity *in vitro* and suppress inflammation has been differently reported. Enzyme inhibition and anti-inflammatory effect has been shown for lipocortin 1 (Cirino *et al.*, 1989; Maridonneau-Parini *et al.*, 1989), lipocortin 2 (Parente *et al.*, 1990) and lipocortin 5 (Errasfa & Russo-Marie, 1989). On the other hand, direct inhibition of PLA_2 by lipocortins has been questioned (Davidson *et al.*, 1987; Ahn *et al.*, 1988) and negative results on the anti-inflammatory effect of lipocortin 1 have been described (Northup *et al.*, 1988).

An independent line of investigation has shown that uteroglobin, a progesterone-induced protein, also inhibits PLA_2 activity (Levin *et al.*, 1986). From the region of high amino acid sequence, similarity between uteroglobin and lipocortin 1 two nine amino acid peptides have been identified. One peptide (antiflammin 1) corresponds to residues 39–47 (MQMKKVLDS) of uteroglobin, the other peptide (antiflammin 2) corresponds to residues 247–255 (HDMNKVLDL) of lipocortin 1. These peptides have potent anti-PLA_2 activity *in vitro* and show anti-inflammatory effects *in vivo* (Miele *et al.*, 1988).

In the present paper a similar computerized approach has been used to align uteroglobin and human recombinant lipocortin 5 (hrLC5). We describe the characterization of a novel nine amino acid peptide from hrLC5 which inhibits PLA_2-dependent processes *in vitro* and exhibits anti-inflammatory effects *in vivo*.

[1] Author for correspondence.

Methods

Computer analysis

The sequence of the monomer of rabbit uteroglobin (Miele *et al.*, 1988) and of hrLC5 (Pepinsky *et al.*, 1988) were analysed by the programme BestFit (Devereux *et al.*, 1984) with a Digital Vax750 computer. BestFit makes an optimal alignment of the best segment of similarity between two sequences using the 'local homology' algorithm of Smith & Waterman (1981).

Phospholipase A_2 assay

Peptides were assayed for PLA_2 inhibitory activity according to Pepinsky *et al.* (1986) with slight modifications. Each tube contained 200 μl of peptides or mepacrine as positive control, 50 μl of buffer (0.7 M Tris-HCl, pH 8.0 + 60 mM $CaCl_2$), 50 μl of buffer containing 50 ng of porcine pancreatic PLA_2 (10 nM final concentration, specific activity 600 units mg^{-1}) and 125 μg of fatty acid-free bovine serum albumin. After 60 min incubation in ice, [^{3}H]-oleic acid-labelled *Escherichia coli* (1 nmol total phospholipids) was added as substrate. The mixture was incubated for 10 min at 4°C. The reaction was stopped by addition of 100 μl 2 N HCl and 100 μl bovine serum albumin (20 mg ml^{-1}). After a further 30 min in ice the tubes were centrifuged for 5 min at 10,000 *g*. The radioactivity in the supernatant was quantified by scintillation counting and adjusted for non-specific release by subtracting the background control value. The peptides were tested for PLA_2 inhibitory activity in a concentration-range from 9 nM to 90 μM. In separate experiments peptides were tested in the presence of trypsin inhibitor (10 μg ml^{-1}) in order to check for contaminating peptidase activity in the assay system.

Smooth muscle contractions

Male Wistar rats (Charles River, Calco, Italy) weighing 225 to 250 g were killed by cervical dislocation and rat isolated stomach strips were prepared as described by Vane (1957). Briefly, tissues were suspended under a 2.5 g load in 20 ml organ chambers and bathed with gassed (95% O_2:5% CO_2) Krebs solution maintained at 37°C. Contractions of the tissue

preparations to different agonists were recorded via isotonic transducers (type 7006, Basile, Comerio, Italy) coupled to a dual channel recorder (type 7070, Basile). All preparations were equilibrated for at least 1 h before starting any experimental procedure.

Preliminary concentration-effect curves for each agonist were performed and concentrations eliciting comparable responses were chosen for subsequent experiments using test compounds, viz., 4.4 $\mu g\,ml^{-1}$ for porcine pancreatic PLA_2, 9 $\mu g\,ml^{-1}$ for arachidonic acid (AA) and 20 $ng\,ml^{-1}$ for PGE_2. These concentrations corresponded to approximately 85% of the maximum response for both PLA_2 and AA and half-maximum response for PGE_2. An incubation period of 30 min was sufficient to test the peptides and indomethacin whereas 2 h was found to be necessary for the experiments using dexamethasone. To standardize the contractions obtained from different tissue preparations, responses were expressed as a percentage of the contraction elicited by the respective agonist prior to incubation with test compounds. In all cases at least two consecutive contractions of similar magnitude were obtained to the respective agonist before evaluating the effect of the peptides or standard drugs.

Prostaglandin E_2 release in vitro

Cultures of human skin fibroblasts and rat peritoneal macrophages were prepared as previously described (Solito & Parente, 1989; Perretti *et al.*, 1989). Briefly, fibroblasts were seeded at 1×10^4 cells per well of cluster[96] plates (Costar, U.S.A.) in culture medium with 10% foetal calf serum and incubated for 72 h at 37°C in a controlled humidifier incubator in 5% CO_2. Fibroblasts were then rinsed three times and incubated with the peptides for 30 min, after which the cells were stimulated with 1 μM bradykinin for 30 min in 0.2 ml final volume of serum-free medium. Rat peritoneal macrophages were plated at 1×10^6 cells per well in cluster[24] plates (Costar) in medium with 20% foetal calf serum. After 2 h incubation at 37°C in 5% CO_2, non-adherent cells were removed by washing with serum-free medium. Adherent macrophages were incubated with the peptides for 30 min, then stimulated with 200 $\mu g\,ml^{-1}$ opsonized zymosan for 60 min in 0.5 ml final volume of serum-free medium. Agonist concentrations and incubation times have been shown to be optimal for eicosanoid formation (Flower *et al.*, 1986; Solito & Parente, 1989). At the end of the incubation, PGE_2 concentrations in the supernatants were determined by specific radioimmunoassay (N.E.N., Dreieich, FRG). Cell viability was always >95% as measured by the trypan blue exclusion test.

Carrageenin oedema

Oedema was induced by subplantar injection of carrageenin (0.1 ml, 1% in 0.9% NaCl) into the right hind paw of male Wistar rats (230–250 g). Peptides dissolved in 100 μl of 25 mM Tris-HCl buffer pH 7.4 were injected into the paw 15 min before the carrageenin. Control animals received 0.1 ml of the vehicle. The paw volume was measured with a water plethysmometer (Basile) at time 0 and every 60 min up to 6 h. All the experiments were performed according to the double blind protocol.

Materials

Peptides were purchased from Proteins & Peptide Research (Reading, U.K.). Purity was always >95% analysed by h.p.l.c.; amino acid composition and M_r were confirmed by mass spectrometry (data from the manufacturer). Peptides were stored as lyophilized powders at 4°C and dissolved in either 25 mM Tris-HCl buffer pH 7.4 or Krebs bicarbonate solution prior to use. The pH was adjusted to 7.4 with 0.1 N NaOH when concentrated solutions (>4 $mg\,ml^{-1}$) were prepared. Arachidonic acid was supplied by BioData Corp. (Hartboro, MA, U.S.A.). All other chemicals and drugs were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Statistics

Statistical significance on original values was assessed by one tail Student's *t* test for unpaired samples (paired for the data obtained in smooth muscle experiments) with $P < 0.05$ regarded as significant.

Results

Computer analysis

A total similarity (identities and conservative replacements) of approximately 40% was found between the monomer of uteroglobin and each repeat of hrLC5. Total similarity (shown in Figure 1) found between fragments 39–47 of uteroglobin (i.e. antiflammin 1) and 204–212 of hrLC5 was 66%. Peptide 204–212 is in close vicinity to the 'consensus sequence' shown previously to be responsible for the binding of calcium ions (Pepinsky *et al.*, 1988). The peptide 204–212 (SHLRKVFDK, $M_r = 1128$) and its fragments were subsequently synthesized and tested for anti-PLA_2 and anti-inflammatory activity.

Phospholipase A_2 assay

In the absence of inhibitors, phospholipid hydrolysis of about 15% has been observed in our experiments (Table 1), similar to the value reported by Davidson *et al.* (1987). Peptide 204–212 of hrLC5 (9 nM to 90 μM) did not reduce PLA_2 activity. The presence of trypsin inhibitor did not significantly modify the effect of the peptide, suggesting that the observed lack of inhibition cannot be ascribed to contaminating peptidase inactivation of the peptide. A similar lack of inhibition of PLA_2 activity was also observed for antiflammin 1 and 2 (not shown). In contrast, the enzyme activity was dose-dependently reduced by mepacrine used as positive control (Table 1).

Smooth muscle contractions

Tracings representative of typical experiments on rat isolated stomach strips contracted by various agonists are shown in Figure 2. Peptide 204–212 (20 $\mu g\,ml^{-1}$) reduced tissue contractions induced by porcine pancreatic PLA_2 (Figure 2a). After washout the recovery of the contractions was almost complete

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174                    LIPOCORTIN 5                    233

QDAQALFQAGELKWGTDEEKFITIFGTRSVS.HLRKVFDKYMTISGFQIEETIDRETSGNL
|  | *|  |     ||*    *       |  | |||**|*         *     ||    | *
HVIENLLLGTPSSYETSLKEFEPDDTMKDAGMQMKKVLDSLPQTTRENIMKLTEKIVKSPL

8                      UTEROGLOBIN                     78
```

Figure 1 Alignment between the sequences of the third repeat of human recombinant lipocortin 5 and rabbit uteroglobin. Asterisks indicate amino acid identities; lines show amino acid conservative replacements.

Table 1 Effect of peptide 204–212 and mepacrine on phospholipase A_2 (PLA_2) activity

Treatment	*μM*	*% hydrolysis*	
None	—	16.5 ± 1.1	15.3 ± 1.3¶
Peptide 204–212	0.009	17.0 ± 1.1	15.2 ± 0.9¶
Peptide 204–212	0.09	16.8 ± 0.7	12.5 ± 1.1¶
Peptide 204–212	0.9	16.5 ± 0.8	16.1 ± 1.8¶
Peptide 204–212	9.0	16.3 ± 1.4	12.5 ± 0.4¶
Peptide 204–212	90.0	16.8 ± 1.3	15.5 ± 0.9¶
None	—	14.3 ± 0.9	
Mepacrine	25	9.5 ± 0.9*	
Mepacrine	250	6.0 ± 0.4**	
Mepacrine	2500	0.7 ± 0.2***	

PLA_2 activity was tested on [^{3}H]-oleic acid-labelled *E. coli* as described in Methods. Peptide 204–212 and mepacrine were added simultaneously with the enzyme.
¶ Assays carried out in the presence of 10 μg ml^{-1} trypsin inhibitor.
Results are mean ± s.e.mean of four assays performed in triplicate ($n = 4$) for the peptide alone and of two assays in triplicate ($n = 2$) for both the peptide + trypsin inhibitor and mepacrine experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

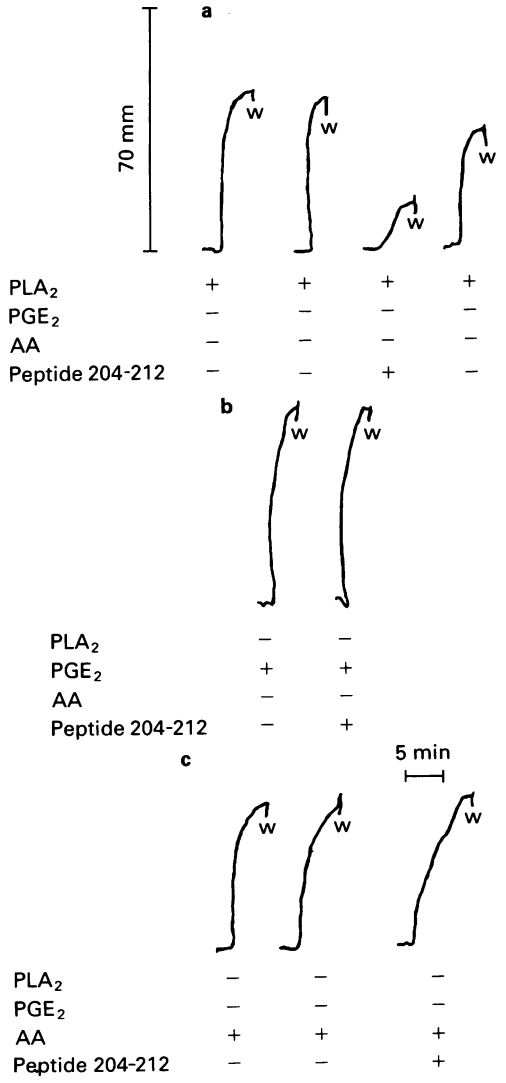

Figure 2 Effect of peptide 204–212 of human recombinant lipocortin 5 (20 μg ml^{-1}) on contractions of rat stomach strips induced by porcine pancreatic phospholipase A_2 (PLA_2, 4.4 μg ml^{-1}, a), prostaglandin E_2 (PGE_2, 20 ng ml^{-1}, b) and arachidonic acid (AA, 9 μg ml^{-1}, c). The peptide was incubated with the tissues for 30 min prior to the addition of the agonist. Tracings are representative of 3–6 similar experiments. W, washout of bathing fluid.

(88.4 ± 4.8%, $n = 5$). Responses elicited by both PGE_2 (Figure 2b) and AA (Figure 2c) were unaffected. The peptide also failed to reduce responses caused by 5-hydroxytryptamine (not shown). The cumulative data of the effect of peptide 204–212 and its fragments on smooth muscle contractions are shown in Table 2. PLA_2-induced contractions were inhibited by peptide 204–212 in a concentration-dependent manner. The seven amino acid fragment 206–212 (LRKVFDK) retained the inhibitory activity whereas the two five amino acid fragments 204–208 (SHLRK) and 208–212 (KVFDK) were ineffective. A control nonapeptide 104–112 (GTNEKVLTE) from hrLC5 was also ineffective. Table 2 also shows that antiflammin 2, but not antiflammin 1, inhibited tissue contractions with similar potency to peptide 204–212. PLA_2-induced responses were significantly reduced by either indomethacin or dexamethasone. The steroid effect was reversed in presence of cycloheximide. Finally both peptide 204–212 and dexamethasone did not affect AA-induced contractions. The basal tone of the preparations was not modified by the peptides alone.

Prostaglandin E_2 release

Rat peritoneal macrophages stimulated for 60 min with opsonized zymosan (200 μg ml^{-1}) released 1.92 ± 0.14 ng ml^{-1} PGE_2 (Table 3). This release was reduced in a concentration-dependent manner by peptide 204–212 (10–100 μg ml^{-1}) and

Table 2 Effect of peptides and drugs on contractions of rat stomach strips

Treatment	*μg ml^{-1}*	*PLA_2*	*n*	*AA*	*n*
None	—	106.0 ± 6.0	7	101.0 ± 2.5	3
Peptide 204–212	20	47.8 ± 7.3***	6	107.2 ± 4.7	3
Peptide 204–212	10	71.9 ± 5.8**	3	ND	
Peptide 206–212	20	38.3 ± 1.1***	3	ND	
Peptide 206–212	10	69.2 ± 2.4***	4	ND	
Peptide 204–208	40	92.7 ± 4.5	3	ND	
Peptide 208–212	40	94.1 ± 3.6	3	ND	
Peptide 104–112	20	102.4 ± 7.0	5	ND	
Antiflammin 1	20	91.0 ± 4.6	3	ND	
Antiflammin 2	20	33.9 ± 3.6***	5	ND	
Indomethacin	2	20.9 ± 6.3***	6	0***	3
Dexamethasone	5	36.6 ± 4.3***	7	97.9 ± 1.5	3
Dexamethasone + cycloheximide	5 10	110.0 ± 2.4	6	ND	

After 60 min equilibration, tissues were incubated for 30 min with peptides or indomethacin prior to the addition of either phospholipase A_2 (PLA_2, 4.4 μg ml^{-1}) or arachidonic acid (AA, 9.0 μg ml^{-1}). Dexamethasone and cycloheximide were preincubated for 2 h.
Results (mean ± s.e.mean) are expressed as % control response.
ND, not done; ** $P < 0.01$; *** $P < 0.001$.

Table 3 Effect of peptides on prostaglandin E_2 (PGE_2) release by rat peritoneal macrophages

Treatment	*μg ml^{-1}*	*PGE_2 (ng ml^{-1})*
None	—	1.92 ± 0.14
Peptide 204–212	100	0.31 ± 0.06***
Peptide 204–212	10	0.72 ± 0.05***
Peptide 206–212	100	0.55 ± 0.04***
Peptide 206–212	10	1.15 ± 0.03**
Peptide 208–212	100	1.60 ± 0.09
Peptide 204–208	100	1.55 ± 0.08

Cells were incubated with the peptides for 30 min then opsonized zymosan (200 μg ml^{-1}) was added for further 60 min. Results are mean ± s.e.mean of four experiments performed in triplicate ($n = 4$).
** $P < 0.01$; *** $P < 0.001$.

its seven amino acid fragment 206–212 (10–100 $\mu g\,ml^{-1}$). The basal PGE_2 release by macrophages ($0.37 \pm 0.06\,ng\,ml^{-1}$) was not significantly modified by peptide 204–212 at $100\,\mu g\,ml^{-1}$ ($0.34 \pm 0.04\,ng\,ml^{-1}$, $n = 4$). Table 2 also shows that the two five amino acid fragments 204–208 and 208–212 were ineffective at $100\,\mu g\,ml^{-1}$.

Human skin fibroblasts stimulated for 30 min with bradykinin (1 μM) released $1.08 \pm 0.30\,ng\,ml^{-1}$ PGE_2. This release was inhibited dose-dependently by peptide 204–212 (0.01–$100\,\mu g\,ml^{-1}$) with an IC_{50} of $1.13\,\mu g\,ml^{-1}$ (Figure 3). The basal PGE_2 release by fibroblasts ($0.23 \pm 0.08\,ng\,ml^{-1}$) was not significantly affected by the peptide at $100\,\mu g\,ml^{-1}$ ($0.39 \pm 0.10\,ng\,ml^{-1}$, $n = 3$).

Carrageenin oedema

A total of seven experiments of carrageenin-induced oedema with five animals in each experimental group were performed. A consistent reduction of the inflammatory response was caused by the local administration of peptide 204–212 ($2\,mg\,kg^{-1}$) in six out of the seven experiments. The mean inhibition of the oedema measured 3 h after the injection of the irritant was $31.4 \pm 2.2\%$ ($n = 30$). At the same dose a control peptide 104–112 of hrLC5 did not inhibit the development of the inflammation (not shown). Additionally, higher doses of this peptide as well as peptide 204–212, locally injected, caused after 60 min a significant increase in paw volume, possibly due to an aspecific osmotic effect.

Figure 4 shows two typical oedema experiments. In (a), the peptide 204–212 ($2\,mg\,kg^{-1}$) and antiflammin 2 ($2\,mg\,kg^{-1}$) inhibited to a similar extent the oedema formation at 3 and 4 h after carrageenin injection. The experiment in (b) shows that the inhibition caused by peptide 204–212 was dose-dependent.

Discussion

The present investigation describes the identification and biological activity of a novel peptide from hrLC5. A computerized comparison has highlighted a striking similarity in an amino acid region between residues 204–212 of hrLC5 and residues 39–47 of uteroglobin, a progesterone-induced PLA_2-inhibitory protein (Levin *et al.*, 1986). It is interesting that in previous studies the uteroglobin fragment has shown high amino acid similarity with residues 247–255 of lipocortin 1. The two peptides from uteroglobin (antiflammin 1) and from lipocortin 1 (antiflammin 2) have exhibited potent anti-PLA_2 inhibitory activity *in vitro* and anti-inflammatory effects

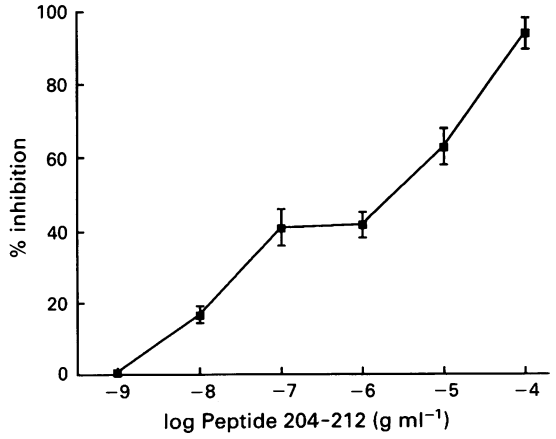

Figure 3 Dose-dependent inhibition by peptide 204–212 of prostaglandin E_2 (PGE_2) release by fibroblasts. Cells were incubated with the peptide for 30 min, then bradykinin (1 μM) was added for a further 30 min. Results are mean (vertical bars show s.e.mean) of two experiments performed in triplicate and are expressed as % inhibition of PGE_2 release by cells in absence of the peptide ($1.08 \pm 0.3\,ng\,ml^{-1}$).

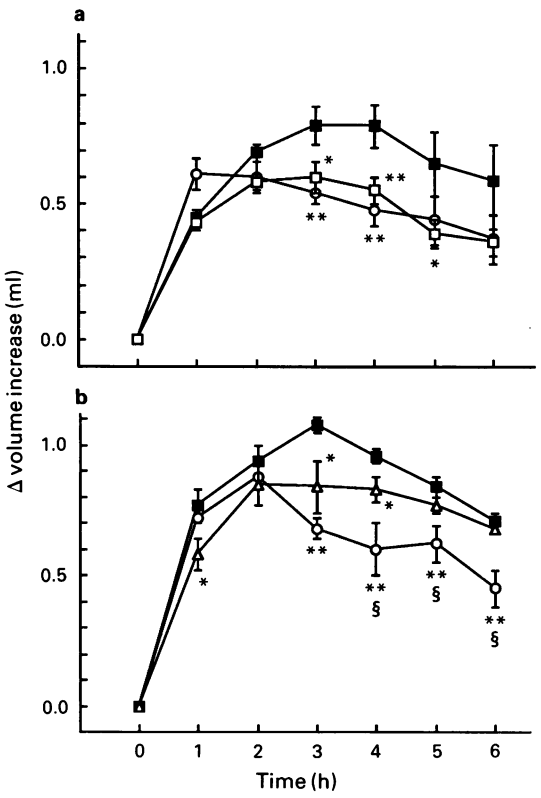

Figure 4 Effect of peptide 204–212 of human recombinant lipocortin 5 and antiflammin 2 on carrageenin-induced paw oedema. Peptides were locally injected 15 min before carrageenin. (a) (■) Control animals; (○) peptide 204–212, $2\,mg\,kg^{-1}$; (□) antiflammin 2, $2\,mg\,kg^{-1}$. (b) (■) Control animals; peptide 204–212 (○) $2\,mg\,kg^{-1}$ and (△) $0.5\,mg\,kg^{-1}$. Results are mean with s.e.mean shown by vertical bars ($n = 5$); * $P < 0.05$; ** $P < 0.01$ vs. controls; § $P < 0.05$ vs. peptide 204–212 $0.5\,mg\,kg^{-1}$.

in vivo (Miele *et al.*, 1988). The data presented here demonstrate that peptide 204–212 of hrLC5 and antiflammin 2 inhibited to a similar extent smooth muscle contractions induced by porcine pancreatic PLA_2. The inhibitory effect of peptide 204–212 was dose-dependent and appeared to be specific since contractions elicited by AA, PGE_2 or 5-hydroxytryptamine were unaffected. Furthermore, the effect of PLA_2-induced contractions was clearly reversible since responses recovered almost completely following the removal of the peptide by washout. It is therefore unlikely that the observed actions of the peptide were due to toxic effects. The contractions caused by porcine pancreatic PLA_2 were substantially reduced by the cyclo-oxygenase inhibitor, indomethacin, indicating albeit indirectly, that the contractions are mediated by prostaglandin-like substances. Additionally, the response to PLA_2 was found to be sensitive to dexamethasone whereas contractions elicited by AA were unaffected, suggesting strongly that the effects on PLA_2 of the steroid were not brought about through reduction of cyclo-oxygenase activity. The inhibitory action of the steroid was completely prevented by cycloheximide supporting further the possible involvement of lipocortin-like molecules mediating the effect of dexamethasone in this system. In fact, lipocortin 1, 2 and 5 have been found in substantial amounts in rat stomach preparations (Smillie *et al.*, 1989).

The hydrolytic activity of porcine pancreatic PLA_2 in a radiochemical assay using labelled *E. coli* membranes was not modified by the peptides. We have recently observed that lipocortin 1 (Solito & Parente, unpublished results) and lipocortin 2 (Parente *et al.*, 1990) are able to inhibit PLA_2 activity in this assay, confirming previous results (Pepinsky *et al.*, 1986; Davidson *et al.*, 1987). This inhibitory activity has been ascribed to substrate depletion rather than to a direct inter-

action with the enzyme (Davidson *et al.*, 1987). On the other hand it has been shown recently that anti-lipocortin 1 monoclonal antibodies which abolish the PLA_2 inhibitory activity are not able to block the binding of the protein to phosphatidylserine (Hayashi *et al.*, 1990). This suggests that different domains of the lipocortins are responsible for PLA_2 inhibition and phospholipid binding. Since the peptides studied are not part of the lipocortin consensus sequence which is thought to be important for calcium and phospholipid binding (Geisow *et al.*, 1986; Kretsinger & Creutz, 1986) it is not totally surprising that they had no effect in the *E. coli* assay. The contrasting effect of the peptides on the same enzyme in two different experimental conditions may be explained by the existence of different pharmacologically active sites on the enzyme, as recently suggested for snake venom PLA_2 (Kini & Evans, 1989). Alternatively, the fact that the peptides inhibit the enzyme only in whole cell systems may suggest the existence of a cell co-factor necessary for the activity.

The inhibitory effect of peptide 204–212 of hrLC5 on PLA_2 activation was supported by the experiments where PGE_2 release was assessed. The peptide reduced the release of PGE_2 by fibroblasts stimulated with bradykinin as well as by macrophages stimulated with zymosan without affecting the basal release. It has been shown that in fibroblasts, bradykinin stimulates prostaglandin synthesis by activating PLA_2 and not phospholipase C (Burch & Axelrod, 1987) and that in macrophages, PLA_2 activity is greatly stimulated by zymosan (Wightman *et al.*, 1981).

The inhibitory activity on both smooth muscle contractions and PGE_2 release by macrophages was retained by the seven amino acid fragment 206–212, but it was lost by the two five amino acid fragments 204–208 and 208–212. It is also noteworthy that fragments of the antiflammins smaller than six amino acids were found to be ineffective (Miele *et al.*, 1988).

Finally, peptide 204–212 of hrLC5 and antiflammin 2 inhibited carrageenin-induced paw oedema in the rat, a classical model of experimental inflammation. The inhibition by peptide 204–212 was dose-dependent and maximal after the third and the fourth hour of the development of the inflammation, when the phlogistic process is sustained by the release of arachidonic acid metabolites (Di Rosa *et al.*, 1971). Taken together these results make the peptide 204–212 a good candidate for the active site of the anti-PLA_2 and anti-inflammatory properties of lipocortin 5 as has been proposed for antiflammin 2 with regard to lipocortin 1 (Miele *et al.*, 1988).

The biological activity of antiflammins has been variously reported. After the first paper by Miele *et al.* (1988) the anti-inflammatory effect of the peptides was confirmed by Ialenti *et al.* (1990). Recently, in an extensive study by Camussi *et al.* (1990) the antiflammins have been shown to inhibit PLA_2 activity, platelet-activating factor release, aggregation and chemotaxis in human neutrophils. Moreover the peptides reduced the increased vascular permeability and leukocyte infiltration in a model of Arthus reaction. On the other hand, negative data on both PLA_2-inhibitory and anti-inflammatory activities have been obtained (van Binsbergen *et al.*, 1989; Marki *et al.*, 1990). In our experiments antiflammin 2 was found to be equipotent with peptide 204–212 of hrLC5, whereas antiflammin 1 showed no activity in any of the experimental models. These controversial results may be ascribed to the sensitivity of antiflammins to oxidation because of the methionines present in their structure. Indeed, Camussi *et al.* (1990) have shown that antiflammins are inactivated by oxidizing agents and antiflammin 1, which has two methionines, is more easily degraded and less inhibitory than antiflammin 2, which has only one methionine. Therefore, peptides devoid of this amino acid, like the fragment 204–212 of hrLC5 described in this paper, may be more stable and exhibit more consistent anti-inflammatory activity.

Note added in proof

Since this paper was submitted the tertiary structure of lipocortin 5 (annexin V) became available (Huber *et al.*, 1990). The polypeptide chain is folded into four domains consistent with the amino acid sequence repeats. Each domain is folded into five α-helices. The peptide 204–212 falls entirely into helix IIIC which appears to be exposed on the surface of the molecule. It is confirmed that the peptide is not part of the calcium phospholipid binding site of the protein.

The authors wish to thank M. Bartalini for RIA measurement and G. Corsi for producing the illustrations.

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(Received July 27, 1990
Revised January 24, 1991
Accepted January 25, 1991)

Br. J. Pharmacol. (1991), **103**, 1333–1338 © Macmillan Press Ltd, 1991

Platelet-leukocyte interaction in adhesion to endothelial cells induced by platelet-activating factor *in vitro*

[1]Masahiko Hirafuji & Hisashi Shinoda

Department of Pharmacology, Tohoku University School of Dentistry, 4-1 Seiryo-machi, Sendai 980, Japan

1 Platelet-activating factor (PAF, 10 nM) did not induce platelet adhesion to endothelial cells cultured in monolayer but it induced their adhesion to protein-coated plastic. However, PAF induced a marked platelet adhesion to endothelial cells when polymorphonuclear leukocytes (PMNs) were present. Lyso-PAF had no effect.

2 Phase-contrast microscopic examination showed that single platelets rather than their aggregates adhered to the endothelial cell surface around aggregating and adhering PMNs.

3 Significant platelet adhesion was induced by PAF at concentrations higher than 0.01 nM with the maximal response at 10 nM. Platelet adhesion occurred within minutes after PAF addition, reaching a maximum approximately after 30 min. Platelet adhesion also occurred significantly at a PMN : platelet ratio of 1 : 800, and linearly up to 1 : 50.

4 The PAF-induced platelet adhesion was suppressed by three structurally unrelated PAF antagonists, WEB 2086, ONO 6240 and BN 52021, in a concentration-dependent manner.

5 PAF also increased PMN adhesion to endothelial cell monolayers, which was further augmented by the presence of platelets.

6 The present study demonstrates that PAF induces platelet adhesion to endothelial cells *in vitro* when PMNs are present and that there is a close interaction between platelets and PMNs in their adhesion to endothelial cells. The present study further suggests that PMNs could play a central role in platelet adhesion to vascular endothlium in certain pathological conditions.

Keywords: Platelet-activating factor (PAF); platelets; leukocytes; endothelial cells; platelet adhesion; vascular injury

Introduction

Cell-cell interactions may play a critical role in the pathogenesis of inflammation, thrombosis, and atherosclerosis. In particular, interaction of platelets and polymorphonuclear leukocytes (PMNs) with the blood vessel wall at sites of vascular injury is considered to be an early event in the pathogenesis of these diseases. Morphological studies of platelet plugs and thrombi in vascular lesions frequently demonstrate that they are also rich in PMNs (Henry, 1965; Bourgain *et al.*, 1984), suggesting a close interaction between platelets and PMNs, and possibly among these cells along with the vascular endothelial cells. However, the molecular mechanisms that mediate the cell-cell interactions are still unclear.

PAF (platelet-activating factor) is a lipid mediator that is produced by various cells types including platelets, PMNs or endothelial cells in response to a variety of immunological or non-immunological stimuli (Snyder, 1985). PAF is also a potent activator of these cells, and induces various cellular responses that may play a critical role in the process of vascular diseases (Vargaftig *et al.*, 1989). Several *in vitro* studies have demonstrated or suggested that newly formed PAF is an important mediator of direct interactions between platelets and PMNs (Benveniste *et al.*, 1972; Shaw & Henson, 1980; Oda *et al.*, 1986; Coëffier *et al.*, 1987), or PMNs and endothelial cells (Zimmerman *et al.*, 1985; Breviario *et al.*, 1988). Cooperation between platelets and PMNs in PAF formation has also been described (Coëffier *et al.*, 1990). However, the role of PAF in the cell-cell interaction between PMNs, platelets and endothelial cells in the complex processes leading ultimately to platelet and PMN adhesion to the endothlium remains to be clarified. Therefore, in the present study, we investigated the effect of exogenous PAF on the adhesion of platelet and PMN mixtures to endothelial cells *in vitro*, to determine if PAF has any influence on platelet and PMN adhesion to endothelial cells, especially if the presence of one cell type might modulate the adhesion of another cell type. Here we demonstrate that PAF induces platelet adhesion to endothelial cells when PMNs are present, and that there is a close interaction between platelets and PMNs in the adhesion process to endothelial cells. Some of these results have been presented at the 63rd Annual Meeting of the Japanese Pharmacological Society (Hirafuji & Shinoda, 1990).

[1] Author for correspondence.

Methods

Endothelial cells

Endothelial cells were isolated from human umbilical cord veins by collagenase digestion as described previously (Hirafuji *et al.*, 1987). Primary endothelial cells were seeded into 24-well culture plates (Primaria, Falcon), and grown until forming a complete monolayer (3–5 days) in Ham's F-12 medium containing 15% foetal calf serum, 50 u ml^{-1} penicillin and 50 $\mu g\,ml^{-1}$ streptomycin. When protein-coated culture plates were used, wells of the plate were incubated overnight at 37°C with the culture medium. Just prior to experiments, endothelial cells and the wells were washed 3 times with Hanks' balanced salt solution containing 10 mM HEPES and 0.25% bovine serum albumin (BSA; pH 7.4; HBSS).

Platelets and polymorphonuclear leukocytes

Blood was collected from the central ear artery of unanaesthetized adult male rabbits and immediately mixed with EDTA (5 mM, final concentration). Platelet-rich plasma (PRP) obtained by centrifugation of the whole blood at 500 *g* for 20 min was then centrifuged at 1,500 *g* for 15 min at room temperature. Platelets were resuspended in calcium-free Tyrode buffer (composition, mM: NaCl 137, KCl 2.6, NaH_2PO_4 0.4, $MgCl_2$ 1.0 and gluccose 5.6, pH 6.5) containing 10 mM HEPES, 0.25% BSA and 0.2 mM EGTA, washed twice with the same buffer, and counted in a Celltac Counter (MEK-

4150, Nihon Kohden, Japan). The platelet pellet from the final wash was resuspended in a small volume of the same buffer, and finally diluted to a concentration of 5×10^8 platelets ml^{-1} with HBSS, and kept at room temperature until use. Contamination by other blood cells in the platelet suspension was negligible, as determined with a haemocytometer.

The cells obtained after removing PRP were mixed with the same volume of 3% dextran (average Mr 2.5×10^5) in physiological saline, and allowed to stand at room temperature for approximately 30 min. During this time the red blood cells sedimented. The supernantant was aspirated, and cells were collected by centrifugation at 230 *g* for 10 min at 4°C. The resultant pellet was resuspended in 1 ml of cold physiological saline. The suspension was added to 10 ml of ice-cold distilled water to lyse contaminating erythrocytes. After 40 s 10 ml of a cold 1.8% NaCl solution was added to restore isotonicity. After centrifugation (at 230 *g* for 10 min at 4°C), the cell pellet was resuspended in 2 ml physiological saline containing 20% autologous platelet-poor plasma, underlayerd with 6 ml Lymphoprep solution and subjected to centrifugation at 500 *g* for 20 min at 20°C. The pellet (PMNs) was washed twice by centrifugation in calcium-, and magnesium-free HBSS, finally resuspended in this buffer, and kept at 0°C until use. The cell-number was adjusted to 1×10^7 cells ml^{-1} after counting in a haemocytometer. Cell purity and viability, as assessed by microscopic examination and exlusion of trypan blue dye, respectively, exceeded 95%. Calcium (1.3 mM) and magnesium (1.0 mM) were added just prior to the experiments.

Labelling of platelets and polymorphonuclear leukocytes

Platelets or PMNs were radiolabelled with [^{3}H]-adenine according to the methods of Curwen *et al.* (1982) and Gimbrone & Buchanan (1982). Platelets in PRP or PMNs in calcium- and magnesium-free HBSS were incubated with 0.5 μCi ml^{-1} [^{3}H]-adenine for 30 min or 2.0 μCi ml^{-1} for 15 min at 37°C, respectively. Platelets and PMNs incorporated 80–90% and 20–30% of the total radioactivities, respectively. After washing out free radioactivity, cold adenine (100 μM) was added to the final suspension of radiolabelled cells.

Adhesion assay

In standard experiments, platelets (7.5×10^7 per well) and/or PMNs (1.5×10^6 per well) were added to culture plates that had been coated with proteins or where endothelial cells had reached confluence and incubated for 20 min at 37°C with gentle shaking (0.5 Hz) in the presence of test compounds in a total volume of 400 μl. At end of the incubation time, the wells were gently washed 3 times with 500 μl HBSS to remove non-adherent cells. In some experiments, the incubation period and the number of PMNs added to the well were varied as indicated in the text. Distilled water (500 μl) containing 1% Triton X-100 was added to each well to lyse the remaining cells. After 30 min incubation at 37°C, the radioactivity in the wells was counted in a scintillation counter. For inverted phase-contrast photomicrographs, cells remaining in the well after washing procedure were fixed overnight with 2% paraformaldehye–2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) at 4°C.

Materials

PAF (1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine), lyso-PAF (1-O-hexadecyl-2-lyso-*sn*-glycero-3-phosphocholine), BSA (fatty acid-free) and adenine were obtained from Sigma, U.S.A.; HEPES from Dojin, Japan; collagenase (*Clostridium histolyticum*) and dextran from Wako, Japan; Hanks' balanced salt solution, calcium- and magnesium-free Hanks' balanced salt solution, Ham's F-12 culture medium, foetal bovine serum, penicillin and streptomycin from Gibco, U.S.A.; Lymphoprep (9.6% sodium metrizoate and 5.6% Ficoll, Nycomed AS, Norway); [^{3}H]-adenine (24 Ci $mmol^{-1}$; 1 μCi = 37 kBq) from Amersham, Buckinghamshire. BN 52021 (3-tert-butyl-hexahydro-4,7b,11-trihydroxy-8-methyl-9H-1,7a-epoxy-methano-1H, 6aH-cyclopenta[c]furo[2,3b]furo[3',2' : 3,4] cyclopenta[1,2-d]furan-5,9,12(4H)trione) was a kind gift from Dr J.M. Mencia-Huerta, Institut Henri Beaufour, France; WEB 2086 (3-[4-(2-chlorophenyl)-9-methyl-6H-thieno[3,2-f][1,2,4]-triazolo-[4,3-a][1,4]-diazepine-2-yl]-1-(4-morpholinyl)-1-propanone) from Dr H. Heuer, Boehringer-Ingelheim KG, FRG; ONO 6240 (1-O-hexadecyl-2**RS**-O-ethyl-3-O-(7-thiazolinoheptyl)-glycerol methanesulphonate) from Ono Pharmaceutical Indust. Ltd., Japan.

PAF and lyso-PAF were dissolved in physiological saline containing 0.25% BSA at a concentration of 10 mM and stored at −20°C. WEB 2086 and ONO 6240 were dissolved in physiological saline. BN 52021 was dissolved in dimethylsulphoxide at a concentration of 20 mM. These stock solutions were diluted to the desired concentrations with the incubation medium before use.

Statistical analysis

Platelet or PMN adhesion was expressed as a percentage of the total radioactivity in cell suspension added to the wells. Results are expressed as mean ± s.e.mean of (n) experiments performed in replicate which agrees within 10% coefficient of variation or of replicate determinations. Statistical analysis was made by ANOVA followed by Student's unpaired t test or Cochran-Cox test, and P values less than 0.05 were considered as significant.

Results

Release of [^{3}H]-adenine from radiolabelled cells

Centrifugation at 2,000 *g* for 3 min at room temperature of the final suspensions of radiolabelled platelets and PMNs showed that 3.2 ± 0.8% (mean ± s.e.mean, $n = 5$) and 3.6 ± 0.1% ($n = 3$), respectively, of the radioactivity was spontaneously released from the cells 1 h after the preparation and less than 10% within the time frame of experiments. Incubation for 20 min at 37°C of radiolabelled PMNs and endothelial cells without or with 10 nM PAF demonstrated that PAF did not increase the radioactivity release (16.2 ± 0.4%, $n = 3$, and 17.2 ± 0.9%, $n = 3$, of the total radioactivity without or with PAF, respectively). When radiolabelled platelets were incubated for 20 min at 37°C with an endothelial cell monolayer, 6.5 ± 1.4 ($n = 3$) and 21.9 ± 2.1 ($n = 3$)% of the radioactivity was released in the absence and the presence of 10 nM PAF, respectively. The presence of PMNs did not affect the release.

Uptake of [^{3}H]-adenine by unlabelled cells

Incubation for 20 min at 37°C of either an endothelial cell monolayer or protein-coated plastic with [^{3}H]-adenine (1×10^5 d.p.m. per well; approximately equivalent to the activity in a typical labelled platelet or PMN suspension) in the presence of excess cold adenine (37.5 μM) demonstrated that less than 1% (0.8 ± 0.08 and 0.5 ± 0.04%, respectively, $n = 3$) of the radioactivity remained associated with the well after the standard washing procedure. Also, incubation for 20 min at 37°C of PMNs (3.75×10^6 ml^{-1}; equivalent to the number used for the adhesion assay) or platelets (1.9×10^8 ml^{-1}) with [^{3}H]-adenine in the presence of excess cold adenine caused negligible incorporation of the radioactivity. Thus, the radioactivity remaining in the well after the washing procedure was a reflection of the number of adherent cells.

Platelet adhesion to protein-coated plastic

Figure 1a illustrates the effects of PAF (10 nM) on platelet adhesion to protein-coated plastic in the presence or the

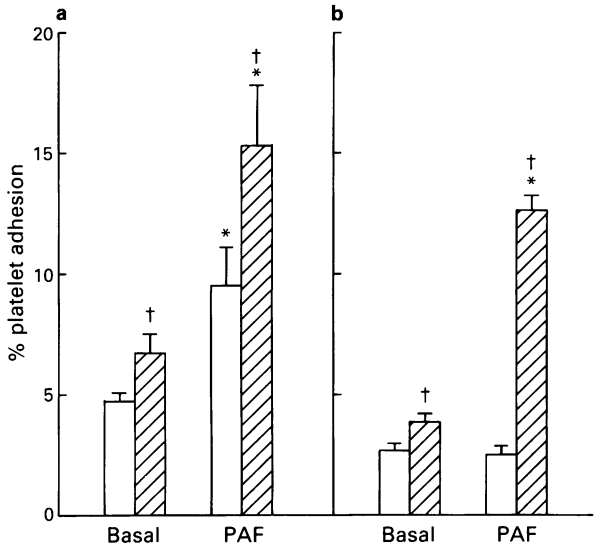

Figure 1 Effect of PAF on platelet adhesion to protein-coated plastic and endothelial cells. Radiolabelled platelets without (open columns) or with polymorphonuclear leukocytes (PMNs, hatched columns) were added to protein-coated culture plate (a) or endothelial cell monolayers (b), and incubated at 37°C for 20 min in the presence or the absence of 10 nM PAF. At the end of incubation time, the wells were washed 3 times with HBSS, and the remaining radioactivity was counted. Results are expressed as percentage of added total radioactivity. Each column represents mean of 4 separate experiments performed in triplicate; vertical bars show s.e.mean; * and † indicate significant differences from each basal and value without PMNs, respectively.

absence of PMNs. Without endothelial cells, PAF significantly increased the platelet adhesion to the protein-coated plastic in the absence of PMNs (4.8 ± 0.2 and 9.5 ± 1.6% platelet adhesion for the control and PAF-treated groups, respectively, $n = 4$ experiments tested in triplicate). When PMNs were coincubated, the basal platelet adhesion slightly but significantly increased (6.8 ± 1.1% platelet adhesion). The platelet adhesion in the presence of PMNs was increased further when PAF was also added to the incubation medium (15.3 ± 2.5% platelet adhesion). Inverted phase-contrast microscopic examination at the end of the incubation period showed that, as well as PMNs, single and mainly aggregated platelets adhered to the plastic surface. This concentration of PAF (10 nM) induced a maximal platelet aggregation when assayed in an aggregometer (83.5 ± 2.9% of light transmittance, $n = 3$).

Platelet adhesion to endothelial cells

In contrast, as illustrated in Figure 1b, PAF had no effect on platelet adhesion to endothelial cell monolayers in the absence of PMNs (2.7 ± 0.2 and 2.5 ± 0.3% platelet adhesion for the control and PAF-treated groups, respectively, $n = 4$ experiments tested in triplicate), a fact that was also confirmed by phase-contrast microscopy. When PMNs were added together with platelets, the basal platelet adhesion was slightly but significantly increased (3.9 ± 0.2% platelet adhesion). However, PAF caused a marked increase in the platelet adhesion when PMNs were also present (12.6 ± 0.5% platelet adhesion). As demonstrated in Figure 2, microscopic examination clearly showed that single platelets rather than small aggregates adhered to endothlial cell surface around aggregating and adhering PMNs. There was no visible endothelial cell lysis or gap formation between the cells during the incubation time. Although it was not easy to determine accurately at this level of resolution, platelets also seemed to adhere to single and possibly aggregated PMNs.

As presented in Figure 3, platelet adhesion was dependent on the PAF concentration, and was significantly induced by 0.01 nM PAF with a maximal response at 10 nM, whereas lyso-PAF, the deacetylated metabolite of PAF, at a concentration of 10 nM, had no effect. Figure 4 presents the time course of PAF-induced platelet adhesion in the presence of PMNs. The platelet adhesion was a rapid response, which was induced

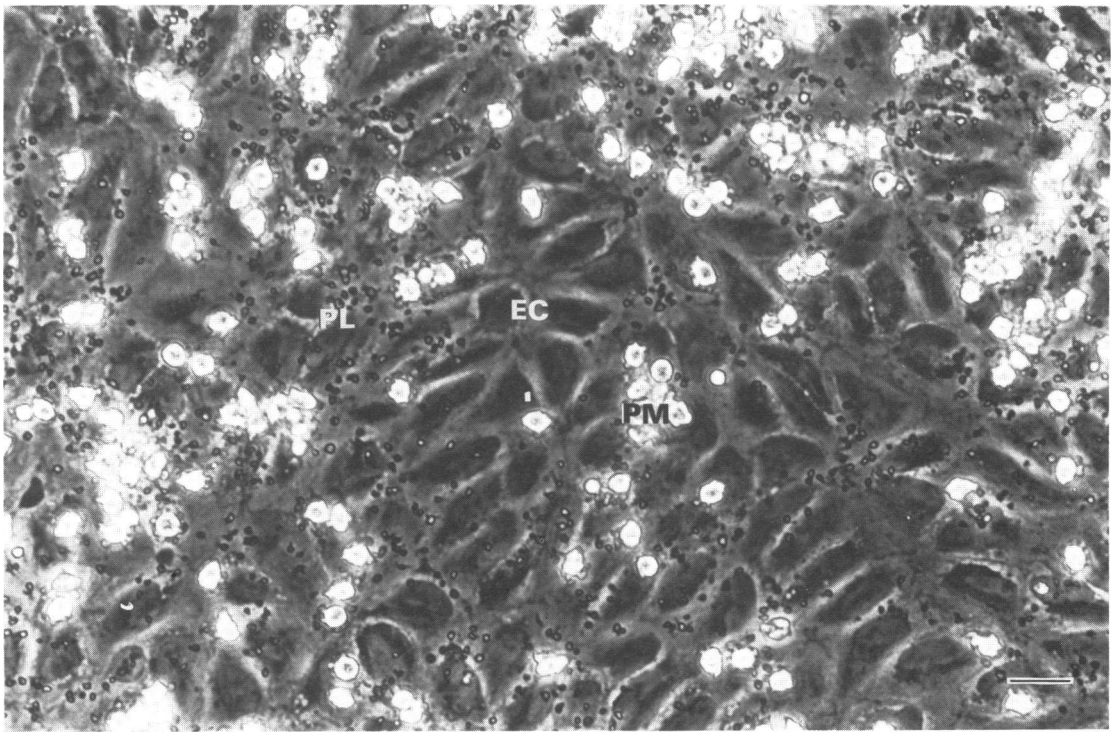

Figure 2 Phase-contrast photomicrograph (original magnification; ×200) of the adhesion of platelets and polymorphonuclear leukocytes (PMNs) to endothelial cells. Endothelial cell monolayer (EC) was incubated for 20 min with platelets (PL), PMNs (PM) and PAF (10 nM). At the end of adhesion assay, the well was washed and fixed with 2% paraformaldehye–2% glutaraldehyde. Scale bar = 20 μm.

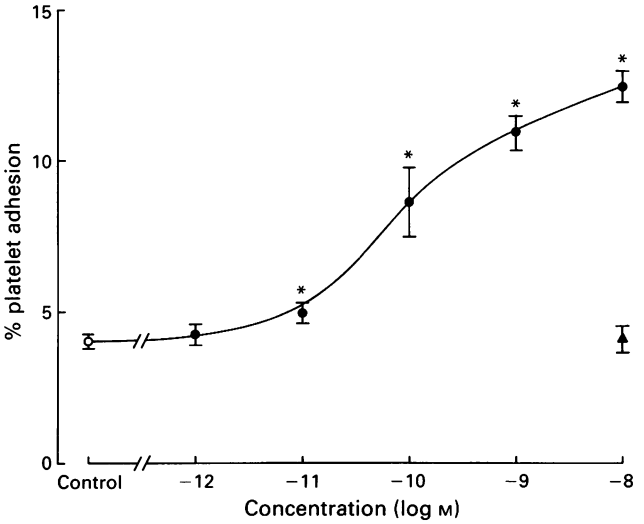

Figure 3 Effects of PAF and lyso-PAF on platelet adhesion to endothelial cells. Radiolabelled platelets were added to endothelial cell monolayers with polymorphonuclear leukocytes (PMNs) and incubated for 20 min without (control, ○) or with indicated concentrations of PAF (●) or lyso-PAF (▲). Mean of 4 and 2 experiments performed in triplicate for PAF and lyso-PAF, respectively; vertical bars show s.e.mean.

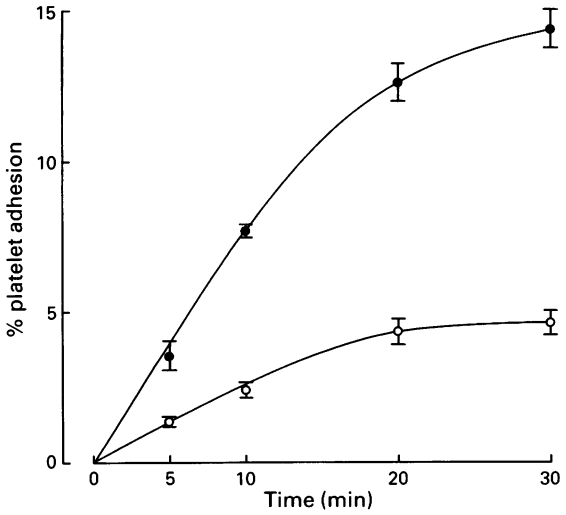

Figure 4 Time course of PAF-induced platelet adhesion to endothelial cells. Radiolabelled platelets were added to endothelial cell monolayers with polymorphonuclear leukocytes (PMNs), and incubated in the absence (○) or the presence (●) of 10 nM PAF. At the indicated time, the wells were washed, and the remaining radioactivity was counted. Mean of 3 experiments in triplicate; vertical bars show s.e.mean.

Table 1 Effect of PAF and platelets on polymorphonuclear leukocyte (PMN) adhesion to endothelial cells

	% PMN adhesion	
	− Platelets	*+ Platelets*
Control	14.6 ± 1.2	20.6 ± 0.2†
PAF 10 nM	22.1 ± 1.4*	36.2 ± 3.2*†

PMNs labelled with [^{3}H]-adenine were added to endothelial cell monolayers with or without platelets and incubated at 37°C for 20 min in the absence (control) or the presence of 10 nM PAF. Results are expressed as percentage of total radioactivity added. Mean ± s.e.mean of a representative experiment performed in quadruplicate. * and † indicate statistical significance compared with control values and the values in the absence of platelets, respectively.

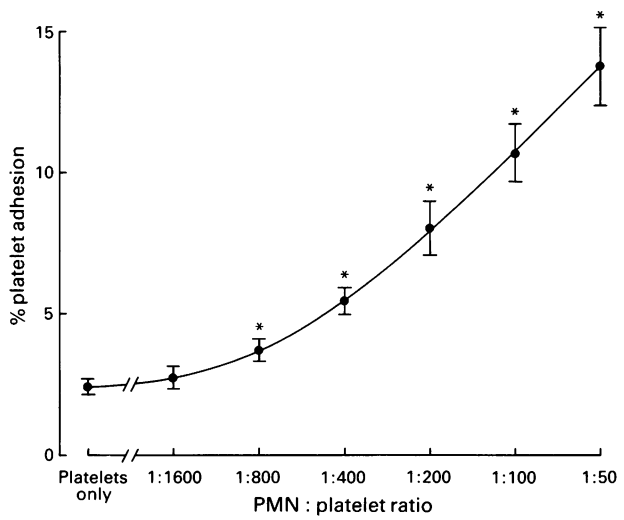

Figure 5 Effect of polymorphonuclear leukocyte (PMN): platelet ratio on PAF-induced platelet adhesion to endothelial cells. Fixed number of radiolabelled platelets (7.5×10^7 per well) with different numbers of PMNs at the indicated PMN : platelet ratio was added to endothelial cell monolayers and incubated for 20 min in the presence of 10 nM PAF. Mean of 3 experiments in duplicate; vertical bars show s.e.mean.* Indicates significant differences from the value with platelets only.

within minutes after PAF addition and reached its maximum by approximately 30 min. Figure 5 demonstrates the effect of PMN number on PAF-induced platelet adhesion to endothelial cells. A significant increase of PMN-dependent platelet adhesion was observed at a PMN : platelet ratio of 1 : 800 when compared to platelets only. At this ratio, platelet adhesion to the endothelial cell surface could be observed microscopically. The platelet adhesion was increased linearly up to a PMN : platelet ratio of 1 : 50.

As shown in Figure 6, the platelet adhesion induced by 10 nM PAF was suppressed in a concentration-dependent manner by three structurally unrelated PAF antagonists, WEB 2086, ONO 6240 and BN 52021.

Polymorphonuclear leukocyte adhesion to endothelial cells

Table 1 presents the effect of PAF (10 nM) on PMN adhesion to endothelial cell monolayers in the absence or in the presence of platelets. PAF induced a 1.5 fold increase in PMN adhesion to endothelial cells in the absence of platelets. When platelets were present, basal PMN adhesion was increased,

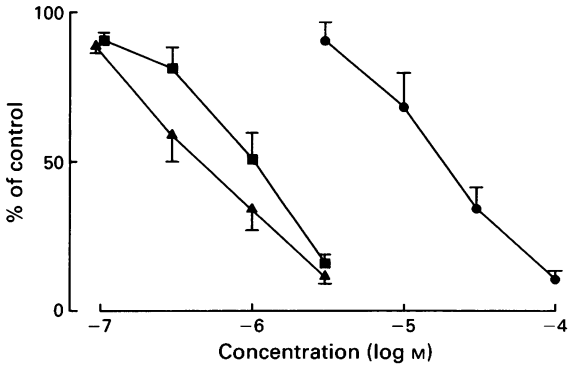

Figure 6 Effect of PAF antagonists on PAF-induced platelet adhesion to endothelial cells. Radiolabelled platelets were added to endothelial cell monolayers with polymorphonuclear leukocytes, preincubated for 1 min with different concentrations of WEB 2086 (▲), ONO 6240 (■) or BN 52021 (●), and then stimulated with 10 nM PAF for 20 min. Results are expressed as percentage of the control platelet adhesion induced by PAF. Each point represents mean of 3 experiments in triplicate; vertical bars show s.e.mean.

and PAF induced a further 1.8 fold increase in PMN adhesion to endothelial cells. As shown in Figure 2, microscopic examination at the end of the incubation period showed that single PMNs and numerous aggregated PMNs were adherent to the endothelial cell surface. In contrast, when PMNs were stimulated by PAF in the absence of platelets, mainly single PMNs adhered to the endothelial cells.

Discussion

Circulating blood platelets normally do not adhere to the intact vascular endothelium. This property of endothelium could be attributed to the ability to produce or release several factors such as prostacyclin (Higgs *et al.*, 1978), 13-hydroxyoctadecadienoic acid (Buchanan *et al.*, 1985), or endothelium-derived relaxing factor (Radomski *et al.*, 1987; Sneddon & Vane, 1988), although the relative contributions of these factors have to be clarified. This anti-platelet property of endothelial cells seems to function efficiently even when PAF, the most potent platelet activating substance, is present. Indeed, this lipid mediator did not induce platelet adhesion to cultured endothelial cells even at a concentration that induced the maximal aggregation and stimulated the platelet adhesion to protein-coated plastic.

However, in the present study, we observed that PAF induced platelet adhesion to endothelial cells when PMNs were present. By phase-contrast microscopy, it was confirmed that single platelets rather than aggregates adhered to the endothelial cell surface around aggregating and adhering PMNs. Platelets are known to be adhesive to PMNs *in vitro* (McGregor *et al.*, 1980). Although it was not clear at this level of resolution, platelets also seemed to adhere to single and possibly agregated PMNs, which can be confirmed only by electron microscopy. Therefore, when platelet adhesion to endothelial cell monolayers was studied in the presence of PMNs, the radioactivity remaining in the wells after the washing procedure should be interpreted as being a total reflection of the number of platelets associated with adhering PMNs as well as those adhered to endothelial cell surface. Since the methodology adopted in the present study cannot discriminate between the relative contribution of these two types of platelet adhesion, we have referred to them as 'platelet adhesion to endothelial cells' for convenience. Furthermore, a critical point of our adhesion assay using platelets labelled with [^{3}H]-adenine might be the slight release of radioactivity in response to PAF, although our technique has some advantages compared with those using ^{51}Cr or ^{111}In-oxine (Curwen *et al.*, 1982; Heaton *et al.*, 1989). Although Curwen *et al.* (1982) found that no detectable release of radioactivity was observed after incubation of [^{3}H]-adenine-labelled PRP with aggregating stimuli, in our hands, the radioactivity released from labelled platelets incubated with an endothelial monolayer was 15.4% higher in the presence of 10 nM PAF (the highest concentration used in the present study) than in control. This means that PAF-dependent platelet adhesion is underestimated by at most 15.4% in our experimental conditions. However, even taking into account this release, it was clear from our study with the parallel microscopic observation that PAF induced platelet adhesion to endothelial cells when PMNs were present.

The platelet adhesion to endothelial cells in the presence of PMNs occurred very rapidly, i.e. within minutes after PAF stimulation, and was dependent on the PAF concentrations and the PMN number coincubated with platelets and endothelial cells. Lyso-PAF, the biologically inactive metabolite of PAF, had no effect. Three structurally unrelated PAF antagonists, WEB 2086 (Casals-Stenzel *et al.*, 1987), ONO 6240 (Miyamoto *et al.*, 1985) and BN 52021 (Braquet *et al.*, 1985), dose-dependently suppressed the platelet adhesion. PAF also activated PMNs, resulting in the stimulation of PMN adhesion to endothelial cell monolayer. These results suggest that the platelet adhesion is mediated by activation of PAF specific membrane receptors, most probably on PMNs. However, PAF is an activator not only of platelets but also of endothelial cells (Bussolino *et al.*, 1987; Hirafuji *et al.*, 1988) so a possibility remains that the simultaneous PAF activation of platelets and/or endothelial cells as well as PMNs is a prerequisite to the platelet adhesion.

Although several mechanisms for the PMN-dependent platelet adhesion to endothelial cells induced by PAF can be postulated, microscopic examination suggests that some factor(s) released from PAF-stimulated PMNs mediates the adhesion by acting on platelets or/and endothelial cells. During direct interaction of platelets and PMNs, activated PMNs are known to produce and release a number of soluble factors that modulate platelet activity; for example, oxygen-derived free radicals (Levine *et al.*, 1976; Clark & Klebanoff, 1980), proteases (Chignard *et al.*, 1986; Del Maschio *et al.*, 1990), arachidonic acid metabolites (Coëffier *et al.*, 1987), or PAF (Benveniste *et al.*, 1972; Coëffier *et al.*, 1987). Most of these factors are also modulators of endothelial cell function (Harlan, 1987). One of the most likely factors would be superoxide anions derived from PMNs, since Salvemini *et al.* (1989) have shown that oxygen radicals enhance platelet adhesion to gelatin-coated plastic and aggregation induced by thrombin, in a process that is inhibited by superoxide dismutase (SOD). However, preliminary experiments have shown that SOD at a high concentration only slightly inhibited the platelet adhesion to endothelial cells (Hirafuji & Shinoda, 1990), suggesting the involvement of other mechanisms. Further experiments are now being undertaken to clarify the mechanism(s) underlying the PMN-dependent platelet adhesion observed in the present study.

The present study also indicated that PAF increased PMN adhesion to endothelial cells, which was augmented *vice versa* by the presence of platelets. This result is in agreement with that of Boogaerts *et al.* (1982), who have shown that platelets release products which enhance granulocyte-endothelial cell adherence. Such an augmentation by direct interaction of PMNs with platelets has also been reported to occur in a nylon-fibre assay of PMN adherence (Rasp *et al.*, 1981).

Thus, the present study demonstrates that PAF induces platelet adhesion to endothelial cells *in vitro* when PMNs are present. As well, a close interaction between platelets and PMNs in the adhesion process to endothelial cells is demonstrated. Our results further support the central role of PMNs in platelet adhesion to the vascular endothelium under certain pathological conditions. In this respect it is noteworthy that the intravenous injection of inflammatory stimuli such as FMLP to the rabbit, triggers leukocyte and platelet deposition at the inflammatory site, which is prevented by leukocyte depletion (Issekutz *et al.*, 1983). The interaction of platelets and PMNs demonstrated in the present study may have a significant relevance to the process of vascular diseases, since the adhesion of these cells to the endothelium is considered to be one of the initial steps in the development of thrombosis, atherosclerosis, or inflammation.

The authors are grateful to Dr T. Adachi and the staff at the Adachi Maternity Hospital for kindly providing the umbilical cords, and to Dr J.M. Mencia-Huerta, Institut Henri Beaufour, France; Dr H. Heuer, Boehringer-Ingelheim KG, FRG; Ono Pharmaceutical Indust., Ltd., Osaka, Japan, for the generous gift of PAF antagonists. This work was supported input by Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

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(Received August 3, 1990
Revised December 28, 1990
Accepted January 23, 1991)

Br. J. Pharmacol. (1991), **103**, 1339–1346 © Macmillan Press Ltd, 1991

Inhibition of eosinophil cyclic nucleotide PDE activity and opsonised zymosan-stimulated respiratory burst by 'type IV'-selective PDE inhibitors

Gordon Dent, Mark A. Giembycz, [1]Klaus F. Rabe & [2]Peter J. Barnes

Department of Thoracic Medicine, National Heart and Lung Institute, Dovehouse Street, London SW3 6LY

1 The cyclic nucleotide phosphodiesterase (PDE) of guinea-pig eosinophils was partially characterized and the effects of selective inhibitors of PDE isoenzymes upon opsonized zymosan (OZ)-stimulated respiratory burst were studied.

2 PDE activity in eosinophil lysates appeared to be membrane-associated, displayed substrate specificity for adenosine 3':5' cyclic monophosphate (cyclic AMP) versus guanosine 3':5' cyclic monophosphate (cyclic GMP) and was insensitive to cyclic GMP or Ca^{2+} and calmodulin.

3 The non-selective PDE inhibitor, 3-isobutyl-1-methylxanthine caused a concentration-dependent inhibition of both OZ-stimulated hydrogen peroxide (H_2O_2) generation and cyclic AMP hydrolysis. The type IV-selective PDE inhibitors, rolipram and denbufylline, also inhibited H_2O_2 generation and cyclic AMP hydrolysis in a concentration-dependent manner whilst SK&F 94120 and Org 9935 (type III-selective) and zaprinast (type Ia or V-selective) were ineffective.

4 Dibutyryl cyclic AMP, a cell-permeable, non-hydrolysable analogue of cyclic AMP, caused a concentration-dependent inhibition of H_2O_2 generation stimulated by OZ. Dibutyryl cyclic GMP was ineffective.

5 It is concluded that eosinophil respiratory burst activity induced by OZ can be regulated by intracellular cyclic AMP and that the levels of cyclic AMP are controlled exclusively by a rolipram- and denbufylline-sensitive PDE isoenzyme that resembles a type IV species.

Keywords: Eosinophils; phosphodiesterases; phosphodiesterase inhibitors; hydrogen peroxide; respiratory burst

Introduction

The second messenger adenosine 3':5' cyclic monophosphate (cyclic AMP) is synthesized from adenosine 5'-triphosphate (ATP) through the activation of adenylyl cyclase by a variety of receptor agonists. Cyclic AMP is known to inhibit the activation of many cell types, including mast cells and basophils (MacGlashan *et al.*, 1983; Undem *et al.*, 1990), lymphocytes and monocytes (Thompson *et al.*, 1976; Elliott & Leonard, 1989), macrophages (Bonta *et al.*, 1984) and neutrophils (Busse & Sosman, 1984; Hoult *et al.*, 1986; Grady & Thomas, 1986). In view of this inhibitory action upon cells involved in inflammatory responses, it has been suggested that agents capable of elevating cyclic AMP levels in such cells may have potential as anti-inflammatory drugs (Persson *et al.*, 1988; Torphy, 1989).

Intracellular levels of cyclic AMP and guanosine 3':5' cyclic monophosphate (cyclic GMP) are regulated by the concerted action of adenylyl and guanylyl cyclase, which catalyse their respective formation, and of 3'-phosphodiesterase (PDE) enzymes which hydrolyse the 3'-ribose phosphate bond to produce the biologically inert 5'-monophosphates. It is now apparent that this latter reaction can be catalysed by a large number of distinct isoenzymes, forming at least five PDE 'families' which may be distinguished by several criteria, including their substrate specificity, kinetic characteristics, tissue distribution, subcellular localization, physico-chemical properties and sensitivity to endogenous cofactors and inhibitors (see Beavo, 1988; Beavo & Reifsnyder, 1990). While non-selective inhibitors of PDEs, such as theophylline and 3-isobuty-1-methylxanthine (IBMX) have been available for some time, potent selective inhibitors have only more recently been identified for certain PDE isoenzymes. For example, zaprinast inhibits the PDE isoenzymes which specifically hydrolyse cyclic GMP, known as type I (Reeves *et al.*, 1987), type Ia (Torphy & Cieslinski, 1990) or type V (Beavo & Reifsnyder, 1990) PDE (Prigent *et al.*, 1988); SK&F 94120, milrinone, cilostamide and Org 9935, among others, inhibit the low K_m, cyclic GMP-inhibited, cyclic AMP-specific (type III) PDE (Gristwood *et al.*, 1986; Harrison *et al.*, 1986; Shahid *et al.*, 1990) and the cyclic GMP-insensitive low K_m, cyclic AMP-specific (type IV) PDE is selectively inhibited by Ro 20,1724, rolipram and denbufylline (Reeves *et al.*, 1987; Nicholson *et al.*, 1989).

It is likely that eosinophils contribute to the pathogenesis of allergic inflammatory diseases, including bronchial asthma, through the release of a range of cytotoxic basic proteins and oxygen-derived free radicals (Venge, 1981; Gleich *et al.*, 1984). We have demonstrated previously that generation of superoxide anions by guinea-pig and human eosinophils can be inhibited by high concentrations of theophylline (Yukawa *et al.*, 1989) and it has been suggested that xanthines, such as theophylline, may be beneficial in asthma due to their anti-inflammatory, as much as to any bronchodilator, action (Kuehl *et al.*, 1987; Persson *et al.*, 1988). Since theophylline may act by inhibiting cyclic nucleotide hydrolysis (Polson *et al.*, 1978), we have investigated the effects of a cyclic AMP analogue and of non-selective and isoenzyme-selective PDE inhibitors upon opsonised zymosan (OZ)-stimulated respiratory burst in guinea-pig eosinophils. In addition, we have characterized the PDE enzyme activity in these cells in order to determine whether cyclic AMP can inhibit eosinophil function and which PDE isoenzyme is responsible for regulation of intracellular cyclic AMP levels in this cell.

Preliminary accounts of some of these data have been presented to the British Pharmacological Society (Dent *et al.*, 1990; Giembycz *et al.*, 1991).

[1] Present address: Krankenhaus Grosshansdorf, Zentrum für Pneumologie und Thoraxchirurgie, Wöhrendamm 80, 2070 Grosshansdorf, Germany.

[2] Author for correspondence.

Methods

Eosinophil isolation

Eosinophils were harvested by peritoneal lavage of anaesthetized (ketamine 25 mg kg^{-1}, xylazine 5 mg kg^{-1}, i.m.) male Dunkin-Hartley guinea-pigs (700–1300 g body weight) rendered eosinophilic by weekly intraperitoneal injection of normal human serum (1 ml per animal). Lavage cells were washed twice with Hanks balanced salts solution (HBSS) and separated by centrifugation on discontinuous Percoll density gradients, as described by Gärtner (1980). Eosinophil-rich fractions were pooled, washed with HBSS and finally resuspended in the appropriate buffer solution.

Measurement of respiratory burst activity

Respiratory burst activity was measured as the rate of production of hydrogen peroxide (H_2O_2) in the presence of a saturating concentration of superoxide dismutase (SOD). H_2O_2 was assayed by the horseradish peroxidase (HRPO)-catalysed oxidation of scopoletin, quantified by its loss of fluorescence, as described by Root *et al.* (1975). Eosinophils were suspended at a density of 10^7 cells ml^{-1} in HEPES-buffered Ca^{2+}/Mg^{2+}-free Krebs-Ringer bicarbonate solution (KRB, composition, mM: NaCl 138, KCl 6, NaH_2PO_4 1, $NaHCO_3$ 5, glucose 5.5 and HEPES 10, pH 7.4) and stored on ice until required; cells prepared in this way remained responsive for up to 7 h. Cells (10^6 in 100 μl) were added to 875 μl KRB containing $MgSO_4$ 1 mM, $CaCl_2$ 1 mM, SOD 30 u, HRPO 1 u and scopoletin 4 μM in polystyrene fluorimeter cuvettes and incubated, with or without drugs, for 2 min at 37°C prior to the addition of 25 μl of zymosan suspension, containing 500 μg of OZ. Kinetic measurements were made at 37°C with a recording spectrofluorophotometer (model RF-5000; Shimadzu Corporation, Kyoto, Japan) at an excitation wavelength of 350 nm and emission wavelength of 460 nm with band widths set to 3 nm. Fluorescence was monitored continuously for 15 min. Rates of reduction of fluorescence were obtained from 1st derivative plots and converted to rates of H_2O_2 generation by comparison with a standard curve constructed for known H_2O_2 concentrations.

Measurement of PDE activity

PDE activity was measured in crude eosinophil lysates by assaying the cleavage of [^{3}H]-cyclic AMP or [^{3}H]-cyclic GMP to its corresponding radiolabelled nucleoside 5'-monophosphate. Eosinophils (10^8 ml^{-1}) were suspended in lysis buffer, consisting of triethanolamine (TEA) 20 mM, EDTA 1 mM, bacitracin 100 μg ml^{-1}, benzamidine 2 mM, leupeptin 50 μM, phenylmethylsulphonyl fluoride (PMSF) 50 μM and soybean trypsin inhibitor (STI) 10 μg ml^{-1} (pH 8.0), and lysed by rapid freezing in liquid nitrogen and slow thawing at room temperature followed by ten repeated extrusions through a 23G needle and two rounds of sonication (10 s at 26 μm amplitude); Triton X-100 was added to the resulting homogenate at a final concentration of 0.5% (v/v). It is acknowledged that eosinophils are difficult cells to disrupt (Spry, 1988) and these exceptionally harsh conditions were required to cause lysis of a substantial proportion of the cells. Effective cell disruption was confirmed by the inability of the cell fragments in the resulting suspension to exclude Trypan blue dye. When required, the soluble and particulate fractions were separated by centrifugation of the lysate at 45,000 *g* for 30 min. In further experiments, the particulate fraction was resuspended in ice-cold lysis buffer made up to 500 mM with KCl or supplemented with either Triton X-100 (0.1%, 0.5% or 1% v/v) or sodium deoxycholate (2 mM or 5 mM). This suspension was mixed continuously for 60 min at 4°C and then centrifuged at 45,000 *g* for 30 min; the resulting supernatant was de-salted (Centriprep-30; Amicon – MWCO 30,000) if necessary, prior to assaying for PDE activity.

PDE activity was assayed according to the method of Thompson & Appelman (1971), as modified by Schwartz & Passonneau (1974). Briefly, 30 μl of eosinophil lysate (or the soluble or particulate fraction thereof) was added to 270 μl of reaction medium containing TEA 40 mM (pH 8.0), EGTA 2 mM, dithiothreitol (DTT) 2 mM, magnesium acetate 5 mM, bovine serum albumin (BSA) 500 μg ml^{-1}, cyclic nucleotide monophosphate supplemented with 2.5–3.5 × 10^5 d.p.m. [^{3}H]-cyclic nucleotide monophosphate, alkaline phosphatase 0.25 u, approximately 5 × 10^3 d.p.m. [^{14}C]-adenosine and the drug under test of the appropriate vehicle. The concentration of cyclic nucleotide monophosphate was 1 μM throughout except for kinetic experiments in which a range of cyclic AMP concentrations from 1 μM to 1 mM was used. In some experiments cyclic AMP hydrolysis was determined in the presence of cyclic GMP (10 μM) or calmodulin (0.25 u plus 10 μM $CaCl_2$; EGTA was not included in these assays). Reactions were terminated after 30 min by the addition of 1 ml Dowex AG 1-X8, methanol and water (1:2:1). Slurries were mixed thoroughly and left to stand for 10 min at 4°C before centrifugation at 12,000 *g* for 4 min. The radioactivity in 800 μl aliquots of the resulting supernatants (representing ^{3}H-nucleoside, the product of dephosphorylation of ^{3}H-nucleotide monophosphate by alkaline phosphatase) was determined by liquid scintillation counting, upon the addition of 2 ml ACS II scintillant, at a counting efficiency of approximately 60%. Enzyme activity is expressed as the formation of nucleoside 5'-monophosphate from cyclic nucleotide monophosphate in 1 min per 10^6 cells at 37°C after correction for the recovery (typically 65–85%) of [^{14}C]-adenosine.

Data analysis

Concentration-response curves were analysed by least-squares non-linear regression with the InPlot curve-fitting programme (GraphPad Software; San Diego, California, U.S.A.). IC_{50} values (the concentration of drug required to cause 50% inhibition) are expressed as arithmetic mean ± s.e.mean from 3–10 experiments. Where probability levels are reported, they were calculated by Student's *t* test; a probability of less than 5% was designated as significant.

Materials

Percoll was purchased from LKB/Pharmacia (Milton Keynes, Bucks.). Alkaline phosphatase, bacitracin, benzamidine, BSA (fraction V), calmodulin, cyclic AMP, cyclic GMP, DTT, EDTA, EGTA, H_2O_2 (30% w/v), HRPO, IBMX, leupeptin, magnesium acetate, N^6-*O*-2-dibutyryl cyclic GMP, PMSF, scopoletin (7-hydroxy-6-methoxy-2H-1-benzopyran-2-one), SOD (type IV, from bovine erythrocytes), sodium deoxycholate, STI, TEA, theophylline, Triton X-100 and zymosan A were obtained from Sigma Chemical Co. (Poole, Dorset). N^6-*O*-2-dibutyryl cyclic AMP was purchased from Aldrich Chemical Co. (Gillingham, Dorset). [2,8-^{3}H]-cyclic AMP, [8-^{3}H]-cyclic GMP and [8-^{14}C]-adenosine were supplied by Amersham International (Little Chalfont, Bucks.). Dowex AG 1-X8 (200–400 mesh, acetate form) was obtained from Bio-Rad (Hemel Hempstead, Herts.). Zaprinast (2-*o*-propoxyphenyl-8-azapurin-6-one) was a kind gift of Rhône-Poulenc Rörer Inc., Dagenham, Essex. Org 9935 (4,5-dihydro-6-(5,6-dimethoxybenzo[b]thien-2-yl)-5-methyl-3(2H)-pyridazinone) and Org 30029 (N'-hydroxy-5,6-dimethoxybenzo[b] thiophene-2-carboxamidamido hydrochloride) were donated by Organon Laboratories, Newhouse, Strathclyde. Rolipram (4-(3'-cyclopentyloxy-4'-methoxyphenyl)-2-pyrrolidone) was a gift from Schering GmbH, Berlin, Germany. SK&F 94120 ((5-acetimidophenyl)pyrazin-(1H)-one) and denbufylline (1,3-di-*n*-butyl-7-(2'-oxopropyl)-xanthine) were donated by SmithKline

Beecham (Welwyn Garden City, Herts.). Zardaverine (6-(4-difluoromethoxy-3-methoxyphenyl)-3[2H]-pyridazinone) was supplied by Byk Gulden Pharmazeutica, Konstanz, Germany. AH 21-132 ((±)-*cis*-6-(*p*-acetamidophenyl)-1,2,3,4,4a,10b-hexahydro-8,9-dimethoxy-2-methyl-benzo-[c[1, 6] naphthyridine) was obtained from Sandoz AG, Basel, Switzerland. Drugs were made up as stock solutions of 100 mM in ethanol (IBMX, rolipram, denbufylline), 100 mM NaOH (zaprinast, Org 9935, SK&F 94120, theophylline), dimethyl sulphoxide (Org 30029, zardaverine) or distilled water (AH 21-132) and diluted in the appropriate assay buffer. Dibutyryl cyclic nucleotides were dissolved in buffer at a stock concentration of 100 mM. Zymosan was opsonised for 30 min at 37°C in HBSS containing 20% pooled guinea-pig serum, washed and resuspended in KRB.

Results

Inhibition of respiratory burst

Opsonised zymosan at a concentration of 500 $\mu g\,ml^{-1}$, a concentration inducing a sub-maximal response, caused a delayed, slow and prolonged generation of H_2O_2 by guinea-pig eosinophils (Figure 1b). The mean peak rate of OZ-stimulated H_2O_2 generation was 2.11 ± 0.18 nmol min^{-1} ($n = 20$). This response was inhibited by dibutyryl cyclic AMP, a cell-permeable, non-hydrolysable analogue of cyclic AMP (Figure 1c). This inhibition was concentration-dependent, with an IC_{50} of 322 ± 97.3 μM ($n = 3$), and reached a maximum of 78.8 ± 8.12% (Figure 2). In contrast, dibutyryl cyclic GMP had little effect at concentrations below 10 mM.

The suppression of H_2O_2 generation by PDE inhibitors is shown in Figure 3 and summarised in Table 1: IBMX and the two type IV-selective inhibitors, rolipram and denbufylline, were effective in reducing OZ-stimulated eosinophil respiratory burst activity while the type V-selective inhibitor zaprinast and type III-selective inhibitors SK&F and Org 9935 were ineffective within the range of concentrations at which they are known to be selective. The concentration-response curves for rolipram and denbufylline were very shallow (Hill coefficient $(n_H) = 0.398 \pm 0.040$ and $n_H = 0.260 \pm 0.049$, respectively) whilst IBMX gave a steeper curve ($n_H = 0.827 \pm 0.062$); IBMX appeared to cause an enhancement of zymosan-stimulated H_2O_2 generation at lower concentrations, although this effect did not attain statistical significance. Extending the pre-incubation period caused a small reduction in the inhibition induced by 10 μM denbufylline (Figure 4), suggesting that the shallowness of the concentration-response curve is unlikely to be due to insufficient equilibration time leading to lower than expected intracellular concentrations of the inhibitor. Concentrations of PDE inhibitors higher than 100 μM could not be used for respiratory burst measurements owing to the inhibitory actions of the solvents used for the drugs at these levels.

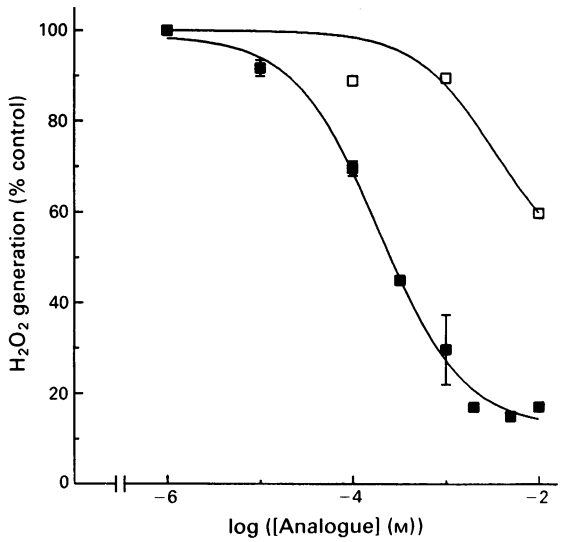

Figure 2 Effect of dibutyryl cyclic AMP (■) and dibutyryl cyclic GMP (□) on guinea-pig peritoneal eosinophil respiratory burst. Conditions were as described in Figure 1. Rates of generation of H_2O_2 were calculated from the rate of loss of fluorescence of scopoletin. Data represent mean (s.e.mean shown by vertical bars) from 3 separate experiments for dibutyryl cyclic AMP and results from 1 experiment for dibutyryl cyclic GMP.

Characterization of PDE activity

Little hydrolysis of either cyclic AMP or cyclic GMP (1 μM) was detected in the soluble fraction of eosinophil homogenates

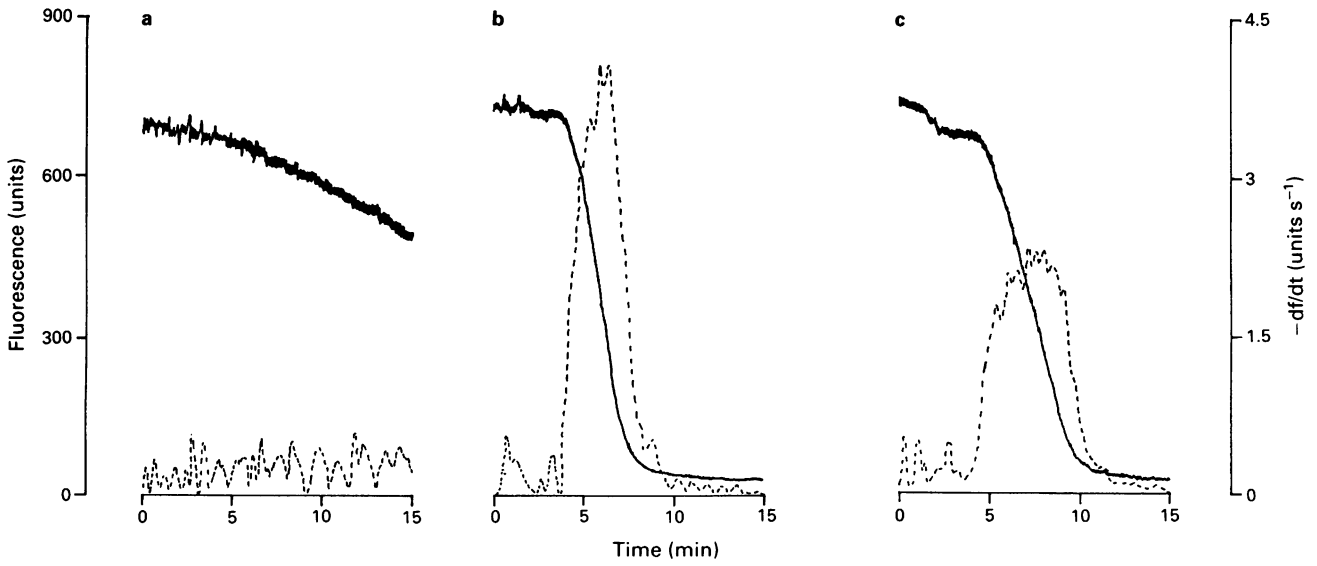

Figure 1 Loss of fluorescence of scopoletin induced by opsonized zymosan (OZ) and its inhibition by a cyclic AMP analogue. Eosinophils (1×10^6) were pre-incubated without (a and b) or with 1 mM dibutyryl cyclic AMP (c) for 2 min at 37°C prior to addition of buffer (a) or 500 μg OZ (b and c). Solid lines represent fluorescence (arbitrary units), broken lines are inverted first derivative curves representing rate of decrease of fluorescence. Figure shows original traces from a single experiment.

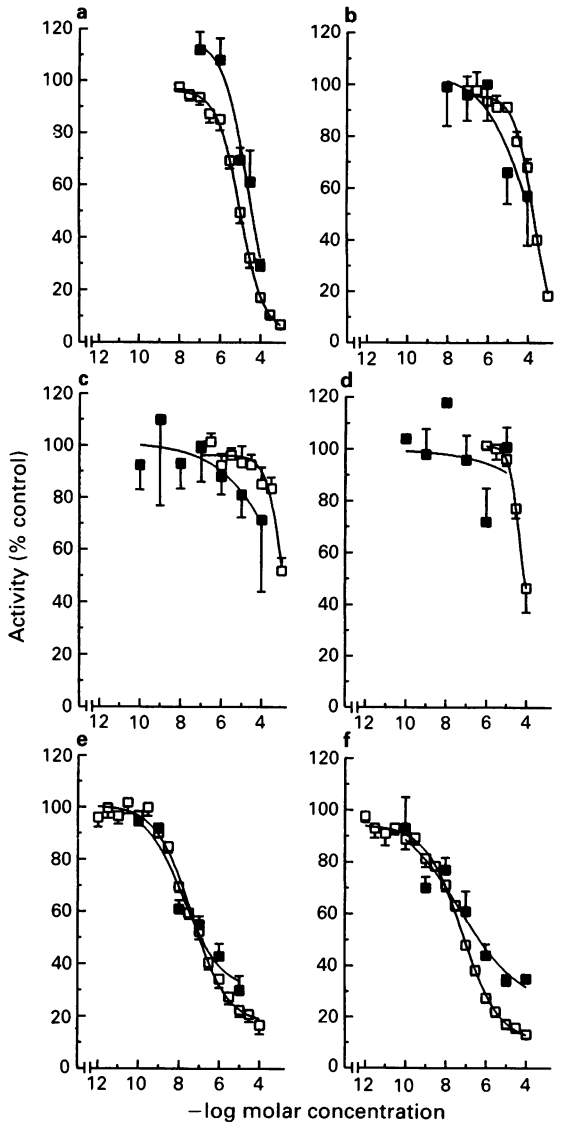

Figure 3 Effect of phosphodiesterase (PDE) inhibitors on opsonised zymosan-stimulated H_2O_2 generation (■) and total PDE activity (□) of guinea-pig peritoneal eosinophils. Conditions for H_2O_2 measurements were as described in Figure 1; cyclic AMP hydrolysis was measured at a substrate concentration of 1 μM over 30 min at 37°C. Data represent mean (s.e.mean shown by vertical bars) from 3–10 separate experiments. (a) 3-Isobutyl-1-methylxanthine – non-selective PDE inhibitor; (b) zaprinast – type V PDE inhibitor; (c) SK&F 94120 – type III PDE inhibitor; (d) Org 9935 – type III PDE inhibitor; (e) rolipram – type IV inhibitor; (f) denbufylline – type IV PDE inhibitor.

Table 1 Inhibition of eosinophil respiratory burst and cyclic AMP hydrolysis by phosphodiesterase inhibitors

	IC_{50} (μM)	
Drug	*vs. respiratory burst*	*vs. cyclic AMP*[1] *hydrolysis*
IBMX	36.0 ± 10.4 (n = 5)	11.2 ± 2.16 (n = 4)
SK&F 94120	>100 (n = 5)	>100 (n = 4)
Org 9935	>100 (n = 3)	≈100 (n = 4)
Rolipram	0.485 ± 0.231 (n = 5)	0.160 ± 0.044 (n = 9)
Denbufylline	0.862 ± 0.474 (n = 5)	0.102 ± 0.016 (n = 10)
Zaprinast	>100 (n = 3)	>100 (n = 4)

Data represent means ± s.e.mean. [1] Cyclic AMP at 1 μM. IBMX: 3-isobutyl-1-methylxanthine

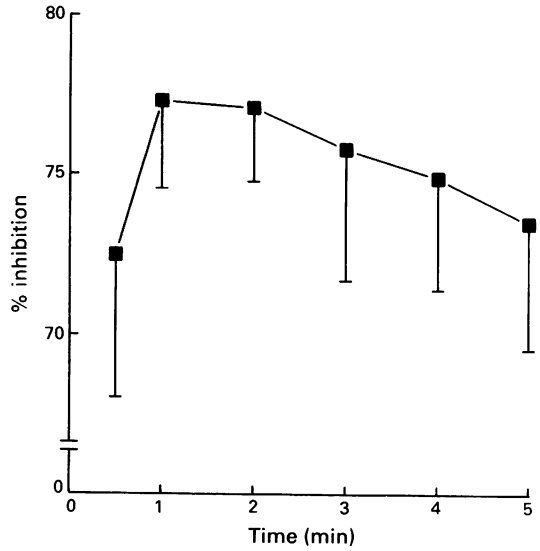

Figure 4 Time-course of inhibition of opsonised zymosan-stimulated H_2O_2 generation by denbufylline (10 μM). Conditions were as described in Figure 1 except that the duration of pre-incubation was varied between 30 s and 4 min. Data points represent mean (s.e.mean shown by vertical bars) from 3 experiments.

(Figure 5a). Increasing the number of cells 10 fold to 10^9 ml^{-1} did not enhance cyclic nucleotide hydrolysis in this fraction. The apparent lack of soluble PDE activity did not appear to be due to proteolysis of the native enzymes occurring during cell lysis since the inclusion of the protease inhibitors bacitracin, benzamidine, leupeptin, PMSF and STI in the lysis buffer did not uncover detectable PDE activity. Similarly, oxidation of the enzyme at reactive sulphydryl moieties was unlikely to explain the low soluble PDE activity, as evidenced by the lack of a protective effect by DTT. In contrast both cyclic AMP and, to a lesser extent, cyclic GMP were readily hydrolysed by a PDE activity present in the particulate material (Figure 5a). Treatment of the particulate fraction with a high ionic strength buffer solution (KCl 500 mM) failed to release PDE activity into the soluble phase, as did treatment with the non-ionic detergent Triton X-100 (0.1–1% v/v) or the ionic detergent sodium deoxycholate (2–5 mM).

With the crude eosinophil lysate as the source of PDE, cyclic AMP was the preferred substrate. At a concentration of 1 μM, cyclic AMP was hydrolysed by eosinophil PDE at a rate of 1.81 ± 0.19 pmol min^{-1} per 10^6 cells (n = 37). This rate was significantly (approximately 16 fold) greater than that achieved when the same concentration of cyclic GMP was used as substrate (0.11 ± 0.01 pmol min^{-1} per 10^6 cells, n = 6) (Figure 5b). At a concentration of 100 μM however, cyclic GMP was substantially hydrolysed (Figure 5b); such a profile of substrate specificity is a characteristic of type IV PDEs (Beavo, 1988). Cyclic AMP hydrolysis was affected by the inclusion of neither cyclic GMP (10 μM) nor calmodulin (25 u) and $CaCl_2$ (10 μM) (Figure 5c).

Figure 3 shows the effect of PDE inhibitors upon hydrolysis of cyclic AMP by crude eosinophil lysates. The inhibition of PDE activity closely mimicked the effects upon respiratory burst: rolipram and denbufylline were potent inhibitors while IBMX was less potent but gave a steeper concentration-response curve (Table 2); SK&F 94120, Org 9935 and zaprinast were ineffective at concentrations below 10 μM. IC_{50} values for inhibition of cyclic AMP hydrolysis by IBMX, rolipram and denbufylline were lower than those for inhibition of OZ-stimulated H_2O_2 generation (Table 1); however, in all cases the maximum inhibition attainable appeared lower for respiratory burst than for PDE activity (Figure 3). Comparison of a range of non-selective and isoenzyme-selective PDE inhibitors (Table 2) showed inhibition of cyclic AMP hydrolysis by eosinophil lysates to be confined to compounds with PDE IV-

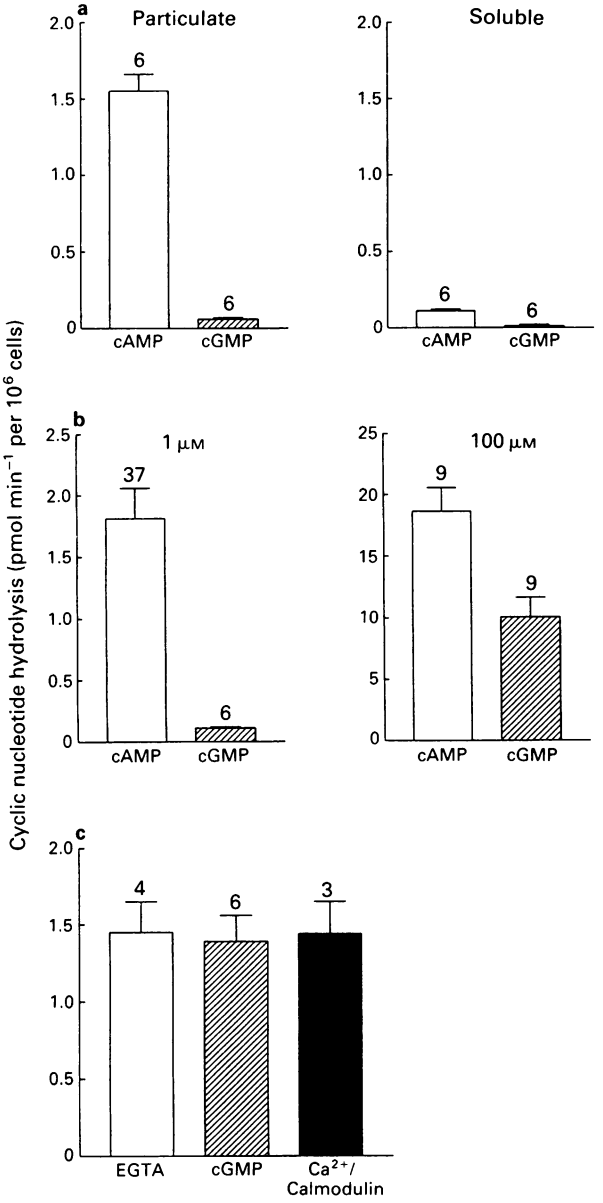

Figure 5 Subcellular distribution, substrate specificity and sensitivity to endogenous factors of guinea-pig eosinophil phosphodiesterase (PDE). Cyclic nucleotide hydrolysis was measured over 30 min at 37°C. (a) Rate of hydrolysis of cyclic AMP (open columns) and cyclic GMP (hatched columns, both at 1 μM) by the particulate (left hand panel) and soluble (right hand panel) fractions of eosinophil lysates prepared from 10^8 cells ml^{-1}; (b) hydrolysis of cyclic AMP (open columns) and cyclic GMP (hatched columns) at concentrations of 1 μM (left hand panel) or 100 μM (right hand panel) by eosinophil lysates; (c) hydrolysis of cyclic AMP under control conditions (open columns) and in the presence of 10 μM cyclic GMP (hatched column) or 10 μM $CaCl_2$ and 25 u calmodulin (solid column). Data points represent mean (s.e.mean shown by vertical bars) from 3–37 experiments.

inhibitory activity while shallow inhibition curves were seen only with the PDE IV-selective drugs.

Kinetic analysis of cyclic AMP hydrolysis by crude eosinophil lysates yielded a curvilinear Hofstee plot (Figure 6), suggesting the presence of either two distinct enzyme activities or a single enzyme with two hydrolytic sites. Resolution of the curves into two components, assuming conformation of both activities to Michaelis-Menten kinetics (Spears *et al.*, 1971), revealed a high-affinity, low capacity activity (K_m = 7.97 μM, V_{max} = 10.0 pmol min^{-1} per 10^6 cells) and a low-affinity, high-capacity activity (K_m = 368 μM, V_{max} = 27.8 pmol min^{-1} per 10^6 cells).

Table 2 Effects of non-selective and isoenzyme-selective phosphodiesterase inhibitors on cyclic AMP hydrolysis by eosinophil lysates

Inhibitor	n	*Selectivity*	IC_{50} (μM)[1]	n_H
Rolipram	10	IV	0.160 ± 0.044	0.444 ± 0.035
Denbufylline	9	IV	0.102 ± 0.016	0.454 ± 0.033
Zardaverine	3	III/IV	1.63 ± 0.38	0.866 ± 0.866
AH 21-132	4	III/IV	2.83 ± 0.54	0.971 ± 0.055
Org 30029	3	III/IV	9.28 ± 1.81	0.774 ± 0.121
IBMX	4	NS[2]	11.2 ± 2.16	0.794 ± 0.053
Theophylline	3	NS[2]	477 ± 98.9	1.175 ± 0.167
Zaprinast	4	V	225 ± 37.3	0.956 ± 0.088
Org 9935	4	III	50.1 ± 3.4%[3]	
SK&F 94120	4	III	32.7 ± 12.1%[4]	
Imazodan	3	III	35.5 ± 8.3%[4]	

Data represent mean ± s.e.mean. [1] Cyclic AMP at 1 μM. [2] Non-selective. [3] % inhibition at 100 μM (maximum concentration attainable owing to poor aqueous solubility). [4] % inhibition at 1 mM.
IBMX: 3-isobutyl-1-methylxanthine.

Discussion

The identification of isoenzymes of cyclic nucleotide phosphodiesterase which are distinct in their cellular and subcellular distribution and in their sensitivity to specific inhibitors may allow such inhibitors to be used to investigate the mechanisms of regulation of cyclic nucleotides within cells as well as to control specific biological processes in discrete cells or tissues (Corda *et al.*, 1990). Certain cell types may express only a single PDE isoenzyme: for instance murine S49 lymphoma cells contain predominantly the cyclic GMP-insensitive, cyclic AMP-specific, low K_m PDE known as the type IV isoenzyme (Brothers *et al.*, 1982), while human monocytes (Thompson *et al.*, 1976; Elliott & Leonard, 1989) and neutrophils (Wright *et al.*, 1990; Nielson *et al.*, 1990) seem to contain this single cyclic AMP PDE isoenzyme exclusively. These observations prompt speculation that selective inhibitors of this family of PDE isoenzymes may be able to suppress immune reactions; the sensitivity of human neutrophil PDE to Ro 20-1724 (Hoult *et al.*, 1986; Grady & Thomas, 1986) also implies a potential anti-inflammatory capacity for selective PDE IV inhibitors, although it has been reported that such drugs have no effect upon human alveolar macrophage activation (Fuller *et al.*, 1988).

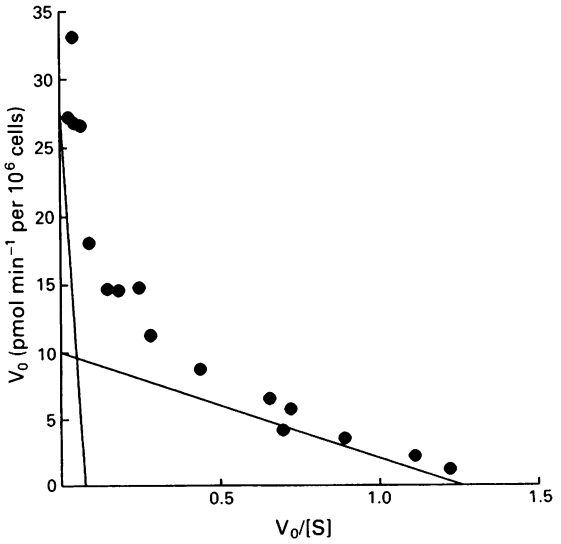

Figure 6 Kinetics of hydrolysis of cyclic AMP (1 μM–1 mM) by guinea-pig eosinophil phosphodiesterase. Enzyme activity was measured in the particulate fraction of an eosinophil homogenate over 30 min at 37°C. Data are presented according to Hofstee (1952) and resolved into two components according to Spears *et al.* (1971). V_0 = initial reaction velocity; [S] = substrate concentration.

In the present study, we have investigated the effects of both non-selective PDE inhibitors and selective inhibitors of the type III, type IV and type V (also known as type I or Ia) PDE isoenzymes upon the opsonised zymosan-stimulated generation of H_2O_2 by guinea-pig peritoneal eosinophils. We have shown this marker of eosinophil activation to be inhibited by non-selective and PDE IV-selective drugs, while the other drugs tested were ineffective within the range of concentrations at which they retain isoenzyme-selectivity. These findings were confirmed by the sensitivity of crude eosinophil PDE activity exclusively to inhibitors of PDE IV. Concentration-response curves for inhibition of cyclic AMP hydrolysis and OZ (500 μg)-induced H_2O_2 generation were almost superimposable. However, IBMX, rolipram and denbufylline all achieved a maximal inhibition of PDE activity which was greater than their respective maximal inhibition of H_2O_2 generation. Since dibutyryl cyclic AMP was capable of inhibiting only up to 80% of stimulated H_2O_2 generation, it is possible that the mechanism of activation of this response is not completely cyclic AMP-sensitive and, consequently, respiratory burst activity cannot be eliminated by total blockade of cyclic AMP hydrolysis by PDE.

It is striking that the inhibition curves for rolipram and denbufylline against both H_2O_2 generation and cyclic AMP hydrolysis have Hill coefficients significantly lower than unity. The most likely explanations for this phenomenon are: (1) an insufficient equilibration time with inhibitor, (2) the presence of two enzymes with different affinities for the inhibitor or (3) the expression of two hydrolytic sites which have different affinities for cyclic AMP. It is unlikely that equilibrium time was insufficient since the shallow curves are seen in the cell-free PDE assay system as well as the cellular activation measurements and thus diffusion time cannot be critical. In addition, extension of the pre-incubation time led to a slight decrease in the inhibition of H_2O_2 generation by denbufylline. Another explanation is that there may be a second PDE isoenzyme present in eosinophils for which rolipram and denbufylline have a lower affinity. (Non-selective and mixed PDE III/PDE IV inhibitors gave steeper inhibition curves which suggests that, if there are two enzymes, these drugs have an equal affinity for both.) The lack of effect of cyclic GMP upon rates of cyclic AMP hydrolysis in eosinophil lysates indicates the absence of either the cyclic GMP-stimulated (type II) or cyclic GMP-inhibited (type III) isoenzymes, while the insensitivity of the eosinophil PDE activity to Ca^{2+} and calmodulin confirms the absence of either of the Ca^{2+}/calmodulin-dependent (type Ib and Ic; Torphy & Cieslinski, 1990) isoenzymes. The great preference for cyclic AMP as substrate, in conjunction with the lack of inhibition by zaprinast, implies that the cyclic GMP-specific (type V) isoenzyme makes no contribution to the PDE activity of guinea-pig eosinophils. Perhaps a more likely interpretation is that two distinct type IV isoenzymes may be present within these cells: all of the inhibition curves for rolipram and denbufylline can be fitted to a two-component model with no significant change in goodness-of-fit, whilst a Hofstee plot of the data from kinetic experiments was clearly curvilinear, suggesting the possible presence of a second enzyme. However, the very high K_m value obtained for the low-affinity cyclic AMP-hydrolytic activity, suggests that this may not represent a genuine PDE enzyme. Our inability to solubilize the particulate PDE isoenzyme(s) present, added to the presence within the cell lysates of highly cationic proteins, has precluded the resolution of separate eosinophil PDE isoenzymes by ion-exchange chromatographic methods.

It is noteworthy that two distinct denbufylline-sensitive PDEs have been isolated from rat gastrocnemius (C.D. Nicholson & R. Wilke, personal communication) and two cyclic GMP-insensitive, rolipram-inhibited PDEs are present in soluble extracts of rat liver (Lavan *et al.*, 1989) and kidney (Hoey & Houslay, 1990). In addition, a second, high-affinity, membrane-associated binding site for [^{3}H]-rolipram has been identified in the central nervous systems of several species but its identity as a PDE enzyme has not been confirmed (Schneider *et al.*, 1986); both binding sites identified in the latter study had similar affinities for IBMX, which would be consistent with the steeper inhibition curves obtained with IBMX (compared to those for denbufylline) in the present study.

An enhancement of activity of a plasma membrane cyclic AMP PDE has been demonstrated as a result of phosphorylation by an insulin receptor-activated tyrosyl kinase (Pyne *et al.*, 1989) and it is possible that particular enzymes may exist in multiple states with different activities. It is apparent that the solubilization and purification of the guinea-pig eosinophil PDE must be achieved in order to determine the number and precise nature of the isoenzyme(s) present.

Unlike membrane-bound PDE isoenzymes in other tissues e.g. type II in rat liver plasma membranes (Pyne *et al.*, 1986) and cardiac ventricle (Timouyasse *et al.*, 1989); type III in rat adipocytes (Degerman *et al.*, 1987) and in canine and Rhesus monkey cardiac ventricle (Weishaar *et al.*, 1987); type IV in rat liver plasma membranes (Pyne *et al.*, 1987) which are effectively solubilized by treatment with various ionic and non-ionic detergents, the membrane-associated PDE present in guinea-pig eosinophils was exceedingly resistant to detergent solubilization. Indeed, both Triton X-100 and deoxycholate were completely ineffective at solubilizing the particulate PDE. This suggests that the eosinophil PDE is an integral membrane component.

The IC_{50} for inhibition of guinea-pig peritoneal eosinophil PDE by IBMX obtained in the present study (11.2 μM) is similar to that reported for the type IV PDE isoenzyme obtained from murine S49 lymphoma cells (14 μM), human monocytes (16 μM) and canine trachea (9.1 μM). In contrast, the IC_{50} obtained for rolipram (0.16 μM) is approximately 10 to 20 fold lower than that reported for type IV PDE of human monocytes (2.7 μM) and canine trachea (1.5 μM) (Brothers *et al.*, 1982; Elliott & Leonard, 1989; Galvan & Schudt, 1990), but only 4.5 fold lower than its IC_{50} for PDE IV from human neutrophils (Nielson *et al.*, 1990). This discrepancy appears to suggest that multiple rolipram-sensitive isoenzymes exist and that an enzyme with greater sensitivity to rolipram predominates in neutrophils and eosinophils. The IC_{50} of rolipram for inhibition of formylmethionyl-leucyl-phenylalanine-stimulated respiratory burst in human neutrophils was 0.71 μM, in close accordance with our figure for the OZ-stimulated eosinophil response (0.49 μM). Consistent with our findings in eosinophils, the only cyclic AMP PDE enzyme activity in human neutrophils was confined exclusively to the particulate fraction with characteristics indicative of it being a type IV isoenzyme. Furthermore, analysis of cyclic AMP hydrolysis by this PDE clearly shows curvilinear kinetics (Wright *et al.*, 1990).

In conclusion, we have now identified a membrane-associated type IV PDE as the predominant isoenzyme in guinea-pig peritoneal eosinophils by demonstration of the inhibition of stimulated respiratory burst and PDE activity exclusively by inhibitors of the type IV isoenzyme. Thus, drugs such as rolipram and Ro 20-1724 which have already been identified as bronchodilators both *in vitro* (Torphy *et al.*, 1988) and *in vivo* (Harris *et al.*, 1988) may also act to reduce airway eosinophilia and inflammation.

We thank the Medical Research Council, Pfizer Central Research (U.K.) and Bayer (Germany) for financial support.

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(Received September 19, 1990
Revised January 22, 1991
Accepted January 28, 1991)

Br. J. Pharmacol. (1991), **103**, 1347–1350 © Macmillan Press Ltd, 1991

Regulation of bradykinin receptor level by cholera toxin, pertussis toxin and forskolin in cultured human fibroblasts

[1]Beth G. Etscheid, Peter H. Ko & Mitchell L. Villereal

Department of Pharmacological and Physiological Sciences, University of Chicago, Chicago, IL 60637, U.S.A.

1 The effect of bacterial toxins on bradykinin-triggered release of arachidonic acid was studied in serum-deprived human foreskin (HSWP) fibroblasts prelabelled with [^{3}H]-arachidonic acid. An 18-h exposure of HSWP cells to cholera toxin, pertussis toxin, or forskolin enhanced the bradykinin-stimulated release of arachidonic acid and metabolites.

2 Prolonged treatment of HSWP cells with these agents also caused a 3 to 4 fold rise in cell surface [^{3}H]-bradykinin binding. The rise was inhibited by concurrent incubation with cycloheximide or actinomycin D. In addition, cholera toxin and foreskolin increased [^{3}H]-bradykinin binding in wildtype PC12 cells, but not in mutant PC12 cells with reduced cyclic AMP-dependent protein kinase type II activity.

3 In conclusion, cholera toxin, pertussis toxin and forskolin enhanced arachidonic acid release in response to bradykinin, and increased the number of bradykinin receptors in HSWP fibroblasts. A cyclic AMP-dependent mechanism appears to mediate the actions of the toxins and forskolin.

Keywords: Bradykinin receptors; cholera toxin; pertussis toxin; arachidonic acid release; human fibroblasts; forskolin

Introduction

Bradykinin, a nine amino acid peptide, is formed during tissue injury and in pathological conditions by the cleavage of inactive precursors, kininogens, that are present throughout mammalian tissues and body fluids. The actions of bradykinin include relaxation of vascular smooth muscle, increasing electrolyte secretion by the kidney and stimulation of pain fibres. Many of these effects appear to be mediated by arachidonic acid metabolites. For example, prostacyclin, which is released from the heart in response to bradykinin, mimics bradykinin induced dilatation of coronary vessels (Needleman *et al.*, 1975); and in the kidney, bradykinin causes the release of prostaglandin E_2, which stimulates salt and water excretion (McGiff *et al.*, 1976).

In cultured fibroblasts, bradykinin elicits both the release of arachidonic acid and its conversion to prostaglandins (Hong & Levine, 1976). That arachidonate is released directly from phospholipids by phospholipase A_2 action and is independent of phospholipase C involvement, has been demonstrated in fibroblasts (Murayama & Ui, 1985; Burch & Axelrod, 1987) and in endothelial cells (Kaya *et al.*, 1989) in culture. The pertussis toxin sensitivity (Murayama & Ui, 1985) and the guanine nucleotide-dependence (Burch & Axelrod, 1987) of bradykinin-stimulated arachidonate and prostaglandin production are evidence that a guanine nucleotide-binding protein couples the bradykinin receptor to phospholipase A_2.

Recently, we showed that bradykinin stimulated both arachidonic acid release and phosphatidylinositol turnover in cultured human foreskin (HSWP) fibroblasts and that cholera toxin and pertussis toxin had the anomolous effect of enhancing inositol phosphate generation in response to bradykinin (Etscheid & Villereal, 1989). In the present study, we investigated the effect of these bacterial toxins on bradykinin-stimulated arachidonic acid release, as well as the mechanism of action underlying the enhancement by the toxins of bradykinin responsiveness in HSWP cells.

Methods

Cell culture

HSWP (human foreskin-derived) fibroblasts were cultured in Eagle's minimum essential medium (EMEM) plus 10% foetal calf serum and gentamicin 1 μg ml^{-1}. For experiments, cells were subcultured onto 60 mm dishes, refed weekly, and allowed to attain confluence (at least 2 weeks) before assay. Normal PC12 (rat phaeochromocytoma) cells and chemically-mutated PC12 cells (A126-1B2; described by Van Buskirk *et al.*, 1985), were subcultured onto polylysine-coated 60 mm dishes in Dulbecco's modified Eagle's medium (DMEM) plus 10% foetal calf serum, 5% horse serum, penicillin 100 units ml^{-1} and streptomycin 100 μg ml^{-1}. They were refed every 2 days and used at 80% confluence. HSWP cells were incubated in EMEM plus 0.1% serum overnight before assays. Where appropriate, the serum-deprivation medium contained forskolin 25 μM, cholera toxin 250 ng ml^{-1}, pertussis toxin 50 ng ml^{-1}, cycloheximide 35 μM or actinomycin D 20 μM. Normal and mutant PC12 cells were treated overnight with these agents, but serum-deprived only for the last 4 h. Assays were conducted at 37°C in a water bath rotating at 60 r.p.m.

Arachidonic acid measurements

Cells prelabelled with [^{3}H]-arachidonic acid 0.1 μCi ml^{-1} overnight in serum-depriving medium were washed 3 times in assay medium consisting of HEPES-buffered, pH 7.45, amino acid-free EMEM with 0.1% fatty acid-free bovine serum albumin, and were incubated for 1 h at 37°C. The assay was initiated by replacing the final wash with 3 ml medium containing bradykinin 100 nM or 10% calf serum. This concentration of bradykinin was shown via dose-response assays to stimulate arachidonic acid release maximally in HSWP cells (Etscheid & Villereal, 1989). At 5 min, 1 ml medium aspirated from the cells was centrifuged to pellet dislodged cells, and was transferred to a vial with 2 ml scintillation fluid for quantitation of radioactivity ($n = 3$). Thin layer chromatography of the medium (by the method of Hojvat *et al.*, 1983) from bradykinin-stimulated cells showed that the major arachidonic acid metabolites were prostaglandin E_2 (PGE_2), PGI_2 (measured as 6-keto-$PGF_{1\alpha}$), and $PGF_{2\alpha}$. Uptake of [^{3}H]-arachidonic acid into phospholipid pools was measured for each experiment and found to be comparable in control and toxin- or forskolin-treated cells. One-way analysis of variance was used to analyze differences between the data from various treatment groups.

[^{3}H]-bradykinin binding

The procedure used was that of Roscher *et al.* (1983), with minor modifications. Briefly, serum-deprived cells previously

[1] Author for correspondence.

exposed to toxins, forskolin and/or protein synthesis inhibitors were washed in chilled phosphate-buffered saline and set in an ice bath. Cells were incubated on ice for 90 min with unlabelled- and ^{3}H-labelled bradykinin in low Na^+, Mg^{2+} and Ca^{2+} Hanks buffered saline plus 0.05% bovine serum albumin and bacitracin 1 mM (to inhibit kininase II). Following 5 washes in cold Tris-buffered saline plus 0.05% albumin, the cells were extracted with 2 ml 0.2% sodium dodecyl sulphate, and the radioactivity was measured by scintillation counting ($n = 3$). The concentration of [^{3}H]-bradykinin used for all assays was approximately 50 pM, which is on the linear portion of the dose-response curve, where changes in binding are most noticeable (Etscheid & Villereal, 1989). To measure non-specific binding in the assays depicted in Figures 2 and 3, an excess of unlabelled bradykinin, 1 μM, was used to displace [^{3}H]-bradykinin from the receptors. Unlabelled bradykinin, 0.25 nM to 1000 nM, was added with 50 pM [^{3}H]-bradykinin to the cells to generate the dissociation curve the parameters of which are listed in Table 1. A weighted, non-linear least-squares analysis of binding data was done with the LIGAND programme of Munson & Rodbard (1980) to determine the dissociation constant (K_d), receptor number (B_{max}) and degree of nonspecific binding (NS) for the data displayed in Table 1. P values were obtained from a one-way analysis of variance using non-parametric testing.

Materials

Cholera and pertussis toxins were obtained from List Biologicals, actinomycin D and cycloheximide from Sigma Chemical Co., forskolin from Calbiochem, and bradykinin from Peninsula. NEN-Dupont supplied [^{3}H]-bradykinin and [^{3}H]-arachidonic acid.

Results

To characterize the toxin-sensitivity of the stimulation by bradykinin of arachidonic acid release, we incubated HSWP fibroblasts overnight in low serum medium containing [^{3}H]-arachidonic acid, with or without cholera toxin or pertussis toxin, before exposing the cells to bradykinin. The data in Figure 1 show that the bradykinin-induced release of [^{3}H]-arachidonic acid and metabolites was significantly increased in HSWP cells incubated with either cholera toxin or pertussis toxin for 18 h. In contrast, arachidonate release in response to serum was not markedly changed by pretreatment with either toxin (Figure 1).

As both cholera and pertussis toxins have been demonstrated to increase cellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels in cultured cells (Moss & Vaughan, 1987), we postulated that a rise in cyclic AMP might underlie the toxin-induced enhancement of bradykinin responsiveness in HSWP cells. To test this, we treated HSWP cells for 18 h with forskolin, an agent that raises cyclic AMP levels via activation of adenylate cyclase, and then exposed the cells to bradykinin or to serum. As shown in Figure 1, forskolin markedly amplified the bradykinin-stimulated liberation of arachidonic acid and metabolites. A smaller increase in serum-stimulated arachidonate release was also observed. Importantly, an acute (5 min) exposure to 50 μM 8-p-chlorophenylthio-cyclic AMP, a membrane-permeant analogue of cyclic AMP, slightly decreased bradykinin- and serum-stimulated release (data not shown). These results suggested that a prolonged increase in cyclic AMP levels might be required for the increased bradykinin-responsiveness of HSWP cells treated with cholera or pertussis toxin.

As the toxin-elicited enhancement was seen with bradykinin stimulation of arachidonic acid release (Figure 1) and phosphatidylinositol turnover (Etscheid & Villereal, 1989), but not with serum stimulation, we speculated that the toxins might induce some change in a component unique to the bradykinin pathway, possibly the bradykinin receptor. As toxin-induced

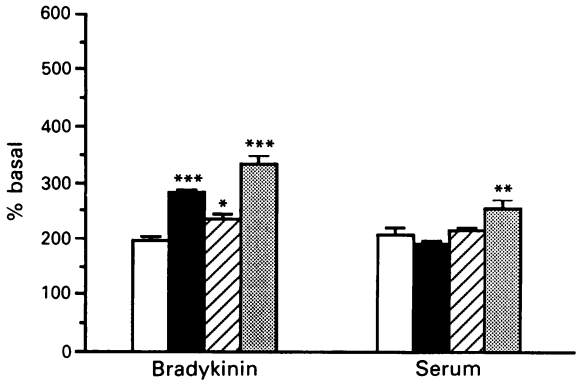

Figure 1 Bradykinin stimulation of [^{3}H]-arachidonic acid release in human foreskin (HSWP) cells pretreated with cholera toxin, pertussis toxin, or forskolin. Cells were incubated simultaneously with [^{3}H]-arachidonic acid and either cholera toxin (250 ng ml^{-1}), pertussis toxin (50 ng ml^{-1}) or forskolin (25 μM) for 18 h, then stimulated with bradykinin (100 nM) for 5 min: open columns, control; solid columns, cholera toxin; hatched columns, pertussis toxin and stippled columns, forskolin. Data are the mean (s.e.mean shown by vertical bars) expressed as percentage of basal, which was (in d.p.m. released per total d.p.m. [^{3}H]-arachidonic acid incorporated) 0.00442 for control cells, 0.00397 for cholera toxin-treated cells, 0.00409 for pertussis toxin-treated cells and 0.00390 for forskolin-treated cells. Three separate experiments yielded similar results. One-way analysis of variance was used to measure differences between groups; * denotes significant difference at $P < 0.05$, ** $P < 0.02$, and *** $P < 0.001$.

alterations in the receptor might be revealed as a change in the binding of its ligand, we conducted assays of [^{3}H]-bradykinin binding in HSWP cells pretreated for 18 h with cholera toxin, pertussis toxin or forskolin. All three treatments dramatically enhanced bradykinin binding to the cells via a 3 to 4 fold increase in receptor number (B_{max}, see Table 1).

A time course of forskolin pretreatment showed that the increase in binding capacity had a 4 h lag and reached a maximum between 18 and 24 h (Figure 2a). Concurrent exposure of the cells to cycloheximide or actinomycin D (inhibitors of protein and messenger RNA synthesis, respectively) blocked the forskolin-induced increment in [^{3}H]-bradykinin binding (Figure 2b).

To investigate further the cyclic AMP-dependence of the rise in bradykinin binding, we assayed binding of [^{3}H]-bradykinin to wildtype PC12 cells and to the chemically-transformed variant, PC12 A126-1B2, which exhibits substantially reduced cyclic AMP-dependent protein kinase type II activity (Van Buskirk et al., 1987). As shown in Figure 3, pretreatment with cholera toxin or forskolin greatly increased [^{3}H]-bradykinin binding to the wildtype PC12, but not to the PC12 A126-1B2 cells. These results indicated that a cyclic AMP-dependent process, possibly cyclic AMP-

Table 1 Binding of [^{3}H]-bradykinin to toxin- and forskolin-pretreated human foreskin HSWP cells

Treatment	K_d (nM)	B_{max} (fmol mg^{-1} protein)
Control	2.2 ± 0.4	80 ± 10
Cholera toxin	2.4 ± 0.5	280 ± 40
Pertussis toxin	2.2 ± 0.5	330 ± 50
Forskolin	2.3 ± 0.4	240 ± 30

Cultured HSWP cells were serum-deprived for 18 h in the presence of cholera toxin 250 ng ml^{-1}, pertussis toxin 50 ng ml^{-1} or forskolin 25 μM. The pretreated cells were incubated with [^{3}H]-bradykinin 50 pM and increasing amounts of unlabelled bradykinin (0.25 nM to 1000 nM) for 90 min to generate a dissociation curve. Binding data were analyzed by the non-linear, least-squares, curve fitting programme, LIGAND (Munson & Rodbard, 1980). The calculated values for the parameters are reported with their coefficients of variance.

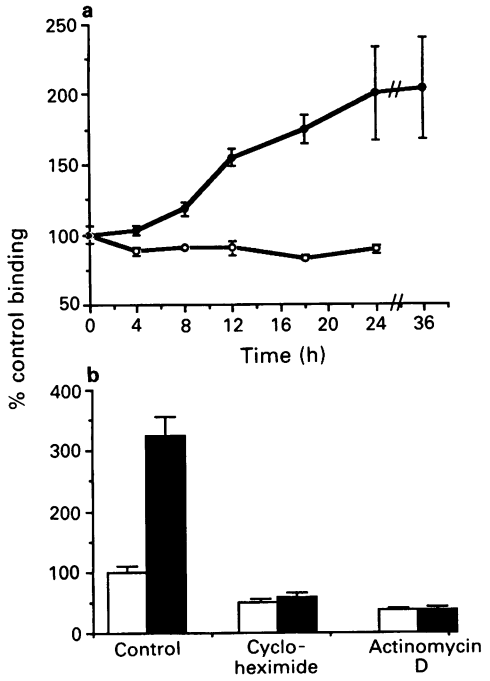

Figure 2 [^{3}H]-bradykinin binding to forskolin-treated human foreskin (HSWP) cells. (a) Time course of the forskolin-induced increase in [^{3}H]-bradykinin binding. Serum-deprived cells were pretreated for 0 to 36 h with 25 μM forskolin (●) or vehicle (○) before exposure to [^{3}H]-bradykinin. Specific binding to the receptors was determined by subtracting nonspecific binding (50 pM [^{3}H]-bradykinin + 1 μM unlabelled bradykinin) from total binding ([^{3}H]-bradykinin alone). This graph represents the mean of three separate time courses; vertical bars show s.e.mean. (b) Inhibition of forskolin-mediated increases in [^{3}H]-bradykinin binding by cycloheximide and actinomycin D. Cells were treated as in (a) for 24 h with forskolin in the presence or absence of cycloheximide (35 μM) or actinomycin D (20 μM), followed by binding with [^{3}H]-bradykinin: open columns, vehicle; solid columns, forskolin-treated.

dependent protein phosphorylation, might be involved in the toxin- and forskolin-induced increase in bradykinin binding.

Discussion

The effect of bacterial toxins on bradykinin-stimulated processes can be inhibitory, stimulatory or nil, depending upon the physiological assay and cell type studied. The impetus for this study was to determine the mechanism by which cholera toxin and pertussis toxin enhanced cellular responsiveness to bradykinin stimulation. The initial report of a toxin-induced enhancement of bradykinin action was made by Malkiel & Hargis (1967), who described the increased lethality of an injection of bradykinin into mice previously treated with pertussis toxin. In 1988, Moss *et al.* demonstrated that the stimulation of prostaglandin production by bradykinin was greater in cultured fibroblasts that were pretreated with various agents, including cholera and pertussis toxin, than in untreated cells. We recently found an increase in bradykinin stimulation of phosphatidylinositol breakdown in cultured foreskin fibroblasts pretreated with either cholera toxin or pertussis toxin (Etscheid & Villereal, 1989). Whereas the mechanism by which cholera toxin and pertussis toxin inhibited various processes had already been well-documented, there were no reports of how these toxins enhanced the actions of bradykinin in cultured cells.

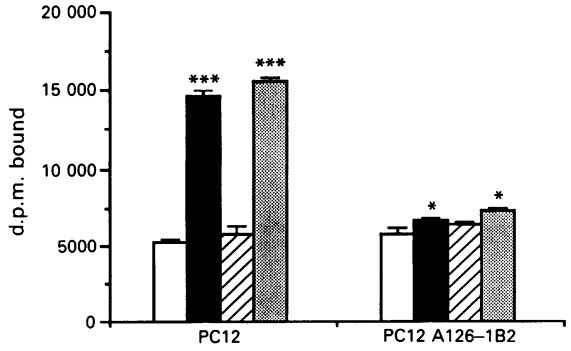

Figure 3 Binding of [^{3}H]-bradykinin to forskolin- or toxin-treated normal and mutant PC12 cells. Cells were exposed for 18 h to forskolin (25 μM), cholera toxin (250 ng ml^{-1}) or pertussis toxin (50 ng ml^{-1}), serum-deprived for 4 h and then incubated with 50 pM [^{3}H]-bradykinin with or without 1 μM unlabelled bradykinin to determine specific binding. Columns show mean with s.e.mean indicated by vertical bars: open columns, control; solid columns, cholera toxin; hatched columns, pertussis toxin; stippled columns, forskolin. *P* values as in Figure 1.

The significance of this study is the finding that the toxin-induced increase in bradykinin sensitivity is paralleled by an increase in bradykinin receptor number. Based upon our findings that the toxins and forskolin enhanced both bradykinin-stimulated arachidonate release (Figure 1) and phosphatidylinositol turnover (Etscheid & Villereal, 1989), but not serum-stimulated processes, we postulated that the site of action of the toxins and forskolin must be unique to the bradykinin pathway and might in fact be the bradykinin receptor. As shown in Table 1, treatment of HSWP cells with cholera toxin, pertussis toxin or forskolin caused a 3 to 4 fold increase in the number of available cell-surface bradykinin receptors. It is interesting to note that: (1) the increase in receptor number is not matched by a 3 to 4 fold increase in bradykinin-stimulated arachidonic acid release, suggesting that the added receptors might not all be coupled to downstream effectors; and that (2) whereas the enhanced bradykinin responsiveness was induced by forskolin > cholera > toxin > pertussis toxin; the receptor number was increased by pertussus toxin > cholera toxin > forskolin, indicating that the toxins and forskolin might have additional (inhibitory) effects on the bradykinin stimulation pathway.

In other cultured cells, both bradykinin responsiveness and receptor number have been found to increase in response to various stimuli. Recently, transformation of 3T3 cells with Kirsten murine sarcoma virus (Parries *et al.*, 1987), overexpression in Rat-1 cells of normal or transforming forms of Ha-, Ki- and N-*ras* proteins (Downward *et al.*, 1988), and differentiation of PC12 cells with nerve growth factor (Volonte *et al.*, 1988) have been shown to increase both the number of bradykinin receptors and the bradykinin stimulation of inositol phosphate accumulation. The source of the additional bradykinin receptors revealed in these studies is not yet known. Whether transformation, differentiation, or treatment of cells with cyclic AMP-elevating agents alters receptor turnover, increases *de novo* receptor synthesis, or recruits extra receptors from an inaccessible pool is unclear. The prolonged lag time before the rise in bradykinin receptor number (Figure 2a), and its inhibition by cycloheximide and actinomycin D (Figure 2b), give evidence that protein synthesis is required. Once the bradykinin receptor gene is cloned and sequenced, probes will become available to test for increases in transcription from the gene following cyclic AMP treatment of HSWP cells.

Agents that raise cellular cyclic AMP levels have been demonstrated to cause an increase in the receptors for insulin (Thomopoulos *et al.*, 1977), β-melanocyte stimulating hormone (DiPasquale *et al.*, 1977), interleukin-2 (Narumiya *et al.*, 1987), and low-density lipoprotein (Golos & Strauss, 1985). In the latter two cases, transcription of the receptor genes was stimulated by elevated cyclic AMP levels (Narumiya *et al.*, 1987; Golos & Strauss, 1987). Using wildtype PC12 and mutant PC12 A126-1B2 cells lacking a functional cyclic AMP-dependent protein kinase type II activity, Montminy *et al.* (1986) demonstrated that a functional kinase was required

for the cyclic AMP induction of transcription of the somatostatin gene. In the present study, we found that wildtype PC12 cells, but not the mutant PC12 cell line (A126-1B2), showed an increase in bradykinin binding in response to cholera toxin and forskolin (Figure 3), indicating that a kinase might be involved in the increase in bradykinin receptor number following toxin and forskolin treatment.

In conclusion, we have demonstrated that pretreatment of HSWP fibroblasts with cholera toxin, pertussis toxin and forskolin enhanced bradykinin-stimulated arachidonic acid release. Treatment with the toxins or with forskolin also increased the number of cell surface bradykinin receptors, probably via a rise in intracellular cyclic AMP levels and the activation of a cyclic AMP-dependent protein kinase. As a role for kinins has been proposed in many pathophysiological conditions, including carcinoid, post-gastrectomy dumping syndrome, hemorrhagic and endotoxic shock, rheumatoid arthritis, and myocardial infarction, investigations into the modulation of bradykinin receptor level may have widespread clinical significance.

We thank S.K. Ambler and T.L. Steck for their critical reading of the manuscript, and M.J. Brady for providing the normal and mutant PC12 cells. This work was supported by NIH grants GM-28359 and GM-41400 to M.L.V. and a Pharmacological Sciences Training Grant GM-07151 to B.G.E.

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(Received November 26, 1990
Revised January 20, 1991
Accepted January 29, 1991)

Br. J. Pharmacol. (1991), **103**, 1351–1356 © Macmillan Press Ltd, 1991

Characterization of catecholamine-mediated relaxations in rat isolated gastric fundus: evidence for an atypical β-adrenoceptor

[1]Daniel P. McLaughlin & [2]Allan MacDonald

Department of Biological Sciences, Glasgow College, Cowcaddens Road, Glasgow G4 0BA

1 Experiments were carried out in order to characterize the receptors mediating relaxant responses to catecholamines in the rat gastric fundus. The effects of noradrenaline, isoprenaline and the 'atypical' or β_3-adrenoceptor agonist, BRL 37344, on methacholine-induced tone were measured. Prazosin, propranolol and cyanopindolol were used as antagonists.

2 Relaxant responses to noradrenaline, in the presence of propranolol (1 μM) were antagonized in a concentration-dependent manner by prazosin (0.01 to 1 μM), although this antagonism was weak and non-competitive in nature. Relaxant responses to isoprenaline, in the presence of prazosin (0.1 μM), were antagonized only by the highest concentration of propranolol (1 μM) giving a pK_B of 6.3. BRL 37344 also relaxed the rat gastric fundus in the presence of prazosin (0.1 μM), and the responses to BRL 37344 were unaffected by propranolol (1 μM).

3 Tachyphylaxis to BRL 37344 was observed, a second concentration-response curve being significantly shifted to the right. Exposure of tissues to BRL 37344 (1 μM) between concentration-response curves also caused an 11 fold rightward shift in the response to isoprenaline.

4 In the presence of prazosin (0.1 μM) and propranolol (1 μM), the rank order of potency of the agonists was: (−)-isoprenaline (1.0) > (−)-noradrenaline (0.39) > BRL 37344 (0.10).

5 Responses to BRL 37344 in the presence of prazosin (0.1 μM) and propranolol (1 μM) were antagonized by (±)-cyanopindolol (1 μM), with a pK_B of 6.56. Responses to isoprenaline, under the same conditions, were antagonized in a competitive manner by (±)-cyanopindolol (0.1–1 μM), with the slope of a Schild plot close to unity and a pA_2 value of 7.44.

6 The resistance to blockade by prazosin and propranolol and the antagonism by cyanopindolol of the responses mediated by isoprenaline and BRL 37344 suggest that atypical β-adrenoceptors similar to 'atypical' β-adrenoceptors in rat adipocytes and other tissues are present in the rat gastric fundus.

Keywords: Atypical β-adrenoceptors; catecholamines; smooth muscle; gastric fundus

Introduction

It is well known that catecholamines generally produce relaxation of non-sphincteric gastrointestinal smooth muscle and that this effect is mediated by postjunctional α- and β-adrenoceptors (Burnstock & Wong, 1981). However, relaxant responses to catecholamines which are resistant to blockade by α- and β-adrenoceptor antagonists have also been reported in a number of gastrointestinal smooth muscle preparations including rabbit stomach (Bristow *et al.*, 1970), rabbit colon (Gillespie & Khoyi, 1977), rat oesophageal smooth muscle (Buckner & Christopherson, 1974), guinea-pig ileum (Wikberg, 1977; Bond *et al.*, 1986; Bond & Clarke, 1987), dog colon (Grivegnee *et al.*, 1984), rat gastric fundus (Dettmar *et al.*, 1986; McLaughlin & MacDonald, 1990a; Kelly & MacDonald, 1990), rat proximal colon (Croci *et al.*, 1988; Manara *et al.*, 1989; Bianchetti & Manara, 1990), rabbit jejunum (Norman & Leathard, 1990), rat jejunum (Van der Vliet *et al.*, 1990) and rat distal colon (McLaughlin & MacDonald, 1989; 1990b). In the guinea-pig ileum, the order and relative potency of catecholamines is consistent with β-adrenoceptors although responses were resistant to propranolol (Bond & Clarke, 1988). In addition, the receptor mediating propranolol-resistant responses is characterized by the relatively high potency of a novel class of β-adrenoceptor agonists (Arch *et al.*, 1984), e.g. BRL 37344 in guinea-pig ileum (Bond & Clarke, 1988) and rat distal colon (McLaughlin & MacDonald, 1990b) and BRL 35135 in guinea-pig gastric fundus (Coleman *et al.*, 1987).

Atypical β-adrenoceptors also exist in non-gastrointestinal tissue, for example in skeletal muscle (Challis *et al.*, 1988) and in heart (Kaumann, 1989), and resistance of catecholamine-mediated responses to classical β-adrenoceptor antagonists and the high potency of BRL 37344 are also features of the atypical β-adrenoceptors mediating lipolysis in rat adipocytes (Stanton, 1972; Harms *et al.*, 1977). It has been suggested that these adipocyte β-adrenoceptors may represent a separate subclass of β-adrenoceptor, which has been termed the 'β_3-adrenoceptor' (Tan & Curtis-Prior, 1983; Arch, 1989). Recently a human gene has been isolated that encodes a product with similar properties (low affinity for classical β-adrenoceptor antagonists and high potency of BRL 37344) and referred to as the 'β_3-adrenergic receptor' (Emorine *et al.*, 1989).

The aim of the present study was to investigate further the prazosin- and propranolol-resistant responses to catecholamines previously reported in rat gastric fundus (Dettmar *et al.*, 1986; Kelly & MacDonald, 1990) by comparing the potencies of isoprenaline, noradrenaline and BRL 37344. In addition the effects of the β-adrenoceptor antagonist cyanopindolol, which has been reported to be a competitive antagonist at atypical β-adrenoceptors in guinea-pig ileum (Blue *et al.*, 1989), were investigated.

A preliminary account of this work has been presented to the British Pharmacological Society (McLaughlin & MacDonald, 1990a).

Methods

Tissue preparation

Male Wistar rats (250–350 g), fed *ad libitum*, were killed by a blow to the head and cervical dislocation. Longitudinal strips of gastric fundic smooth muscle (15 mm long, 3 mm wide) were dissected by the method of Vane (1957) and were suspended in

[1] Present address: Department of Neuroscience, Institute of Psychiatry, De Crespigny Park, Denmark Hill, London.
[2] Author for correspondence.

organ baths in 30 ml Krebs physiological saline solution (PSS) under an initial isometric tension of 1 g. The Krebs PSS was maintained at 37°C and was bubbled continuously with a mixture of 95% O_2 and 5% CO_2. The composition of the Krebs PSS was as follows (mM): NaCl 118, $CaCl_2$ 2.5, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2 and glucose 11.1. The Krebs solution contained cocaine (3 μM) to block neuronal uptake of noradrenaline and ascorbic acid (30 μM) and EDTA (30 μM) to offset oxidation of the catecholamines. Tissues were allowed to equilibrate for a period of at least 30 min before experimental procedures were begun.

Concentration-response curves to noradrenaline were constructed in the presence of propranolol (1 μM) to allow investigation of the contribution of α-adrenoceptors to responses. Concentration-response curves to isoprenaline were carried out in the presence of prazosin (0.1 μM) to remove any contribution from α_1-adrenoceptors.

Concentration-response curves (CRCs)

The relaxant action of the agonists was determined by measuring the percentage relaxation of methacholine (MCh)-induced tone produced by an agonist. Initially, a MCh CRC was constructed for each tissue, to determine a concentration producing approximately 50% of maximal tone. In most cases, this was between 0.3 and 1 μM. CRCs to agonists were constructed by cumulative addition (0.5 log unit increments) to MCh-contracted strips until a stable response was observed. In antagonist studies of isoprenaline and noradrenaline, four consecutive CRCs were constructed at 1 h intervals; one control CRC followed by three CRCs with increasing concentration of antagonist. Antagonists were added at the end of each CRC, allowing 45 min equilibration time. Some tissues received no antagonist and served as controls to assess time-dependent changes. In antagonist studies with BRL 37344 only one CRC to BRL 37344 could be carried out in any one tissue due to desensitization. In these experiments tissues were exposed to BRL 37344 in the presence and absence of antagonist.

Schild plots

Agonist concentration-ratios (CRs) were determined from EC_{50} values with or without antagonist and were corrected for time-dependent effects by dividing by the apparent shift in tissues in which no antagonist was present.

The plot of log (agonist concentration ratio − 1.0) versus log [antagonist] (Arunlakshana & Schild, 1959) was analysed by linear regression. Antagonism was considered to be simple competitive in nature if the slope of the regression line was not significantly different from unity. In such cases a mean pA_2 value was obtained from individual estimates from the equation:

$$pA_2 = \log(\text{agonist CR} - 1) - \log[\text{antagonist}]$$

after verifying that there was no significant regression of pA_2 on antagonist concentration (MacKay, 1978).

In some experiments only one concentration of antagonist was tested and in these cases the value obtained from the above equation is a pK_B rather than a pA_2 value.

Drugs used

The following drugs were dissolved in distilled water (with the exception of (±)-cyanopindolol which was dissolved in 0.1 M tartaric acid): acetyl-β-methyl choline (methacholine) chloride (Sigma); BRL 37344 (sodium-4-[-2-[2-hydroxy-2-(-3-chlorophenyl) ethylamino] propyl] phenoxyacetate) (Beecham Research Laboratories); cocaine hydrochloride (Thornton and Ross Ltd.); (±)-cyanopindolol (Sandoz); (−)-isoprenaline (+)-bitartrate (Sigma); (−)-noradrenaline (+)-bitartrate (arterenol) (Sigma); prazosin hydrochloride (synthesized by Reckitt and Colman plc); (±)-propranolol hydrochloride (Sigma).

Statistical analysis

Results are expressed as mean ± s.e.mean with the number of observations, n, in parentheses with the exception of regression line slopes which are expressed as slope ± 95% confidence limits. Statistical significance between two data sets was tested by either Student's t test or a paired t test. A probability level of $P < 0.05$ was considered to be statistically significant.

Results

Responses to noradrenaline

In the presence of propranolol (1 μM), noradrenaline relaxed MCh-induced tone in the rat gastric fundus, with a pD_2 of 6.7 ± 0.15 ($n = 8$). Responses to noradrenaline consisted of two phases (Figure 1a). An initial, 'sharp' phase was followed by a secondary partial recovery of the tone. The initial 'sharp' phase of relaxation in response to noradrenaline was prevented by prazosin (0.01 to 0.1 μM, Figures 1b and c). The relaxant responses to noradrenaline were reproducible with time, and no statistically significant time-dependent decrease in the sensitivity of tissues to noradrenaline was observed over the 3 h time period (Figure 2a).

CRCs to noradrenaline were antagonized in a concentration-dependent manner by prazosin (0.01 to 1 μM, Figure 2b). A Schild plot (Figure 2c) revealed that this antagonism did not appear to be simple competitive in nature, the slope of the plot being significantly less than unity (0.53 ± 0.28, 95% C.L., $n = 23$).

Responses to isoprenaline

In the presence of prazosin (0.1 μM), isoprenaline relaxed MCh-tone, with a pD_2 of 7.7 ± 0.21 ($n = 6$). Responses to iso-

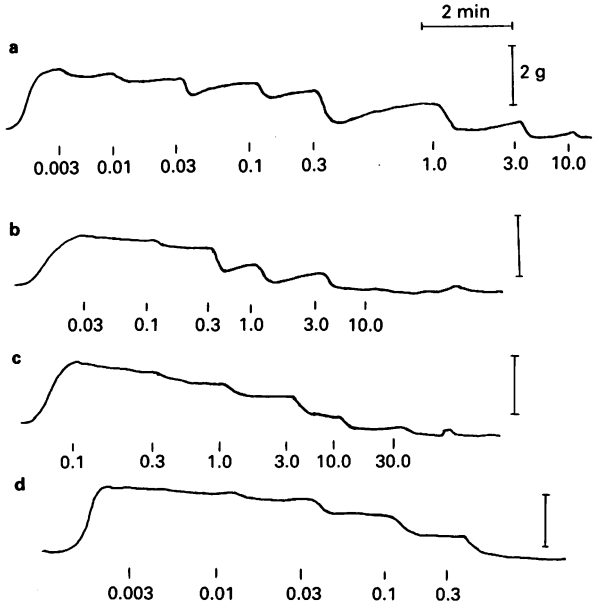

Figure 1 Representative tracings showing relaxations of rat isolated gastric fundus elicited by catecholamines. In each case tone was induced by addition of 1 μM methacholine. Lines indicate addition of catecholamines and values are concentration (μM). (a) Noradrenaline in the presence of propranolol (1 μM); (b) noradrenaline in the presence of propranolol (1 μM) and prazosin (0.01 μM); (c) noradrenaline in the presence of propranolol (1 μM) and prazosin (0.01 μM); (d) isoprenaline in the presence of prazosin (0.1 μM).

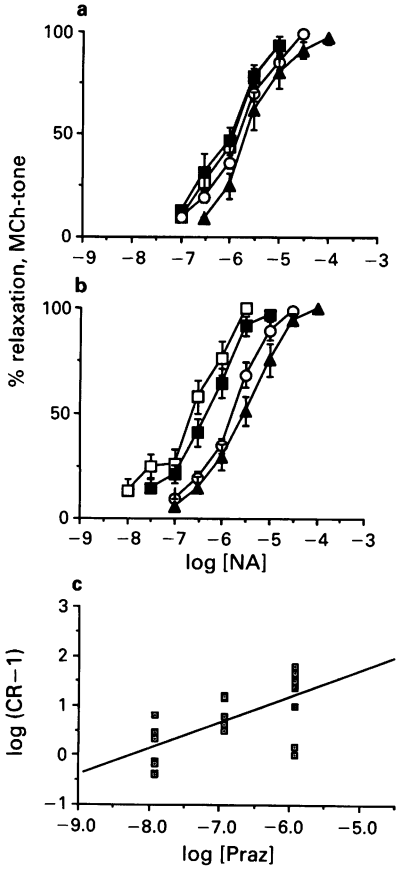

Figure 2 The effect of prazosin on concentration-response curves to noradrenaline in the rat gastric fundus in the presence of propranolol (1 μM). Tone was raised by the addition of methacholine (1 μM) to the organ baths. (a) Time control responses to noradrenaline. First (□, $n = 4$), second (■, $n = 4$), third (○, $n = 4$) and fourth (▲, $n = 4$) concentration-response curves to noradrenaline, constructed at hourly intervals. (b) Effect of prazosin (Praz): (□), controls ($n = 8$); (■), 0.01 μM prazosin ($n = 8$); (○), 0.1 μM prazosin ($n = 8$); (▲), 1 μM prazosin ($n = 8$). (c) The Schild plot of data from (b).

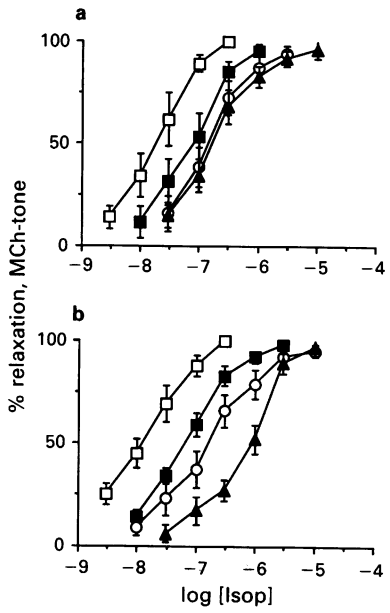

Figure 3 The effect of propranolol on concentration-response curves to isoprenaline (Isop) in the rat gastric fundus in the presence of prazosin (0.1 μM). Tone was raised by the addition of methacholine (1 μM) to the organ baths. (a) Time control responses to isoprenaline. First (□, $n = 4$), second (■, $n = 4$), third (○, $n = 4$) and fouth (▲, $n = 4$) concentration-response curves to isoprenaline, constructed at hourly intervals. (b) Effect of propranolol. (□), controls ($n = 6$); (■), 0.01 μM propranolol ($n = 6$); (○), 0.1 μM propranolol ($n = 6$); (▲), 1 μM propranolol ($n = 6$).

prenaline consisted of a slow relaxation of the tissue with no initial 'sharp' phase of relaxation as seen with noradrenaline (Figure 1d). A time-dependent decrease in the sensitivity of the tissues to isoprenaline (Figure 3a) was taken into consideration when calculating shifts produced by propranolol (see Methods). After correction for time-dependent changes, the shifts produced by propranolol 0.01 and 0.1 μM were insignificant (Figure 3b). Propranolol, 1 μM, did produce a significant shift of the CRC to isoprenaline (Figure 3b, 3.5 fold shift of pD_2 value, $P < 0.05$) giving a pK_B of 6.30 ± 0.14 ($n = 6$).

Responses to BRL 37344

In the presence of prazosin (0.1 μM), BRL 37344 relaxed MCh-induced tone with a pD_2 of 5.22 ± 0.09 ($n = 8$). Propranolol (1 μM) had no effect on the CRC to BRL 37344, the pD_2 of BRL 37344 being 5.30 ± 0.15 ($n = 15$) in the presence of prazosin and propranolol (1 μM).

Tachyphylaxis to BRL 37344 was observed, although this was difficult to quantify, since in second CRCs the highest concentration of BRL 37344 which could be produced in the organ baths (100 μM) often failed to produce even 30% relaxation of the tissue (Figure 4a). In the presence of prazosin (0.1 μM) and propranolol (1 μM), exposure of the tissue to BRL 37344 (1 μM) for a 3 min period between CRCs to isoprenaline produced a significant rightward shift in the CRC to isoprenaline (mean 11 fold shift of pD_2 values, $P < 0.01$, Figure 4b).

Order of potency of agonists in the presence of α- and β-adrenoceptor blockade

In the presence of prazosin (0.1 μM) and propranolol (1 μM), the pD_2 values of isoprenaline, noradrenaline and BRL 37344 were respectively, 6.30 ± 0.17 ($n = 6$), 5.89 ± 0.08 ($n = 8$) and

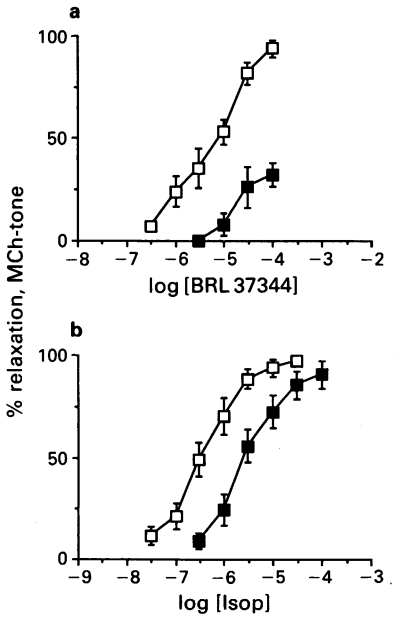

Figure 4 Tachyphylaxis and cross-desensitization to BRL 37344 in the rat gastric fundus in the presence of prazosin (0.1 μM) and propranolol (1 μM). (a) First (□, $n = 5$) and second (■, $n = 5$) concentration-response curves to BRL 37344, constructed at an interval of 1 h. (b) The effect of exposure of portions of gastric fundus to 1 μM BRL 37344 for a 3 min period between concentration-response curves to isoprenaline: mean concentration-response curves to isoprenaline (Isop) before (□, $n = 8$) and after (■, $n = 8$) exposure.

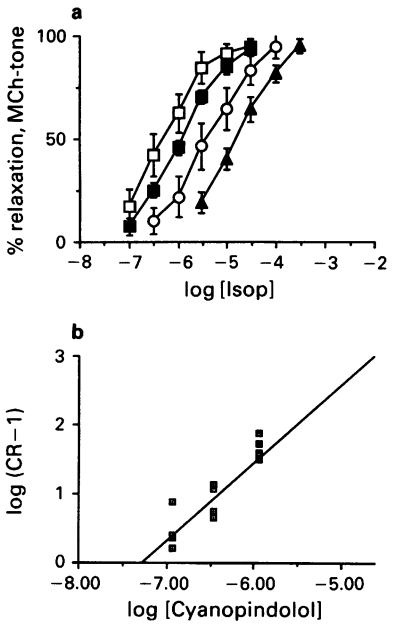

Figure 5 The effect of cyanopindolol (0.1 to 1 μM) on mean concentration-response curves to isoprenaline (Isop) in the rat gastric fundus in the presence of 0.1 μM prazosin and 1 μM propranolol. (a) Mean concentration-response curves to isoprenaline in the absence (□, $n = 4$) and presence of 0.1 μM (■, $n = 4$), 0.3 μM (○, $n = 4$) and 1 μM (▲, $n = 4$) cyanopindolol. (b) Schild plot of the antagonism of isoprenaline action by cyanopindolol.

5.30 ± 0.15 ($n = 15$), giving a rank order of potency of isoprenaline (1.0) > noradrenaline (0.39) > BRL 37344 (0.10).

Effect of cyanopindolol on responses to BRL 37344 and isoprenaline under conditions of α- and β-adrenoceptor blockade

In the presence of prazosin (0.1 μM) and propranolol (1 μM), responses to BRL 37344 were antagonized by cyanopindolol (1 μM) (mean pD_2 values in absence and presence of cyanopindolol in paired tissues: 5.55 ± 0.40 and 4.75 ± 0.25, $n = 5$, $P < 0.05$) giving a pK_B for cyanopindolol against BRL 37344 of 6.56 ± 0.32 ($n = 5$). Also, cyanopindolol (0.1 to 1 μM) antagonized responses to isoprenaline in a concentration-dependent manner (Figure 5a). The antagonism appeared to be simple competitive in nature, since the slope of the Schild plot (Figure 5b) was not significantly different from unity (1.22 ± 0.37, 95% CL, $n = 12$). The pA_2 value for cyanopindolol was 7.44 ± 0.07 ($n = 12$), significantly higher than the pK_B of 6.56 obtained with BRL 37344 as the agonist ($P < 0.001$).

Effect of cyanopindolol on muscle tone

In the experiments described above, cyanopindolol pretreatment had no effect on the degree of tone induced by MCh (tone in cyanopindolol-treated strips as % of control, $n = 4$: 0.1 μM, 123 ± 3; 0.3 μM, 113 ± 6; 1 μM, 97 ± 9).

In separate experiments in which cyanopindolol was added to MCh-contracted strips, higher concentrations than those used for antagonist studies produced some relaxation (% relaxation of MCh-contracted strips by cyanopindolol, $n = 4$: 3 μM, 1 ± 1; 10 μM, 6 ± 4; 30 μM, 21 ± 6; 100 μM, 33 ± 8).

Discussion

In the presence of propranolol to block classical β-adrenoceptors, noradrenaline produced relaxant responses of the rat gastric fundus which were rapid in onset and poorly maintained, a pattern shown previously to occur with α-adrenoceptor-mediated inhibition in rabbit intestine (Bowman & Hall, 1970). The response to noradrenaline was antagonized by prazosin, confirming the presence of postjunctional inhibitory α_1-adrenoceptors reported previously (Verplanken *et al.*, 1984; Dettmar *et al.*, 1986; Kelly & MacDonald, 1990). However, although postjunctional α_2-adrenoceptors are absent from this tissue (MacDonald *et al.*, 1990), the action of noradrenaline could not be attributed wholly to an action at α_1-adrenoceptors since the antagonism by prazosin was not simple competitive, as seen by the low slope of the Schild plot. Verplanken *et al.* (1984) also reported non-competitive antagonism of noradrenaline (but not phenylephrine) by prazosin in the presence of propranolol in rat gastric fundus and suggested that high concentrations of noradrenaline might be overcoming propranolol blockade. This seems unlikely since raising the propranolol concentration from 2 μM to 30 μM failed to affect the prazosin-resistant response (Kelly & MacDonald, 1990). In any case, the previous findings of a prazosin-resistant component of the response to noradrenaline in the presence of propranolol are confirmed (Verplanken *et al.*, 1984; Dettmar *et al.*, 1986; Kelly & MacDonald, 1990).

In the presence of prazosin to block α_1-adrenoceptors, isoprenaline relaxed the fundal strips with a response that was slow in onset and well maintained, characteristic of β-adrenoceptor-mediated inhibition in the gut (Bowman & Hall, 1970). This response was antagonized by propranolol. Lefebvre *et al.* (1984) also reported antagonism of isoprenaline by propranolol and by selective β_1- and β_2-adrenoceptor antagonists. It seems therefore that at least part of the response to isoprenaline in this preparation may be mediated by classical β_1- and β_2-adrenoceptors. However the antagonism produced by propranolol was weak, with a significant shift of the CRC occurring only with the highest concentration (1 μM). The pK_B of 6.3, calculated from the single (1 μM) concentration of propranolol, is low compared with 8.2–8.8 for classical β_1-adrenoceptors mediating atrial stimulation and 8.3–8.6 for β_2-adrenoceptors mediating tracheal relaxation (Wilson *et al.*, 1984) suggesting an action at atypical β-adrenoceptors. A similarly low pK_B was reported for propranolol at atypical β-adrenoceptors mediating lipolysis (6.6, Wilson *et al.*, 1984) or relaxation in rat distal colon (6.57, McLaughlin & MacDonald, 1990b).

BRL 37344, a selective agonist at β_3-adrenoceptors in rat adipocytes (Arch *et al.*, 1984), also produced relaxation which was resistant to antagonism by propranolol, suggesting similarities between the receptor mediating relaxation in the rat gastric fundus and that mediating lipolysis.

BRL 37344 caused tachyphylaxis to itself and to isoprenaline. Tachyphylaxis was previously reported in guinea-pig gastric fundus with BRL 35135, the methyl ester of BRL 37344, (Coleman *et al.*, 1987) and with BRL 37344 in rat distal colon (McLaughlin & MacDonald, 1990b). The tachyphylaxis does not appear to be a non-specific effect since it has been shown previously in rat distal colon that relaxant responses to papaverine were unaffected (McLaughlin & MacDonald, 1990b). The significance of the tachyphylaxis is unclear since it has not been reported to occur in studies of lipolysis.

The relaxant effects of BRL 37344 and isoprenaline in the presence of prazosin and propranolol were antagonized by cyanopindolol. A Schild regression was not carried out for BRL 37344 but the antagonism of isoprenaline by cyanopindolol was consistent with competitive antagonism with the Schild slope close to unity. The mean pA_2 value of 7.44 is similar to the value of 7.63 reported by Blue *et al.* (1989) for cyanopindolol in guinea-pig ileum and to the value of 7.12 in rat distal colon (McLaughlin & MacDonald, 1990b). Thus the receptor mediating responses to isoprenaline in rat gastric fundus appears similar to the atypical β-adrenoceptor in guinea-pig ileum and rat distal colon. The pK_B calculated from the single concentration of cyanopindolol against BRL 37344 (6.56) was significantly lower than the pA_2 value obtained with isoprenaline as agonist (7.44). Lower pA_2 values

for antagonists against BRL compounds have previously been shown (Stock & Sudera, 1988; Jones *et al.*, 1989; Arch, 1989; McLaughlin & MacDonald, 1990b) and attributed to allosteric modification of the binding site by BRL compounds, resulting in lowered antagonist affinity (Arch, 1989).

Cyanopindolol itself produced relaxation of the rat gastric fundus at high concentrations. Some β-adrenoceptor antagonists including pindolol analogues, have previously been shown to act as partial agonists in rat adipocytes (Engel *et al.*, 1981) and at atypical β-adrenoceptors in the heart (Walter *et al.*, 1984; Kaumann, 1989). In the heart the agonism occurred at concentrations higher than required for blockade of conventional β_1- and β_2-adrenoceptors, suggesting an action at a third site, possibly the atypical β-adrenoceptor. However, in the present study the effect of cyanopindolol was only obtained at concentrations considerably higher than those producing blockade of the propranolol-resistant responses. As partial agonists should produce agonist effects at the same concentrations as blockade if the same receptor is involved (Kenakin, 1987), this would suggest that the partial agonist activity of cyanopindolol is at a receptor other than the atypical β-adrenoceptor. On the other hand, pindolol also acted as a partial agonist of adenosine 3':5'-cyclic monophosphate (cyclic AMP) accumulation in Chinese hamster ovary cells containing no β_1- or β_2-adrenoceptors and transfected with the human gene encoding the 'β_3-adrenergic receptor' and this action occurred at concentrations approximately 14 times higher than the dissociation constant at the β_3-site obtained from receptor binding (Emorine *et al.*, 1989). Thus there may be a significant threshold effect involved in the response (over 50% receptor occupancy required before a response is observed) (Kenakin, 1987). It is therefore possible that the agonist effect of high concentrations of cyanopindolol observed in the present study could be due to a similar phenomenon and is at atypical β-adrenoceptors.

The relative potency of BRL 37344 under conditions of α- and β-adrenoceptor blockade, isoprenaline (1.0) > noradrenaline (0.39) > BRL 37344 (0.10), is less than that reported at rat adipocyte β_3-adrenoceptors (5 fold more potent than isoprenaline, Arch *et al.*, 1984) or at atypical β-adrenoceptors in guinea-pig ileum (2.5 fold more potent than isoprenaline, Bond & Clarke, 1988) or rat distal colon (equipotent with isoprenaline, McLaughlin & MacDonald, 1990b). This may be due to tissue factors, such as efficiency of receptor coupling, which can affect agonist potency ratios (Kenakin, 1987). Alternatively the atypical β-adrenoceptor in rat gastric fundus may not be identical with atypical β-adrenoceptors reported elsewhere. There is ample evidence for differences in sites labelled as atypical e.g. alprenolol antagonized responses to isoprenaline at atypical β-adrenoceptors in rat proximal colon with a pA_2 value of 7.6 (Bianchetti & Manara, 1990), was more than an order of magnitude less potent (pA_2 6.5) at atypical β-adrenoceptors in guinea-pig ileum (Blue *et al.*, 1990) and failed to antagonize cyclic AMP accumulation by isoprenaline in Chinese hamster ovary cells transfected with the 'β_3-adrenergic receptor' gene (Emorine *et al.*, 1989). Further examples of discrepancies (Zaagsma & Nahorski, 1990) suggest that the atypical β-adrenoceptor may represent a class of receptor rather than a single receptor subtype. An interesting proposal by Clarke and co-workers (Blue *et al.*, 1990) is that the atypical β-adrenoeptor may represent a population of β_1-type receptor sites located in a different domain of the receptor molecule. These authors propose that such a site would not differ from the β_1-site in recognition of small agonist molecules (noradrenaline, adrenaline, isoprenaline) binding only to the receptor site, but large molecules with additional binding sites such as antagonists and synthetic agonists would have different affinities for the two sites due to differences in the domain of the receptor site. Further pharmacological characterization of atypical β-adrenoceptors in different locations is required.

In conclusion, the resistance of β-adrenoceptor relaxant responses to propranolol and the antagonism of BRL 37344 and isoprenaline by cyanopindolol suggest the presence of atypical β-adrenoceptors in rat gastric fundus. The relatively low potency of BRL 37344 at this site compared with other atypical β-adrenoceptors may be due to differences in coupling efficiency or may indicate receptor heterogeneity.

BRL 37344 was kindly supplied by Dr M.A. Cawthorne, Beecham Pharmaceuticals Research Division, Great Burgh, Epsom.

D.P.McL. was a Glasgow College Research Student. The authors are also grateful to the College for financial support.

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(Received January 9, 1991
Revised January 23, 1991
Accepted January 30, 1991)

Br. J. Pharmacol. (1991), **103**, 1357–1362 © Macmillan Press Ltd, 1991

Selective impairment of hindquarters vasodilator responses to bradykinin in conscious Wistar rats with streptozotocin-induced diabetes mellitus

R.J. Kiff, [1]S.M. Gardiner, A.M. Compton & T. Bennett

Department of Physiology and Pharmacology, Medical School, Queen's Medical Centre, Nottingham NG7 2UH

1 Male, Wistar rats were treated with streptozotocin (STZ, 70 mg kg^{-1}, i.p.) or saline and chronically instrumented with pulsed Doppler probes and intravascular catheters (implanted under sodium methohexitone anaesthesia) to allow assessment of haemodynamics in the conscious state 28 days later.

2 Control and STZ-treated rats received bolus doses of glyceryl trinitrate (10–80 nmol kg^{-1}), acetylcholine (0.1–5 nmol kg^{-1}) and bradykinin (0.3–30 nmol kg^{-1}).

3 Although, as reported previously, STZ-treated rats had normal mean arterial blood pressure together with renal and mesenteric vasodilatations and hindquarters vasoconstriction relative to control rats, both groups showed similar hypotensive and regional haemodynamic responses to glyceryl trinitrate and acetylcholine. However, while the depressor effects of bradykinin were similar in control and STZ-treated rats, the former showed a hindquarters vasodilator response to bradykinin that was absent in the STZ-treated rats.

4 A loss of bradykinin-mediated vasodilatation in the hindquarters vascular bed in STZ-treated rats in the presence of normal, hindquarters vasodilator responses to other agents and normal bradykinin-mediated vasodilator responses in other vascular beds is consistent with existing evidence that the vasodilatation elicited by bradykinin in the hindquarters vascular bed is particularly dependent on nitric oxide synthesis and that this is impaired selectively in STZ-treated rats.

Keywords: Bradykinin; glyceryl trinitrate; acetylcholine; haemodynamics; streptozotocin-induced diabetes

Introduction

There is evidence for endothelial cell dysfunction in clinical diabetes mellitus (Porta *et al.*, 1987; Stout, 1987) and it is, therefore, possible that this abnormality contributes to the several cardiovascular complications that can occur in people with this disease (Porta *et al.*, 1987; Stout, 1987). Growing awareness of the importance of endothelial cell integrity for the maintenance of normal cardiovascular homeostasis (Kaiser & Sparks, 1987; Gryglewski *et al.*, 1988; Moncada *et al.*, 1989) has led to a series of studies concerned with the possibility that endothelium-dependent vasorelaxation is abnormal in diabetes mellitus (e.g. Oyama *et al.*, 1986; Bhardwaj & Moore, 1988; Altan *et al.*, 1989). While the majority of these investigations has been concerned with *in vitro* vasorelaxation of tissues obtained from rats with streptozotocin (STZ)-induced diabetes mellitus, the results are not consistent. For example, aortic rings isolated from rats with STZ-induced diabetes mellitus, when precontracted with noradrenaline, have been found to show reduced relaxation to acetylcholine, ADP, histamine and the calcium ionophore, A23187, but not to sodium nitroprusside, glyceryl trinitrate, papaverine or atrial natriuretic peptide (Oyama *et al.*, 1986; Pieper & Gross, 1988; Kamata *et al.*, 1989). These findings indicate selective impairment of endothelium-dependent vasorelaxation. However, in other studies, also concerned with acetylcholine-induced relaxations on aortic rings precontracted with noradrenaline, responses of tissues from STZ-treated rats have been found to be normal (Head *et al.*, 1987; Wakabayashi *et al.*, 1987; Harris & MacLeod, 1988; Mulhern & Docherty, 1989). There are no systematic differences between these two groups of studies regarding dose of STZ or duration of diabetes mellitus.

It is feasible that the disparity between these results is due to the *in vitro* experimental conditions. Thus, none of the investigations cited allowed for the marked differences in composition of extracellular fluid in STZ-treated and control rats (Hebden *et al.*, 1986). In this context it is notable that *in vivo* studies have shown impaired renal vasodilator responses to acetylcholine and bradykinin (Ha & Dunham, 1987) and diminished cerebral arteriolar vasodilator responses to adenosine 5'-diphosphate (ADP) and 5-hydroxytryptamine (Mayhan, 1989) in STZ-treated rats. However, Ha & Dunham (1987) found that the renal vasodilator response to sodium nitroprusside was also impaired in STZ-treated rats, whereas Mayhan (1989) reported a normal cerebral vasodilator response to glyceryl trinitrate in rats with STZ-induced diabetes mellitus. It is noteworthy that both these investigations were performed in anaesthetized animals.

Recently, we found that conscious, Wistar rats with STZ-induced diabetes mellitus had resting renal and mesenteric vasodilatations and hindquarters vasoconstriction, relative to control rats (Kiff *et al.*, 1991). Furthermore, there appeared to be a selective impairment of the hindquarters vasoconstrictor response to N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide production from L-arginine (Moore *et al.*, 1990; Rees *et al.*, 1990), consistent with the resting hindquarters vasoconstriction being due to reduced production of, and/or sensitivity to, endothelium-derived nitric oxide. In the present work our objective was to investigate the possibility that administration of a range of vasodilator agents might reveal additional abnormalities in STZ-treated rats. Therefore, we extended our previous observations by comparing the depressor and the regional vasodilator responses to glyceryl trinitrate, acetylcholine and bradykinin in control and STZ-treated Wistar rats. These vasodilators were chosen because they have differential regional haemodynamic actions and we have background information on the influence of L-NAME on their effects in normal rats (Gardiner *et al.*, 1990b).

Methods

Male Wistar rats (3–4 months old) were injected with STZ (70 mg kg^{-1}) dissolved in 0.9% NaCl ($n = 22$) or with saline

[1] Author for correspondence.

alone ($n = 9$). Rats that had blood glucose levels of greater than 12 mmol l^{-1}, measured on a tail vein sample with a Reflomat (Boehringer, Mannheim) 4 days after STZ injection, were considered to have diabetes mellitus and were entered into the experiment. Fourteen days later rats were anaesthetized (sodium methohexitone 40–60 mg kg^{-1}, i.p., supplemented as required) and pulsed Doppler flow probes (Haywood *et al.*, 1981) were sutured around the left renal and superior mesenteric arteries and the distal abdominal aorta (below the ileocaecal artery to measure hindquarters flow). Following surgery, the rats were kept in their own cages. After a further 14 days the animals were re-anaesthetized (sodium methohexitone, 40 mg kg^{-1} i.p., supplemented as required). Venous catheters were implanted into the right jugular vein and an intra-arterial catheter was placed in the distal abdominal aorta (via the ventral caudal artery). The animals were allowed to recover and were housed individually in their home cages. The following day the animals were connected to a pressure transducer and a pulsed Doppler flow-meter (Crystal Biotech, VF-1, Holliston, USA) for continuous recordings of heart rate, blood pressure and Doppler shift, which was taken as an index of flow (Haywood *et al.*, 1981). The Doppler flow-meter was modified to operate with a pulse repetition frequency of 125 kHz and was fitted with high velocity modules to avoid the problem of signal aliasing (Gardiner *et al.*, 1990a). Vascular conductances were calculated by dividing the mean Doppler shift values by the mean blood pressure (Gardiner *et al.*, 1990b) and were expressed as arbitrary units.

After a 30 min baseline recording period, incremental bolus doses of glyceryl trinitrate (10–80 nmol kg^{-1}), acetylcholine (0.1–5 nmol kg^{-1}) and bradykinin (0.3–30 nmol kg^{-1}) were given in random order, with at least 10 min between each dose.

Blood glucose levels were determined at the end of the experimental period. All the STZ-treated rats had blood glucose levels in excess of 20 mmol l^{-1} (i.e. the upper limit of the Reflomat).

Data analysis

All agents used caused dose-dependent hypotension, but they influenced regional blood flows differently. Thus, glyceryl trinitrate caused marked mesenteric hyperaemia with little change in renal and hindquarters blood flow; acetylcholine increased renal blood flow but not mesenteric or hindquarters blood flows whereas bradykinin increased all blood flows (in control rats), with the most marked effect on the mesenteric vascular bed. Hence, we elected to compare the results in control and STZ-treated rats by considering the nadirs in mean arterial blood pressure and the peaks in regional vascular conductances, irrespective of when these occurred. However, in both groups these events generally coincided, with the notable exception that the maximum rise in mesenteric blood flow and vascular conductance following administration of glyceryl trinitrate occurred when mean arterial blood pressure had returned to baseline.

Results were analyzed by Wilcoxon's ranks sums test in the Mann-Whitney U test for paired and unpaired data, respectively. $P < 0.05$ was taken as significant.

Drugs and peptides

Acetylcholine chloride (Sigma), bradykinin (Bachem U.K.), glyceryl trinitrate (Tridil, DuPont, U.K.) and streptozotocin (Sigma) were all dissolved (or diluted in the case of glyceryl trinitrate) in isotonic saline. For bradykinin, the saline contained 1% bovine serum albumin (Sigma). Steptozotocin was made up immediately before use.

All i.v. injections were given in 0.1 ml aliquots and flushed in with 0.15 ml isotonic saline. The volume of saline alone had no consistent cardiovascular effects.

Results

Changes in mean arterial blood pressure

Glyceryl trinitrate, acetylcholine and bradykinin all caused dose-dependent reductions in mean arterial blood pressure (Figure 1). There were no significant differences between the depressor responses to each of these agents in control and STZ-treated rats (Figure 1).

Changes in renal vascular conductance

Although there was a resting renal vasodilatation in STZ-treated rats relative to the control animals (Figure 2), both control and experimental groups showed vasodilator

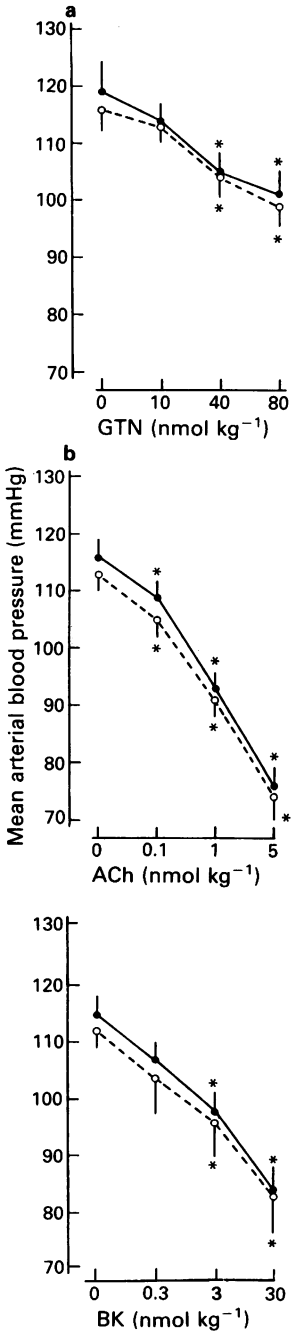

Figure 1 Resting values and maximum changes in mean arterial blood pressure in response to bolus doses of (a) glyceryl trinitrate (GTN), (b) acetylcholine (ACh) and (c) bradykinin (BK) in control (●, $n = 9$) and in streptozotocin-treated (○, $n = 11$), Wistar rats. * $P < 0.05$ versus baseline.

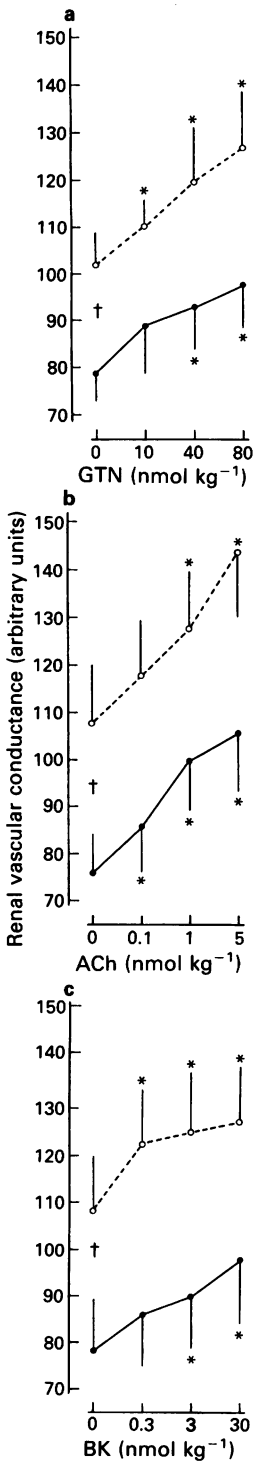

Figure 2 Resting values and maximum changes in renal vascular conductance in response to bolus doses of (a) glyceryl trinitrate (GTN), (b) acetylcholine (ACh) and (c) bradykinin (BK) in control (●, $n = 9$) and in streptozotocin (STZ)-treated (○, $n = 11$), Wistar rats. * $P < 0.05$ versus baseline; † $P < 0.05$ control versus STZ-treated (resting values).

responses to glyceryl trinitrate, acetylcholine and bradykinin. The responses in the two groups were not significantly different, except for the response to bradykinin which reached a maximum in the STZ-treated group but not in the control group (Figure 2).

Changes in mesenteric vascular conductance

There was a difference in mesenteric vascular conductance between control and STZ-treated rats that persisted in the presence of the vasodilators (Figure 3). Glyceryl trinitrate, acetylcholine and bradykinin elicited similar mesenteric vasodilatations in control and STZ-treated rats (Figure 3), although the mesenteric vasodilator response to bradykinin came to a plateau at the higher doses in STZ-treated rats (Figure 3).

Changes in hindquarters vascular conductance

There was a resting hindquarters vasoconstriction in the STZ-treated rats relative to the control animals (Figure 4). In

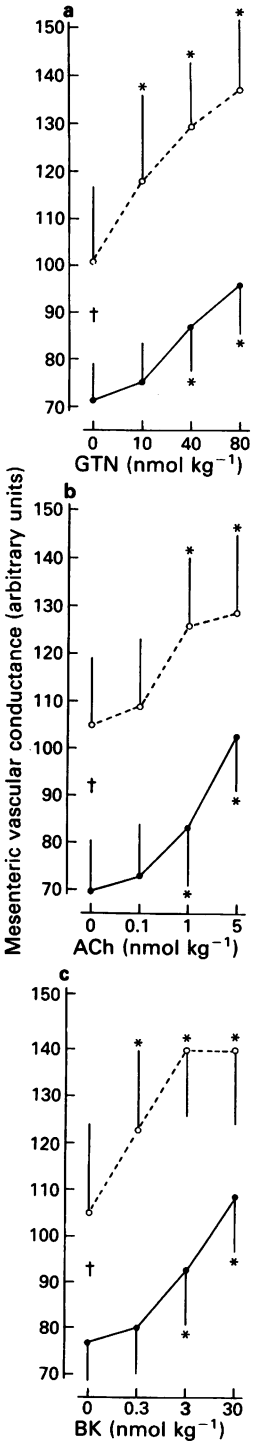

Figure 3 Resting values and maximum changes in mesenteric vascular conductance in response to bolus doses of (a) glyceryl trinitrate (GTN), (b) acetylcholine (ACh) and (c) bradykinin (BK) in control (●, $n = 9$) and in streptozotocin (STZ)-treated (○, $n = 11$), Wistar rats. * $P < 0.05$ versus baseline; † $P < 0.05$ control versus STZ-treated (resting values).

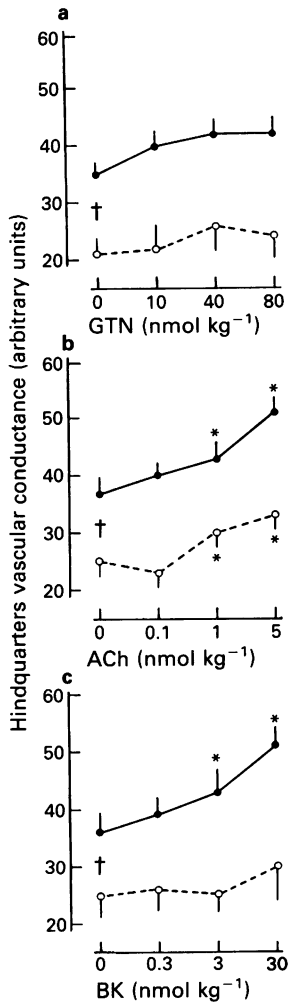

Figure 4 Resting values and maximum changes in hindquarters vascular conductance in response to bolus doses of (a) glyceryl trinitrate (GTN), (b) acetylcholine (ACh) and (c) bradykinin (BK) in control (●, $n = 9$) and in streptozotocin (STZ)-treated (○, $n = 11$), Wistar rats. * $P < 0.05$ versus baseline; † $P < 0.05$ control versus STZ-treated (resting values).

neither group did glyceryl trinitrate cause a significant hindquarters vasodilatation (Figure 4), whereas there was a small vasodilator response to acetylcholine that was similar in control and STZ-treated rats (Figure 4). However, while control rats showed a significant hindquarters vasodilator response to bradykinin, this was absent in the STZ-treated rats (Figure 4).

Discussion

The present work has shown that, although STZ-treated rats may have apparently normal hypotensive responses to glyceryl trinitrate, acetylcholine and bradykinin, there can be very specific abnormalities in the underlying haemodynamic changes. There were increases in renal vascular conductance in control and in STZ-treated rats in response to glyceryl trinitrate, acetylcholine and bradykinin, and in each case the responses in the two groups were not different (although the response to bradykinin in the STZ-treated group reached a plateau whereas it did not in the control rats). In contrast, Bhardwaj & Moore (1988) found that perfused kidneys from STZ-treated rats showed enhanced vasodilator responses to acetylcholine, whereas the vasodilator responses to sodium nitroprusside were the same as in control animals. However, these experiments were carried out in the presence of indomethacin and noradrenaline and so are difficult to compare to the present observations. Moreover, it is notable that Bhardwaj & Moore (1988) found no evidence of the resting renal vasodilatation observed here in conscious STZ-treated rats. However, their studies were performed only 12 days after STZ treatment, whereas our animals had been treated with STZ 28 days before measurements were made. Hence, it is possible that renal hyperaemia is a relatively late development following STZ-treatment. If renal hyperperfusion is associated with renal hypertrophy (Christiansen *et al.*, 1981) this would be consistent with the observation of Bhardwaj & Moore (1988) that kidney weight was not different in the control and STZ-treated animals they studied (i.e. there was no evidence of renal hypertrophy), although there is evidence for renal hypertrophy occurring as early as 2 days after induction of experimental diabetes mellitus (Steer *et al.*, 1985).

Ha & Dunham (1987) studied animals 4 weeks after STZ treatment and made measurements of renal blood flow *in vivo*. They found a significant reduction in resting renal blood flow and vascular conductance in STZ-treated rats but they made these measurements under pentobarbitone anaesthesia. Thus, their results cannot be extrapolated to the conscious state, particularly since it is evident that barbiturate anaesthesia abolishes the polyuria and high electrolyte excretion seen in conscious, STZ-treated rats (Musabayane, 1990). In addition, we have observed that renal blood flow in control and, particularly, in STZ-treated rats under barbiturate (sodium methohexitone) anaesthesia is substantially less than in the same animals when they are conscious (unpublished observations). The use of anaesthesia in the experiments of Ha & Dunham (1987) might account also for their observations of reduced renal vasodilator responses to acetylcholine, bradykinin and sodium nitroprusside in STZ-treated rats, since in the present experiments in conscious, STZ-treated animals, all renal vasodilator responses were normal. While there is discussion about the extent to which 'endothelium-dependent' vasodilatation in response to acetylcholine and bradykinin *in vivo* involves nitric oxide (Gardiner *et al.*, 1990b), the present findings of normal renal vasodilator responses to acetylcholine and to bradykinin in conscious, STZ-treated rats is consistent with the normal renal vasoconstrictor responses to L-NAME seen in these animals (Kiff *et al.*, 1991).

In the mesenteric vascular bed there was no difference between the vasodilator responses to glyceryl trinitrate, to acetylcholine, or to bradykinin in the control and STZ-treated rats. However, the vasodilator responses to bradykinin reached a maximum with the higher doses of the peptide in the STZ-treated rats whereas no plateau was achieved in control animals, but this could be accounted for entirely by the elevation in resting mesenteric vascular conductance in the former group (Kiff *et al.*, 1991).

The hindquarters was the only vascular bed that showed evidence of impaired vasodilator responses in STZ-treated rats. Moreover, this defect was apparent only in response to bradykinin, with no difference in the hindquarters vasodilator responses to acetylcholine, or to endothelin-1 (Kiff *et al.*, 1991), and with neither control nor STZ-treated rats showing hindquarters vasodilatation to glyceryl trinitrate. These observations raise several questions. Why should there be a selective reduction only of the bradykinin-induced hindquarters vasodilator response in STZ-treated rats? Our previous findings of a resting hindquarters vasoconstriction and diminished vasoconstrictor response to L-NAME in STZ-treated rats led us to propose that there might be a localized impairment in the production of, and/or sensitivity to, nitric oxide in the hindquarters vascular bed (Kiff *et al.*, 1991), possibly due to local interference by L-glutamine of the synthesis of nitric oxide from L-arginine (Swierkosz *et al.*, 1990). The present findings of a selective diminution of the bradykinin-induced hindquarters vasodilatation in STZ-treated rats is consistent with the observation that, under normal conditions, this response is particularly sensitive to L-NAME (Gardiner *et al.*, 1990b), and hence may be more dependent on synthesis of nitric oxide than the responses to other vasodilators or the responses to bradykinin in other vascular beds (Gardiner *et al.*, 1990b). An observation that may be relevant to the latter

proposal is that vasodilator responses to bradykinin may involve more than one mechanism (Cowan & Cohen, 1990), and, hence, the extent of their involvements may vary in different vascular beds. The finding that hindquarters vasodilator responses to acetylcholine (this paper) and to endothelin-1 (Kiff *et al.*, 1991) were intact when bradykinin-mediated responses were impaired in STZ-treated rats is consistent with the relative resistance of the former responses to inhibition by L-NAME (Gardiner *et al.*, 1990b). Elsewhere (Gardiner *et al.*, 1990b) we have discussed the possibility that this is due to release of nitric oxide from a pre-formed pool and/or the involvement of nitric oxide-independent mechanisms (Aisaka *et al.*, 1989; Long & Berkowitz, 1989).

Another important question that arises from the present results is: what is the explanation for the lack of hindquarters vasodilator response to glyceryl trinitrate in control and in STZ-treated rats? Although this phenomenon might relate to dose and experimental protocol, it was clear from the experiments reported here that glyceryl trinitrate caused marked mesenteric hyperaemia and vasodilatation when hindquarters blood flow and vascular conductance were unchanged. There is evidence that the regional haemodynamic effects of glyceryl trinitrate are influenced by the reflex responses to the reductions in preload and afterload it causes (Vatner *et al.*, 1978), but it is notable that administration of a dose of endothelin-1 (Kiff *et al.*, 1991) that had similar hypotensive effects to glyceryl trinitrate in the present experiments did elicit marked hindquarters vasodilatation. Thus, it is possible that the differential regional vasodilator effects of glyceryl trinitrate relate more to differences in local biotransformation of the drug (Kawamoto *et al.*, 1990).

The regionally selective and agonist specific, vasodilator abnormalities noted in the present work highlight the problems associated with *in vitro* studies of aortic ring preparations when these are used as models of generalized endothelial dysfunction in diabetes mellitus (see Introduction). However, considering *in vitro* findings showing aortic endothelial cell abnormalities in STZ-treated rats, and the present results indicating normal vasodilator responses in renal and superior mesenteric vascular beds together with selective impairment of bradykinin-mediated vasodilatation only in the hindquarters vascular bed, it would be of interest to compare aortic compliance (Cruickshank *et al.*, 1991) in control and STZ-treated rats to determine if this is a more sensitive *in vivo* correlate with the *in vitro* findings.

This work was supported by a grant from Wellcome Research Laboratories.

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(Received December 14, 1990
Revised January 29, 1991
Accepted January 30, 1991)

Br. J. Pharmacol. (1991), **103**, 1363–1366 © Macmillan Press Ltd, 1991

The effects of thromboxane receptor antagonists on oestrogen-induced uterotrophic responses in the spontaneously hypertensive rat

M.B. Kerr, K. Marshall & [1]J. Senior

Postgraduate Studies in Pharmacology, School of Pharmacy, University of Bradford, BD7 1DP

1 The possible role of thromboxane in the uterotrophic response to oestrogen, in the spontaneously hypertensive rat was investigated by use of the thromboxane receptor antagonists EP092, AH23848 and BM 13.505.

2 The parameters studied were uterine blood flow (measured by the microsphere technique), uterine wet and dry weights and the concentrations of cytosolic and nuclear oestrogen receptors.

3 The antagonists attenuated oestradiol-induced uterine blood flow and significantly reduced both wet and dry uterine weight. These changes were accompanied by decreases in nuclear oestrogen receptor levels.

4 The results suggest a supportive role for thromboxane in oestradiol-induced uterine growth.

Keywords: Uterus; hypertensive; thromboxane; oestrogen; thromboxane receptor antagonists; spontaneously hypertensive rat; oestrogen receptor

Introduction

The spontaneously hypertensive rat (SHR) is an established strain that develops hypertension several months after birth (Okamoto, 1969). The SHR is considered to be the most acceptable experimental model thus far developed of essential hypertension since neither drug treatment nor surgical intervention are required to induce a chronic hypertensive state. The exact aetiology of the hypertension is unknown but it probably involves a balance of cardiac and peripheral factors. Increased vascular resistance has been attributed to a plethora of factors: encroachment into the vessel lumen caused by arterial wall hypertrophy (Folkow *et al.*, 1973); augmented humoral sensitivity (Lais *et al.*, 1974); sympathetic nerve hyperactivity (Bohlen, 1979) and arterial rarefaction (Hutchins, 1979).

In this work the role of thromboxane, the production of which is most commonly associated with potent platelet aggregation and induction of vascular and extra-vascular smooth muscle contraction, in the uterotrophic response to oestrogen in the non-pregnant SHR is investigated with the view to extending this study to the pregnant animal. The contribution made by thromboxane was assessed by the use of known thromboxane receptor blocking drugs namely: EP092 (Armstrong *et al.*, 1984); AH23848 (Brittain *et al.*, 1985) and BM 13.505 (Stegmeier *et al.*, 1986). Comparisons are made to earlier results from this laboratory using the normotensive CD.

Methods

Mature, virgin, female spontaneously hypertensive rats, SHR strain (BP > 180 mmHg) from the animal house, University of Bradford, were used throughout and were housed in light (07 h 00 min–19 h 00 min) and temperature (18°C) controlled rooms, food and water were available *ad libitum*. All the animals used in this study were bilaterally ovariectomized at least 21 days before further experimentation and were randomly assigned to groups. All the animals used were of similar body weight (around 240 g); there was no significant difference between the weights within the groups or between groups.

[1] Author for correspondence.

Measurement of blood flow

The microsphere technique (previously described by Phaily & Senior, 1978a) was used to determine blood flow in the rat anaesthetized with sodium pentobarbitone (60 mg kg^{-1}). This dose was selected to ensure anaesthetic control throughout the experimental procedure and minimal cardiovascular changes. The microspheres used were 15 μm in diameter (NEN-Trac, New England Nuclear, Boston, MA, U.S.A.), uniformly labelled with Sc^{46} and suspended in 10% (w/v) dextran containing 0.1% (w/v) Tween 80. Uterine blood flows were calculated from wet weight of tissue. In all cases blood flow between the adrenals and kidneys was balanced, indicating a uniform distribution of microspheres. Cardiac output was also unaffected by the treatments. When dry weights are quoted the tissue was dried until the weight remained constant.

Measurement of cytoplasmic and nuclear oestrogen receptors by [^{3}H]-oestradiol exchange

Homogenization, cytosol and nuclear pellet preparation and determination of oestrogen receptors therein was carried out by the method of Marshall & Senior (1987). Briefly, animals were killed by cervical dislocation and the uteri were rapidly removed and homogenized in PE buffer (10 mM phosphate buffer, pH 7.4, containing 1.5 mM EDTA and 2 mM mercaptoethanol). All procedures were performed at 4°C unless otherwise stated. The exchange conditions employed were found to be optimal with respect to maximal exchange with minimal loss of binding sites. Homogenates were centrifuged at 100,000*g* for 1 h. Aliquots of cytosol were incubated at 30°C for 1 h with 1–20 nM of [^{3}H]-oestradiol ([2,4,6,7-^{3}H]-oestradiol, 86–110 Ci mmol^{-1}) (Radiochemical Centre, Amersham, Bucks) plus or minus 100 fold excess of oestradiol. Separation of bound from free ligand was achieved by the use of dextran-coated charcoal (0.5% dextran (BDH, Poole), 5% Norit A acid washed charcoal w/v in phosphate buffer). The radioactivity in the supernatant was determined by liquid scintillation counting. The assay of nuclear oestrogen receptors involved the preparation of a low speed nuclear pellet (prepared by centrifugation at 800*g* of homogenate). Aliquots of the nuclear suspension were treated in the same way as in the cytosolic assay. Separation was achieved with a 60% hydroxylapatite suspension in phosphate buffer. After ethanolic extraction of the pellet, radioactivity was determined by liquid scintillation counting (45% efficiency).

Table 1 The effect of EP092 (10 mg kg^{-1} i.v., 2 doses) given 10 min before and 1 h 30 min after oestradiol, AH23848 (2.5 mg kg^{-1} i.v., single dose) and BM 13.505 (20 mg kg^{-1} i.v., single dose) given 10 min before oestradiol on uterine blood flow and weight in the ovariectomised SHR

Pretreatment	No. of rats	Uterine blood flow (ml min^{-1} 100 g^{-1})	Uterine weight (mg) Wet	Dry
None (control)	6	15 ± 5	83 ± 6	17 ± 1
Oestradiol alone	6	277 ± 50***	111 ± 9*	25 ± 1**
EP092	6	150 ± 40*	56 ± 3†††**	11 ± 1†††
AH23848	6	160 ± 11†***	92 ± 4	19 ± 0.3†††
BM 13.505	6	148 ± 47*	82 ± 7†	16 ± 3†

Measurements were made 3 h after oestradiol (0.5 μg kg^{-1} i.v., single dose) administration.
Values are mean ± s.e.mean: * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$ values are significantly different from those of control group; † $P < 0.05$, ††† $P < 0.001$ values are significantly different from those of oestradiol alone group.

In this work the term cytoplasmic receptor is used: this refers to the receptor form that separates into the cytoplasmic cell fraction after homogenisation. It represents the unoccupied inactive form of the receptor thought to be located in the nucleus *in vivo* (King & Greene, 1984). The nuclear or activated form of the receptor remains in the nuclear compartment after fractionisation. Although receptor localisation is important, in terms of this study receptor measurements made are used in a qualitative manner to assess the effect of drug treatments on the receptor population as a whole, rather than as an attempt to compartmentalise the oestrogen receptor.

Drug administration

Oestradiol (oestr-1,3,5-(10)-triene-3,17β-diol; BDH, Poole, U.K.) was dissolved in a 10% (v/v) solution of propylene glycol and was administered intravenously at a dose level of 0.5 μg kg^{-1} (single dose) into the tail vein of the conscious rat 3.0 h before measurements were made. The following compounds were gifts and are gratefully acknowledged: EP092 ((+) - 5 - endo(6' - carboxy - hex - 2'z - enyl) - 6 - exo - (1'' - [N - (phenylthiocarbamoyl) - hydrazono] - ethyl - bi - cyclo[2,2,1] heptane) (Pharmacology Dept., University of Edinburgh) was dissolved in absolute ethanol (5 mg ml^{-1}) plus 2.5 μM sodium hydroxide per mg EP092; the ethanol was then evaporated under nitrogen and the resultant sodium salt was dissolved in aqueous sodium hydroxide (M/400)/sodium chloride (0.9%). EP092 was given 1 h 30 min and 3 h 10 min before measurements were made at a dose level of 10 mg kg^{-1}. AH23848 ([1α(z),2β,5α] - (+) - 7 - [5-[[(1,1' - biphenyl) - 4 - yl]methoxy] - 2-(4-morphinoyl)-3-oxocyclopentyl]-4-heptenoic acid) (Glaxo Group Research) was dissolved in 1% (w/v) sodium bicarbonate in sodium chloride 0.9% (w/ v). BM 13.505 (4-(2-(4-chlorobenzenesulphonamide)-ethyl)-benzene acetic acid) (Boehringer Mannheim, GmBH) was obtained in a stock solution of BM 13.505 100 mg; meglumin 65 mg; mannitol 164 mg; Tritriplex III 1 mg in water for injection to 5 ml, all subsequent dilutions were made in water for injection. Both AH23848 (2.5 mg kg^{-1}) and BM 13.505 (20 mg kg^{-1}) were given intravenously 3 h 10 min before measurements were made. The doses of the thromboxane antagonists were chosen after preliminary studies which indicated that at these dosage levels the uterotrophic response was significantly altered. The drug solvents used did not have any effects on the parameters measured and these results have been grouped as controls.

Statistical analysis

The results (expressed as mean ± s.e.mean) were compared by analysis of variance and Student's *t* test (two tailed) (Snedecor & Cochran, 1979).

Results

In the absence of any exogenous oestradiol the thromboxane antagonists had no significant effect on the parameters studied.

Effect of EP092, AH23848 and BM 13.505 on oestradiol-stimulated blood flow (Table 1)

The vehicles used for the thromboxane antagonists had no effect upon the oestradiol response. All three antagonists were observed to cause a reduction in oestradiol-stimulated uterine blood flow; the hyperaemic response was significantly attenuated by AH23848 alone.

Cardiovascular function was not affected by treatment with any of the antagonists; cardiac output and heart rate remained unchanged. Likewise, thromboxane antagonist pretreatment did not affect blood flow, to the organs studied.

As previously reported by Kerr *et al.* (1990a) it was observed that in approximately 40% of hypertensive rats a significant increase in radioactivity in the lung was noted. This enhanced accumulation of microspheres in the lung did not appear to affect flow to any other organ examined. Animals exhibiting pulmonary pooling were not included in the study.

Effect of EP092, AH23848 and BM 13.505 on oestradiol-stimulated uterine weight (Table 1)

All three thromboxane antagonists induced a significant reduction in oestradiol stimulated wet and dry uterine weight. In the case of EP092 the uterine weight was reduced to a level below that seen in the untreated control group.

Effect of EP092, AH23848 and BM 13.505 on oestrogen receptor distribution in the oestrogen stimulated rat uterus (Figure 1)

Cytosolic receptor levels were not significantly affected by the thromboxane antagonists compared with oestradiol alone. All three antagonists showed a tendency to reduce nuclear receptor levels; this attenuation was significant in the case of EP092 (compared with oestradiol alone).

Discussion

It is known that a single injection of oestradiol stimulates a wide range of metabolic events (Clark & Peck, 1979) in the uterus of the ovariectomized rat. Since a time delay is apparent between the administration of oestradiol and the onset of uterine hyperaemia a number of vasoactive mediators have been implicated in the induction of the response to the steroid. Phaily & Senior (1978a,b) suggested that oestradiol-induced

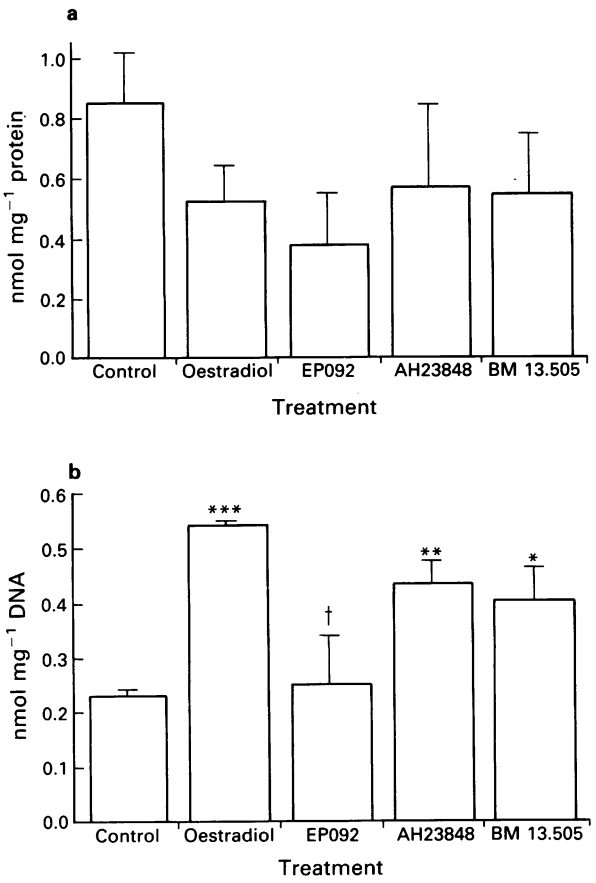

Figure 1 The effect of EP092 (10 mg kg^{-1}) given 10 min before and 1 h 30 min after oestradiol, AH23848 (2.5 mg kg^{-1} i.v., single dose) and BM 13.505 (20 mg kg^{-1} i.v., single dose) given 10 min before oestradiol on cytosolic (a) and nuclear (b) oestrogen receptor distribution in the ovariectomized SHR. Measurements were made 3 h after oestradiol (0.5 μg kg^{-1} i.v., single dose) administration. Each column represents the mean of 3–6 determinations, with 6 uteri per determination; vertical bars show s.e.mean. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$ values are significantly different from those of control group. † $P < 0.05$ value is significantly different from oestradiol alone group.

hyperaemia is mediated through the complex interaction of several vasoactive substances: histamine; kinins and prostaglandins. Stimulation by oestrogen of prostaglandin synthesis has been widely accepted (Ramwell *et al.*, 1977). An oestradiol-induced increase in thromboxane content of the ovariectomized rat uterus has also been reported (Jouanen *et al.*, 1985). Ramwell *et al.* (1977) speculated that the latter may be attributed to increased uterine blood flow with a concomitant rise in platelet number. However, direct stimulation of thromboxane production by oestrogen, in the uterine tissue of ovariectomized rats has been reported (Brown & Poyser, 1985). Therefore, it would be expected that in this study the addition of a thromboxane antagonist (at a dose known to block receptors; Armstrong *et al.*, 1984; Brittain *et al.*, 1985; Stegmeier *et al.*, 1986) to the oestradiol regimen would potentiate the hyperaemic response. However, the converse was found to be true, in that the inclusion of the antagonists attenuated the blood flow response to oestradiol. In the cases of AH23848 and BM 13.505, this phenomenon could be ascribed to the fact that both these compounds have been shown to be partial agonists (Brittain *et al.*, 1985; Patscheke *et al.*, 1987). However, there have been no reports of EP092 displaying partial agonist activity and in the normotensive CD strain this compound was found to enhance the oestradiol-stimulated hyperaemia (Kerr *et al.*, 1991). It is possible that due to the time lag between drug administration and blood flow analysis, adaptive regulatory vascular changes have occurred in the SHR which counteract the influence of the thromboxane receptor blockade. Thus while it would appear that basal vascular thromboxane levels show no significant strain differences (when the antagonists were used without oestradiol they had no effect on blood flow in either species), the thromboxane synthetic capacity of the SHR may exhibit altered reactivity under oestrogen stimulated conditions.

Oestradiol-induced water imbibition also appears to be more sensitive to the inhibitory actions of the thromboxane antagonists than the equivalent response in the CD rat (Kerr *et al.*, 1991). Thromboxane has been implicated in the induction of lysosomal instability (Dorn & Halushka, 1985) and such activity may contribute to the eosinophil-mediated (Tchernitchin *et al.*, 1989) uterine oedematous response. Alternatively thromboxane may exert an anti-chemotactic influence on the eosinophils so preventing the initial migration of their cells to the uterus.

The addition of the thromboxane antagonists to the oestradiol regimen caused a marked reduction in uterine dry weight, which corresponded with decreased levels of nuclear receptor. This phenomenon was also observed, but to a lesser extent, in the CD (Kerr *et al.*, 1991). Interestingly, the total receptor level was lower in the SHR uterus than the CD, even though the weight of the uterus in the SHR is higher than that of the CD when normalised for body weight (Kerr *et al.*, 1991). This paucity of oestrogen receptors in the SHR may explain the increased sensitivity seen to thromboxane antagonists in inhibiting the dry weight response in the uterus. These results suggest that oestradiol may mobilize cellular thromboxane; there is contradictory evidence as to whether this event is genomically mediated as a study by Castracane & Jordan (1976), using systemically administered protein synthesis inhibitors found that these agents did not prevent the stimulation by oestrogen of uterine prostaglandin synthesis. However, Poyser (1979) and Poyser *et al.* (1987) have presented some contradictory evidence in this respect in the guinea-pig, but in turn this prostanoid contributes to the induction of oestrogen-induced uterine growth, presumed to be genomically mediated. This train of events may be of significance in other hormonally regulated tissues.

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(Received December 4, 1990
Revised January 29, 1991
Accepted January 30, 1991)

Br. J. Pharmacol. (1991), **103**, 1367–1372 © Macmillan Press Ltd, 1991

Human interleukin-1 induces a rapid relaxation of the rabbit isolated mesenteric artery

[1]François Marceau, Eric Petitclerc, Denis DeBlois, *Philippe Pradelles & **Patrice E. Poubelle

Centre de recherche de l'Université Laval, Hôtel-Dieu de Québec, Québec, Canada G1R 2J6, *Département de biologie, CEN-Saclay, France and **Centre de recherche Inflammation et Immunologie-Rheumatologie, Centre Hospitalier de l'Université Laval, Ste-Foy, Canada G1V 4G2

1 Strips of rabbit superior mesenteric artery, precontracted with phenylephrine, relaxed when exposed to human recombinant interleukin-1 (IL-1) of the α or β types. The effect was observed within 10 min, was optimal 32 min after the application of the cytokines and concentration-dependent (12–290 pM).

2 IL-1α and IL-1β were equipotent in relaxing the rabbit mesenteric artery. A synthetic fragment corresponding to IL-1β 163-171 was approximately one million fold less active than IL-1β. The tripeptide Lys-D-Pro-Thr, an analogue of IL-1β 193-195, was inactive as an antagonist of IL-1β on the preparation.

3 Indomethacin (2.8 μM) prevented or acutely reversed IL-1-induced relaxations in the rabbit mesenteric artery. Purified haemoglobin (10 μM) or the removal of endothelium had no effect on relaxations elicited by IL-1β.

4 The preparation exhibited some selectivity for IL-1 as recombinant human tumour necrosis factor-α (TNF-α), IL-2 or IL-6 failed to influence it. TNF-α was not synergistic with a subthreshold concentration of IL-1β.

5 Immunoreactive 6-keto-prostaglandin $F_{1\alpha}$ and prostaglandin E_2 were increased in the bathing fluid of isolated mesenteric arteries exposed to IL-1β as compared to controls.

6 A supernatant of lipopolysaccharide-stimulated human monocytes produced a relaxation of the preparation with a profile similar to that produced with IL-1s and there was a good quantitative agreement between the extent of the relaxation and the enzyme immunoassay measurements of IL-1α and IL-1β in the supernatant. Furthermore the relaxation of crude monocyte IL-1 was prevented by preincubating with antibodies to IL-1α and IL-1β. This experiment illustrates the possible use of the preparation for bioassay of IL-1.

7 It is concluded that either form of IL-1 relaxes the precontracted rabbit mesenteric artery by a prostaglandin-dependent, nitric oxide-independent mechanism. The model is also useful for distinguishing the mechanism of IL-1-induced hypotension *in vivo* in rabbits.

Keywords: Interleukin-1-induced vascular relaxation; prostaglandin-induced vascular relaxation; rabbit mesenteric artery; effect of interleukin-1

Introduction

Interleukin-1 (IL-1) is a powerful endogenous mediator capable of reproducing many symptoms and signs of inflammation and sepsis, such as fever, alteration of acute phase protein concentration in blood, eicosanoid production in many cell types and activation of various forms of leukocytes (Dinarello, 1988). Two homologue 17 kDa molecular forms, termed IL-1α and IL-1β, have been characterized and shown to possess qualitatively similar actions and, probably, share a common type of cellular receptor (Dower & Urdal, 1987).

Recombinant human IL-1β has been shown to be a powerful and long-acting hypotensive agent in rabbits (Okusawa *et al.*, 1988); this effect was unusually rapid for this cytokine (< 30 min) and was prevented by ibuprofen treatment, suggesting the involvement of vasodilator prostaglandins. We used a rabbit isolated artery known to be sensitive to such prostaglandins, the mesenteric artery (Simmet & Hertting, 1980), to analyze mechanistically the relationship between IL-1, prostaglandin production and the time course of the vasorelaxation in the absence of circulating blood cells. An additional expected benefit was to develop and characterize a smooth muscle bioassay specific for IL-1 that could be useful to investigators testing IL-1 inhibitors and developing analogues and antagonists of IL-1.

[1] Author for correspondence.

Methods

Tissues

Superior mesenteric arteries were isolated from New Zealand white rabbits (1.5–2.5 kg) of either sex; the first 5 mm segment proximal to the aorta was not used and the following 8–10 mm was removed. Only one strip (approximately 2 × 10 mm) per animal was obtained; other fresh tissues from these rabbits were used in different experiments not described in this study to make maximal use of this animal resource. The tissues were suspended in 5 ml tissue baths containing warmed (37°C) and oxygenated (95% O_2 : 5% CO_2) Krebs solution. A basal tension of 1 g was imposed on tissues and isometric changes in the tissue tension following stimulation with agents were recorded with Harvard transducers (model 52-9545) on a chart recorder. The composition of Krebs solution was (mM): NaCl 117.5, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.18, $CaCl_2$ 2.5, $NaHCO_3$ 25.0 and D-glucose 5.5.

Protocols

Muscle responses to IL-1 were essentially relaxations and this was optimally demonstrated by pre-contracting the tissues. Tissues were equilibrated for 1 h, then stimulated once with phenylephrine (PE; 4 μM; ED_{70} approximately, Churchill & Ward, 1986), washed extensively with fresh Krebs and left to equilibrate for an additional 90 min. Then the tissues were exposed again to PE (4 μM) and 10–15 min later, when a stable tonic contraction was obtained, either form of IL-1, tumour-

necrosis factor-α (TNF-α), IL-2 or IL-6 were injected into the baths. The contraction profile was then recorded for one additional hour without washing. The tissues were not used subsequently (except for testing the acute effect of indomethacin as in Figure 1).

The results are expressed as the percentage of remaining PE-induced contraction plateau at a given time and are expressed as means ± standard errors of the mean (s.e.mean). The spontaneous relaxation of the preparation has been evaluated in a control group of tissues. In this group, the beginning of the observation period, corresponding to a stable PE-induced plateau, was marked by the observer without any intervention on the tissues. The numerical analysis usually is limited to the time 32 min, where the difference between the spontaneous relaxation and the cytokine-induced relaxation was maximal (see results).

In some experiments, the tissues were exposed to indomethacin (2.8 μM), to a tripeptide analogue of IL-1 (10 μM, see below) or to purified haemoglobin (10 μM) 30 min before the second PE application in order to monitor the effect of these drugs on the cytokine-induced vasorelaxation. This concentration of haemoglobin completely inhibits the endothelium-dependent relaxations of rabbit isolated aortae (Martin *et al.*, 1985), probably by binding the mediator endothelium-derived relaxing factor (EDRF), tentatively identified with nitric oxide (Palmer *et al.*, 1987). In some experiments, the endothelial lining of rabbit vascular strips was removed by gently rubbing the intimal surface of strips with a rounded metal spatula; the loss of substance P-induced relaxation in those preparations was monitored as an indication of a successful procedure as endothelial cells are necessary for this response in this preparation (deBlois & Marceau, 1987; see also Results).

Prostaglandin release by fragments of fresh mesenteric arteries

Three isolated mesenteric arteries from different rabbits were each cut into 4 rings (5–10 mg wet weight). These tissues were suspended between metal hooks in 5 ml tissue baths under a tension of 1 g. Baths contained vigorously bubbled Krebs solution, as described above. In order to compare prostaglandin production and release into the bathing fluid, sets of 4 tissues were assigned to one of the following stimuli: IL-1β (290 pM), TNF-α (670 pM), PE (4 μM) or control (no stimulus). After a 2.5 h equilibration period, the tissues were extensively washed and the stimuli were applied at that time without precontracting the tissues. Aliquots of the bathing solution (250 μl) were taken periodically during the following 4 h of observation without washing the tissues. Prostaglandin E_2 (PGE_2) and 6-keto-$PGF_{1\alpha}$ concentrations were determined in the unextracted Krebs solution by two specific enzyme immunoassays based on a colorimetric reaction catalyzed by acetylcholinesterase (Pradelles *et al.*, 1985). Results are expressed as the concentration of prostaglandin in the bathing fluid at a given time and are the means of triplicate determinations from different animals.

Drugs

Recombinant human IL-1α and IL-1β were gifts of Dr A. Shaw, Biogen S.A. (Geneva, Switzerland). Some recombinant IL-1α was purchased from R & D Systems (Minneapolis, MN, U.S.A.). Recombinant human TNF-α was a gift from the Knoll Corp. (Whippany, NJ, U.S.A.). Indomethacin, purified human haemoglobin and recombinant human IL-2 were purchased from Sigma (St. Louis, MO, U.S.A.). Phenylephrine hydrochloride was from Winthrop (Aurora, Canada). A synthetic fragment of IL-1β (position 163 to 171; Boraschi *et al.*, 1988) and Lys-D-Pro-Thr, a tripeptide analogue of IL-1β (position 193 to 195) proposed as an IL-1 antagonist (Ferreira *et al.*, 1988), were obtained from Peninsula Laboratories (Belmont, CA, U.S.A.). Human recombinant IL-6 was a gift from Genetics Institute (Boston, MA, U.S.A.).

Indomethacin was dissolved in 0.1 M Na_2CO_3 and other drugs, in pyrogen-free 0.9% saline. Drugs were injected into the tissue baths at the appropriate time in a small volume (10 μl or less).

Preparation of a crude source of human interleukin-1

Normal human monocytes were purified by counterflow centrifugal elutriation as previously described (Poubelle *et al.*, 1987). Purified monocytes (10^6 ml^{-1}) were incubated at 37°C for 24 h in Hank's balanced salt solution containing 5% foetal bovine serum (Gibco) in the presence of *E. coli* lipopolysaccharide (LPS, Difco Labs, Detroit, MI, U.S.A.) (5 μg ml^{-1}) and indomethacin (2.8 μM). At the end of the incubation period, the supernatant was removed and stored at −20°C. The concentration of both IL-1α and IL-1β was measured separately by two selective enzyme immunoassays recently developed (Grassi *et al.*, 1989). The concentrations found were 0.46 and 1.49 nM, respectively. The supernatant was used in some experiments on isolated mesenteric arteries to evaluate the relaxation profile and amplitude produced by crude IL-1. The final dilutions of the supernatant at the tissue level were 1:100 and 1:500. The exclusive role of IL-1 in the supernatant was assessed by immunoneutralization of IL-1. Fifty μl samples of the supernatant were incubated at 0°C for 1.5 h in the presence of 10 μg each of two polyclonal antibodies targeted on both types of IL-1s (neutralizing sera from R & D Systems, Minneapolis, MN, U.S.A.); this material was then injected into a 5 ml bath for a 1:100 dilution. The composition of Hank's balanced salt solution was (mM): $CaCl_2$ 1.26, KCl 5.36, KH_2PO_4 0.4, $MgCl_2$ 0.5, $MgSO_4$ 0.4, NaCl 137, $NaHCO_3$ 4.16, Na_2HPO_4 0.34 and D-glucose 5.55.

Results

Relaxant effect of interleukin-1, concentration-effect relationship, time course

The rabbit isolated mesenteric artery responded to PE (4 μM) with an immediate contraction that reached a plateau at a somewhat lower level than the peak of the response (Figure 1a, upper tracing). However the plateau was fairly stable over an extended period of time, exhibiting a decline of 20.8 ± 4.6% in the 32 min period after reaching a stable plateau ($n = 6$; see Methods). By contrast, isolated mesenteric arteries exposed to human recombinant IL-1α or IL-1β, applied when a stable PE-induced plateau was recorded, exhibited a sustained vasorelaxant response that was concentration-dependent (Figure 1 typical tracings; time course and concentration-effect relationship, Figure 2). Only the difference between this control curve and the ones obtained in the presence of IL-1 is interpreted as the effect of IL-1. The 2.4 pM concentration of either form of IL-1 did not produce significant effects on the mesenteric artery; however concentrations in the range 12–290 pM produced concentration-dependent relaxations that were clearly superior to the control loss of tone. Virtually complete relaxation of the PE-induced contraction was obtained with 290 pM of IL-1α or IL-1β 48 min after their application (Figure 1).

The time course of IL-1-induced relaxation was similar according to the IL-1 type tested. Both forms induced a slow-developing effect with credible relaxation within the first 10 min only at the higher concentration levels (Figures 1 and 2).

Effect of synthetic fragments of interleukin-1β

The synthetic fragment 163-171 of IL-1β produced a relaxant response of the mesenteric artery at 10 μM (Figure 2b). The fragment was approximately one million fold less potent than the parent protein. The relaxation in the presence of the frag-

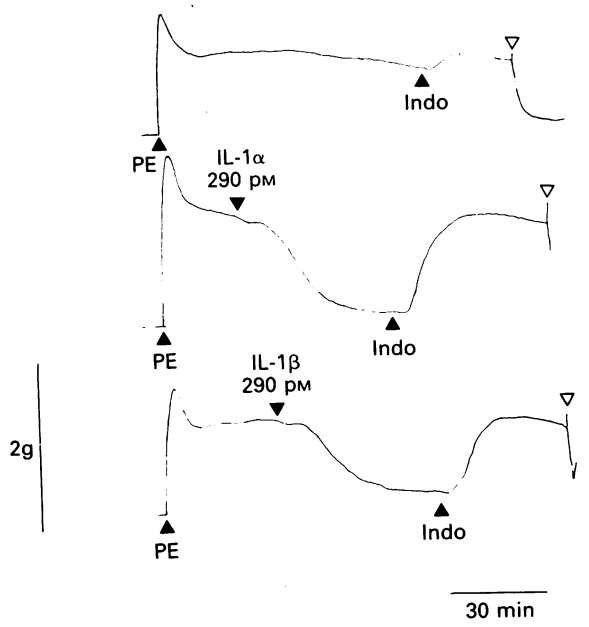

Figure 1 Effect of interleukin-1 (IL-1) of the α and β types on typical preparations of rabbit isolated mesenteric artery. Tissues were precontracted with phenylephrine (PE, 4 μM) before the application of the IL-1. The top tracing illustrates the spontaneous evolution of the PE-induced plateau over the observation period. At the end of the observation period, indomethacin (Indo, 2.8 μM) was injected into the baths without washing the tissues. Abscissa scale: time; ordinate scale: isometric contraction, g. Closed symbols refer to the application of agents and open symbols, to washout of stimulants.

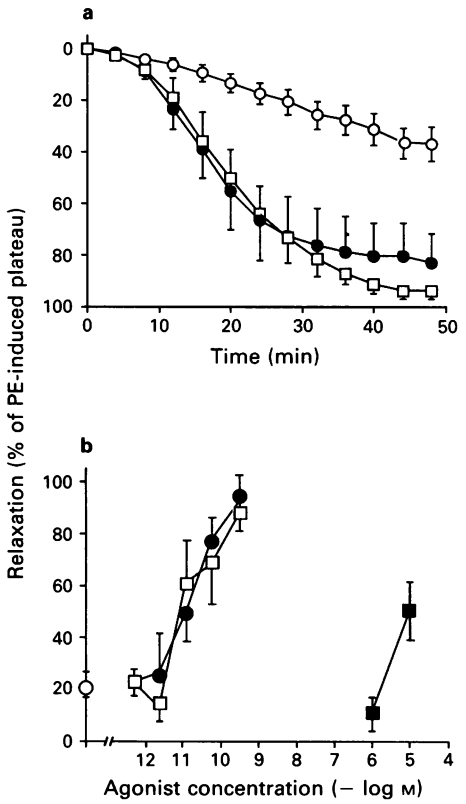

Figure 2 (a) Time course for interleukin-1 (IL-1)-induced relaxations in rabbit isolated mesenteric arteries. Tissues were precontracted with phenylephrine (4 μM). The spontaneous relaxation of the preparation has been evaluated in a set of tissues (○). Other tissues were exposed to either IL-1α (●) or IL-1β (□) (290 pM). Abscissa scale: time; ordinate scale: residual contraction, expressed as a percent of the phenylephrine-induced plateau. Results are the mean of 4 to 6 determinations; vertical bars show s.e.mean. (b) Concentration-effect relationship for IL-1-induced relaxations in rabbit isolated mesenteric arteries. Tissues were precontracted with phenylephrine (4 μM) and results are expressed as the relaxation observed at time 32 min in control preparation (○) or in tissues exposed to one concentration of IL-1α (●), IL-1β (□) or the 163–171 fragment of IL-1β (■).

ment (10.4 ± 4.6% at 12 min, 46.5 ± 10.5% at 32 min, $n = 4$) was slow developing, as for IL-1β-induced response (compare with Figure 2a).

The tripeptide Lys-D-Pro-Thr, applied at 10 μM before IL-1β (290 pM), failed to antagonize the relaxation induced by this cytokine (Figure 3). This concentration of the tripeptide had no relaxant effect by itself on the preparation.

Effect of drugs or endothelium removal on interleukin-1-induced relaxations

The application of indomethacin (2.8 μM) at the end of the observation period for the IL-1-induced vasorelaxation elicited a contraction of the mesenteric artery (Figure 1). The spontaneous relaxation of the tissue over long periods was also partly reversed following the application of indomethacin (Figure 1, top tracing, typical of 6 experiments). These findings suggest that prostaglandins submitted to a rapid turnover might be involved in both the spontaneous and the IL-1-induced relaxation.

Pretreatment of the tissues with indomethacin (2.8 μM) inhibited significantly the relaxant effect of both types of IL-1, tested at 290 pM, on the precontracted mesenteric artery (Figure 3). Indomethacin-treated tissues contracted with PE but not exposed to IL-1 relaxed over time to the same extent as control tissues (Figure 3), a contrasting finding with the effect of indomethacin applied at the end of the spontaneous relaxation (Figure 1, top tracing).

The neuropeptide substance P was used as a reference endothelium-dependent relaxing agent on isolated mesenteric arteries (deBlois & Marceau, 1987 and Figure 4). The relaxation induced by substance P (74 nM) developed in less than 2 min and eventually faded away. The relaxing effect of the neuropeptide (29.0 ± 5.6% of plateau, $n = 7$) was totally prevented when tissues were pretreated with purified human haemoglobin (10 μM) (Figure 4 representative of 7 experiments, $P < 0.01$ by Student's t test for paired samples). The same stock of haemoglobin (10 μM) failed to modify the profile of either IL-1β-induced relaxation or the spontaneous relaxation in tissues precontracted with PE (Figure 3). The endothelial lining was removed from other sets of tissues. These preparations failed to relax when exposed to substance P ($n = 8$). The relaxing response to IL-1β or the spontaneous relaxation of tissues not exposed to IL-1 were similar to controls with endothelium (Figure 3).

Effect of other human cytokines on the isolated mesenteric artery

Recombinant cytokines with human sequences were applied to the preparation in order to determine its selectivity for IL-1. TNF-α, at a highly active concentration in several *in vitro* systems (670 pM; e.g. Poubelle *et al.*, 1990), failed to induce rapid or slow relaxations in PE-precontracted tissues (Figure 5). The synergistic hypotensive effect of human TNF-α with sub-threshold doses of IL-1β in rabbits has been reported (Okusawa *et al.*, 1988). The isolated mesenteric artery did not relax significantly in the presence of TNF-α (670 pM) and a sub-threshold concentration of IL-1β (2.4 pM, Figure 5). Human recombinant IL-2 or IL-6 also failed to influence the rabbit arterial preparation (Figure 5) at concentration levels that are very active in various *in vitro* systems (100 and 1000 u ml^{-1}, respectively; Ritchie & Fuller, 1983; Frasier-Scott *et al.*, 1988; Mizutani *et al.*, 1989).

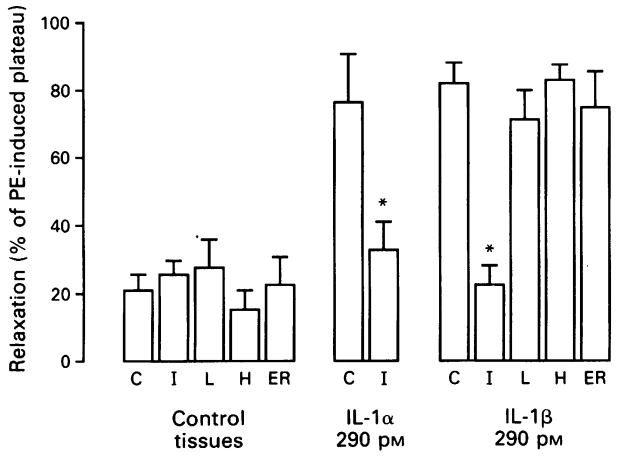

Figure 3 Effect of drugs or endothelium removal on interleukin-1 (IL-1)-induced or spontaneous relaxation of the rabbit mesenteric artery recorded at time 32 min. Results are expressed as the mean relaxations, as a percentage of the phenylephrine-induced plateau. 'Control tissues' illustrate the spontaneous relaxation of the preparation, with or without the treatments tested against IL-1s. Results are the mean of 3 to 6 determinations; vertical bars show s.e.mean. One-way analysis of variance applied to control tissue values failed to show significant differences between groups. The same tests applied to tissue groups exposed to IL-1β demonstrated significant differences between groups ($P < 0.01$). Dunnett's test (Tallarida & Murray, 1987) was then applied to compare treated groups with the one exposed to IL-1β only: $^*P < 0.01$. The values recorded in the 2 tissue groups exposed to IL-1α were compared by Student's t test: $^*P < 0.01$. Abbreviations for treatments: C, control; I, indomethacin (2.8 μM); L, Lys-D-Pro-Thr (10 μM); H, haemoglobin (10 μM); ER, endothelium removal.

Prostaglandin release by stimulated mesenteric arteries

Both PGE_2 and 6-keto-$PGF_{1\alpha}$ concentrations increased in the bathing solution of isolated mesenteric arteries during a 4 h observation period, the latter prostaglandin being more abundant at all times (Table 1). IL-1β (290 pM) increased the release of both prostaglandins, statistical significance being reached for PGE_2 30 min after stimulation and for both types of prostaglandin at 4 h. TNF-α (670 pM) or PE (4 μM) had no significant effect on prostaglandin release by fresh arteries under the conditions applied.

Effect of crude human monocyte interleukin-1 on the rabbit isolated mesenteric artery

The supernatant of LPS-stimulated human monocytes contained 1.95 nM of total IL-1 (α and β forms combined, see Methods), as determined by enzyme immunoassays. Therefore the 1:100 and 1:500 dilutions of this supernatant in the organ baths corresponds to calculated IL-1 concentrations of 20 and 3.9 pM, respectively. These dilutions of the supernatant produced slow-developing relaxations of the PE-induced plateau (at 4 min: 0% for both dilutions; at 32 min: 60 $\pm$ 13% and 28 $\pm$ 11%, $n = 3$ and 5 respectively). Both forms of recombinant IL-1 were shown to be equipotent, on a molar basis, in eliciting a vasorelaxant response in the rabbit isolated mesenteric artery. Thus, the amplitude of the relaxations at 32 min can be converted into IL-1 concentrations using the concentration-effect curves obtained with recombinant standards (Figure 2b) and it can be seen that there is a good quantitative agreement between the enzyme immunoassays and this bioassay. Preincubation of the supernatant with neutralizing antibodies to both types of IL-1 reduced the effect of the 1:100 supernatant dilution to that of control tissues (17.5 $\pm$ 5.2% at 32 min, $n = 4$). LPS-containing medium that had not been incubated with monocytes had a negligible effect on the precontracted mesenteric artery at the 1:100 dilution (relaxation of 17.0 $\pm$ 4.6% at 32 min, $n = 3$).

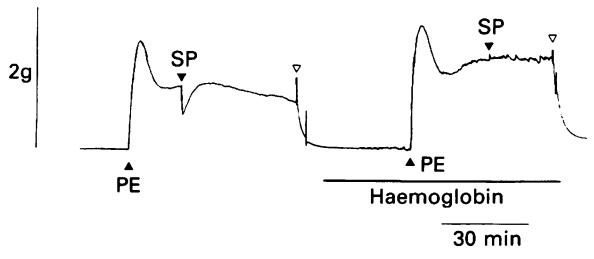

Figure 4 Relaxing effect of substance P (SP) (74 nM) on a typical preparation of rabbit isolated mesenteric artery. The tissue was precontracted with phenylephrine (PE, 4 μM) before the application of substance P. The second recording was made in the same tissue in the presence of human purified haemoglobin (10 μM), which prevented the relaxing effect of substance P. Abscissa scale: time; ordinate scale: isometric contraction, g. Closed symbols refer to the application of agents and open symbols, to washout of stimulants.

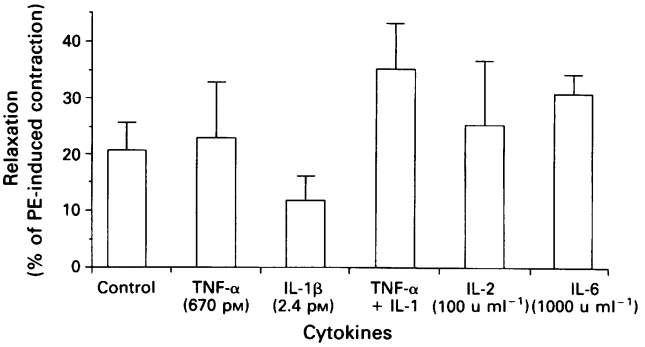

Figure 5 Effect of various cytokines on the phenylephrine (PE)-induced contraction plateau of rabbit isolated mesenteric artery. Tumour necrosis factor-α (TNF-α, 670 pM), a sub-threshold concentration of interleukin-1β (IL-1β) or a combination of both were tested. IL-2 (100 u ml^{-1}) and IL-6 (1000 u ml^{-1}) were also tested. The relaxations at 32 min after cytokine application are expressed as a percentage of the contraction plateau and are compared with the spontaneous relaxation of the preparation (control). No significant differences between the groups were found. Results are the means of 4 to 6 determinations; vertical bars show s.e.mean.

Discussion

Human recombinant IL-1s were shown to relax the rabbit isolated mesenteric artery. The concentration-range for an effect greater than the spontaneous relaxation of the preparation compares favorably with various *in vitro* bioassays for IL-1. The inhibitory effect of indomethacin on the relaxation suggests that in this system, as in many others (Burch *et al.*, 1988; Dinarello, 1988), IL-1 promotes the release of cyclo-oxygenase products. The smooth muscle of the rabbit mesenteric artery is known to relax to PGE_2 (Simmet & Hertting, 1980), consistent with the mechanical effect observed. We have produced evidence that PGE_2 and PGI_2 are produced relatively rapidly by the tissue under IL-1 stimulation, by measuring immunoreactive PGE_2 and 6-keto-$PGF_{1\alpha}$, a degradation product of PGI_2, in the bathing solution (Table 1). Other agents, such as kinins, also produce prostaglandin-dependent relaxation of this preparation (Churchill & Ward, 1986; deBlois & Marceau, 1987); however, these responses developed faster than those elicited by either type of IL-1 and were not as sustained as those recorded with IL-1s.

There is an apparent discrepancy in the inhibitory effect of indomethacin applied before phenylephrine (Figure 3) or at the end of a recording (Figure 1) on relaxations of the precontracted artery, either spontaneous or IL-1-induced. The drug applied at the end of a recording appears to reverse com-

Table 1 Prostaglandin concentration in the bathing fluid of rabbit isolated mesenteric arteries undergoing various types of stimulation

Time after stimulation	*Prostaglandin*	*Control*	*Stimuli* *IL-1β* (290 pM)	*TNF-α* (670 pM)	*PE* (4 μM)
15 min	PGE_2	28 ± 7[a]	39 ± 5	33 ± 6	24 ± 7
	6-keto-$PGF_{1\alpha}$	83 ± 8	126 ± 21	88 ± 25	95 ± 36
30 min	PGE_2	29 ± 5	69 ± 8*[b]	55 ± 16	32 ± 10
	6-keto-$PGF_{1\alpha}$	121 ± 21	248 ± 54	165 ± 38	141 ± 72
4 h	PGE_2	141 ± 22	512 ± 16**	324 ± 71	187 ± 18
	6-keto-$PGF_{1\alpha}$	531 ± 133	1581 ± 353*	666 ± 91	627 ± 136

[a] Prostaglandin concentration (pg ml^{-1}) in the bathing Krebs solution. Results are the means ± s.e.mean of triplicate determinations from different animals.

[b] Results from stimulated tissues were compared with controls at each time by Student's *t* test: * $P < 0.05$; ** $P < 0.01$.

IL-1β, interleukin-1β; TNF-α, tumour necrosis factor-α; PE, phenylephrine.

pletely both types of relaxations, whereas some relaxation occurs in tissues preexposed to indomethacin and exposed later to IL-1. These results can be reconciled if one considers that the α-adrenoceptor agonist, PE, is a significant stimulus for prostaglandin release in rabbit isolated blood vessels (Förstermann *et al.*, 1984), although not as potent as IL-1 under the conditions applied in our prostaglandin release assay (Table 1). The contraction plateau might be somewhat reduced by PE-induced prostaglandins in tissues not pretreated with indomethacin; if indomethacin was applied only at the end of a recording, the tone would return to a hypothetical higher plateau, itself exhibiting some fading away over time.

As discussed previously, the time course of IL-1-induced phospholipase A_2 or cyclo-oxygenase synthesis in most *in vitro* systems studied involving mesenchymal cells is not fast enough (4–24 h) to account for some relatively rapid (10–30 min) prostaglandin-mediated effect of IL-1, such as fever and haemodynamic effects (Dinarello, 1988; Burch *et al.*, 1988). The present results based on both mechanical effects and secretory studies suggest that the prostaglandin-mediated component of IL-1-induced relaxation of the mesenteric artery is quite rapid relative to various cultured cell types. Even if the prostaglandin output was not maximal during the 32 min observation period for the relaxant effect, the 69 ng ml^{-1} level of PGE_2 recorded in the bathing fluid at 30 min is sufficient to produce a large relaxant response in several rabbit isolated arteries (Förstermann *et al.*, 1984).

IL-1 is not apparently an endothelium-dependent relaxing agent in the mesenteric artery as intimal damage or treatment with a functional endothelium-derived relaxing factor (EDRF) inhibitor, haemoglobin, failed to modify the response to the cytokine. The endothelium is probably not a major source of relaxing prostanoids in this system, assuming that the response to IL-1 is essentially mediated by these lipids.

There was a 10^6 factor between the potency of recombinant IL-1β and of its fragment 163-171. This factor is in good agreement with its reported relative immunostimulant potency in mice (Antoni *et al.*, 1986; Nencioni *et al.*, 1987) and indicates that the oligopeptide retains only marginally the effect of the complete protein. The fragment has been developed as a non-inflammatory analogue of IL-1, possessing only immunostimulant activity (Boraschi *et al.*, 1988). As such, it may possess a selectivity for lymphocytes and be inactive on mesenchymal cells and on neutrophils (Ogle *et al.*, 1990). The tripeptide analogue, proposed as an antagonist selective for IL-1β in an *in vivo* model of hyperalgesia (Ferreira *et al.*, 1988), is not active in our model as an antagonist. The model of hyperalgesia in which it was reportedly active may be atypical because it was unusually selective for IL-1β.

The haemodynamic effect of human recombinant IL-1β has been analyzed in some detail in the rabbit (Okusawa *et al.*, 1988). A prostaglandin-dependent and durable hypotensive effect followed bolus injections of infusions of IL-1β. This was related to a drop of peripheral resistance. The present model illustrates mechanistically several features of the *in vivo* model by showing (1) that some cell type(s) present in the vessel wall responds with a durable prostaglandin-mediated relaxation in the absence of circulating blood cells; (2) the time course of IL-1-induced hypotension is very similar to the *in vitro* model and exceptionally rapid for this cytokine. However, in our model, human recombinant TNF-α does not act by itself or synergistically with a sub-threshold concentration of IL-1β. This contrasts with the *in vivo* model. An action of TNF-α on circulating cells or cells in remote organs (e.g. reticuloendothelial cells) may explain this difference. The fact that IL-1 response is relatively rapid, that TNF-α does not reproduce the IL-1 effect, and that the immunostimulant IL-1β fragment has some activity in our model may also suggest that the primary target cell type for IL-1 in the mesenteric artery is perhaps a type of leukocyte present in the connective structure or sequestrated in the blood vessel wall.

The lack of effect of human IL-2 and IL-6 on the rabbit isolated mesenteric artery may only indicate a species barrier for some of the cytokines; such a barrier does not exist for IL-1α or IL-1β as the rabbit molecules share 64% and 81% homology with their human counterparts, respectively (Furutani *et al.*, 1985; Cannon *et al.*, 1989). Interestingly, the sequence corresponding to IL-1β 163–171 is identical in the human and rabbit form of the protein (Cannon *et al.*, 1989). Such a selectivity of the preparation may be turned to an advantage in the screening and development of drugs active on IL-1 receptors, or for measuring IL-1 in biological fluids. The observation of the correct relaxation time course and amplitude after the application of crude monocyte IL-1 is interesting in that context because LPS-stimulated monocytes also produce TNF-α and IL-6 (Kawakami *et al.*, 1982; Ritchie *et al.*, 1983). Classical bioassays of IL-1 based on thymocyte proliferation are prone to much interference from IL-6 (Helle *et al.*, 1988; Mizutani *et al.*, 1989).

Supported by a grant (MA-9358) from the Medical Research Council of Canada (MRCC). F.M. and P.E.P. are Scholars of the Fonds de la recherche en Santé du Québec (FRSQ) and D.D., the recipient of a Studentship from the FRSQ. The authors thank Ms Johanne Bouthillier for technical assistance.

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(Received November 7, 1990
Revised January 28, 1991
Accepted January 31, 1991)

Br. J. Pharmacol. (1991), **103**, 1373–1376 © Macmillan Press Ltd, 1991

The spider toxin, argiotoxin$_{636}$, binds to a Mg^{2+} site on the N-methyl-D-aspartate receptor complex

Ian J. Reynolds

Department of Pharmacology, University of Pittsburgh, Pittsburgh PA 15261, U.S.A.

1 The mechanism of action of the arylalkylamine spider toxin, argiotoxin$_{636}$, on the N-methyl-D-aspartate (NMDA) receptor was investigated by use of [^{3}H]-dizocilpine binding to well-washed membranes obtained from rat brain.

2 Argiotoxin$_{636}$ decreased [^{3}H]-dizocilpine binding with an apparent potency of about 3 μM. The inhibition of [^{3}H]-dizocilpine by argiotoxin$_{636}$ was insensitive to the concentration of glutamate, glycine and spermidine in the assay.

3 Argiotoxin$_{636}$ alone had no effect on the dissociation of [^{3}H]-dizocilpine. However, argiotoxin$_{636}$ reversed the actions of Mg^{2+} on the dissociation of [^{3}H]-dizocilpine by decreasing the apparent potency of Mg^{2+}. Argiotoxin$_{636}$ also reversed the action of Ca^{2+} on the dissociation of [^{3}H]-dizocilpine.

4 These results suggest that argiotoxin$_{636}$ exerts a novel inhibitory effect on the NMDA receptor complex by binding to one of the Mg^{2+} sites located within the NMDA-operated ion channel.

Keywords: NMDA receptor; argiotoxin$_{636}$; [^{3}H]-dizocilpine; Mg^{2+}

Introduction

A variety of arylalkylamine toxins have been isolated from the venom of orb-weaving and funnel web spiders (Jackson & Usherwood, 1988; Jackson & Parks, 1989). These include joro spider toxin (JSTX), a family of α-agatoxins from the funnel web spider *Agelenopsis aperta* (Quistad *et al.*, 1990) and a series of argiotoxins from the *Argiope* genus of orb weaving spiders (Adams *et al.*, 1987). Their biological activity is believed to reside in their potent antagonism of arthropod glutamate receptors. These toxins are not competitive with respect to the actions of glutamate and act primarily by blocking glutamate-operated cation channels (Jackson & Usherwood, 1988). Thus, these toxins produce neuromuscular blockade and paralysis.

Glutamate, although not employed at neuromuscular junctions, is an important neurotransmitter in the mammalian central nervous system. Mammalian glutamate receptors are pharmacologically distinct from their arthropod counterparts (Shinozaki, 1988). However, the principal glutamate receptors are also intimately associated with cation-conducting ion channels (Collingridge & Lester, 1989). Thus, it is possible that arylalkylamine spider toxins might also be effective channel blocking agents at mammalian glutamate receptors (Jackson & Usherwood, 1988). Several studies have demonstrated that JSTX selectively and non-competitively blocks AMPA and kainate receptors (Saito *et al.*, 1985; Akaike *et al.*, 1987). In addition, argiotoxin$_{636}$ selectively blocks N-methyl-D-aspartate (NMDA) receptors by a use- and voltage-dependent mechanism, which suggests a site of action within the NMDA-operated ion channel (Priestley *et al.*, 1989).

The NMDA receptor complex has been extensively characterized both electrophysiologically and in ligand binding studies. These studies have demonstrated the presence of several distinct binding sites on the receptor, including recognition sites for glutamate and NMDA, glycine, Zn^{2+}, Mg^{2+}, phencyclidine-like drugs and most recently polyamines (Collingridge & Lester, 1989). We have previously demonstrated that the action of drugs at each of these binding sites can be differentiated by utilizing [^{3}H]-dizocilpine ([^{3}H]-MK801) binding to the phencyclidine site (Reynolds *et al.*, 1987; Reynolds & Miller, 1988; Reynolds, 1990a,b). The finding that argiotoxin$_{636}$, which incorporates a polyamine-like moiety as a part of its structure (Figure 1), was a specific NMDA receptor antagonist raised the possibility that argiotoxin$_{636}$ might act by inhibiting the positive modulatory actions of polyamines on the NMDA receptor complex (Ransom & Stec, 1988). To examine this hypothesis, the interaction of argiotoxin$_{636}$ with the NMDA receptor was monitored in ligand binding studies in which [^{3}H]-dizocilpine was used to label the receptor.

Methods

In [^{3}H]-dizocilpine binding experiments well-washed membranes from rat brain were used prepared as previously described (Reynolds & Miller, 1989). Assay tubes contained 100–150 μg membrane protein, 0.2–0.3 nM [^{3}H]-dizocilpine and drugs as appropriate in a final volume of 1 ml 10 mM HEPES-NaOH, pH 7.4. Assays were incubated for 2 h at room temperature (21–25°C), after which tubes were vacuum filtered over glass fibre filters (number 32, Schleicher and Schuell, Keene, NH, U.S.A.). After three 5 ml washes with HEPES buffer, filters were placed in scintillant (Ecolite, ICN Biomedicals, Cleveland, OH, U.S.A.) for at least 90 min before liquid scintillation counting. Assays performed in this way do not reach equilibrium within 2 h unless saturating concentrations of glutamate, glycine and spermidine have been added. This non-equilibrium condition permits the assay of the NMDA receptor activators and positive allosteric modulators (Reynolds *et al.*, 1987; Foster & Wong, 1987; Bonhaus & McNamara, 1988). Solutions of synthetic argiotoxin$_{636}$ (Natural Product Sciences, Salt Lake City, UT, U.S.A.) were prepared in HEPES buffer on the day of use, as stock solutions become discoloured within 24 h of storage at −20°C.

The estimation of the actions of argiotoxin$_{636}$ on the dissociation of [^{3}H]-dizocilpine was performed essentially as previously described (Reynolds & Miller, 1989) with a minor modification. Briefly, [^{3}H]-dizocilpine (15–20 nM) was

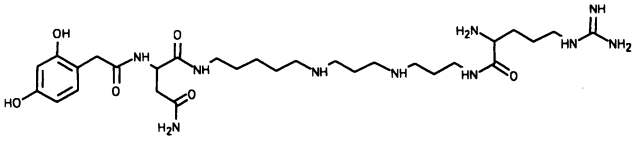

Figure 1 Structure of argiotoxin$_{636}$.

incubated with membranes (10 mg ml^{-1} protein) for 2 h. Aliquots (10 μl) of this mixture were then added to assay tubes containing 100 μM glutamate, 30 μM glycine and drugs as appropriate in a final volume of 1 ml HEPES-NaOH, pH 7.4. A smaller volume was used than in the previous study in order to minimize the quantity of argiotoxin$_{636}$ necessary for this series of experiments. However, the dilution factor (100 fold) was similar to that previously employed. Moreover, the addition of dizocilpine after 2 h did not further decrease the levels of binding below that produced by dilution alone, suggesting that the dilution was sufficient to eliminate the association reaction. After a further 60 min incubation, the assay mixture was filtered and radioactivity determined as described above.

Concentration-response curves with argiotoxin$_{636}$ were analysed with the EBDA and LIGAND programmes as previously described. In the presence of 100 μM glutamate and 30 μM glycine, the dissociation rate of [^{3}H]-dizocilpine is 0.00384 min^{-1} (Reynolds & Miller, 1989). After 60 min, 56 $\pm$ 3.3% of the total binding remained. Results from these experiments were expressed as a proportion of the binding remaining after 60 min.

Results

Argiotoxin$_{636}$ inhibited basal [^{3}H]-dizocilpine binding with a potency of about 3 μM (Figure 2). The addition of saturating concentrations of glutamate (100 μM) and glycine (30 μM) did not significantly modify the potency, while spermidine significantly increased the potency of argiotoxin$_{636}$. Interestingly, in the presence of high levels of glutamate and glycine spermidine had no effect on the potency of argiotoxin$_{636}$. A summary of the data for the inhibition of [^{3}H]-dizocilpine binding by argiotoxin$_{636}$ is given in Table 1. Hill slopes did not significantly differ from unity. The failure of glutamate, glycine and spermidine to decrease the apparent potency of argiotoxin$_{636}$ precludes an action of the toxin at the NMDA, glycine and polyamine sites, in spite of the similarity in structure between argiotoxin$_{636}$ and spermine.

To probe further the mechanism of action of argiotoxin$_{636}$, the effects of the toxin on the dissociation of [^{3}H]-dizocilpine were monitored. Argiotoxin$_{636}$ had little effect on the dissociation of [^{3}H]-dizocilpine at concentrations ranging from 0.5–200 μM (Figure 3a). The addition of 0.5 mM Mg^{2+} greatly reduced [^{3}H]-dizocilpine binding remaining after 60 min. Interestingly, although argiotoxin$_{636}$ had little effect alone, it effectively reversed the actions of Mg^{2+}. Additionally, argiotoxin$_{636}$ also reversed the effects of Ca^{2+} on the dissociation of [^{3}H]-dizocilpine. Half maximal reversal of the effects of Mg^{2+} were observed at about 30 μM, and approximately half maximal reversal of the action of Ca^{2+} at 100 μM. Dizocilpine at concentrations up to 1 μM was unable to reverse the actions of Mg^{2+} (data not shown). The concentration-dependence of the actions of Mg^{2+} and Ca^{2+} are illustrated in Figure 3b. These cations increase the

Table 1 Effects of argiotoxin$_{636}$ on [^{3}H]-dizocilpine binding to well-washed rat brain membranes

Condition	IC_{50}, μM	nH
0.1 μM Glu + 0.03 μM Gly	3.03 $\pm$ 0.74	1.22 $\pm$ 0.16
100 μM Glu + 30 μM Gly	3.36 $\pm$ 0.52	1.34 $\pm$ 0.13
0.1 μM Glu + 0.03 μM Gly + 50 μM Spd	0.84 $\pm$ 0.11**	1.06 $\pm$ 0.08
100 μM Glu + 30 μM Gly + 50 μM Spd	2.67 $\pm$ 0.47	0.96 $\pm$ 0.03

Results are the mean ($\pm$ S.E.M.) of 3–5 experiments performed in duplicate as described in methods.
** Value significantly different ($P < 0.05$) from IC_{50} in the presence of 100 μM Glu + 30 μM Gly (ANOVA with Bonferroni correction for multiple comparisons).

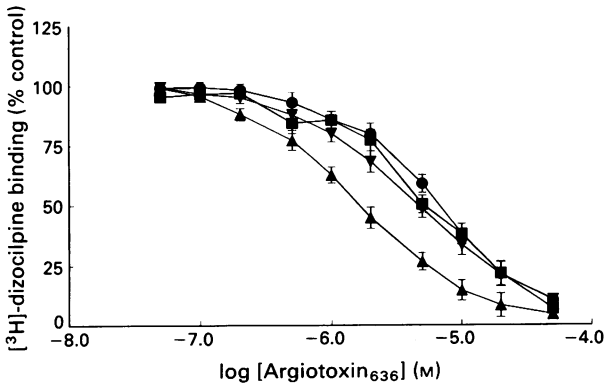

Figure 2 Effect of glutamate, glycine and spermidine (Spd) on the inhibition of [^{3}H]-dizocilpine binding by argiotoxin$_{636}$. Concentration-response curves were generated in the presence of 0.1 μM glutamate and 0.03 μM glycine (■), 100 μM glutamate and 30 μM glycine (●), 0.1 μM glutamate, 0.03 μM glycine and 50 μM spermidine (▲) or 100 μM glutamate, 30 μM glycine and 50 μM spermidine (▼). Non-equilibrium binding assays were performed as described in methods. Results represent the mean of 3–6 experiments performed in duplicate. Vertical bars represent s.e.mean although some error bars are smaller than the appropriate symbols.

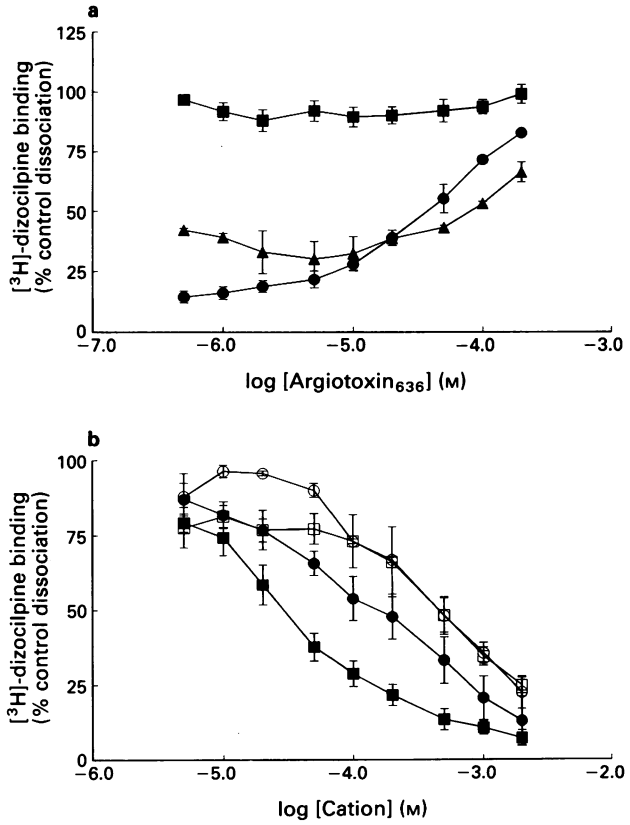

Figure 3 Effects of (a) argiotoxin$_{636}$ and (b) divalent cations on the dissociation of [^{3}H]-dizocilpine from the NMDA receptor. Data are expressed as a proportion of [^{3}H]-dizocilpine binding remaining after 60 min. At this time 56 $\pm$ 3.3% of the total binding remained. Curves in (a) were performed in the absence of cations (■) or in the presence of 0.5 mM Mg^{2+} (●) or 0.5 mM Ca^{2+} (▲). Curves in (b) were performed using Mg^{2+} (squares) or Ca^{2+} (circles) in the absence (closed symbols) and presence (open symbols) of 50 μM argiotoxin$_{636}$ as indicated. The results represent the mean of 3–4 experiments performed in duplicate as described in methods. Vertical bars represent s.e.mean.

dissociation of [^{3}H]-dizocilpine at similar concentrations to those at which they inhibit non-equilibrium [^{3}H]-dizocilpine binding (Reynolds & Miller, 1988). The addition of 50 μM argiotoxin$_{636}$ decreased the apparent potency of both Mg^{2+} and Ca^{2+} in this assay although Mg^{2+} was more sensitive to the effects of the toxin.

Discussion

A primary goal of this study was to determine the site and mechanism of action of the arylalkylamine spider toxin, argiotoxin$_{636}$. These experiments permit several conclusions to be drawn regarding the likely site of action of the toxin. It is unlikely that argiotoxin$_{636}$ binds to the NMDA or glycine binding sites on the NMDA receptor as glutamate and glycine did not alter the apparent potency of the toxin. Polyamines exert two prominent effects on [^{3}H]-dizocilpine binding. Low concentrations (5–100 μM) increase [^{3}H]-dizocilpine binding, while higher concentrations have an inhibitory effect (Reynolds & Miller, 1989; Williams *et al.*, 1989). The concentration of spermidine used is sufficient to occupy fully the polyamine site responsible for increasing [^{3}H]-dizocilpine binding and will significantly decrease the apparent potency of competitive polyamine antagonists such as arcaine (Reynolds, 1990a). This concentration of spermidine did not decrease the potency of argiotoxin$_{636}$, thus precluding an action at this polyamine site. Indeed, in the presence of low levels of glutamate and glycine, spermidine actually increased the potency of argiotoxin$_{636}$. The mechanism underlying this effect is not clear.

The investigation of the actions of argiotoxin$_{636}$ on the dissociation of [^{3}H]-dizocilpine was most revealing. Argiotoxin$_{636}$ alone had little effect on the dissociation of [^{3}H]-dizocilpine. This distinguishes it from Zn^{2+}-like drugs. The latter agents, by closing the NMDA-operated ion channel, delay the egress of [^{3}H]-dizocilpine and thereby result in more binding remaining after 60 min, compared to control. In addition, argiotoxin$_{636}$ does not simply mimic the actions of Mg^{2+}. As demonstrated in Figure 3, Mg^{2+} profoundly decreases the levels of [^{3}H]-dizocilpine binding remaining after 60 min, presumably as a consequence of an allosteric interaction between the Mg^{2+} and the phencyclidine binding sites. It is also unlikely that argiotoxin$_{636}$ mimics dizocilpine as, unlike argiotoxin$_{636}$, dizocilpine was unable to reverse the actions of Mg^{2+}.

On the basis of these inferences it seems probable that argiotoxin$_{636}$ binds to the Mg^{2+} site on the receptor that is responsible for accelerating the dissociation of [^{3}H]-dizocilpine, but without exerting an allosteric effect on the phenycyclidine site. Argiotoxin$_{636}$ might therefore be considered as a Mg^{2+}-like compound that lacks efficacy, that is, a Mg^{2+} antagonist. However, this term should be used with caution, as argiotoxin$_{636}$ itself is an antagonist of the function of the receptor. If argiotoxin$_{636}$ binds to a Mg^{2+} site but does not produce an allosteric effect it is reasonable to ask how argiotoxin$_{636}$ inhibits non-equilibrium [^{3}H]-dizocilpine binding as shown in Figure 2. The mechanism for this inhibition has not been determined. However, as the Mg^{2+} site, like the phencyclidine site, is located within the ion channel and the argiotoxin$_{636}$ molecule is relatively large, it is possible that inhibition of binding occurs through stearic hindrance.

This conclusion is consistent with several prior observations. Priestley and colleagues (1989) showed that argiotoxin$_{636}$ was a use- and voltage-dependent antagonist of the NMDA receptor. The finding that argiotoxin$_{636}$ interacts with a Mg^{2+} site is fully consistent with these observations. Moreover, the potency of argiotoxin$_{636}$ was similar to that found in this study. Preliminary studies that monitored the actions of argiotoxin$_{636}$ on [^{3}H]-glycine binding showed that argiotoxin$_{636}$ increased [^{3}H]-glycine binding (Gullak *et al.*, 1989). This is consistent with the lack of a competitive interaction between argiotoxin$_{636}$ and glycine observed in this study. Interestingly, Mg^{2+} also stimulates glycine binding (Marvizon & Skolnick, 1988; Gullak *et al.*, 1989). Moreover, Mg^{2+} effectively displaced [^{125}I]-argiotoxin$_{636}$ binding to rat brain membranes (Mena *et al.*, 1989). In a recent study, it was concluded that the low affinity inhibitory actions of polyamines were mediated by an action at a Mg^{2+}-like binding site (Reynolds, 1990a). The structural similarity between argiotoxin$_{636}$ and spermine support the possibility that the inhibitory effects of the two compounds are mediated by the same binding site.

The precise identity of the Mg^{2+} site to which argiotoxin$_{636}$ binds cannot be clearly ascertained. The most prominent action of Mg^{2+} is to effect a voltage-dependent blockade of the NMDA-operated ion channel (Nowak *et al.*, 1984; Mayer *et al.*, 1984), and it has generally been assumed that the actions of Mg^{2+} observed in [^{3}H]-dizocilpine and [^{3}H]-TCP (N-(1-thienyl)-cyclohexyl-3,4-piperidine binding assays are a consequence of Mg^{2+} binding to this site. However, a second Mg^{2+} binding site within the channel was recently reported. Mg^{2+} approaches this site from inside the cell, and displays a reversed voltage-dependence compared to the external site (Johnson & Ascher, 1990). It is not clear which of these sites is responsible for the actions of Mg^{2+} on [^{3}H]-dizocilpine binding, nor is it clear to which Mg^{2+} site argiotoxin$_{636}$ binds. However, the voltage-dependence observed by Priestley *et al.* (1989) is not consistent with an action only at the internal Mg^{2+} site. Further studies will be necessary to resolve this issue.

The gift of argiotoxin$_{636}$ from Natural Product Sciences, Salt Lake City, UT, U.S.A. is gratefully acknowledged.

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(Received August 30, 1990
Revised January 4, 1991
Accepted February 4, 1991)

Br. J. Pharmacol. (1991), **103**, 1377–1380 © Macmillan Press Ltd, 1991

Maintenance of functional activity of human pulmonary arteries after cryopreservation

P. Ellis & [1]E. Müller-Schweinitzer

Pharmacological Research, Sandoz Pharma AG, 4002 Basel, Switzerland

1 Human intrapulmonary arteries have been investigated *in vitro* in fresh tissue or after storage at −190°C in foetal calf serum containing 1.8 M dimethyl sulphoxide.

2 After cryopreservation of the arteries, maximal contractile force was reduced to 76%. This was assessed by the responses (in g) to 10 nM of the thromboxane analogue, U 46619.

3 Constricting agonists such as noradrenaline, 5-hydroxytryptamine, histamine and U 46619 stimulated fresh and frozen/thawed arteries producing pD_2 values similar to the respective values determined on fresh tissues.

4 Endothelium-independent relaxant responses of U 46619-precontracted arteries to prostacyclin (PGI_2), aminophylline and papaverine were generally unchanged after storage. The same was true for relaxant response to the potassium channel activator P-1075 whereas the pD_2 values for SDZ PCO 400, RP 49356 and cromakalim were somewhat diminished.

5 Nevertheless, a significant correlation was obtained when the apparent pD_2 values for all agonists on fresh and frozen/thawed tissues were compared ($P < 0.001$).

6 The evidence suggests that after cryopreservation of human intrapulmonary arteries at −190°C, mechanisms of both contraction and relaxation are well-maintained.

Keywords: Human pulmonary arteries; cryopreservation; K^+ channel activators

Introduction

Animal tissue has been shown in many cases to be of use in human pharmacology and is widely used. Naturally, though, if human tissues were made available, the results obtained from experiments would be the most accurate and predictable for human pharmacology. Human tissue can be obtained from surgery or during autopsy. However, not all human tissue so obtained can be used as it may be diseased when healthy tissue is required or it may be damaged during surgery, by ischaemic periods during and after surgery, by patient age, medication or anaesthesia. Therefore, besides obtaining tissue, the main problems are the diversity of the tissue types available and the irregularity of supply. Once removed from the patient, the tissue has a very short life span and so experiments should start as soon as possible, if not immediately, but this is not always convenient. These factors make studies with human tissue difficult to conduct. Hence the advantage of a simple and reliable method of storing human tissues in such a way as to preserve their functional attributes, is readily apparent. Storage in such a way would also mean that tissue need not all be used at once and several days of experiments could be carried out from the same tissue divided into samples.

Various cryoprotective agents and freezing methods have been tested taking the maximal contractile force of canine saphenous vein strips as a parameter for the maintenance of cell integrity during the freezing-thawing procedure. In those studies the best recovery was obtained when venous segments were immersed in foetal calf serum containing 1.8 M dimethyl sulphoxide (DMSO), slowly frozen to −70°C and then stored at −190°C (Müller-Schweinitzer & Tapparelli, 1987; Müller-Schweinitzer, 1988). Validation studies for this method of cryopreservation have been performed with various blood vessels from different animals. These showed that in addition to functional responses to different agonists this cryopreservation method may also sustain mitochondrial enzyme activity and endogenous prostaglandin synthesis (Müller-Schweinitzer & Tapparelli, 1986), adrenergic nerve function (Müller-Schweinitzer & Tapparelli, 1986; 1987; Thompson *et al.*, 1989), calcium uptake mechanisms (Ebeigbe *et al.*, 1988) and endothelial function (Thompson *et al.*, 1989; Schoeffter & Müller-Schweinitzer, 1990). Moreover, the same method has been applied successfully to preserve specimens of human saphenous veins (Müller-Schweinitzer *et al.*, 1986) and bronchi obtained from surgery and samples of human basilar and cerebral arteries obtained during autopsy (Müller-Schweinitzer, 1988). In all these studies a very good correlation between the affinity parameters for various agonists and antagonists on fresh and frozen/thawed vessels was obtained, indicating the usefulness of this method for the storage of human material for pharmacological studies.

The present study demonstrates that cryopreservation of small human pulmonary arteries in the presence of DMSO is not only able to preserve attributes such as contractile responses but also relaxant effects in response to potassium channel activation.

Methods

Tissue preparation and storage method

Specimens of lung segments, obtained from patients who had undergone surgery for removal of lung carcinoma, were placed in ice cold Krebs-Henseleit solution (composition mM: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, EDTA 0.03) and transported to the laboratory within 1–2 h after removal. Small pulmonary vessels (outer diameter ≈ 1–3 mm) were carefully removed and divided into two groups. Group 1 consisted of 'fresh vessels' which were stored in Krebs-Henseleit solution at 4°C and used within 20 h of surgery. The samples of Group 2, 'frozen/thawed vessels', were placed in 2 ml liquid Nitrogen Storage Ampoules (Gibco AG, Basel, Switzerland) filled with foetal calf serum (FCS) containing 1.8 M dimethylsulphoxide (DMSO) as cryoprotecting agent. The ampoules were placed in a polystyrol box (11 × 11 × 22 cm) and slowly frozen at a mean cooling rate of about 0.6°C min^{-1} in a freezer maintained at −70°C. After 3–20 h the ampoules were transferred

[1] Author for correspondence.

into liquid nitrogen (−190°C) where they were stored until use. Before use the tissues were exposed for 20–60 min to −70°C before being thawed within 2.5 min by placing the ampoules in a 37°C water bath. Thereafter the vessel segments were rinsed in a dish containing Krebs-Henseleit solution at 37°C, cleaned of loose connective tissue and cut into rings (about 2–3 mm in length).

Experimental procedures

The rings were mounted between two stainless steel wire hooks and suspended in 10 ml organ baths containing Krebs-Henseleit solution at 37°C, gassed continuously with 5% CO_2 in O_2. The tension of the rings was recorded isometrically under a resting tension of about 1 g with electromechanical transducers (Statham model UC 3) and a potentiometric recorder. The preparations were allowed to equilibrate for 2–3 h in the bathing medium. During this time the preparations were challenged twice with noradrenaline (1 μM) and the baseline tension of the rings was readjusted to 1 g. Concentration-response curves for agonists were established by cumulative additions, each concentration being added when the maximum effect had been produced by the previous concentration. The contractile response curves were normalized in terms of the percentage of maximal contraction. When relaxant responses to drugs or solvents were investigated, active tone was induced by adding 10 nM of the thromboxane analogue, U 46619. At the end of these concentration-response curves papaverine (300 μM) was added to induce complete relaxation of the vascular rings. This effect was taken as 100% relaxation.

Drugs

The following pharmacological agents were used: (−)-noradrenaline hydrogentartrate, histamine dihydrochloride (Fluka, Buchs, Switzerland), 5-hydroxytryptamine creatinine sulphate (5-HT), U 46619 (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$), papaverine hydrochloride, aminophylline (Sigma, Munich, F.R.G.), PGI_2 (Iloprost, Schering, Berlin, F.R.G.), cromakalim (Beecham Pharmaceuticals, Harlow, Essex, U.K.), P-1075 (N-tert-pentyl-N'-3-pyridyl-N''-cyanoguanidine, Leo Pharmaceuticals, Ballerup, Denmark), RP 49356 (N-methyl-2-(3-pyridyl)-tetrahydrothiopyran-2-carbothioamide-1-oxide, Rhône-Poulenc Vitry, France). SDZ PCO 400 ((−)-(3S,4R)-3,4-dihydro-3-hydroxy-2,2-dimethyl-4-(3-oxo-cyclopent-1-enyloxy)-2H-1-benzopyran-6-carbonitrile) was synthesized at Sandoz. Both U 46619 and cromakalim were dissolved in ethanol and diluted in distilled water to give 1 mM solutions containing 60% ethanol. Both P-1075 and RP49356 were dissolved in ethanol and diluted to give 1 mM solutions containing 5% ethanol. SDZ PCO 400 was dissolved in DMSO (Merck-Schuchardt, Hohenbrunn, F.R.G.) and diluted to give a 1 mM solution containing 5% DMSO. Further dilutions were performed in 5% glucose or physicological salt solution. Aliquots of U 46619 (1 μM) were kept frozen at −20°C. All other compounds were prepared just before use.

Data analysis

Concentration-response curves were analyzed with a linear computer programme and E_{max} (maximal effects) and pD_2 values (negative logarithm of the molar concentration of the agonist producing 50% of E_{max}) were derived from this analysis. Data are given as means ± s.e.mean. Statistical analysis was performed by the unpaired t test with a P value < 0.05 considered to be significant.

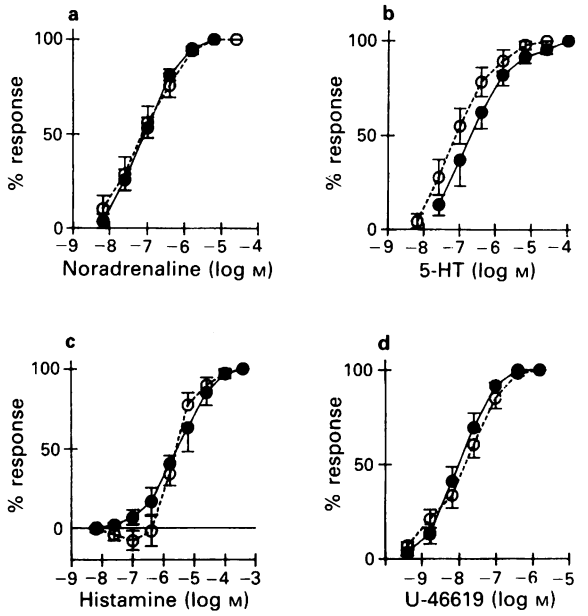

Figure 1 Cumulative concentration-response curves on rings from unfrozen (●) and frozen/thawed (○) human pulmonary arteries for contractile responses to (a) noradrenaline, (b) 5-hydroxytryptamine (5-HT), (c) histamine and (d) U 46619 expressed as percentages of the maximal effect. The bars represent s.e.mean, for each point, n = 5–8.

Results

Contractile responses

The contractile force as assessed by the response in g to 10 nM U 46619 of rings from human pulmonary arteries was diminished to 76% in preparations from frozen/thawed arteries (0.84 ± 0.05 g, n = 72) compared to that produced by fresh tissues (1.11 ± 0.08 g, n = 56, P < 0.005, means ± s.e.mean). Nevertheless all constricting agents tested, i.e., noradrenaline (Figure 1a), 5-HT (Figure 1b), histamine (Figure 1c) and the stable thromboxane analogue U 46619 (Figure 1d) stimulated the vascular preparations of both groups with similar potencies (Figure 1, Table 1). When histamine was investigated, a small relaxant response to low concentrations of the agonist was observed in tissues from 2 out of 4 patients in the group of frozen/thawed arteries (Figure 1c).

Relaxant responses

Responses to agents known to relax vascular smooth muscle were investigated on rings from fresh and frozen/thawed human pulmonary arteries which had been precontracted by a submaximal concentration (approximately EC_{50}) of 10 nM U 46619. The potassium channel activators RP 49356 (Mondot *et al.*, 1988; Figure 2a), P-1075 (Petersen *et al.*, 1978; Figure 2b) and SDZ PCO 400 (Quast *et al.*, 1990; Figure 2c) produced about 40–50% relaxation of precontracted arterial rings from both groups and the apparent pD_2 values were slightly reduced in frozen/thawed arteries (Table 1). Marked differences of both efficacy and potency between fresh and frozen/thawed arteries were observed when cromakalim (Hamilton *et al.*, 1986) was investigated (Figure 2d). This potassium channel activator was significantly more potent and more efficacious on fresh arteries than on frozen/thawed pulmonary vessels. This difference, however, might have been due to the solvent employed, reaching 1% ethanol at the highest cromakalim concentration tested. Solvent controls indicated that ethanol was a significantly more efficacious relaxant agent on fresh than on frozen/thawed arteries (Figure 2f), whereas DMSO was equieffective on arteries from both groups (Figure 2e). Like papaverine and aminophylline, PGI_2 also elicited

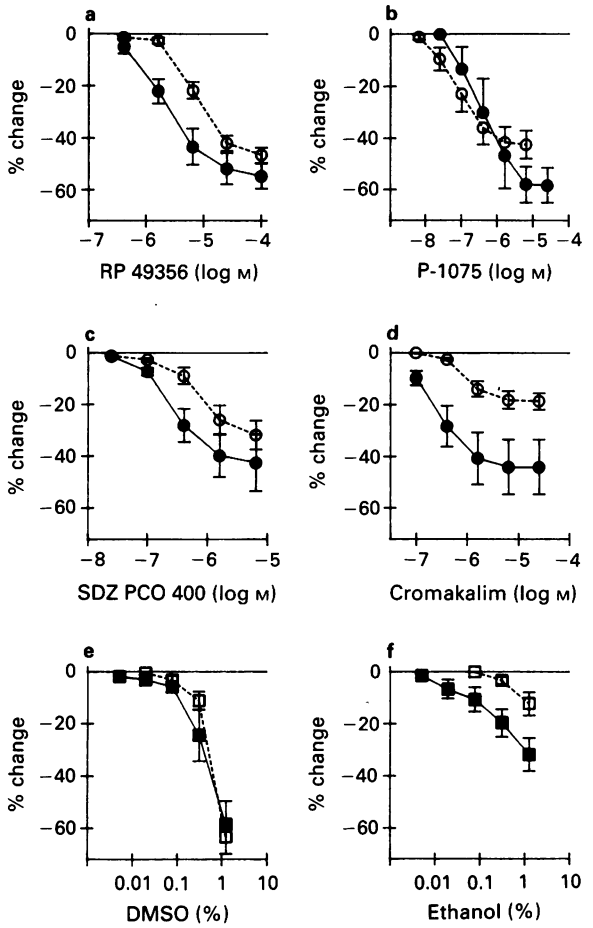

Figure 2 Cumulative concentration-response curves on rings from unfrozen (●) and frozen/thawed (○) human pulmonary arteries for relaxant responses to (a) RP 49356, (b) P-1075, (c) SDZ PCO 400, (d) cromakalim, and the solvents (e) dimethylsulphoxide (DMSO) and (f) ethanol. Active tone was induced by 10 nM U 46619 and the relaxant effects were expressed as percentages of the maximal relaxation induced by 300 μM papaverine. The bars represent s.e.mean; for each point, $n = 4$–9.

marked relaxation of the precontracted rings being the most potent relaxing agonist tested and equipotent on both fresh and frozen/thawed arteries (Table 1). A significant correlation ($r = 0.95$, $P < 0.001$) was obtained when the apparent pD_2 values for all compounds on fresh and frozen/thawed arteries were compared (Figure 3).

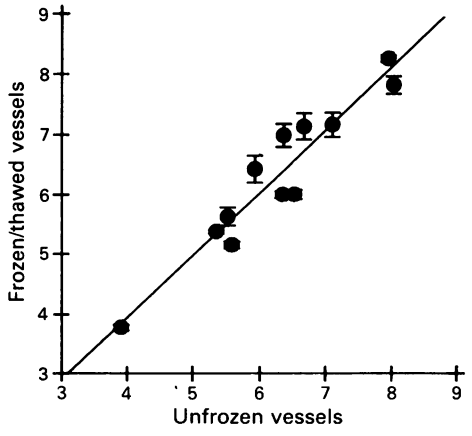

Figure 3 Correlation between pD_2 values for contractile and relaxant agonists on rings from unfrozen (abscissa scale) and frozen/thawed (ordinate scale) human pulmonary arteries. Data were compared by linear regression analysis. $y = 1.04x - 0.22$; $r = 0.95$; $P < 0.001$.

Table 1 Parameters for various contractile and relaxant effects on rings from fresh and frozen/thawed human pulmonary arteries

	Fresh arteries	*Frozen/thawed arteries*
pD_2 values for contractile agents		
Noradrenaline	7.10 ± 0.12 (8)	7.16 ± 0.21 (6)
5-HT	6.67 ± 0.21 (7)	7.13 ± 0.22 (5)
Histamine	5.52 ± 0.33 (4)	5.63 ± 0.16 (8)
U 46619	8.03 ± 0.15 (7)	7.82 ± 0.15 (7)
Carbachol	5.92 ± 0.42 (4)	6.42 ± 0.23 (6)
pD_2 values for relaxant agents		
SDZ PCO 400	6.35 ± 0.14 (6)	6.00 ± 0.06 (9)*
P-1075	6.36 ± 0.31 (4)	6.98 ± 0.20 (6)
RP 49356	5.58 ± 0.14 (6)	5.16 ± 0.06 (6)**
Cromakalim	6.52 ± 0.11 (6)	6.00 ± 0.08 (9)***
PGI_2	7.95 ± 0.24 (6)	8.26 ± 0.06 (7)
Aminophylline	3.91 ± 0.14 (3)	3.71 ± 0.08 (4)
Papaverine	5.35 ± 0.30 (3)	5.38 ± 0.03 (3)
E_{max} (%) of relaxant responses		
SDZ PCO 400	47 ± 9% (6)	37 ± 6% (9)
P-1075	58 ± 7% (4)	44 ± 5% (6)
RP 49356	55 ± 5% (6)	47 ± 3% (6)
Cromakalim	44 ± 11% (6)	19 ± 3% (9)*
PGI_2	73 ± 9% (6)	82 ± 4% (7)
Aminophylline	92 ± 3% (3)	75 ± 11% (3)
Papaverine	100 ± 0% (3)	100 ± 0% (3)

E_{max} = maximal relaxation expressed as percentage of maximal response to papaverine; pD_2 = negative logarithm of the molar concentration producing 50% of maximum response. Data are given as means ± s.e.mean. Number of determinations in parentheses. Differences from fresh control significant at * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Discussion

The present data confirm and extend the usefulness of cryopreservation for storing human vascular tissue for subsequent pharmacological studies. As observed in previous studies on animal (Müller-Schweinitzer, 1988, Thompson *et al.*, 1989; Schoeffter & Müller-Schweinitzer, 1990) and human vascular tissues (Müller-Schweinitzer, 1988), the maximal contractile responses of frozen/thawed human pulmonary arteries was somewhat diminished compared with fresh arteries. However, the calculated apparent pD_2 values for the contractile agents, which were in good agreement with those published by other groups (Houghton & Phillips, 1973; Boe & Simonsson, 1980; Boe *et al.*, 1980; Goldie *et al.*, 1982; Schellenberg & Foster, 1984), proved to be similar on both fresh and frozen/thawed tissues.

The relaxant responses of frozen/thawed arteries to agents acting through various endothelium-independent mechanisms were largely comparable to those produced by fresh tissues. Thus after cryopreservation the maximal relaxant responses, expressed as percentages of that induced by papaverine, to PGI_2, aminophylline and the potassium channel activators SDZ PCO 400, RP 49356 and P-1075 were not significantly different from those obtained with fresh arteries. Only with the potassium channel activator cromakalim was the difference between the relaxant efficacies in both groups of arteries significant. However, this discrepancy might have been due to the relatively high concentration of the solvent ethanol, the relaxant efficacy of which was significantly stronger on fresh than on frozen/thawed arteries. Nevertheless, with the exception of the potassium channel activators cromakalim, RP 49356 and SDZ PCO 400, the potencies in terms of apparent pD_2 values for all relaxant agonists, proved to be similar in arteries from both groups.

One interesting finding was a relaxant response to low concentrations of histamine in frozen/thawed tissues from 2 out of 4 patients. The histamine-induced relaxation in human pul-

monary arteries has been shown to be endothelium-dependent and mediated through enhanced PGI_2 production via stimulation of H_1 histamine receptors (Schellenberg *et al.*, 1986). The present observation, therefore, strongly suggested that the endothelial function was also preserved during cryo-storage. When human material is used, an endothelium-dependent relaxation can be shown only in approximately 50% of the vessels studied (Thom *et al.*, 1987). This variability may be due to various factors such as tissue handling during surgery, ischaemic periods during and after surgery, time between surgery and organ bath study, patient age and disease, pre- and perioperative medication and anaesthesia. Further experiments are planned, to investigate whether the well documented endothelial function of human pulmonary arteries (Furchgott & Zawadzki, 1980; Schellenberg & Foster, 1984; Hadházy *et al.*, 1985; Thom *et al.*, 1987; Greenberg *et al.*, 1987a,b; Dinh Xuan *et al.*, 1989a,b; Crawley *et al.*, 1990) can be maintained during the freezing/thawing process.

In conclusion, the present experiments suggest that the cryopreservation technique used, preserves both contractile and relaxant mechanisms in human intrapulmonary arteries.

The authors gratefully acknowledge the valuable technical assistance of M. Bretz.

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(Received November 26, 1990
Revised January 18, 1991
Accepted February 4, 1991)

Br. J. Pharmacol. (1991), **103**, 1381–1384 © Macmillan Press Ltd, 1991

Inhibition by galanin and by high K^+ of human basophil histamine release triggered by calcium ionophores but not responses induced by anti-IgE, chemotactic peptide or phorbol ester

[1]Håkan Bergstrand, Britta Lundquist & *Bo Ahrén

Department of Pharmacology, AB Draco, Lund and *Department of Pharmacology, University of Lund, Lund, Sweden

1 Galanin concentration-dependently blocked human leukocyte histamine release triggered by the calcium ionophores A23187 and ionomycin. Almost complete inhibition of release was recorded at 410 nM whereas 41 nM galanin mediated close to 50% inhibition of responses.

2 Pretreatment of the cells with pertussis toxin did not influence the inhibitory effects of galanin.

3 Leukocyte responses triggered by anti-IgE, the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (FMLP) or 4β-phorbol 12-myristate 13-acetate (PMA) were not affected by galanin.

4 Ionophore-induced basophil histamine release, but not responses triggered by anti-IgE, FMLP or PMA, was inhibited when cells were challenged in medium containing high potassium (120 mM).

5 We conclude that galanin and a depolarizing medium selectively inhibit ionophore-induced basophil histamine release.

Keywords: Basophil; galanin; histamine release; hyperpolarization; pertussis toxin; potassium

Introduction

Galanin is a widely distributed 29 amino acid residue neuropeptide (Rökaeus, 1987). A high level of galanin-containing nerve fibres is associated with pancreatic islets (Ahrén *et al.*, 1990). The peptide is thought to contribute to the regulation of islet hormone secretion (Ahrén *et al.*, 1988; Dunning & Taborsky, 1988). Galanin inhibits basal and stimulated insulin release; proposed modes of action include (i) an increase in the activity of adenosine 5'-triphosphate (ATP)-dependent K^+ channels with a concomitant hyperpolarization of the cell membrane and opening of voltage-dependent channels for calcium influx (Ahrén *et al.*, 1988; 1989; Dunne *et al.*, 1989; Nilsson *et al.*, 1989), (ii) inhibition of adenylate cyclase (Sharp *et al.*, 1989), (iii) a direct interference with the pertussis-sensitive G protein-linked exocytotic event (Amiranoff *et al.*, 1989; Dunne *et al.*, 1989; Nilsson *et al.*, 1989) and (iv) a calcium-independent mechanism probably involving protein kinase C (PKC) (Amiranoff *et al.*, 1989; Ullrich & Wollheim, 1989).

Degranulation of human basophils with a concomitant histamine release is another functionally well-defined exocytotic process. It can be triggered by different secretagogues including receptor-associating agents like anti-IgE and the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (FMLP), the ionophores A23187 and ionomycin, and the protein kinase C (PKC) activators 4β-phorbol 12-myristate 13-acetate (PMA) and 1,2-isopropylidene-3-decanoyl-*sn*-glycerol ($IpOCOC_9$). These secretagogues all seem to act through different signal transduction pathways some of which possibly involve opening of Ca^{2+} channels (Conroy *et al.*, 1987; Warner *et al.*, 1987; Bergstrand *et al.*, 1988, Warner & McGlashan 1989).

To gain further insight into possible mode(s) of action of galanin, we assessed modulatory effects of the peptide in the basophil histamine release system and compared these to effects on release observed in a depolarizing medium.

[1] Author for correspondence at Department of Pharmacology, Research and Development Laboratories, AB Draco, Box 34, S-22100 Lund, Sweden.

Methods

Human leukocyte histamine release was assessed as detailed elsewhere (Bergstrand *et al.*, 1988) except that reactions were performed half scale. In brief, the leukocyte-rich plasma layer obtained after dextran-glucose-induced red cell sedimentation of EDTA-treated venous blood from healthy volunteers was harvested and the cells were washed in buffer without added Ca^{2+} and Mg^{2+}. The leukocytes were resuspended in HEPES buffer containing 0.6 mM Ca^{2+}, 1.0 mM Mg^{2+} and 0.03% human serum albumin (buffer HACM). The HEPES buffer normally contained 120 mM NaCl and 5 mM KCl; when indicated, experiments were performed in a HEPES buffer containing 5 mM NaCl and 120 mM KCl (high potassium). Volumes of 60 μl cell suspension were placed in plastic tubes (each tube contained basophils in the order of 10^3 to 10^4). Volumes of 0.5 ml of a solution of galanin (in HACM) were added. Control tubes received vehicle. After incubation for 5 min at 37°C, 60 μl of a solution containing the pertinent stimulant was added. Release was allowed to proceed for 40 min. Each assay was run in duplicate. Histamine secreted into the medium and that obtained from the cell pellet by extraction with perchloric acid was assayed spectrophotofluorimetrically after condensation with *o*-phthalaldehyde by the automated procedure essentially as described earlier (Bergstrand *et al.*, 1988). Means of the duplicates of each assay were calculated and the net histamine released into the supernatant was expressed as a percentage of the total cellular histamine content after correction for spontaneous release (rarely exceeding 5%).

Compound 1,2-isopropylidene-3-decanoyl-*sn*-glycerol ($IpOCOC_9$) was synthesized as described previously (Bergstrand *et al.*, 1988). Galanin was obtained from Peninsula Lab, Belmonte, CA, U.S.A. and pertussis toxin from List

Table 1 Effect of galanin on human basophil histamine induced by various secretagogues

	Net histamine release (%)								
Secretagogue	*Control*	*Galanin* (4.1 nM) (%)		*Control*	*Galanin* (41 nM) (%)		*Control*	*Galanin* (410 nM) (%)	
Anti-IgE (1 : 10000)	42.0 ± 14.0 (3)	43.0 ± 13.8 (3)	102	44.2 ± 7.7 (6)	43.2 ± 8.3 (6)	98	42.0 ± 14.0 (3)	42.3 ± 14.7 (3)	101
A23187 (0.06 μM)	6.0 ± 3.8 (3)	0.7 ± 1.5 (3)	11	21.7 ± 7.7 (6)	7.5 ± 3.0 (6)	35*	6.0 ± 3.8 (3)	0 ± 0.6 (3)	0
A23187 (0.2 μM)	40.0 ± 14.8 (3)	39.3 ± 13.1 (3)	98	49.5 ± 8.6 (6)	36.5 ± 8.7 (6)	74*	40.0 ± 14.8 (3)	−1.7 ± 2.3 (3)	0
Ionomycin (0.15 μM)	25.7 ± 15.2 (3)	22.0 ± 15.0 (3)	86	39.8 ± 9.8 (6)	23.0 ± 8.4 (6)	58*	25.7 ± 15.2 (3)	5.3 ± 2.4 (3)	21
FMLP (1 μM)	33.0 ± 15.5 (3)	33.7 ± 15.9 (3)	102	26.5 ± 8.1 (6)	26.3 ± 7.5 (6)	99	33.0 ± 15.5 (3)	30.7 ± 15.2 (3)	93
PMA (160 nM)	48.3 ± 19.9 (3)	49.0 ± 19.1 (3)	101	43.0 ± 9.4 (6)	42.7 ± 8.9 (6)	99	48.3 ± 19.9 (3)	46.7 ± 18.8 (3)	97
$IpOCOC_9$ (206 μM)				49.3 ± 8.2 (3)	49.7 ± 6.6 (3)	101			

Basophil histamine release induced by the indicated concentration of each secretagogue was assessed as described in Methods. Mean ± s.e.mean of net histamine release (expressed as percent of total cellular histamine content) in control cultures and corresponding figures for net histamine release in cultures exposed to the indicated concentration of galanin from the indicated number of experiments are shown for each concentration of galanin. The table also gives histamine release in the presence of galanin as a percentage of control release.

* $P < 0.05$ Wilcoxon's signed rank test on paired observations.

For abbreviations, see text.

Labs., Campbell, CA, U.S.A.; it was reconstituted and handled as recommended by the manufacturer. The sources of other reagents were as specified in Bergstrand et al., (1988).

Results

We initially examined the effects of galanin on human leukocyte histamine release in the concentration-range where the peptide inhibits insulin release from pancreatic β-cells and permeabilized rat insulinoma (RINm5F) cells (Rökeues, 1987; Ahrén *et al.*, 1988; Dunne *et al.*, 1989; Nilsson *et al.*, 1989). We found that galanin at 4.1 nM, 41 nM or 410 nM did not induce a detectable degree of histamine release *per se* (results not shown). However, the peptide concentration-dependently and selectively reduced the release triggered by the calcium ionophores A23187 and ionomycin without affecting responses triggered by anti-IgE, FMLP, PMA or $IpOCOC_9$ (Table 1).

We thereafter showed that pretreatment of cells with 250 ng ml^{-1} pertussis toxin for 2 h at 37°C reduced responses triggered by A23187 (to 52 and 81%), by ionomycin (to 74%) and by FMLP (to 68%). However, pertussis toxin did not reduce the inhibition of ionophore-induced release seen in the presence of galanin; if anything, the inhibitory action of galanin was enhanced with pertussis toxin-treated cells (Table 2).

Finally, the effect of depolarization on histamine release was examined by conducting the challenge in high potassium medium (120 mM KCl). No histamine release *per se* was observed under these circumstances; however, basophil responses triggered by A23187 and ionomycin were completely blocked whereas responses triggered by anti-IgE, FMLP and PMA (Table 3) and $IpOCOC_9$ (not shown) were not affected.

Table 2 Effect of pertussis toxin (PT) treatment of leukocytes on inhibitory effects of galanin on ionophore-triggered human basophil histamine release

	Net histamine release (%)					
	In absence of PT			*In presence of PT*		
Secretagogue	*Control*	*Galanin*	(%)	*Control*	*Galanin*	(%)
A23187 (0.06 μM)	5.2 ± 2.3 (13)	1.8 ± 1.0 (13)	34*	2.7 ± 1.9 (13)	0.5 ± 1.1 (13)	18
A23187 (0.2 μM)	37.7 ± 8.8 (13)	26.6 ± 7.4 (13)	70*	30.7 ± 8.8 (13)	20.3 ± 7.3 (13)	66*
Ionomycin (0.15 μM)	22.9 ± 4.4 (13)	6.9 ± 2.5 (13)	30*	16.9 ± 6.4 (13)	3.3 ± 2.7 (13)	20*
FMLP (1 μM)	44.5 ± 8.7 (6)	44.5 ± 8.4 (6)	100	30.3 ± 10.0 (6)	29.0 ± 10.5 (6)	96

Leukocytes were treated with 250 ng ml^{-1} of pertussis toxin or vehicle for 2 h at 37°C before pretreatment for 5 min with 41 nM galanin or vehicle and challenge with the indicated concentration of A23187, ionomycin or FMLP. Column symbols as in Table 1. For abbreviations, see text.

* $P < 0.05$ Wilcoxon's signed rank test on paired observations.

Table 3 Effect of high potassium medium on human basophil histamine induced by the indicated secretagogues

	Net histamine release %		
Secretagogue	*Control*	*High* K^+	*Change* (%)
Anti-IgE (1 : 10000)	47.7 ± 10.0	50.8 ± 9.3	107
A23187 (0.09 μM)	5.0 ± 4.8	0.8 ± 1.5	16
A23187 (0.28 μM)	32.2 ± 13.2	1.7 ± 1.0	5*
Ionomycin (0.15 μM)	21.8 ± 10.4	1.2 ± 1.4	6*
FMLP (1 μM)	41.7 ± 8.7	33.0 ± 7.1	79
PMA (160 μM)	40.5 ± 10.6	52.7 ± 9.7	130

Basophil histamine release induced by the indicated concentration of each secretagogue was assessed as described in the text. Figures given are means ± s.e.mean ($n = 6$) of net histamine release (expressed as a percentage of total cellular histamine content) in control cultures and the corresponding figures for net histamine release in cultures performed in medium containing 120 mM potassium and 5 mM sodium. The Table also gives release in high K^+ treated cells as % of controls.

* $P < 0.05$ Wilcoxon's signed rank test on paired observations.

Discussion

Galanin inhibits basal and stimulated insulin secretion (for references, see Introduction). Proposed signal transduction pathways for this effect include opening of ATP-dependent K^+ channels leading to a hyperpolarization of the β-cell membrane (which in turn inhibits triggering of the β-cell by secretagogues depolarizing it), an adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and Ca^{2+}-independent mechanism probably involving PKC, and a direct effect on exocytosis. All these actions of galanin seem to be mediated by pertussis-sensitive G-protein(s) (Amiranoff *et al.*, 1989; Dunne *et al.*, 1989; Nilsson *et al.*, 1989; Sharp *et al.*, 1989; Ullrich & Wollheim 1989).

In the present work, we used the human basophil histamine release system to examine whether galanin exerts similar action(s) in another secretory system. We have shown that galanin inhibits human basophil histamine release triggered by the calcium ionophores A23187 and ionomycin in a way which is not affected by pertussis toxin pretreatment. However, galanin does not reduce nor enhance basophil responses induced by four other differently acting secretagogues, i.e., anti-IgE, FMLP, $IpOCOC_9$ or PMA. Moreover, this pattern of selective inhibition by galanin of ionophore-triggered histamine release is mimicked when challenge of cells is performed in depolarizing high potassium medium.

Together with previous reports, these data suggest that galanin inhibits selected secretory responses of pancreatic islets/RINm5F cells and human basophils by different mechanism(s). Thus, insulin release from pancreatic β-cells is a calcium-dependent process and galanin-induced reduction of stimulated insulin release is accompanied by a reduction in intracellular calcium levels (Ahrén *et al.*, 1988; Amiranoff *et al.*, 1989; Dunne *et al.*, 1989). This process most likely involves voltage-sensitive Ca^{2+}-channels (Ahrén *et al.*, 1988). In comparison, anti-IgE- and FMLP-triggered basophil histamine release also express an absolute requirement for extracellular calcium probably made available to the intracellular compartment via putative receptor-operated calcium channels (Bindslev Jensen *et al.*, 1987); these processes are not affected by galanin. Secondly, galanin inhibition of Ca^{2+}-induced insulin secretion from permeabilized RINm5F cells is abolished by pertussis toxin treatment of the cells (Ullrich & Wollheim, 1989) whereas galanin inhibition of ionophore-triggered histamine release is not affected by pertussis toxin treatment. The possibility that the lack of effect of pertussis toxin we record in this respect is due to experimental failure(s) is less likely since pertussis toxin-treated cells display reduced histamine release when triggered by FMLP. Thirdly, PKC activators like PMA and $IpOCOC_9$ trigger insulin as well as histamine release by both calcium-dependent and calcium-independent processes (Bergstrand *et al.*, 1988; Amiranoff *et al.*, 1989). However, whereas PMA-induced insulin release is reduced by galanin, PMA- and $IpOCOC_9$-triggered basophil responses are not. Hence, galanin inhibits histamine release from basophils by a mechanism that seems to be independent of PKC activity, G-proteins and voltage-dependent Ca^{2+} channels. Fourthly, insulin secretion is reduced by galanin with half maximal responses occurring at concentrations below 10 nM independent of the secretagogue employed, whereas galanin inhibition of histamine release is recorded at somewhat higher concentrations and is selective for ionophore triggered cells. Whether these differences in action of galanin reflect interaction of the peptide with different receptor populations on the different cells is a possibility which remains to be examined. In this respect it may be recalled that pertussis toxin treatment converts galanin receptors in RINm5F cells to a state with 5 fold lower affinity for the peptide (for references, see Sharp *et al.*, 1989). Thus, a possibility is that the putative galanin receptors in human basophils are similar to those in RINm5F cells but are not functionally coupled to a pertussis toxin-sensitive G-protein.

Galanin conceivably acts by influencing the basophil membrane potential. Therefore, we compared the pattern of inhibition of secretagogue-triggered release seen with galanin to that observed in a high potassium medium presumably inducing membrane depolarization. In contrast to the findings of a previous report that depolarization reduces IgE receptor-dependent mediator release from rat basophilic leukemia cells (Mohr & Fewtrell, 1987), we found that the selective inhibition by galanin of ionophore-triggered release is exactly mimicked in a high potassium medium.

In conclusion, galanin selectively inhibits basophil histamine release induced by calcium ionophores but not responses triggered by anti-IgE, FMLP or PMA. A similar pattern of inhibition is seen in high potassium medium. This suggests that galanin may inhibit human basophil histamine release by inducing depolarization.

We thank Mrs Ingegerd Källén for revising the text and Mai Broman for excellent typing.
AB Draco is a subsidiary of AB Astra, Södertälje.

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(Received November 1, 1990
Accepted February 5, 1991)

Br. J. Pharmacol. (1991), **103**, 1385–1392

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The pharmacological specificity of N-methyl-D-aspartate receptors in rat cerebral cortex: correspondence between radioligand binding and electrophysiological measurements

Sarah Grimwood, [1]Alan C. Foster & John A. Kemp

Merck Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Eastwick Road, Harlow, Essex, CM20 2QR

1 The pharmacological specificity of N-methyl-D-aspartate (NMDA) receptors has been investigated in the rat cerebral cortex by use of radioligand binding and electrophysiological techniques.

2 A comparison was made between a functional assay (NMDA-induced depolarizations in a rat cortical slice preparation) and NMDA-sensitive L-[^{3}H]-glutamate binding in the same brain region and species, to provide accurate affinity values for agonists and antagonists at the NMDA recognition site.

3 In a preparation of crude postsynaptic densities (PSD) from rat cortex, L-[^{3}H]-glutamate bound with high affinity to an NMDA-sensitive population of sites with K_D (geometric mean (−s.e.mean, +s.e.mean) = 120 (114, 126) nM, B_{max} (mean ± s.e.mean) = 11.4 ± 0.8 pmol mg^{-1} protein and Hill coefficient (mean ± s.e.mean) = 1.2 ± 0.17 (n = 3).

4 There was a good agreement between the relative affinities in radioligand binding and electrophysiological assays for the receptor agonists NMDA, N-methyl-L-aspartate, quinolinate and *trans*-2,3-piperidine dicarboxylate, which are poor substrates of acidic amino acid transport systems. However, agonists which are good substrates for high affinity uptake systems (L- and D-glutamate, L- and D-aspartate, D-aspartate-β-hydroxamate and L-glutamate-γ-hydroxamate) were much weaker in the electrophysiological experiments.

5 Schild analysis of the antagonism of NMDA responses in the rat cortical slice by DL-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonate, D- and DL-2-amino-5-phosphonovalerate, D- and DL-2-amino- 7-phosphonoheptanoate, D-β-aspartylaminomethylphosphonate, D-γ-glutamylglycine and D-α-aminoadipate (D-AA) indicated a competitive interaction with respective pA_2 values of 6.17, 5.62, 5.24, 5.28, 5.20, 5.00, 4.43 and 3.97.

6 In the radioligand binding experiments the same antagonists inhibited only the NMDA-sensitive component of L-[^{3}H]-glutamate binding. IC_{50} values showed a good correlation with the pA_2 values (correlation coefficient = 0.96), with the exception of D-AA which was more potent than anticipated in the binding experiments (IC_{50} = 9.8 μM).

7 These results confirm that NMDA-sensitive L-[^{3}H]-glutamate binding sites represent the NMDA recognition site of the NMDA receptor and provide affinity values for both agonists and antagonists in the rat cerebral cortex, agreeing well with previous estimates in this and other tissues.

Keywords: NMDA; glutamate; aspartate; cortex; radioligand binding; cortical slice; NMDA receptor

Introduction

N-methyl-D-aspartate (NMDA) preferentially activates a subtype of receptors for the excitatory amino acid neurotransmitter L-glutamate (Watkins & Evans, 1981; Fagg *et al.*, 1986). NMDA receptors are a focus of considerable interest due to their specialized role in neuronal signalling, plasticity and proposed involvement in certain neuropathological conditions including epilepsy, spasticity and cerebral ischaemia (Watkins & Collingridge, 1989). The NMDA receptor possesses at least three functional domains: a recognition site for acidic amino acids such as L-glutamate or NMDA, a recognition site for neutral amino acids such as glycine and a cationic channel (Foster & Fagg, 1987b).

The structure-activity requirements of the recognition site for NMDA have been investigated for several years (see Watkins & Olverman, 1987). Accurate measurement of receptor affinities can best be made *in vitro* where the concentrations of drugs are easily controlled. In general, two approaches can be used to obtain such information, analysis of radioligand binding to membrane fragments or measurements of functional responses to receptor activation in tissue preparations. Both types of experiment have inherent advantages and disadvantages. In radioligand binding assays, a direct measurement of affinity for the receptor can be made in conditions under which cellular influences such as uptake and metabolism are eliminated or minimized. However, the value of this technique rests upon the assumption that the site being labelled is the receptor of interest, a supposition which must be supported by a careful comparison of the pharmacology of the binding site and functionally-defined receptor. A functional assay has the advantages that the response measured is evoked by activation of a receptor in 'living tissue', and that the efficacy of pharmacological agents can be determined (e.g. agonist, partial agonist, antagonist). Nevertheless, estimation of agonist affinity in a functional assay is complicated by differing degrees of efficacy, receptor reserve, receptor desensitization, uptake and metabolism of compounds and in the case of excitatory amino acids, neurotoxic effects. Voltage- and patch-clamp studies give a more direct measure of agonist affinity and efficacy but suffer from the disadvantage that they are usually performed on isolated cultured cells taken from immature animals or expressed in the foreign environment of a *Xenopus* oocyte. Ideally, quantitative determination of receptor affinities should be obtained with radioligand binding and functional assays in parallel, although this is rarely attempted.

Binding sites which have the pharmacological characteristics expected of NMDA receptors have been identified by use of several radioligands and mammalian central nervous

[1] Author for correspondence.

system (CNS) preparations. Thus, NMDA-sensitive binding sites for the agonist L-[^{3}H]-glutamate have been demonstrated in synaptic plasma membranes, postsynaptic densities (PSD) and brain sections (Fagg & Matus, 1984; Monaghan & Cotman, 1986; Foster & Fagg, 1987a; Monahan & Michel, 1987), and pharmacologically-similar sites have been detected in crude PSD for the agonists D-[^{3}H]-aspartate and [^{3}H]-NMDA (Foster & Fagg, 1987a). The competitive antagonists D- (and DL-) [^{3}H]-2-amino-5-phosphonovalerate (AP5), DL-[^{3}H]-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonate (CPP) and [^{3}H]-*cis*-4-phosphonomethyl-2-piperidine carboxylate (CGS 19755) also label NMDA-sensitive sites in similar preparations (Olverman *et al.*, 1984; 1986; 1988a,b; Murphy *et al.*, 1987; 1988; Foster & Fagg, 1987a).

In the present study, we have obtained estimates of the affinity of compounds for NMDA receptors in rat cerebral cortex tissue using both radioligand binding and electrophysiological techniques. This was achieved by measuring the NMDA-sensitive binding of L-[^{3}H]-glutamate to crude postsynaptic densities (PSD) from rat cerebral cortex and depolarizing responses in rat cortical slices evoked by NMDA receptor agonists. By comparing values obtained by these two approaches in the same brain region and species, we sought direct evidence that NMDA-sensitive L-[^{3}H]-glutamate binding sites are the transmitter recognition sites of the NMDA receptor and to provide accurate affinity values for agonists and antagonists at cerebro-cortical NMDA receptors. A preliminary account of some of this work has appeared (Foster *et al.*, 1986).

Methods

Preparation of crude postsynaptic densities

Crude PSD from rat cerebral cortex were prepared by a modification of previously described methods (Cohen *et al.*, 1977; Matus & Taff-Jones, 1978; Fagg & Matus, 1984; Foster & Fagg, 1987a). For each preparation, 20 male Sprague-Dawley rats were killed and their whole cerebral cortices dissected and placed immediately in a solution of 0.32 M sucrose, 5 mM Tris-acetate buffer (pH 7.4) on ice. All further procedures were carried out at 4°C. The tissue was homogenized in 10 volumes (w/v) of 0.32 M sucrose, 5 mM Tris-acetate buffer (pH 7.4) and centrifuged at 1000*g* for 10 min. The supernatant (S_1) was collected, the pellet resuspended in a small volume of the sucrose solution and recentrifuged at 1000*g* for 10 min. The second supernatant was added to S_1, and the mixture centrifuged at 17000*g* for 20 min. The pellet (P_2) was suspended in 20 volumes (with respect to original tissue weight) of 5 mM Tris-acetate (pH 8.0) and stirred on ice for 60 min, then centrifuged at 48000*g* for 30 min. The pellet was suspended in 5 mM Tris-acetate (pH 7.0) and an equal volume of 2.4 M sucrose in 5 mM Tris-acetate (pH 7.0) added to give a final sucrose concentration of 1.2 M. An aliquot (15 ml) of this suspension was placed in the bottom of each of 6 Beckman Ultra Clear tubes, and overlayered with 15 ml of 0.9 M sucrose, 5 mM Tris-acetate (pH 7.0), and finally centrifuged in a Beckman SW 28.1 swing-out rotor for 90 min at 83000*g* in a Beckman L8-M Ultra centrifuge. The synaptic plasma membranes were collected from the 0.9–1.2 M sucrose interface, diluted to 80 ml with 5 mM Tris-acetate (pH 7.0) and stirred on ice; 20 ml of 2% Triton X-100 in 5 mM Tris-acetate (pH 7.0) was slowly added, the mixture stirred for a further 15 min and then 100 ml of 5 mM Tris-acetate (pH 7.0) added before centrifugation at 48000*g* for 60 min. The pellets were suspended in approximately 20 ml of 5 mM Tris-acetate buffer (pH 7.0) and frozen at −20°C for at least 15 h. After thawing, membranes were washed a further 3 times by resuspension in 5 mM Tris-acetate (pH 7.0) and centrifugation at 48000*g* for 30 min, and finally resuspended in a small volume of the same buffer (approx. 4 mg membrane protein ml^{-1}) and stored in aliquots at −80°C. Membrane protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin used as a standard.

L-[^{3}H]-glutamate binding assay

Assays were carried out in 1.5 ml polypropylene microfuge tubes, and the incubation mixture consisted of crude PSD (routinely 50 μg protein) and 50 nM L-[^{3}H]-glutamate (except in the case of saturation analyses) in 50 mM Tris-acetate buffer (pH 7.0) in a final volume of 500 μl. Test compounds were added to the incubation tubes on ice before addition of the radioligand and 1 mM L-glutamate was used to determine non-specific binding. Tubes were incubated at 32°C for 30 min and then centrifuged for 4 min in an Eppendorf microfuge. The supernatant was aspirated, the pellets solubilized in 0.5 ml of 2% sodium dodecyl sulphate and radioactivity determined by scintillation counting.

Cortical slice preparation

For *in vitro* neurophysiological experiments, cerebro-cortical slices of rat brain were prepared in a manner similar to that described by Harrison & Simmonds (1985). Coronal sections (500 μm thick) through the level of the corpus callosum were cut in artificial cerebrospinal fluid (aCSF) at room temperature, with an Oxford Vibratome. The aCSF (composition (mM): NaCl 124, $MgSO_4$ 2, KCl 2, KH_2PO_4 1.25, $NaHCO_3$ 25, $CaCl_2$ 2 and glucose 10) was gassed continuously with an O_2/CO_2 (95:5, vol/vol) mixture. Cortical wedges ($\simeq$1 mm wide) were cut from these slices and mounted in a two-compartment bath, with the ventral margin of the cortical tissue traversing a greased slot which separated the chambers, so that the cortical tissue lay almost entirely within one compartment, and the white matter entirely within the other.

The chamber containing the cortical tissue (volume 0.3 ml) was continuously perfused at a rate of 1.5–2 ml min^{-1} with Mg^{2+}-free aCSF, containing 0.1 μM tetrodotoxin to prevent spontaneous depolarizing potentials. The d.c. potential between the two compartments was monitored via Ag/AgCl electrodes with a high-input impedance amplifier and continuously displayed on a chart recorder.

Data analysis

In the radioligand binding experiments, inhibition curves were analysed for a 1-site model by the equation $\%I = \%I_{max}/1 + (IC_{50}/[L])^{nH}$ and for a 2 site model by the equation $\%I = (\%I_{max}(1) \times [L]/IC_{50}(1) + [L]) + (\%I_{max}(2) \times [L]/IC_{50}(2) + [L])$. IC_{50} is the concentration of ligand giving 50% inhibition of specific binding, [L] is the ligand (inhibitor) concentration and nH is the Hill coefficient. Calculations were performed with the data manipulation and analysis software RS1 (Bolt, Beranek and Newman, Inc.).

In the electrophysiological experiments, all of the competitive antagonists studied produced parallel shifts to the right of the NMDA concentration-response curve. Control responses to 5, 10 and 20 μM NMDA were obtained and then the antagonist was continuously perfused for at least 30 min, to allow it to reach equilibrium, before reapplying NMDA. The shift to the right of the concentration-response curve was usually measured at half the control 20 μM NMDA response level. Arunlakshana-Schild plots (Arunlakshana & Schild, 1959) were produced from the concentration-ratios obtained in this way. Linear regression lines were fitted by the method of least squares (RS1).

Materials

L-[^{3}H]-glutamate (40–60 Ci mmol^{-1}) was obtained from Dupont-New England Nuclear. D- and L-glutamate, quinolinate, D-α-aminoadipate (D-AA), D- and L-aspartate, D-aspartate-β-hydroxamate, L-glutamate-γ-hydroxamate, DL-threo-3-hydroxyaspartate, kainate and dihydrokainate were pur-

chased from Sigma. Quisqualate was obtained from Cambridge Research Biochemicals (Cambridge). All other drugs were obtained from Tocris Neuramin (Essex).

Results

L-[^{3}H]-glutamate binding experiments

Saturation analyses Initial experiments using crude PSD from rat cerebral cortex indicated that the specific binding of L-[^{3}H]-glutamate (50 nM) was linear with membrane protein over the range 20–200 μg/assay tube, had a broad pH optimum between pH 6.8 and 7.4 and had attained equilibrium by 30 min at 32°C (results not shown). Routine assay conditions chosen for all subsequent experiments were 50–100 μg membrane protein, pH 7.0 and incubation at 32°C for 30 min. In agreement with previous results obtained with crude PSD from rat whole forebrain (Foster & Fagg, 1987a), the specific binding of L-[^{3}H]-glutamate at a radioligand concentration of 50 nM represented 70% of the total binding, and 76% of the specific binding was maximally displaced by NMDA (see Table 1).

Saturation analyses were carried out over an L-[^{3}H]-glutamate concentration range of 20–1000 nM in the presence and absence of 100 μM NMDA, a concentration which gives maximal displacement of NMDA-sensitive L-[^{3}H]-glutamate binding (Table 1). Figure 1 shows a typical saturation curve and values obtained from three separate experiments indicated the following kinetic values: specific binding: K_D (nM) = 201 (181, 222) (geometric mean, −s.e.mean, +s.e.mean); B_{max} (pmol mg^{-1} protein) = 21.2 ± 2.2 (mean ± s.e.mean); Hill coefficient = 1.0 ± 0.05 (mean ± s.e.mean). NMDA-sensitive binding: K_D = 120 (114, 126); B_{max} = 11.4 ± 0.8; Hill coefficient = 1.2 ± 0.17. Thus, L-[^{3}H]-glutamate bound with higher affinity to the NMDA-sensitive sites which comprised approximately 50% of the total number of specific binding sites.

Inhibition studies A detailed analysis was made of the ability of a number of acidic amino acids to inhibit L-[^{3}H]-glutamate (50 nM) binding to crude PSD from rat cerebral cortex (Tables 1 and 2, Figure 2). In agreement with previous reports (Fagg & Matus, 1984; Foster & Fagg, 1987a), compounds known to be selective NMDA receptor ligands were unable to cause complete inhibition of specific L-[^{3}H]-glutamate binding. Although saturation analyses indicated that the total number of NMDA-sensitive and -insensitive sites were equal (see above), L-[^{3}H]-glutamate had a higher affinity for NMDA-sensitive sites and consequently these comprise the majority of sites labelled at a radioligand concentration of 50 nM. NMDA, N-methyl-L-aspartate (NMLA), D-aspartate, *trans*-2,3-piperidine dicarboxylate (*trans*-2,3-PDA), quinolinate, CPP, D-AP5, D-2-amino-7-phosphonoheptanoate (D-AP7), D-β-aspartylaminomethylphosphonate (D-β-AspAmp) and D-AA all gave maximal inhibition values of 65–90% (Tables 1 and 2). This is consistent with the idea that these compounds inhibit mainly the NMDA-sensitive component of L-[^{3}H]-glutamate binding.

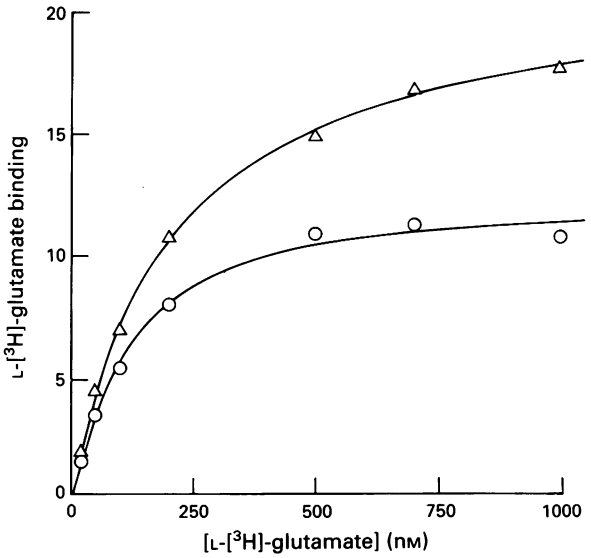

Figure 1 Typical saturation curves for L-[^{3}H]-glutamate binding to crude postsynaptic densities (PSD) from rat cerebral cortex. Assays were carried out over a range of radioligand concentrations from 20–1000 nM in the presence and absence of 100 μM N-methyl-D-aspartate (NMDA). (△) L-[^{3}H]-glutamate binding inhibited by 1 mM L-glutamate (i.e. total specific binding), (○) L-[^{3}H]-glutamate binding inhibited by 100 μM NMDA (i.e. NMDA-sensitive binding).

Compounds known to be 'broad spectrum' excitatory amino acid receptor ligands such as L- and D-glutamate and *cis*-2,3-PDA caused complete inhibition of L-[^{3}H]-glutamate binding, indicating an interaction with both NMDA-sensitive and -insensitive populations of sites. For *cis*-2,3-PDA and D-

Table 1 Inhibition of L-[^{3}H]-glutamate binding to crude postsynaptic densities (PSD) from rat cortex by acidic amino acid agonists

		IC_{50} (μM)	% I_{max}	nH
Glutamate	L	0.31 (0.27, 0.35)	99 ± 1	0.98 ± 0.04
	D	23.0 (18.1, 29.4)	98 ± 2	0.70 ± 0.03
Aspartate	L	1.91 (0.82, 1.62)	89 ± 2	0.75 ± 0.08
	D	1.18 (0.86, 1.57)	82 ± 2	0.90 ± 0.04
N-methyl-aspartate	L	14.5 (11.6, 18.1)	80 ± 5	0.94 ± 0.20
	D	3.68 (2.62, 5.20)	76 ± 6	0.67 ± 0.06
trans-2,3-PDA	DL	6.52 (4.77, 8.91)	77 ± 5	0.78 ± 0.07
cis-2,3-PDA	DL	89.6 (67.5, 119)	102 ± 5	0.64 ± 0.08
Aspartate-β-hydroxamate	L	143 (137, 150)	94 ± 5	0.85 ± 0.13
	D	42.2 (39.1, 45.4)	84 ± 4	1.07 ± 0.22
Glutamate-γ-hydroxamate	L	35.3 (29.5, 42.1)	101 ± 7	0.79 ± 0.11
threo-3-hydroxyaspartate	DL	14.7 (13.6, 15.9)	91 ± 7	0.81 ± 0.20
Quinolinate		153 (122, 193)	90 ± 6	0.77 ± 0.17
Quisqualate	DL (i)	0.04 (0.01, 0.07)	22 ± 2	—
	(ii)	59.6 (57.4, 61.9)	73 ± 2	—
Kainate	(i)	0.40 (0.20, 0.77)	22 ± 2	—
	(ii)	389 (299, 507)	58 ± 8	—
Dihydrokainate	(i)	6.0 (4.8, 7.4)	16 ± 2	—
	(ii)	448 (459, 497)	79 ± 1	—

IC_{50} values are the concentration of drug which inhibited 50% of specific L-[^{3}H]-glutamate binding and are the geometric mean (−s.e.mean, +s.e.mean) of ≥3 separate experiments. % I_{max} is the percentage maximal inhibition of L-[^{3}H]-glutamate binding and nH is the Hill coefficient, these are expressed as arithmetic mean ± s.e.mean.

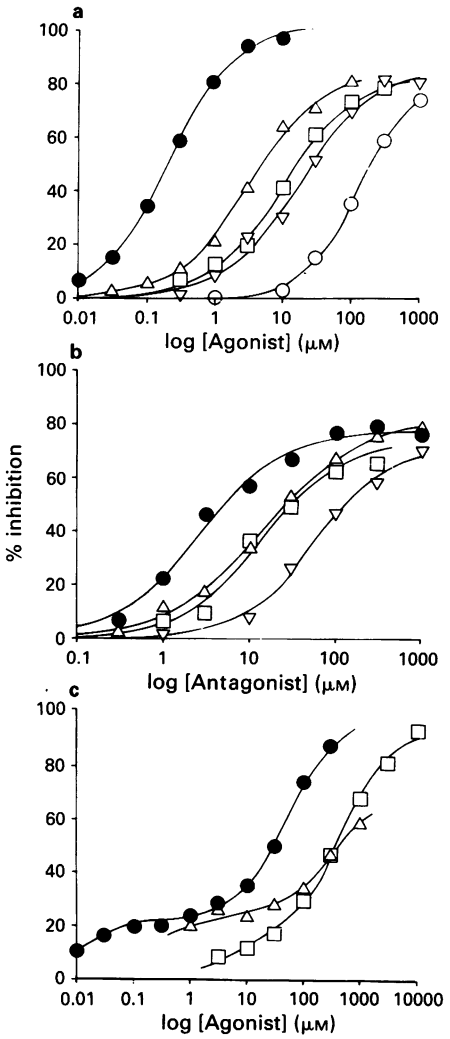

Figure 2 Inhibition of L-[^{3}H]-glutamate binding to crude postsynaptic densities (PSD) from rat cerebral cortex by acidic amino acids. Radioligand concentration was 50 nM. Inhibition curves for: (a) the agonists L-glutamate (●), NMDA (△), *trans*-2,3-PDA (□), NMLA (▽), quinolinate (○); (b) the antagonists D-AP5 (●), D-β-AspAmp (△), D-AP7 (□), D-GG (▽); (c) the agonists quisqualate (●), kainate (△) and dihydrokainate (□). Each curve was from a separate experiment which was repeated 2–3 times with similar results (see Tables 1 and 2 for affinity values). For graphs (a) and (b) percentage inhibition data were analysed using a 1-site fit model; for (c) shallow inhibition curves were best fitted by a 2-site model. Note that L-glutamate, quisqualate and dihydrokainate caused complete inhibition of L-[^{3}H]-glutamate binding, indicating affinity at both NMDA and non-NMDA receptors. For abbreviations, see text.

glutamate, inhibition curves were shallow as reflected by the low Hill coefficients (Table 1). This was also the case with quisqualate, kainate and dihydrokainate, the inhibition curves for which could be resolved into 2 components (Figure 2c). For each of these compounds the low affinity component predominated, representing 58–79% of the binding sites, approximating the proportion expected of NMDA-sensitive sites (see above). The high-affinity component comprised 16–22% of the sites, approximating the proportion of NMDA-insensitive L-[^{3}H]-glutamate binding sites (Table 1). α-Amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) inhibited a minor component of L-[^{3}H]-glutamate binding sites, giving 17.3 ± 2.6% inhibition ($n = 9$) at 100 μM and 29.3 ± 12.2% inhibition ($n = 3$) at 1000 μM. Thus, AMPA appeared to inhibit selectively the NMDA-insensitive population of L-[^{3}H]-glutamate binding sites. However, the small degree of inhibition observed precluded an accurate estimation of the affinity of AMPA.

Table 2 Inhibition of L-[^{3}H]-glutamate binding to crude postsynaptic densities (PSD) from rat cortex by acidic amino acid antagonists

		IC_{50} (μM)	% I_{max}	nH
CPP	DL	1.57 (0.74, 3.21)	68 ± 9	0.81 ± 0.21
AP5	L	49.9 (37.4, 66.4)	80 ± 3	0.83 ± 0.14
	D	2.69 (2.27, 3.12)	84 ± 5	0.71 ± 1.12
	DL	5.10 (4.11, 6.38)	80 ± 3	0.85 ± 0.08
AP7	L	62.9 (50.2, 78.9)	82 ± 2	0.66 ± 0.12
	D	6.17 (3.46, 11.0)	65 ± 6	0.82 ± 0.08
	DL	11.6 (10.9, 12.5)	73 ± 5	0.93 ± 0.01
AA	D	9.8 (9.6, 10.6)	80 ± 1	0.63 ± 0.05
β-AspAmp	D	11.6 (9.07, 14.9)	85 ± 2	0.68 ± 0.06
GG	D	71.0 (54.9, 91.9)	69 ± 2	0.79 ± 0.18
		% inhibition at 300 μM		
AP4	DL	27.8 ± 13.9		
AP6	DL	32.1 ± 9.4		
AP8	DL	18.0 ± 3.9		
GAMS	D	15.4 ± 9.5		
AS	DL	39.5 ± 8.0		

IC_{50}, % I_{max} and nH values are as defined in Table 1 and are the means of ⩾3 separate experiments. For abbreviations, see text.

L-Glutamate was the most potent inhibitor of L-[^{3}H]-glutamate binding to crude PSD from rat cortex and was 74 times more active than D-glutamate. No stereoselectivity was apparent for aspartate, both isomers being potent inhibitors of the binding (Table 1). Amongst selective NMDA agonists, NMDA was most potent followed by *trans*-2,3-PDA, NMLA and quinolinate. Of the antagonists, CPP was the most potent, followed by D-AP5, D-AP7 and D-β-AspAmp; DL-2-amino-4-phosphonobutyric acid (DL-AP4), DL-2-amino-6-phosphonohexanoic acid (DL-AP6) and DL-amino-8-phosphonooctanoic acid (DL-AP8) were all poor inhibitors, as was D-γ-glutamylaminomethylsulphonate (D-GAMS). The L-isomers of AP5 and AP7 were weaker than the corresponding D-isomers. All of the compounds which selectively inhibited NMDA-sensitive binding sites had shallow inhibition curves as reflected by the low Hill coefficients. This was apparent for the agonists NMDA, *trans*-2,3-PDA and quinolinate as well as the competitive antagonists CPP, D-AP5, D-AP7 and D-β-AspAmp.

Four compounds which are inhibitors of glutamate and aspartate uptake were tested for their ability to inhibit L-[^{3}H]-glutamate binding (Table 1). DL-Threo-3-hydroxyaspartate was the most active of these compounds having a potency comparable with NMLA. The maximal inhibition shown by D-aspartate-β-hydroxamate was suggestive of a selective interaction with NMDA-sensitive binding sites, whereas the remaining three compounds caused complete displacement of L-[^{3}H]-glutamate binding.

Cortical slice experiments

Agonists It was not possible to construct full concentration-response curves for acidic amino acid agonists since exposure of cortical slices to high concentrations invariably led to a general reduction of responsiveness, presumably due to neurotoxic effects of the excitatory amino acids. Therefore, a comparison of agonist potencies was made relative to NMDA as a reference compound (Table 3). Amongst agonists thought to be selective for the NMDA receptor, *trans*-2,3-PDA was 2.5 times weaker than NMDA, whereas NMLA was 10 fold weaker and quinolinate 60 fold weaker. These relative potency values were similar to those determined from the receptor binding experiments (Table 3). The isomers of glutamate and aspartate, D-aspartate-β-hydroxamate and L-glutamate-γ-hydroxamate were considerably less potent than NMDA and their relative potency values versus NMDA were all much

Table 3 Relative potencies of acidic amino acid agonists in the rat cortical slice preparation

	Cortical slice *Relative potency*[1]	*Inhibition of* L-[^{3}H]-*glutamate binding to crude PSD from rat cortex* *Relative potency*[1]
NMDA	1	1
NMLA	9.9 ± 0.4	3.9
trans-2,3-PDA	2.8 ± 0.1	1.8
Quinolinate	59.6 ± 4.1	41.6
L-Glutamate	63.1 ± 8.1	0.1
D-Glutamate	39.8 ± 3.0	6.3
L-Aspartate	75.7 ± 9.2	0.5
D-Aspartate	58.7 ± 7.1	0.3
D-Aspartate-β-hydroxamate	89.6 ± 3.0	11.5
L-Glutamate-γ-hydroxamate	139.4 ± 3.6	9.6

[1] NMDA = 1

Relative potencies in the cortical slice were calculated as described in the text from ⩾3 separate experiments. Relative potencies in the L-[^{3}H]-glutamate binding experiments were calculated from the mean values of IC_{50} in Table 1.

greater than would be predicted from the binding assay results (Table 3).

Antagonists Antagonist affinities were obtained by Schild analysis (Arunlakshana & Schild, 1959) of experiments from which concentration-response ratios were calculated from rightward shifts in the NMDA concentration-response curve caused by various concentrations of antagonist (Figure 3). The majority of antagonists tested blocked NMDA responses with

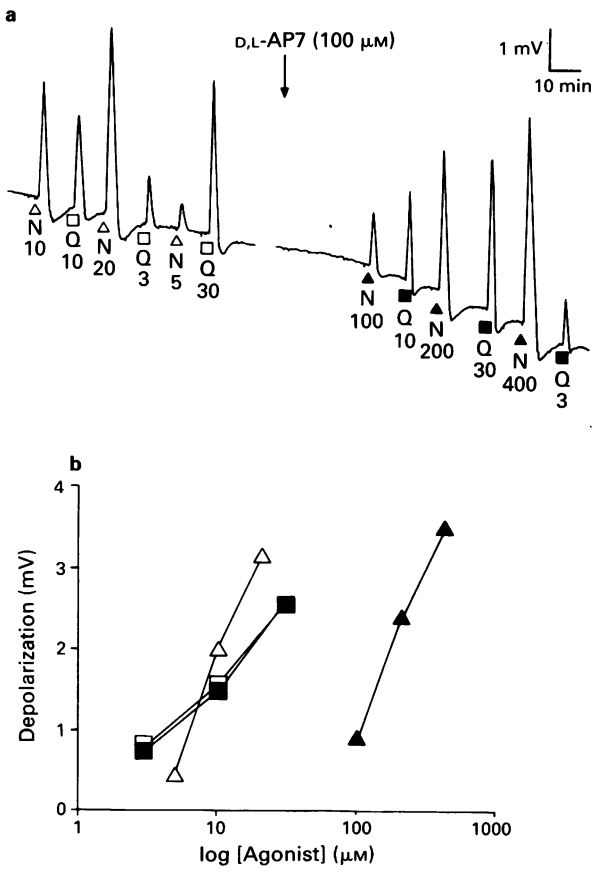

Figure 3 Inhibition of N-methyl-D-aspartate (NMDA) induced depolarizations in the rat cortical slice by D,L-AP7. (a) Trace of depolarizations produced by 1 min applications of NMDA (N) and quisqualate (Q) (numbers indicate concentration in μM). D,L-AP7 (100 μM, from arrow) reduced the responses to NMDA but not quisqualate. (b) Concentration-response curves to NMDA (triangles) and quisqualate (squares) in the absence (open symbols) and presence (filled symbols) of 100 μM D,L-AP7. D,L-AP7 antagonized the NMDA responses, resulting in a parallel shift of the concentration-response curve to the right, whilst it had no effect on the quisqualate responses. For abbreviations, see text.

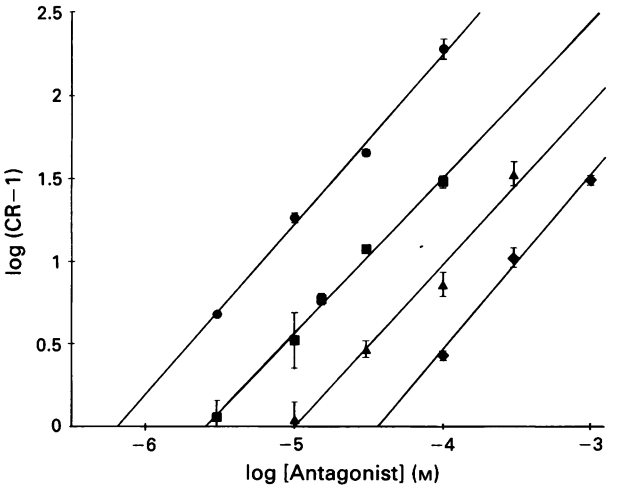

Figure 4 Schild plots for the antagonism of N-methyl-D-aspartate (NMDA) depolarizations in rat cortical slices by CPP (●), D-AP5 (■), D-β-AspAmp (▲) and D-γ-glutamyl glycine (◆). Each point is the mean of at least four experiments. Slopes were not significantly different from 1, indicating a competitive antagonism of NMDA receptors. For abbreviations, see text.

Table 4 Inhibition values for antagonism of NMDA responses in the rat cortical slice by acidic amino acids

	pA_2	*Slope of Schild plot* (±95% confidence limits)
CPP	6.17	1.03 ± 0.08
D-AP5	5.62	0.95 ± 0.17
D-AP7	5.28	0.99 ± 0.16
DL-AP5	5.24	1.04 ± 0.11
DL-AP7	5.20	1.04 ± 0.13[1]
D-β-AspAmp	5.00	0.97 ± 0.16
D-GG	4.43	1.06 ± 0.16
D-AA	3.97	1.14 ± 0.15
D-GAMS	<4	—

[1] Slope for 10–100 μM, 100–300 μM slope = 0.51 ± 0.23.

pA_2 values were derived from Schild analysis as described in the text. For abbreviations, see text.

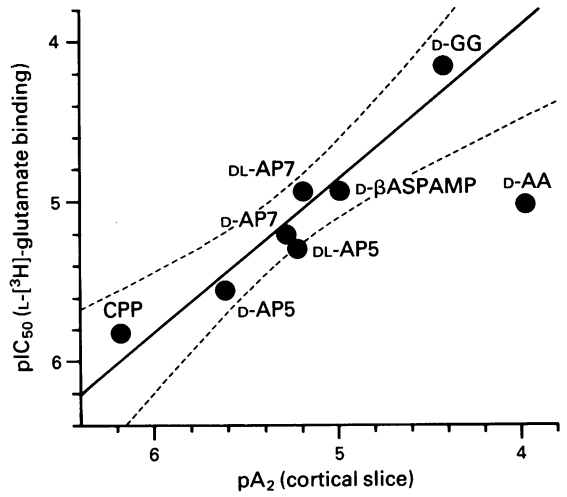

Figure 5 Correlation of affinity values obtained in radioligand binding and electrophysiological experiments for competitive N-methyl-D-aspartate (NMDA) receptor antagonists. pIC_{50} values obtained from NMDA-sensitive L-[^{3}H]-glutamate binding are compared to pA_2 values obtained using the cortical slice preparation. When D-AA was excluded from the analysis (see text) there was an excellent correlation with a correlation coefficient of 0.96. The dotted line indicates the 95% confidence limits. For abbreviations, see text.

no effect on those for quisqualate (Figure 3b) and gave Schild plots with slopes not significantly different from 1 (see Figure 4 and Table 4), indicating a selective, competitive antagonism of NMDA receptors. Table 4 summarizes the pA_2 and Schild plot values obtained. CPP was the most potent antagonist and the D-isomers of AP5 and AP7 were more potent than the racemates, in agreement with the stereoselectivity expected of the NMDA receptor. D-γ-Glutamylglycine (D-GG) was a weak NMDA antagonist which, at the concentrations tested, had little effect on quisqualate or kainate responses (concentration-ratios at 100 μM; quisqualate = 1.27 ± 0.02; kainate = 1.15 ± 0.10, $n = 4$). D-GAMS was a very weak antagonist, having a similar effect at 100 μM against NMDA, quisqualate and kainate responses, with respective concentration-ratios of 1.38 ± 0.17, 1.62 ± 0.16 and 1.46 ± 0.17 ($n = 4$). DL-α-Aminosuberate (DL-AS) was also a weak NMDA receptor antagonist with an apparent pA_2 value of 4.14 ± 0.026 ($n = 4$), calculated from the shift in the NMDA concentration-response curve produced by 100 μM DL-AS.

Affinity values for competitive NMDA receptor antagonists calculated from both electrophysiological and radioligand binding experiments are compared in Figure 5. When D-AA was excluded from the analysis, an excellent correlation was apparent between the two sets of data with a correlation coefficient of 0.96.

Discussion

This study was designed to investigate the pharmacology of NMDA recognition sites in the rat cerebral cortex by both radioligand binding and electrophysiological techniques. With this combined approach we have obtained convincing evidence that NMDA-sensitive L-[^{3}H]-glutamate binding sites in crude PSD are the recognition sites of the NMDA receptor and have provided values of agonist and antagonist affinity at this receptor. In general, these values are in good agreement with previous results obtained with several radioligands (Foster & Fagg, 1987a; Lehmann *et al.*, 1987; Fagg & Baud, 1988; Murphy *et al.*, 1988; Olverman *et al.*, 1988a,b) and in electrophysiological experiments using several CNS regions (Evans *et al.*, 1979; 1982; Jones *et al.*, 1984; Harrison & Simmonds, 1985; Davies *et al.*, 1986).

In rat cortex PSD, L-[^{3}H]-glutamate binds with high affinity to an NMDA-sensitive population of sites which comprise approximately 50% of the total number of L-[^{3}H]-glutamate binding sites labelled under our assay conditions. The K_D of 120 nM for NMDA-sensitive L-[^{3}H]-glutamate binding sites is a lower value (i.e. higher affinity) than those typically reported by other groups who have measured L-[^{3}H]-glutamate binding under conditions where NMDA-sensitive sites predominate (K_D values range from 240–620 nM; Fagg & Matus, 1984; Monaghan & Cotman, 1986; Foster & Fagg, 1987; Monahan & Michel, 1987). In these earlier studies, K_D and B_{max} determinations were made for total L-[^{3}H]-glutamate binding sites with no attempt to differentiate NMDA-sensitive and -insensitive populations. It cannot be assumed, because NMDA inhibits the majority of L-[^{3}H]-glutamate binding sites at 'routine' assay concentrations (usually well below the K_D value), that both K_D and B_{max} values obtained reflect predominantly those of NMDA-sensitive L-[^{3}H]-glutamate binding sites. Indeed, in the present experiments L-[^{3}H]-glutamate bound with higher affinity to the NMDA-sensitive population of sites and at a radioligand concentration of 50 nM, NMDA inhibited 76% of the total specific binding. However, saturation analyses over a range of radioligand concentrations indicated that the total number of NMDA-sensitive and -insensitive sites was equal. Our values for the K_D and B_{max} values of the NMDA-sensitive component of L-[^{3}H]-glutamate binding agree closely with those recently reported by Thedinga *et al.* (1989) for NMDA-sensitive L-[^{3}H]-glutamate binding sites in synaptic membranes from rat telencephalon.

The contention that NMDA-sensitive L-[^{3}H]-glutamate binding sites are recognition sites of the NMDA receptor is based on a comparison of agonist and competitive antagonist potencies in radioligand binding and electrophysiological experiments. In general, estimation of agonist potencies in electrophysiological experiments using intact tissue is made difficult by such factors as uptake and metabolism of compounds, receptor desensitization, varying efficacy and neurotoxic effects. In the case of excitatory amino acids the uptake systems which transport these compounds into neurones and glia (see Fagg & Lane, 1979) are probably the major confounding factor (Ariel *et al.*, 1984; Garthwaite, 1985). In the present experiments, a good agreement between radioligand binding and electrophysiological data was obtained for NMDA, NMLA, *trans*-2,3-PDA and quinolinate, all of which are selective NMDA receptor agonists (Watkins & Evans, 1981; Davies *et al.*, 1982), and which are poor substrates of acidic amino acid transport systems (Balcar & Johnston, 1972; Foster *et al.*, 1983). This was not true, however, of the isomers of glutamate and aspartate, L-glutamate-γ-hydroxamate and D-aspartate-β-hydroxamate, all of which were considerably weaker as agonists on the cortical slice than in the radioligand binding experiments. This can be explained by the fact that all of these compounds are good substrates for acidic amino acid uptake systems in CNS tissue (Balcar & Johnston, 1972; Roberts & Watkins, 1975). Interestingly, D-glutamate was the least discrepant between the two assays and, of these compounds, is the poorest substrate for the high-affinity uptake system but is transported by a low affinity system (Balcar & Johnston, 1972; Benjamin & Quastel, 1976). Nevertheless, the 6 fold difference between the relative potencies of D-glutamate in the electrophysiological and binding experiments indicates, as suggested previously (Cox *et al.*, 1977; Lodge, 1979), that low affinity transport systems also exert a considerable influence on the potency of excitatory amino acids in intact CNS tissue. Thus, it is clear that the affinity of agonists for the NMDA receptor can best be determined by radioligand binding experiments or in situations where uptake systems cannot interfere e.g. concentration-jump experiments on isolated voltage-clamped neurones.

The determination of affinities for competitive antagonists provides a better means of comparing binding sites with receptors. By use of the Schild equation (Arunlakshana & Schild, 1959), affinity values can be derived from electrophysi-

ological data which are theoretically equivalent to affinity values obtained from inhibition data in radioligand binding experiments. An additional advantage for study of NMDA receptors is that the competitive antagonists available are generally poor substrates for acidic amino acid uptake systems (Cross *et al.*, 1986; Lehmann *et al.*, 1987). CPP, AP5, AP7, D-AA and D-β-AspAmp have all been reported as selective competitive NMDA antagonists (Evans *et al.*, 1979; 1982; Jones *et al.*, 1984; Davies *et al.*, 1986; Lehmann *et al.*, 1987). This was confirmed in the present electrophysiological experiments by the fact that these compounds had little or no effect on depolarizations induced by quisqualate or kainate and gave Schild plots with slopes of 1. D-GG was a less selective antagonist, but appeared to be competitive versus NMDA responses. The excellent correlation obtained by comparing the affinities of competitive NMDA receptor antagonists (with the exception of D-AA, see below) in radioligand binding and electrophysiological experiments strongly suggests that NMDA-sensitive L-[^{3}H]-glutamate binding sites are the NMDA recognition sites of the NMDA receptor. The essential structure-activity profile of these competitive NMDA antagonists in our experiments was a preference for D-isomers and longer-chain derivatives such that, amongst the series of α-amino-ω-phosphonocarboxylates, maximal activity was carried by the 5 and 7 carbon members (AP5 and AP7), features, which have been well-documented previously (Evans *et al.*, 1982).

D-AA was an atypical NMDA receptor antagonist having a 10 fold greater affinity in the radioligand binding experiments than in the cortical slice. This may be explained by uptake of D-AA into the slice (Charles & Chang, 1981), which would reduce the effective concentration at the receptor. However, a further anomaly was observed with the analogue DL-AS. In agreement with previous results with spinal cord slices (Evans *et al.*, 1979) this compound was more potent than D-AA as an NMDA antagonist in the cortical slice, but was a poor inhibitor of L-[^{3}H]-glutamate binding (Table 2, see also Fagg & Baud, 1988). Perhaps the antagonism of NMDA responses by DL-AS is mediated by more than one site within the receptor complex.

It is not clear whether the NMDA-insensitive binding sites for L-[^{3}H]-glutamate represent non-NMDA excitatory amino acid receptors. Since L-glutamate is a mixed agonist, and in autoradiographic experiments has been demonstrated to bind to sites which resemble each of the three excitatory amino acid receptor sub-types (Monaghan *et al.*, 1983), the NMDA-insensitive binding of L-[^{3}H]-glutamate may result from an interaction with AMPA and/or kainate receptors. Fagg & Matus (1984) proposed that NMDA-insensitive sites in PSD from rat brain were AMPA receptors based on their sensitivity to inhibition by this compound. The present data tend to support this idea since quisqualate was a good inhibitor of NMDA-insensitive sites (IC_{50} = 40 nM), and was more potent than kainate and dihydrokainate (see Table 1). AMPA itself appeared to be a selective inhibitor of NMDA-insensitive L-[^{3}H]-glutamate binding. Thus, the NMDA-insensitive binding of L-[^{3}H]-glutamate to PSD may represent an interaction with the AMPA receptor, although it should be noted that this population of sites may be heterogeneous and despite the absence of added ions during the assay, could include chloride/calcium-dependent glutamate transport sites (Pin *et al.*, 1984), which are very sensitive to inhibition by quisqualate (Fagg *et al.*, 1983).

The majority of compounds with inhibition profiles suggestive of a selective inhibition of NMDA-sensitive binding sites had shallow inhibition curves as reflected by the low Hill coefficents. This was particularly evident for NMDA itself and the selective NMDA antagonists D-AP5 and D-AP7 (see Table 1). Interestingly, low Hill coefficients were observed for the inhibition of [^{3}H]-CPP binding by a number of acidic amino acids (Murphy *et al.*, 1987) and two components of [^{3}H]-CGS 19755 binding have been identified (Murphy *et al.*, 1988). On the basis of autoradiographic studies of L-[^{3}H]-glutamate and [^{3}H]-CPP binding, Monaghan *et al.* (1988) have suggested the existence of agonist and antagonist-preferring NMDA receptors which have a distinct anatomical localization. It is possible, therefore, that the shallow inhibition curves observed here reflect a heterogeneous population of NMDA receptor types or states in the cortical tissue used for the present studies. If this is the case, it is likely that the present results reflect mainly the agonist-preferring state of the receptor, since we have used L-[^{3}H]-glutamate as a radioligand. A further point of interest is that estimation of the affinity of competitive NMDA receptor antagonists in antagonist binding assays (Murphy *et al.*, 1987; 1988; Olverman *et al.*, 1988) yield values which are up to 10 fold lower (i.e. higher affinity) than those reported here or by other authors using L-[^{3}H]-glutamate as the radioligand. This may suggest that different forms of the NMDA recognition site predominate when labelled by an agonist or antagonist radioligand. If so, the close correspondence in absolute values for the affinities of competitive antagonists between radioligand binding and electrophysiological experiments reported here would suggest that it is the predominant form of NMDA receptor labelled by L-[^{3}H]-glutamate (agonist-preferring?) which is functionally the more important in the rat cerebral cortex.

All of the inhibitors of high-affinity L-glutamate/L-aspartate uptake tested in the present experiments also possess significant affinity for NMDA-sensitive L-[^{3}H]-glutamate binding sites. The most potent of these was DL-threo-3-hydroxyaspartate, which appeared to inhibit selectively NMDA-sensitive L-[^{3}H]-glutamate binding sites. Since these compounds inhibit glutamate transport with affinities in the low micromolar range (Roberts & Watkins, 1975; Benjamin & Quastel, 1976), they cannot be used as selective blockers of high-affinity L-glutamate/L-aspartate uptake.

In conclusion, by directly comparing results from radioligand binding and electrophysiological experiments we have demonstrated that NMDA-sensitive L-[^{3}H]-glutamate binding sites represent the NMDA recognition sites of the NMDA receptor. We have also provided affinity values for both agonists (from the binding experiments) and competitive antagonists (from both binding and electrophysiological experiments) at NMDA receptors in rat cerebral cortex.

We are grateful to Tony Priestley for help and advice with the electrophysiological experiments, to Sue Burton and Eleanor Brawn for expert secretarial assistance and to Andy Butler for his artistic skills.

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(Received October 31, 1990
Revised January 31, 1991
Accepted February 5, 1991)

Br. J. Pharmacol. (1991), **103**, 1393–1398 © Macmillan Press Ltd, 1991

Blockade by the local anaesthetic, tetracaine, of desensitization of Ca-induced Ca release after muscarinic stimulation in smooth muscle

[1]Shigeru Hishinuma & [2]Masaatsu K. Uchida

Department of Molecular Pharmacology, Meiji College of Pharmacy at Setagaya, 1-35-23 Nozawa, Setagaya, Tokyo 154, Japan

1 Desensitization of contractile responses dependent on release of intracellularly stored Ca elicited by carbachol, histamine or caffeine was measured after desensitizing treatment with carbachol or histamine in the presence or absence of local anaesthetics in Ca-free solution containing 2 mM EGTA in the smooth muscle of guinea-pig taenia caecum.

2 Histamine-induced homologous desensitization was inhibited by tetracaine and procainamide. Dibucaine did not exert an inhibitory effect on the desensitization. This is consistent with our previous findings concerning the effects of local anaesthetics on the densitization of histamine H_1-receptors measured under normal physiological conditions.

3 Carbachol induced a functional change of intracellular Ca stores which resulted in heterologous desensitization. Tetracaine completely blocked carbachol-induced desensitization of the caffeine-elicited contraction, but in the case of carbachol-induced desensitization of carbachol- and histamine-elicited contractions, this blocking effect of tetracaine was very weak and absent, respectively. The other local anaesthetics used did not affect the desensitization. These results suggest that the Ca-induced and inositol trisphosphate-induced Ca release mechanisms were both desensitized by carbachol and that the desensitization of the Ca-induced Ca release mechanism was selectively blocked by tetracaine.

Keywords: Desensitization; smooth muscle; muscarinic acetylcholine receptor; histamine H_1-receptor; intracellular Ca store; inositol trisphosphate-induced Ca release; Ca-induced Ca-release; caffeine; tetracaine; dibucaine

Introduction

Desensitization, the phenomenon of diminished cellular responses after prolonged or repeated stimulation with an agonist, is thought to play an important role in cellular homeostasis, but sometimes causes disorders and troubles during drug thereapy. Therefore, it is important to clarify the mechanisms of desensitization and to determine drugs which inhibit the development of desensitization.

Desensitization is classified into two types; homologous, i.e. specific for a given agonist, and heterologous, i.e. not specific for the agonist. In the adenylate cyclase system, it is now generally accepted that receptor phosphorylation is involved in both types of desensitization (Sibley & Lefkowitz, 1985).

Desensitization mechanisms in the signal transduction system via stimulation of phosphatidylinositol turnover (PI turnover) have not been investigated extensively in comparison with those in the adenylate cyclase system, except in the case of α_1-adrenoceptors (Leeb-Lundberg *et al.*, 1987; Bouvier *et al.*, 1987). We have been investigating muscarinic and histamine desensitization of smooth muscle contraction. We have found that contractions dependent on the release of intracellularly stored Ca elicited by carbachol, histamine and caffeine were heterologously desensitized by carbachol, while histamine homologously desensitized the histamine-elicited contraction (Hishinuma & Uchida, 1988). We concluded that desensitization induced by carbachol and histamine was due to the functional changes of intracellular Ca stores and histamine H_1-receptors, respectively. This is consistent with our subsequent findings that carbachol did not desensitize PI turnover via activation of muscarinic and H_1-histamine receptors and that histamine desensitized PI turnover only via activation of the histamine H_1-receptors (Hishinuma & Uchida, 1989).

Local anaesthetics are known to modify the extent of desensitization, for example, they increase the desensitization of nicotinic acetylcholine receptors (Heidmann & Changeux, 1978) and inhibit the desensitization of muscarinic acetylcholine receptors (Magaribuchi *et al.*, 1973; Higuchi *et al.*, 1983). We found in smooth muscle of guinea-pig taenia caecum that histamine-induced homologous desensitization was prevented by some local anaesthetics under normal physiological conditions and that their effects were produced by their specific action rather than by their nonspecific membrane-stabilizing effect (Hishinuma & Uchida, 1987). Local anaesthetics were also shown to have some effects on voltage-dependent Ca channels in the plasma membrane (Spedding & Berg, 1985), on inositol trisphosphate (IP_3)-induced and Ca-induced Ca release channels in intracellular Ca stores (Ahn & Karaki, 1988; Ueno *et al.*, 1987) and on K^+ channels in smooth muscle cells (Ohya *et al.*, 1987).

In this study, we tested whether the carbachol-induced change in intracellular Ca stores and the histamine-induced change in the ability of histamine H_1-receptors to release intracellularly stored Ca were modified by local anaesthetics.

Methods

Guinea pigs of either sex, weighing 250–400 g, were killed by a blow on the neck and were exsanguinated. Strips of taenia caecum were suspended in 10 ml organ baths bubbled with air at 30°C. The bathing solution was normal Locke-Ringer solution of the following composition (mM): NaCl 154, KCl 5.63, $CaCl_2$ 2.16, $MgCl_2$ 2.10, $NaHCO_3$ 5.95, and glucose 5.55. $CaCl_2$ was omitted and 2 mM EGTA was added to the Ca-free solution. The 42 mM K^+-rich solution had the same composition as normal solution but with 117.63 mM NaCl and 42 mM

[1] Present address: Department of Pharmacology, Meiji College of Pharmacy at Tanashi, 1-22-1 Yato, Tanashi, Tokyo 188, Japan.
[2] Author for correspondence.

KCl. Contractile responses were recorded isometrically according to the methods reported by Ohashi *et al.* (1974; 1975) and by Casteels & Raeymaekers (1979) with a slight modification. The muscles were equilibrated in normal solution for 1 h with a resting tension of 0.5 g. As shown in Figure 1, after the muscles were incubated in Ca-free solution for 1 h with a resting tension of 0.05 g, they were soaked in K^+-rich solution and K^+-induced contractions were elicited for 10 min to load Ca into the intracellular Ca stores. The muscles were then replaced in Ca-free solution and exactly 2 min later, carbachol, histamine, or caffeine was added for about 20 s until the peak of the transient tension development was reached (at final concentrations of 10^{-4} M carbachol and histamine, and 5×10^{-2} M caffeine). Muscle tensions generated by high K^+, carbachol, histamine and caffeine were about 6, 5, 3 and 1 g, respectively. These contractile responses in Ca-free solution are induced by release of Ca from the intracellular Ca stores. The muscle strips were then resuspended in normal solution for about 45 min before the next cycle. This cycle was repeated, and the responses in the second cycle were taken as controls. During the third cycle, desensitizing treatment with 10^{-4} M carbachol or 10^{-4} M histamine was performed for 30 min either alone (desensitization), or in the presence of local anaesthetics (desensitization + drug) in Ca-free solution before the K^+-induced contraction (muscles were pretreated with the drug for 5 min before the start of the desensitizing treatment), and then after washing out the drugs with Ca-free solution, K^+-induced contractions and the test responses were recorded. Local anaesthetics were used in a concentration-range such that they did not reduce the K^+-induced contraction. Contractile responses in Ca-free solution were expressed as percentages of the control values in the second cycle. The contractions elicited by carbachol, histamine and caffeine without any local anaesthetic treatment in the third cycle were $100.7 \pm 9.5\%$ ($n = 4$), $104.8 \pm 2.7\%$ ($n = 7$) and $100.8 \pm 8.2\%$ ($n = 5$) of the control values, respectively. These values were used as control for statistical analysis of the effects of local anaesthetics on the contraction in Ca-free solution, when applied without the desensitizing dose of carbachol or histamine (drug alone). Statistical significance was evaluated by Student's t test, and $P = 0.05$ was taken as the upper limit of significance.

Drugs used were histamine dihydrochloride, carbamylcholine chloride and procainamide hydrochloride obtained from Sigma, and caffeine, tetracaine hydrochloride and dibucaine hydrochloride from Wako Pure Chemical Industries, Ltd.

Results

Effects of local anaesthetics on the carbachol-induced desensitization of intracellular Ca stores

Table 1 shows contractile responses in Ca-free solution elicited by 10^{-4} M carbachol, 10^{-4} M histamine and 5×10^{-2} M caffeine after desensitizing treatment with 10^{-4} M carbachol for 30 min in the presence or absence of local anaesthetics in Ca-free solution. After the desensitizing treatment with carbachol in the absence of local anaesthetics (desensitization), contractile responses in Ca-free solution elicited by carbachol, histamine and caffeine were reduced to about 60%, 40%, and 60% of the control, respectively. This heterologous desensitization induced by carbachol was thought to occur due to changes in the intracellular Ca stores (Hishinuma & Uchida, 1988). When high concentrations of local anaesthetics were used, they remained in the muscles even after washing, and reduced K^+-induced contractions probably due to their inhibitory effects on voltage-dependent Ca channels (Spedding & Berg, 1985). Although in the taenia intracellular Ca stores were filled with Ca quite well even in normal solution, contractions in Ca-free solution were larger if high K^+ solution was used to load Ca into the stores (Ohashi *et al.*, 1974; Casteels & Raeymaekers, 1979) and were reduced by the presence of Ca antagonists during Ca-loading procedure with high K^+ solution (Ohashi *et al.*, 1975). These observations indicate that reduction of K^+-induced contractions may bring about failure of Ca-loading into intracellular Ca stores. Therefore, local anaesthetic drugs were used in a concentration-range such that they did not affect the maximal contraction induced by high K^+. There was no significant reduction in the carbachol-, histamine- and caffeine-elicited contractions after treatment with local anaesthetics alone (drug alone), except in the case of carbachol-elicited contraction after treatment with 3×10^{-5} M dibucaine and histamine-elicited contractions after treatment with tetracaine and dibucaine. Desensitization in the absence of local anaesthetics (desensitization) was compared with that in the presence of the local anaesthetics

Table 1 Effects of local anaesthetics on carbachol-induced heterologous desensitization of the contractile response in Ca-free solution elicited by carbachol, histamine or caffeine

			Contractile response elicited by		
Drug	*Conc.* (M)		*Carbachol* (*n*) (% of control)	*Histamine* (*n*) (% of control)	*Caffeine* (*n*) (% of control)
Tetracaine	10^{-4}	Desensitization	56.4 ± 5.6 (12)	30.7 ± 4.3 (4)	69.0 ± 9.7 (4)
		Desensitization + drug	75.7 ± 5.2 (12)*	22.6 ± 5.3 (4)	113.6 ± 12.1 (4)*
		Drug alone	87.1 ± 12.0 (4)	79.2 ± 6.2 (4)†	91.2 ± 9.7 (6)
Procainamide	10^{-3}	Desensitization	62.3 ± 0.8 (4)	45.8 ± 3.0 (3)	57.6 ± 6.4 (4)
		Desensitization + drug	66.9 ± 2.2 (4)	36.3 ± 5.2 (4)	66.8 ± 6.7 (4)
		Drug alone	84.5 ± 7.0 (4)	90.2 ± 11.4 (4)	140.1 ± 10.0 (4)†
Dibucaine	10^{-5}	Desensitization	57.8 ± 5.9 (3)	33.3 ± 2.6 (4)	46.9 ± 3.6 (4)
		Desensitization + drug	60.4 ± 5.0 (4)	30.5 ± 4.9 (4)	45.5 ± 3.9 (4)
		Drug alone	97.3 ± 2.7 (4)	82.4 ± 5.1 (4)†	100.9 ± 5.3 (4)
	3×10^{-5}	Desensitization	56.9 ± 2.1 (6)	44.7 ± 7.5 (4)	71.3 ± 1.0 (4)
		Desensitization + drug	46.9 ± 4.9 (6)	23.3 ± 7.6 (4)	67.8 ± 17.1 (4)
		Drug alone	61.6 ± 3.6 (4)†	77.3 ± 6.3 (4)†	82.9 ± 9.0 (4)

For conditions see Methods. Contractile responses in Ca-free solution after treatment with local anaesthetics for 35 min in the absence of the desensitizing dose of carbachol (drug alone) or after desensitizing treatment with 10^{-4} M carbachol for 30 min in the presence (desensitization + drug) or absence (desensitization) of local anaesthetics in the third cycle were expressed as percentages of the contraction in the second cycle (control) before the drug treatment. Values are means ± s.e.

* $P < 0.05$: compared with desensitization.

† $P < 0.05$: compared with the contraction without any drug treatment in the third cycle whose value is described in Methods.

(desensitization + drug) in each experiment, because the development of desensitization varied to some extent. We found that carbachol-induced desensitization was only blocked by tetracaine, not by dibucaine or procainamide (Table 1). This blocking effect of tetracaine was strikingly observed during the desensitization of the caffeine-elicited contraction, namely on the desensitization of the Ca-induced Ca-release mechanism. On the other hand, the carbachol-induced desensitization of contraction elicited by carbachol was significantly, but not markedly blocked and that elicited by histamine was not blocked by tetracaine. At a lower concentration (3×10^{-5} M), tetracaine did not affect carbachol-induced desensitization of carbachol-elicited contractions (data not shown). Therefore, it appears that tetracaine selectively blocked the desensitization of the Ca-induced Ca release mechanism rather than that of IP_3-induced (receptor-mediated) Ca release mechanism. This block must have occurred through a specific action rather than by its nonspecific membrane-stabilizing action which the other local anaesthetics also possess. It should be noted that carbachol-induced desensitization of the caffeine-elicited contraction was not blocked by procainamide even though the caffeine-elicited contraction was augmented by treatment with procainamide alone.

Effects of local anaesthetics on carbachol-induced change in K^+-induced contraction

Traces of K^+-induced contractions after desensitizing treatment with carbachol or histamine in the presence or absence of local anaesthetics are shown in Figures 1 and 2. We have found that the maximal tension generated by high K^+ was not changed but the shape of its trace was altered by carbachol pretreatment (Hishinuma & Uchida, 1988). That is, the K^+-induced contraction was more rapid and the tonic tension was not maintained after treatment with carbachol as shown in Figure 1. On the other hand, there was no change in the K^+-induced contractions in the histamine-induced desensitized state as shown in Figure 2 and in our previous paper (Hishinuma & Uchida, 1988). All of the local anaesthetics tested (tetracaine, dibucaine and procainamide) reversed the carbachol-induced change in the shape of the K^+-induced contraction to the control shape, although dibucaine and procainamide did not affect carbachol-induced desensitization of

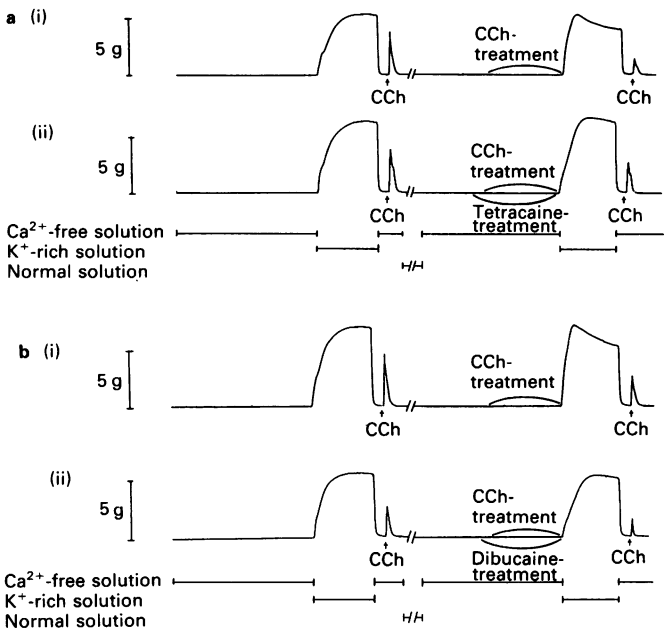

Figure 1 K^+-induced contraction and contractile response in Ca-free solution elicited by carbachol after desensitizing treatment with carbachol in the presence or absence of local anaesthetics in guinea-pig taenia caecum. For conditions see Methods. (a) (i) Traces of the second (control) and third cycles before and after desensitizing treatment with 10^{-4} M carbachol (CCh) for 30 min, respectively. These responses were measured for comparison with the desensitization in the presence of local anaesthetics in the same muscle isolated from the same guinea-pig. One cycle was as follows: the muscle was suspended in Ca-free solution for 1 h, contracted by 42 mM K^+-rich solution for 10 min, and resuspended in Ca-free solution. Then the contractile response in Ca-free solution was elicited by 10^{-4} M carbachol exactly 2 min later. The carbachol was present for about 20 s. The chart speed for about 15 min during the contractile responses was 2.5 times faster than that during preincubation in Ca-free solution for 1 h. The height indicates the tension in g. (a) (ii) Conditions were as in (a(i)) except that the desensitizing treatment with 10^{-4} M carbachol was performed in the presence of 10^{-4} M tetracaine. Muscles were pretreated with tetracaine for 5 min before the start of the desensitizing treatment. (b) (i) Conditions were as in (a(i)). (b) (ii) Conditions were as in (a(ii)) except that 3×10^{-5} M dibucaine was used instead of tetracaine.

Table 2 Effects of local anaesthetics on histamine-induced homologous desensitization of contractile response in Ca-free solution elicited by histamine

Drug	*Conc.* (M)		*Contractile response elicited by histamine* (*n*) (% of control)
Tetracaine	10^{-4}	Desensitization	16.6 ± 1.6 (6)
		Desensitization + drug	40.9 ± 4.4 (6)*
		Drug alone	79.2 ± 6.2 (4)†
Procainamide	10^{-3}	Desensitization	15.8 ± 1.6 (4)
		Desensitization + drug	27.9 ± 2.5 (4)*
		Drug alone	90.2 ± 11.4 (4)
Dibucaine	10^{-5}	Desensitization	17.4 ± 1.4 (8)
		Desensitization + drug	20.9 ± 1.7 (8)
		Drug alone	82.4 ± 5.1 (4)†
	3×10^{-5}	Desensitization	16.1 ± 3.0 (4)
		Desensitization + drug	17.7 ± 2.0 (4)
		Drug alone	77.3 ± 6.3 (4)†

For conditions see Methods. Contractile responses in Ca-free solution after treatment with local anaesthetics for 35 min in the absence of the desensitizing dose of histamine (drug alone) or after desensitizing treatment with 10^{-4} M histamine for 30 min in the presence (desensitization + drug) or absence (desensitization) of local anaesthetics in the third cycle were expressed as percentages of the contraction in the second cycle (control) before the drug treatment. Values are means ± s.e.

** $P < 0.01$ and *** $P < 0.001$, compared with desensitization.

† $P < 0.05$, compared with the contraction without any drug treatment in the third cycle whose value is described in Methods.

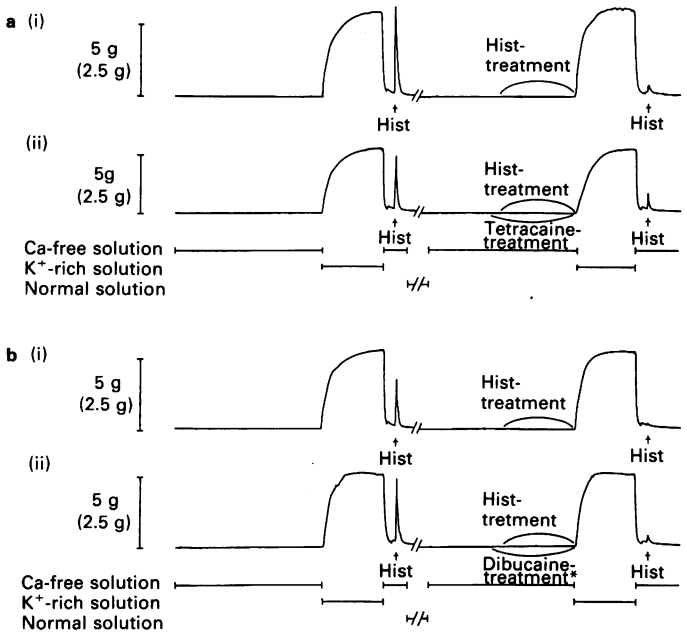

Figure 2 K^+-induced contraction and contractile response in Ca-free solution elicited by histamine after desensitizing treatment with histamine in the presence or absence of local anaesthetics. Conditions were as for Figure 1, except that 10^{-4} M histamine (Hist) was used instead of 10^{-4} M carbachol for the desensitizing treatment and contractile responses in Ca-free solution and that 10^{-5} M dibucaine was used instead of 3×10^{-5} M dibucaine. The tension calibration in parentheses (i.e. 2.5 g) is only for the histamine-elicited contraction after K^+-induced contraction.

the intracellular Ca stores (Table 1). These results indicate that change in the K^+-induced contraction after desensitizing treatment with carbachol was inhibited by the membrane-stabilizing action common to the local anaesthetics tested, differentially from the desensitization of Ca channels in intracellular Ca stores.

Effects of local anaesthetics on histamine-induced desensitization of histamine H_1-receptors

Table 2 shows contractile responses in Ca-free solution elicited by 10^{-4} M histamine after desensitizing treatment with 10^{-4} M histamine for 30 min in the presence or absence of local anaesthetics in Ca-free solution. After the desensitizing treatment with histamine in the absence of local anaesthetics, contractile responses in Ca-free solution elicited by histamine were reduced to less than 20% of control. Since the histamine-induced desensitization was homologous, it may be produced by a functional change in the histamine H_1-receptors. This desensitization was prevented by tetracaine and procainamide, suggesting that tetracaine and procainamide blocked the change in the histamine H_1-receptor itself during the histamine-induced homologous desensitization. In the case of dibucaine, no apparent inhibitory effect on the histamine-induced desensitization was detected. It seems probable that dibucaine suppressed the desensitization, but that it is masked by the additional effect of dibucaine suppressing the contraction on its own. Tetracaine, however, obviously inhibited the desensitization in spite of its inhibitory effect on the histamine-elicited contraction itself. Therefore, we consider that dibucaine has a much smaller inhibitory effect on the histamine-induced desensitization.

Discussion

We have shown that short-term desensitization induced by carbachol was heterologous and occurred at intracellular Ca stores without concomitant reduction in PI turnover by activation of muscarinic and H_1-histamine receptors, whereas that by histamine was homologous and occurred at histamine H_1-receptors with reduction in PI turnover by activation of histamine H_1-receptors in smooth muscle of guinea-pig taenia caecum (Hishinuma & Uchida, 1989).

In carbachol-induced heterologous desensitization, there are two possible causes that could induce reduction of the contraction elicited by release of intracellularly stored Ca: one is prevention of filling of intracellular Ca stores with Ca and the other is changes in the mechanisms of Ca release from the intracellular Ca stores. Receptor-mediated Ca release may be IP_3-induced because (1) PI turnover was stimulated by carbachol during carbachol-elicited contraction in Ca-free solution after the Ca-loading procedure with high K^+ (unpublished observation); (2) muscarinic agonists McN-A-343 and AHR-602, which were reported not to stimulate PI turnover in this muscle preparation (Gardner *et al.*, 1988), did not elicit any tension development in Ca-free solution (unpublished observations); and (3) IP_3 released Ca from intracellular Ca stores in the skinned muscle of guinea-pig taenia caecum (Iino, 1987). Caffeine is thought to release Ca from intracellular stores by enhancing the Ca-induced Ca release mechanism. These two Ca release mechanisms, the IP_3-induced and the Ca-induced release mechanisms, both exist in this smooth muscle preparation. Casteels & Raeymaekers (1979) reported that contraction in Ca-free solution elicited by histamine or caffeine was smaller than that elicited by carbachol probably due to an incomplete release of Ca from the same intracellular Ca store. Iino (1987) and Iino *et al.* (1988) reported that intracellular Ca stores of smooth muscle of guinea-pig taenia caecum consist of two classes: one with both Ca-induced Ca release and IP_3-induced Ca release mechanisms, and the other only with the IP_3-induced Ca release mechanism. These indicate that caffeine releases Ca from IP_3-sensitive Ca stores. If prevention of filling of intracellular Ca stores with Ca after muscarinic stimulation causes desensitization and is blocked by tetracaine, tetracaine would have blocked all the desensitization of contractions elicted by carbachol, histamine and caffeine. Tetracaine, however, blocked only the desensitization of caffeine-elicited contraction (Table 1). Therefore, we consider that IP_3-induced and Ca-induced Ca release mechanisms were both desensitized and that the desensitization of the Ca-induced Ca release mechanism was selectively blocked by tetracaine. It is unclear why tetracaine blocked the desensitization of the carbachol-elicited contraction but did not block that of the histamine-elicited one. One possibility is that carbachol mobilizes intracellularly stored Ca via both IP_3-induced and Ca-induced Ca release, but histamine does it only via IP_3-induced Ca release. From the fact that carbachol induced a much larger PI response and contraction due to release of intracellularly stored Ca than histamine, it is likely that a large amount of Ca, the concentration of which around caffeine-sensitive intracellular Ca stores becomes high enough to cause Ca-induced Ca release mechanism, was released from intracellular Ca stores via muscarinic receptor-mediated signal transduction but not via the H_1-histamine receptor-mediated one.

Local anaesthetics have been reported to inhibit the binding of muscarinic agonists and antagonists to muscarinic receptors at the same agonist site or at some accessory site (Richelson *et al.*, 1978; Taylor *et al.*, 1980; Aguilar *et al.*, 1980; Fairhurst *et al.*, 1980). Contractions elicited by 10^{-4} M carbachol in the presence of tetracaine in normal physiological conditions containing Ca were about 95% of the control values in its absence in smooth muscle of guinea-pig taenia caecum. If the inhibitory effects of local anaesthetics on the agonist binding to muscarinic receptors contribute to the inhibitory effect of tetracaine on development of desensitization by carbachol, the mechanism of IP_3-induced Ca release would have to be desensitized much more easily than that of Ca-induced Ca release, because IP_3-induced Ca release was still desensitized by carbachol even in the presence of tetracaine. However, the other local anaesthetics, dibucaine and procainamide, did not block the desensitization, although

they also have the inhibitory effects on the agonist binding to muscarinic receptors. Therefore, there must be some other factors involved in the blocking effect of tetracaine on the desensitization of the Ca-induced Ca release mechanism. It is more likely that local anaesthetics act on Ca release channels in a structure-specific manner and some of them inhibit the desensitization of the Ca-induced Ca release mechanism. This idea is supported by reports that local anaesthetics act widely on Ca channels including voltage-dependent Ca channels in plasma membrane and IP_3-induced and Ca-induced Ca release channels in intracellular Ca stores (Spedding & Berg, 1985; Ahn & Karaki, 1988; Ueno *et al.*, 1987).

We also found that local anaesthetics inhibited the carbachol-induced change in the K^+-induced contraction (Figure 1). We first thought that the change in the K^+-induced contraction might secondarily cause reduction in contraction dependent on release of intracellularly stored Ca (i.e. desensitization of intracellular Ca stores), because K^+-induced contraction was used for loading of Ca into intracellular Ca stores in our experimental procedure and reduction in K^+-induced contraction was reported to result in reduction in the release of Ca from intracellular Ca stores (Ohashi *et al.*, 1975). If so, all of the local anaesthetics tested should block the desensitization of intracellular Ca stores, because they blocked the change in K^+-induced contraction. However, not all of the local anaesthetics blocked the carbachol-induced desensitization (Table 1). Therefore, it appears that carbachol-induced desensitization of intracellular Ca stores and change in the K^+-induced contraction occurred independently. Acetylcholine has been reported to increase intracellular Na content (Joiner, 1973) and to activate nonselective cation channels in smooth muscle cells (Benham *et al.*, 1985; Inoue & Isenberg, 1990). Increase in intracellular Na will trigger Na/Ca exchange and the Na pump (Bolton, 1979: Brading, 1981). It has been reported that change in distribution of Na, K or Ca across the cell membrane was involved in cholinergic desensitization in intestinal smooth muscle cells (Magaribuchi *et al.*, 1973; Joiner, 1973; James-Kracke & Roufogalis, 1981; Aboulafia *et al.*, 1987). It is possible that change in the K^+-induced contraction after desensitizing treatment with carbachol (Figure 1) was caused by change in ionic distribution across the cell membrane: Na/Ca exchange triggered by the increase in intracellular Na could account for the more rapid rise in tension induced by high K^+ after muscarinic stimulation. The subsequent fade in K^+-induced contraction is likely to be due to the loss of the intracellular Na. Local anaesthetics are known to exert inhibitory effects on ion movements across the membrane. For example, local anaesthetics inhibit Na channels (Lee, 1976; Heidmann & Changeux, 1979), Ca channels (Spedding & Berg, 1985), K channels (Ohya *et al.*, 1987), Na, K-ATPase (Sidek *et al.*, 1984), Ca, Mg-ATPase (Garcia-Martin & Gutierrez-Merino, 1986) and Na/Ca exchange (Michaelis & Michaelis, 1983). It is possible that change in the K^+-induced contraction after muscarinic stimulation was inhibited by these inhibitory effects of local anaesthetics on transmembrane ion movements.

In histamine-induced homologous desensitization, we previously found that tetracaine and procainamide, but not dibucaine, blocked histamine-induced desensitization of the histamine elicited contraction measured under normal physiological conditions and that their effects on the desensitization were not caused by nonspecific membrane-stabilizing actions (Hishinuma & Uchida, 1987). In the presence of extracellular Ca, histamine as well as carbachol is thought to elicit contractions by both the influx of extracellular Ca and the release of intracellularly stored Ca as a result of the signal transduction via activation of histamine H_1-receptors. Therefore, if histamine desensitized histamine H_1-receptors and these local anaesthetics blocked desensitization of the histamine-elicited contraction in normal physiological conditions by inhibiting the change in the function of histamine H_1-receptors, they must also block the desensitization of the contraction dependent on the release of intracellularly stored Ca. As we expected, tetracaine and procainamide blocked the desensitization of the contraction dependent on the release of Ca from intracellular Ca stores, and dibucaine did not exert an apparent inhibitory effect on the desensitization (Table 2). These results suggest that tetracaine and procainamide blocked the functional change in histamine H_1-receptors in histamine-induced homologous desensitization.

We have no information about the desensitizing factor(s) in this muscle, but the observation that carbachol and histamine induced a small sustained contraction about 0.03 g and 0.005 g, respectively, during desensitizing treatment in Ca-free solution (data not shown) in conditions in which PI turnover was not stimulated by these agonists (Hishinuma & Uchida, 1989) indicate that signals of receptor activation may be transduced into the cell in some way including activation of unknown desensitizing factor(s) during the desensitizing treatment even during severe Ca-free conditions. It is possible that these local anaesthetics act on this unknown desensitizing factor(s) to inhibit development of desensitization.

This work was supported by a Grant-in-Aid for Scientific Research (No. 60571062) from the Ministry of Education, Science and Culture of Japan.

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(Received December 7, 1990
Revised January 30, 1991
Accepted February 6, 1991)

Br. J. Pharmacol. (1991), **103**, 1399–1406 © Macmillan Press Ltd, 1991

Naloxone-induced cardiovascular depression in rats that had received chronic morphine-treatment

[1]Soter Dai & [2]Yu Wang

Department of Pharmacology, Faculty of Medicine, University of Hong Kong, 5 Sassoon Road, Hong Kong

1 Cardiovascular changes in response to intravenous injection of naloxone were studied in pentobarbitone-anaesthetized rats which had been given morphine in their drinking water for 1–21 days. The mechanisms of the observed changes were investigated in intact animals and in isolated hearts and tail arteries.

2 In rats that had received chronic morphine-treatment, intravenous administration of naloxone caused immediate decreases in blood pressure, heart rate, left ventricular pressure and $dLVP/dt_{max}$ which were followed by the occurrence of atrial or ventricular extrasystoles and other signs of opiate withdrawal such as faecal passage and muscle twitching.

3 The intensities of the naloxone-precipitated cardiovascular changes were directly related to the duration of chronic morphine pretreatment, reaching statistically significant levels on day 2 or 3 and maximal levels on day 7 or 14. This phenomenon disappeared on days 3 to 14 following opiate withdrawal in animals which had been treated previously with morphine for 21 days.

4 Either atropine or clonidine pretreatment significantly prevented the occurrence of faecal passage or muscle twitching during naloxone-precipitated opiate withdrawal. However, clonidine, but not atropine or yohimbine, abolished the decreases in various haemodynamic parameters. The occurrence of cardiac extrasystoles was not affected.

5 In isolated heart or tail artery preparations from chronically morphine-treated rats, naloxone administration did not elicit reactions which differed from those of the preparations from naive animals.

6 These findings suggest that, under pentobarbitone anaesthesia, the cardiovascular systems of rats that had received chronic morphine treatment exhibit inhibitory, instead of excitatory, reactions to naloxone-precipitated opiate withdrawal. The mechanisms remain unclear, but are unlikely to be due to cholinergic or noradrenergic hyperactivity, nor due to the direct withdrawal reactions of the cardiac or vascular tissues.

Keywords: Naloxone; morphine; circulation; clonidine

Introduction

Opiates are among the oldest pharmacological substances known to man; their pharmacodynamic and pharmacokinetic properties have been extensively studied. However, scientific studies on their cardiovascular effects, especially the circulatory consequences of abrupt withdrawal following the development of physical dependence, have lagged behind other areas of opiate research (Holaday, 1983).

It is generally known that mydriasis, perspiration, tachycardia and elevated blood pressure are symptoms of opiate withdrawal in man (Kolb & Himmelsbach, 1938). Marked increases in blood pressure have also been observed in opiate-dependent patients following administration of the narcotic antagonist, naloxone (Wikler *et al.*, 1953). A similar phenomenon has been observed in experimental animals. The increases in arterial blood pressure produced by naloxone administration in opiate-dependent rats have been thought to serve as a simple, objective, and sensitive measurement in the assessment of narcotic dependence liability (Marshall & Buccafusco, 1985). These observations were made in conscious man or animals. Labrecque & Domino (1974) reported that, in halothane-anaesthetized morphine-dependent cats, naloxone i.v. caused an initial elevation and a subsequent decrease in blood pressure. This indicates the possibility that the pattern of cardiovascular responses of the opiate-dependent animals to abrupt withdrawal may vary between species, and be influenced by the state of consciousness during withdrawal or anaesthetics used.

The present investigation examines the cardiovascular responses of rats that had received chronic morphine-treatment to naloxone administration under pentobarbitone anaesthesia and their relation to the duration of opiate treatment or withdrawal. For studying the mechanisms of the observed changes, similar experiments were carried out in animals pretreated with clonidine, atropine or yohimbine. The responses of the isolated hearts and tail arteries, prepared from chronically morphine-treated rats, to naloxone administration were also studied.

Methods

Animals

Male Sprague-Dawley rats were used. They were housed in a temperature (23 ± 1°C)- and humidity (65 ± 5%)-controlled room and exposed to 12 h light:12 h dark cycle. The animals were allowed free access to tap water and a standard laboratory diet of rat chow (Ralston Purina, U.S.A.).

Chronic morphine treatment

A previously described method for inducing opiate dependence (Ko *et al.*, 1988) was employed for chronic administration of morphine. Rats, weighing 200–400 g, were used. They were given morphine sulphate (Macfarlan Smith, Middlesex) in drinking tap water for 1, 2, 3, 7, 14 or 21 days so that their body weight, when they were used for experiments, was finally 400–450 g. The drug was given in increasing concentrations (48 h apart) of 0.1, 0.2, 0.3 and finally 0.4 mg ml^{-1} (expressed as the salt). The final concentration of morphine sulphate was then continued until the end of the respective treatment periods. Naive animals, which weighed

[1] Author for correspondence at present address: Faculty of Pharmaceutical Sciences, The University of British Columbia, 2146 East Mall, Vancouver, B.C., Canada V6T 1W5.

[2] Present address: Department of Pharmacology, Nanjing Medical University, Nanjing, China.

400–450 g and were never given morphine, were used as day 0 controls.

For studying the responses to naloxone challenge following opiate withdrawal, a group of rats were first given chronic morphine treatment in drinking water for 21 days and then tap water without the opiate to drink for 3, 7 or 14 days before they were used for experiments.

In studies with isolated heart and isolated tail artery preparations, the tissues from the chronically morphine-treated rats were bathed and perfused with physiological solution containing morphine sulphate 0.5 $\mu g\,ml^{-1}$, in order to avoid the possible occurrence of withdrawal effects during the experiment. This concentration of morphine was similar to plasma morphine levels found in dependent rats (Leung *et al.*, 1986). Control preparations, which were isolated from naive animals, did not have morphine in their bathing or perfusing fluid.

Cardiovascular responses to naloxone in intact rats

The rats were anaesthetized with pentobarbitone sodium (Abbot) 60 $mg\,kg^{-1}$ i.p. and were kept warm with a heating lamp. Tracheostomy was performed to ensure a patent airway. The right femoral artery was cannulated for measuring arterial blood pressure by use of a Statham P23ID pressure transducer. A polyethylene tube was introduced into the left ventricle via the exposed right carotid artery and the left ventricular pressure (LVP) was recorded with another Statham P23ID pressure transducer. The index of myocardial contractility, $dLVP/dt_{max}$, was triggered from LVP by a differentiator coupler (Narco Bio-system). The electrocardiogram (ECG) was monitored via standard limb lead II by the use of a Hi-gain preamplifier (E & M Instrument Co., Inc.). The heart rate (HR) was triggered from ECG waveforms by a biotachometer coupler (Narco Bio-system). These variables were continuously displayed on a physiograph (E & M Instrument Co., Inc.).

The anaesthetized animals were then allowed 15 min for equilibrium. Naloxone HCl (Endo) 0.5 $mg\,kg^{-1}$ (expressed as the salt) was then injected i.v. through the cannulated left femoral vein. The cardiovascular parameters, as well as other signs of opiate withdrawal such as muscle twitching and faecal passage, were continuously observed and recorded for 1 h.

For studying mechanisms of action, the effects of various drug pretreatments were examined in separate groups of rats which had been treated chronically with morphine in drinking water for 21 days. They were treated with either 0.9% w/v NaCl (saline) 1 $ml\,kg^{-1}$, atropine sulphate (Merck) 0.5 or 1.0 $mg\,kg^{-1}$, clonidine HCl (Boehringer Ingelheim) 0.05 $mg\,kg^{-1}$, or yohimbine HCl (Sigma) 0.15 $mg\,kg^{-1}$ at 15 min after the cannulation procedure was completed. Fifteen min later, naloxone HCl 0.5 $mg\,kg^{-1}$, i.v. was injected. They were also observed for 1 h. All drugs were freshly dissolved in saline immediately before use, and were injected i.v. through the cannulated left femoral vein. The doses of drugs were expressed as their salts.

Isolated heart preparation

The Langendorff preparation was employed as previously described (Dai, 1989). The rats were killed by a sharp blow on the head. Their hearts were rapidly removed and mounted on a conventional Langendorff apparatus. They were immediately perfused through their aorta with prefiltered Krebs solution which was continuously gassed with 95% O_2 and 5% CO_2 and was kept at 37°C. The composition of the Krebs solution was (mM): NaCl 117.9, $NaHCO_3$ 25.0, KCl 4.7, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.3, dextrose 11.1. Perfusion was provided by a peristaltic pump (Minipuls 2 Gilson, France) at a rate of 13 $ml\,min^{-1}$, maintaining a coronary perfusion pressure (CPP) of 40–60 mmHg which was measured from a side arm of the perfusion cannula with a Statham P23ID pressure transducer. The surface electrocardiogram (ECG) was recorded from needle electrodes positioned on the right atrium and the apex using an Universal coupler (Narco Bio-Systems). Force of ventricular contraction (FC) was measured by an isometric myograph (F-60, Narco Bio-Systems) which was connected with a thread to the apex of the rat heart. Heart rate (HR) was triggered from the strokes of the FC recording by a biotachometer (Narco Bio-Systems). These variables were displayed on a physiograph (MK-IV, Narco Bio-Systems).

Experiments were started after a 15 min period of equilibration. Increasing doses of naloxone HCl 5, 10, 20, 40 and 80 μg (expressed as the salt) were administered at 5 min intervals in a fixed volume of 0.1 ml to the hearts through a fine polyethylene tube which terminated at the tip of the aortic cannula. Naloxone was freshly dissolved in Krebs solution before use. A similar volume of Krebs solution was administered in the same manner as a control before drug administration.

Isolated tail artery

The method described by Xiao & Rand (1989) was used for preparing the rat isolated tail artery. The rats were killed by a sharp blow on the head. Segments of the tail artery, approximately 1 cm long, were dissected out. The artery segment was cannulated at the proximal end; the distal end was tied and an opening made in the wall just below the tie. The segment was then mounted vertically, distal end uppermost, in an organ bath filled with modified Krebs solution which had the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.45, $NaHCO_3$ 25, KH_2PO_4 1.03, dextrose 11.1, disodium edetate 0.067. The artery was then perfused, by use of a peristaltic pump (Minipuls 2, Gilson, France), with the same physiological solution at a rate of about 0.5 $ml\,min^{-1}$ which maintained a perfusion pressure of 45 mmHg. The perfusion pressure was measured with a Statham P23ID pressure transducer and recorded on a physiograph (MK-IV, Narco Bio-Systems). The modified Krebs solutions in the organ bath and in the supply reservoir for perfusion was continuously gassed with 95% O_2 and 5% CO_2. The preparations were kept at 37°C. A 15 min period of perfusion-superfusion was allowed to elapse before the experiments were carried out.

Increasing bolus doses of naloxone HCl 5, 10, 20, 40 and 80 μg (expressed as the salt) were added at 5 min intervals to the organ bath, and any changes in perfusion pressure noted. An equivalent volume of vehicle (modified Krebs solution), acting as a control, was added before naloxone administration. In order to facilitate observations on any possible vasodilator responses to naloxone administration, which would be demonstrated by decreases in perfusion pressure, similar experiments were carried out in separate preparations which were perfused with modified Krebs solution containing noradrenaline bitartrate (Sigma) 1.6×10^{-9} M. The perfusion pressure was raised to, and stabilized at, about 100 mmHg before naloxone administration started.

Statistical analysis

The baseline values of mean blood pressure (MBP), HR, LVP, $dLVP/dt_{max}$ of the anaesthetized rats, and the maximal changes in these variables were expressed as means ± s.e.mean and were analysed by Student's unpaired *t* test. Data obtained from the isolated heart preparations, including the percentage changes in FC, HR and CPP, were expressed and analysed in the same way. The incidence of occurrence of atrial or ventricular extrasystoles and of opiate withdrawal signs in the intact animal studies were analysed by the Chi squared test.

Results

Before naloxone administration

At 15 min after the cannulation procedure was completed, the cardiovascular parameters of the pentobarbitone-

anaesthetized rats were quite stable. The basal values of MBP, HR, LVP and $dLVP/dt_{max}$ before naloxone injection in rats which had been pretreated with morphine in drinking water for various times, as well as those subjected to opiate withdrawal after 3-week chronic morphine treatment, were not significantly different from those in naive animals (Table 1).

After naloxone administration

In naive rats, i.v. injection of naloxone HCl 0.5 mg kg^{-1} caused only negligible changes in either MBP, HR, LVP or $dLVP/dt_{max}$. In chronically morphine-treated animals, there were immediate decreases in all 4 measured parameters following i.v. injection of naloxone (Figure 1). The changes reached their peaks at 30–60 s before slowly recovering to pre-injection levels by 45 min. Some rats exhibited atrial or ventricular extrasystoles and signs of opiate withdrawal, such as defaecation and muscle twitching involving the trunk and limbs. They started at 1–2 min after naloxone injection and occurred in a sporadic and irregular manner throughout the whole 1 h observation period.

Relation to the duration of morphine treatment

Figures 2–5 show the maximal changes in MBP, HR, LVP and $dLVP/dt_{max}$ following naloxone injection in various groups of rats. Only minimal changes were recorded in naive animals. The chronic morphine-treated groups exhibited decreases in all 4 measured parameters in a treatment duration-dependent manner. Statistically significant decreases were observed on day 2–3 and the magnitudes of changes

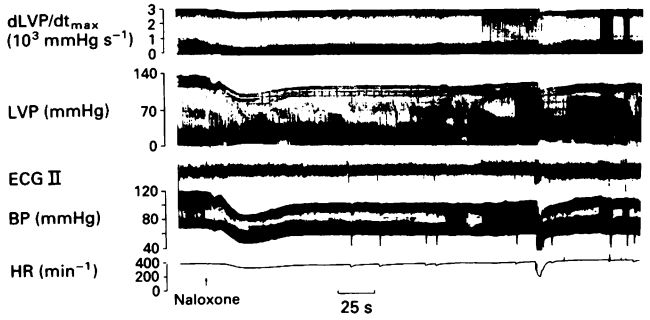

Figure 1 Responses of systemic blood pressure (BP), heart rates (HR), left ventricular pressure (LVP), $dLVP/dt_{max}$ and electrocardiogram (ECG) to naloxone 0.5 mg kg^{-1} i.v. in a pentobarbitone-anaesthetized rat previously treated with morphine in drinking water for 21 days.

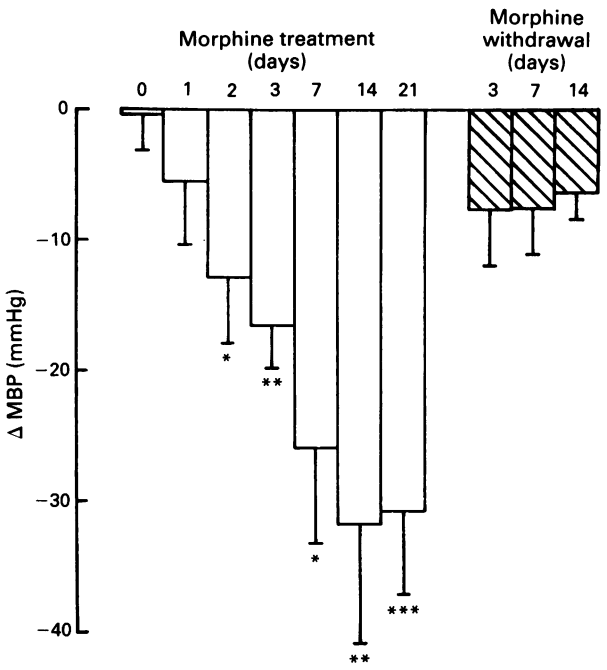

Figure 2 Maximal changes in mean blood pressure (ΔMBP) following i.v. injection of naloxone 0.5 mg kg^{-1} in rats chronically treated with morphine in drinking water for various durations (open columns) and in animals subjected to opiate withdrawal after a 21-day chronic morphine treatment (hatched columns). The values plotted are the means of 7–10 experiments and vertical bars indicate s.e.mean. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ when compared with the control value on day 0.

increased with treatment duration. However, they reached plateau levels on day 7 or 14; a further increase in treatment duration to 21 days did not produce a greater degree of marked changes. In rats subjected to opiate withdrawal after a 3-week chronic morphine treatment, there were noticeable decreases in MBP, LVP and $dLVP/dt_{max}$ after naloxone administration on day 3 after opiate withdrawal; however, the intensities of reductions were not significantly different from those in naive animals. There were further decreases in the magnitudes of these parameters with time and only negligible changes were recorded on day 14. The HR responses to naloxone injection were variable in these groups of rats.

The number of rats exhibiting atrial or ventricular extrasystoles and opiate withdrawal signs in response to naloxone injection also increased in a treatment duration-dependent manner (Table 2). However, the incidence of occurrence of

Table 1 Basal values of the cardiovascular parameters in chronically morphine-treated rats, during the treatment period and after withdrawal, under pentobarbitone anaesthesia

Treatment (duration)	*Number of rats*	*MBP* (mmHg)	*HR* (min^{-1})	*LVP* (mmHg)	*dLVP/dt$_{max}$* (mmHg s^{-1})
Naive	9	118 ± 7	408 ± 41	161 ± 9	3207 ± 184
Morphine (days)					
1	7	132 ± 6	437 ± 12	173 ± 3	3981 ± 19
2	7	111 ± 4	420 ± 13	159 ± 5	3566 ± 28
3	8	121 ± 4	447 ± 13	163 ± 4	3465 ± 67
7	9	105 ± 6	458 ± 14	168 ± 3	3900 ± 67
14	10	121 ± 5	407 ± 10	161 ± 5	3408 ± 179
21	8	113 ± 6	438 ± 10	159 ± 6	3688 ± 133
Morphine withdrawal (days)					
3	8	127 ± 9	420 ± 8	181 ± 12	3029 ± 71
7	8	123 ± 9	446 ± 7	169 ± 3	2863 ± 27
14	8	127 ± 5	447 ± 8	168 ± 6	2825 ± 35

MBP = mean blood pressure: HR = heart rate; LVP = left ventricular systolic pressure; $dLVP/dt_{max}$ = maximal rate of increase of LVP as a measure for myocardial contractility. The values are the means ± s.e.mean.

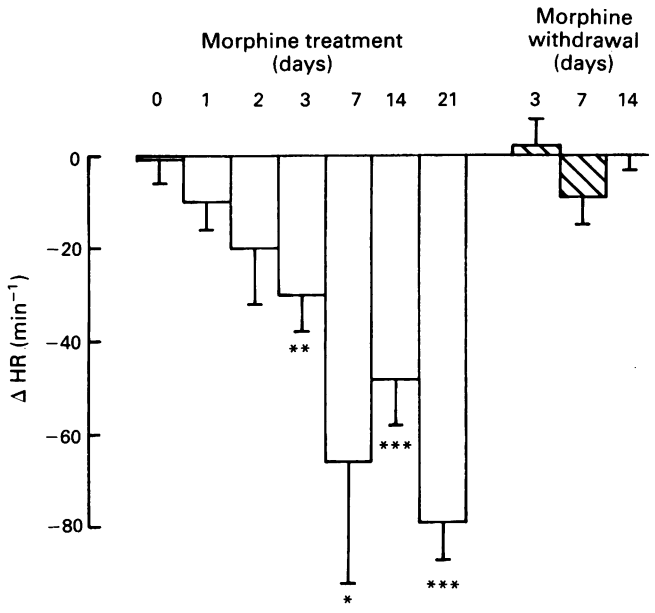

Figure 3 Maximal changes in heart rate (ΔHR) following i.v. injection of naloxone 0.5 mg kg^{-1} in rats chronically treated with morphine in drinking water for various durations (open columns) and in animals subjected to opiate withdrawal after a 21-day chronic morphine treatment (hatched columns). The values plotted are the means of 7–10 experiments and vertical bars indicate s.e.mean. * $P < 0.02$, ** $P < 0.01$, ** $P < 0.001$ when compared with the control value on day 0.

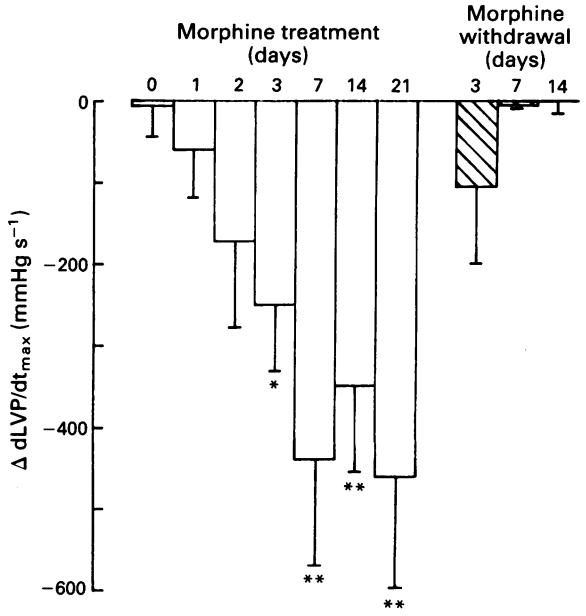

Figure 5 Maximal changes in $dLVP/dt_{max}$ ($\Delta dLVP/dt_{max}$) following i.v. injection of naloxone 0.5 mg kg^{-1} in rats chronically treated with morphine in drinking water for various durations (open columns) and in animals subjected to opiate withdrawal after a 21 day chronic morphine treatment (hatched columns). The values plotted are the means of 7–10 experiments and vertical bars indicate s.e.mean. * $P < 0.02$, ** $P < 0.01$ when compared with the control value on day 0.

cardiac extrasystoles had already reached its peak level of 50–60% after day 3, and no further increases in incidence were noticed even in animals which had been treated for 21 days. On the contrary, there was a steady and progressive increase in the incidence of occurrence of opiate withdrawal signs in relation to the duration of morphine treatment. The highest incidence was recorded in the 21 day treatment group. Following opiate withdrawal, only a small number of the previously morphine-treated rats showed cardiac extrasystoles or signs of opiate withdrawal after naloxone administration and the incidences of their occurrence decreased to zero percent or to levels which were not significantly different from those of the naive group.

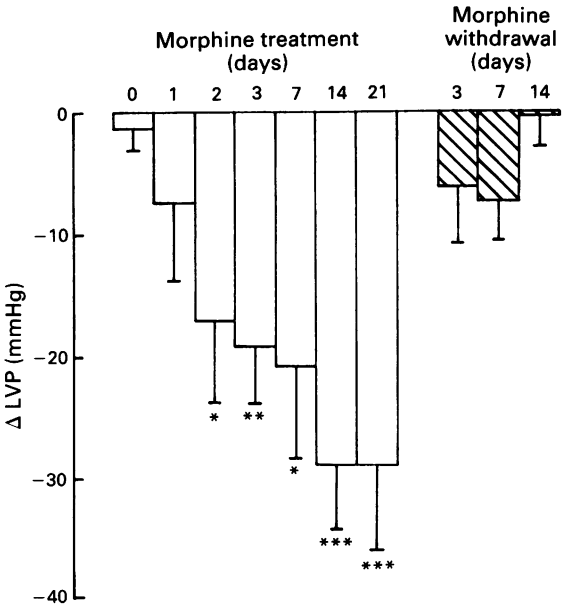

Figure 4 Maximal changes in left ventricular pressure (ΔLVP) following i.v. injection of naloxone 0.5 mg kg^{-1} in rats chronically treated with morphine in drinking water for various durations (open columns) and in animals subjected to opiate withdrawal after a 21-day chronic morphine treatment (hatched columns). The values plotted are the means of 7–10 experiments and vertical bars indicate s.e.mean. * $P < 0.02$, ** $P < 0.01$, *** $P < 0.001$ when compared with the control value on day 0.

Effects of drug pretreatments

The basal values of the cardiovascular parameters of the chronically morphine-treated rats under pentobarbitone anaesthesia at 15 min after the i.v. pretreatments with various drugs are shown in Table 3. In animals pretreated with either atropine 0.5 or 1.0 mg kg^{-1} or yohimbine 0.15 mg kg^{-1}, the values of their MBP, HR, LVP and $dLVP/dt_{max}$ were not significantly different from those of the saline-pretreated controls.

Table 2 Incidences of occurrence of atrial or ventricular extrasystoles and of opiate withdrawal signs after naloxone injection in chronically morphine-treated rats, during the treatment period and after withdrawal, under pentobarbitone anaesthesia

		Incidence of occurrence (%)	
Treatment (duration)	*Number of rats*	*Atrial or ventricular extrasystoles*	*Opiate withdrawal signs*
Naive	9	0	0
Morphine (days)			
1	7	14	0
2	7	43	14
3	8	63*	25
7	9	56*	30
14	10	60*	50*
21	8	63*	63*
Morphine withdrawal (days)			
3	8	0	25
7	8	13	25
14	8	13	0

* $P < 0.05$ when compared with the corresponding values in the naive group.

Table 3 Basal values of the cardiovascular parameters of chronically morphine-treated rats under pentobarbitone anaesthesia at 15 min after pretreatment

Pretreatment (i.v.)	*Number of rats*	*MBP* (mmHg)	*HR* (min^{-1})	*LVP* (mmHg)	*dLVP/dt$_{max}$* (mmHg s^{-1})
Saline 1 ml kg^{-1}	7	119.3 ± 5.5	397.7 ± 21.8	161.1 ± 5.9	4062.9 ± 120.5
Atropine (mg kg^{-1})					
0.5	6	117.5 ± 7.8	428.0 ± 16.0	161.8 ± 9.5	3866.7 ± 121.0
1.0	7	113.6 ± 5.7	390.0 ± 4.0	147.7 ± 7.1	3875.7 ± 97.6
Clonidine					
0.05 mg kg^{-1}	7	85.4 ± 5.0**	324.0 ± 12.0*	123.4 ± 3.9***	3212.7 ± 165.9**
Yohimbine					
0.15 mg kg^{-1}	7	106.6 ± 5.4	414.9 ± 18.5	145.3 ± 5.4	3854.3 ± 96.6

MBP = mean blood pressure; HR = heart rate; LVP = left ventricular systolic pressure; dLVP/dt$_{max}$ = maximal rate of increase of LVP as a measure for myocardial contractility. The values are the means ± s.e.mean.
* $P < 0.02$; ** $P < 0.01$; *** $P < 0.001$ when compared with the corresponding values in the saline-pretreated controls.

In contrast, the clonidine-pretreated rats exhibited significantly lower values in all 4 parameters ($P < 0.02$–0.001).

The effects of various drug pretreatments on the cardiovascular responses to naloxone injection and on the occurrence of opiate withdrawal signs are shown in Tables 4 and 5. In the saline-pretreated controls, naloxone 0.5 mg kg^{-1} i.v. caused immediate decreases in MBP, HR, LVP and dLVP/dt$_{max}$ which were followed by slow recovery to pre-injection levels. Some of the animals exhibited atrial or ventricular extrasystoles (71.4%) and signs of opiate withdrawal (71.4%). The atropine- or yohimbine-pretreated rats had essentially similar cardiovascular responses to naloxone administration. The intensities of decreases in MBP, HR, LVP and dLVP/dt$_{max}$ and the incidence of occurrence of atrial or ventricular extrasystoles in these animals were not significantly different from those of the saline-pretreated controls. However, the incidence of occurrence of opiate withdrawal signs were significantly lower in rats pretreated with atropine 0.5 or 1.0 mg kg^{-1} ($P < 0.05$ or 0.001, respectively) while the incidence in the yohimbine-pretreated animals was not significantly different from that in the saline-pretreated controls. In rats pretreated with clonidine 0.05 mg kg^{-1}, naloxone i.v. caused relatively few changes in the measured cardiovascular parameters. The responses of their MBP, LVP and dLVP/dt$_{max}$ were significantly different from those of the saline-pretreated controls. None of them showed signs of opiate withdrawal. However, atrial or ventricular extrasystoles still occurred in 3 out of 7 animals (42.9%).

Isolated heart preparations

In isolated hearts prepared from naive rats, administration of saline 0.1 ml caused slight decreases in FC, HR and CPP (Figure 6). Increasing doses of naloxone did not produce a significantly different pattern or degree of changes in HR or CPP, but tended to increase the FC. The responses of FC to naloxone 20 and 80 μg were significantly different from that to saline administration ($P < 0.05$ for both).

The basal values of FC, HR and CPP before naloxone administration in isolated hearts prepared from chronically

Table 4 Effects of atropine, clonidine or yohimbine pretreatment on the maximal changes in cardiovascular parameters following naloxone i.v. in chronically morphine-treated rats under pentobarbitone anaesthesia

		Maximal changes			
Pretreatment (i.v.)	*Number of rats*	*MBP* (mmHg)	*HR* (min^{-1})	*LVP* (mmHg)	*dLVP/dt$_{max}$* (mmHg s^{-1})
Saline 1 ml kg^{-1}	7	−28.0 ± 5.4	−49.7 ± 12.4	−29.1 ± 5.3	−480.0 ± 95.5
Atropine (mg kg^{-1})					
0.5	6	−28.8 ± 7.2	−30.0 ± 10.2	−31.8 ± 6.1	−445.0 ± 158.6
1.0	7	−22.9 ± 2.7	−28.0 ± 7.7	−28.9 ± 5.0	−302.9 ± 45.5
Clonidine					
0.05 mg kg^{-1}	7	+0.9 ± 3.0**	−12.0 ± 11.4	−0.6 ± 4.5*	+16.1 ± 83.9*
Yohimbine					
0.15 mg kg^{-1}	7	−21.0 ± 4.7	−59.3 ± 11.0	−18.6 ± 5.2	−446.3 ± 83.1

MBP = mean blood pressure; HR = heart rate; LVP = left ventricular systolic pressure; dLVP/dt$_{max}$ = maximal rate of increase of LVP as a measure for myocardial contractility. The values are the means ± s.e.mean.
* $P < 0.01$; ** $P < 0.001$ when compared with the corresponding values in the saline-pretreated controls.

Table 5 Effects of atropine, clonidine or yohimbine pretreatment on the incidence of occurrence of atrial or ventricular extrasystoles and of opiate withdrawal signs after naloxone administration in chronic morphine-treated rats under pentobarbitone anaesthesia

		Incidence of occurrence (%)	
Pretreatment (i.v.)	*Number of rats*	*Atrial or ventricular extrasystoles*	*Opiate withdrawal signs*
Saline 1 ml kg^{-1}	7	71.4	71.4
Atropine (mg kg^{-})			
0.5	6	66.7	16.6*
1.0	7	42.9	0**
Clonidine			
0.05 mg kg^{-1}	7	42.9	0**
Yohimbine			
0.15 mg kg^{-1}	7	57.1	42.9

* $P < 0.05$; ** $P < 0.001$ when compared with the corresponding values in the saline-treated controls.

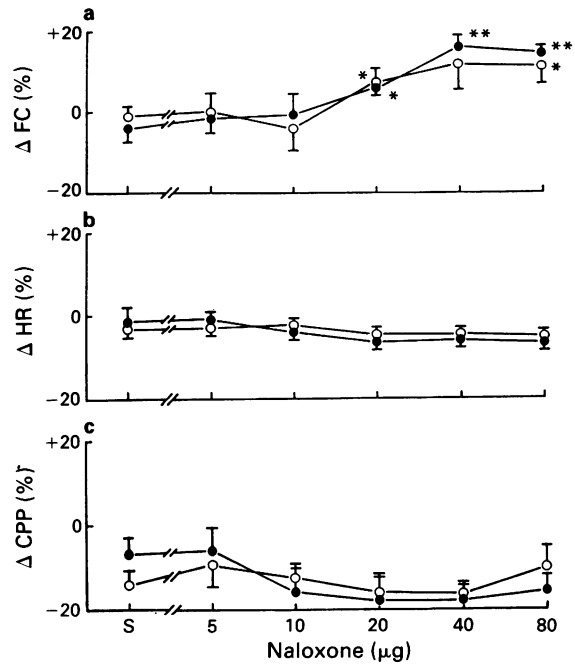

Figure 6 Changes in (a) force of contraction (Δ FC), (b) heart rate (Δ HR) and (c) coronary perfusion pressure (Δ CPP) in response to administration of saline (S) or graded doses of naloxone in the isolated hearts prepared from the naive (○) or chronically morphine-treated (●) rats. $n = 9$ in both groups. The values plotted are the means with s.e.mean shown by vertical bars. * $P < 0.05$, ** $P < 0.01$ when compared with the responses of the same group of preparation to saline administration.

morphine-treated rats were essentially similar to those in the naive controls. Following naloxone administration, the changes in their FC, HR and CPP were also similar to those of the naive controls. Naloxone 20, 40 and 80 μg caused significant increases in FC ($P < 0.05$, 0.01 and 0.01 respectively). However, the magnitudes of their responses to naloxone were not significantly different from those of the naive controls (Figure 6).

In both groups of preparations, administration of naloxone, even at the highest dose of 80 μg, did not markedly affect the cardiac rhythm.

Isolated tail artery preparations

In the isolated tail arteries prepared from either naive or chronically morphine-treated rats, addition of naloxone 5, 10, 20, 40 or 80 μg did not produce any changes in perfusion pressure. In the presence of noradrenaline 1.6×10^{-9} M in the perfusion fluid, the tissues in both groups exhibited similar vasoconstrictor responses. Increasing doses of naloxone also did not induce any changes in their perfusion pressures (data not shown). The viability of these tissues was verified at the end of the experiments because their vasoconstrictor responses to noradrenaline bitartrate 2 μg (expressed as the salt), which was added to the organ bath, could be demonstrated by increases in perfusion pressure of 10–20 mmHg.

Discussion

The present method of administering morphine in drinking water has been shown to be effective in inducing opiate dependence (Badawy *et al.*, 1982). A previous study has revealed that a significant degree of the withdrawal syndrome precipitated by naloxone can be observed at 2 days after starting administration of morphine (Leung *et al.*, 1986). It is, therefore, reasonable to believe that the chronically morphine-treated rats used in the present study were indeed opiate-dependent and that their physiological changes following naloxone administration resulted from the naloxone-precipitated opiate withdrawal.

The current investigation revealed that the baseline values of the cardiovascular parameters of the chronically morphine-treated rats were not significantly different from those of naive animals. This is not at variance with those previously reported (Ko *et al.*, 1988). It was found in this study that rats chronically treated with morphine exhibited remarkable decreases in MBP, HR, LVP and $dLVP/dt_{max}$ and occurrence of cardiac extrasystoles in response to naloxone 0.5 mg kg^{-1}, i.v. It has also been shown that these cardiovascular changes are the manifestations of the opiate withdrawal syndrome occurring in the pentobarbitone-anaesthetized rats. Firstly, naloxone administration causes only negligible changes in the measured cardiovascular parameters in naive animals. This finding is not different from those observed in squirrel monkey (Byrd, 1983). Secondly, the intensities of these reactions increase with the duration of chronic morphine treatment and disappear following opiate withdrawal. Thirdly, the occurrence of other signs after naloxone injection, such as faecal passage and muscle twitching, also demonstrates that the opiate withdrawal syndrome was developing in these animals during the observation period.

Physiological changes during abrupt or naloxone-precipitated opiate withdrawal are very complex. The underlying mechanisms are not clearly understood. During narcotic withdrawal, there is central cholinergic hyperactivity, augmented sympathetic outflow, and enhanced effects of a number of neurotransmitters and neuromodulators in certain organs such as gut (Chang *et al.*, 1984; Marshall & Buccafusco, 1987). These, as well as other endogenous changes, have been implicated in the production of some withdrawal symptoms. However, it is possible that different mechanisms are involved in the expression of the various withdrawal symptoms and signs, and drugs tested for potential anti-withdrawal properties may act selectively on one or more of these mechanisms (Buccafusco, 1983). It was found in the present study that atropine pretreatment dose-dependently reduced the incidence of occurrence of faecal passage and muscle twitching following naloxone injection. This is in agreement with the findings of Chang *et al.* (1984) and Marshall & Buccafusco (1987). However, the current investigation also revealed that atropine pretreatment did not markedly affect the circulatory depression or the occurrence of cardiac extrasystoles after naloxone administration. This indicates that cholinergic hyperactivity is not involved in the cardiovascular responses of chronically morphine-treated rats to naloxone-precipitated opiate withdrawal. It may not be reasonable to compare this postulation with that of Marshall & Buccafusco (1987) who made their observations in conscious and freely moving morphine-dependent rats. Based on the preventive effects of intrathecal injection of atropine or hemicholinium-3, they suggested that the blood pressure increases induced by naloxone administration resulted from central cholinergic hyperactivity which evoked an augmentation of sympathetic activity.

Clonidine is known to exert an anti-withdrawal action both clinically (Gold *et al.*, 1978) and in opiate-dependent rats (Fielding *et al.*, 1978). However, the mechanism of its action is still not understood. Since many of the symptoms and signs of narcotic withdrawal are due to heightened autonomic responsiveness, it has been suggested that the anti-withdrawal action of clonidine may be related to its central sympatholytic effects (Buccafusco, 1983). It was reported that clonidine pretreatment produced a dose-related inhibition of the pressor reaction to naloxone administration in conscious morphine-dependent rats (Buccafusco, 1983). However, the cardiovascular responses of chronic morphine-treated rats under pentobarbitone anaesthesia to naloxone administration were shown in the present study to be inhibitory. It is difficult to relate this circulatory depression to sympathetic excitation. Nevertheless, the current investigation revealed that clonidine pretreatment not only effectively prevented the occurrence of faecal passage and muscle twitching, but also abolished the

decreases in MBP, HR, LVP, and $dLVP/dt_{max}$ in response to naloxone injection. This may be attributed to the α_2-adrenoceptor agonistic action of clonidine in the central nervous system, which leads to central sympatholytic effect, or in the peripheral tissues. In order to evaluate the importance of α_2-adrenoceptors in the production of circulatory depression during naloxone-precipitated opiate withdrawal, similar experiments were carried out in animals pretreated with yohimbine, an α_2-adrenoceptor antagonist. It was found that yohimbine did not significantly aggravate or alleviate the cardiovascular changes as well as the occurrence of other withdrawal signs. It is, therefore, suggested that the effects of clonidine may not be due to its α_2-adrenoceptor agonistic action. Clonidine has an analgesic effect in rats. Tolerance develops to this analgesic effect, and there is cross-tolerance to morphine (Jaffe, 1985). It is possible that this property of clonidine may contribute to its anti-withdrawal effects observed in the present study. However, the occurrence of cardiac extrasystoles during naloxone-precipitated opiate withdrawal was not significantly suppressed by clonidine pretreatment. The reason for this is not clear. It may be due to an inadequate dose of clonidine or to its inability to inhibit other mechanisms which trigger the genesis of cardiac extrasystoles. Therefore, further investigations are required before any conclusion can be made.

The narcotic withdrawal syndrome not only occurs in intact animals but can also be demonstrated in isolated tissues. It has been shown that the addition of naloxone produces muscle contractions and increased peristaltic activity in isolated segments of small intestine from opiate-dependent guinea-pigs (Ehrenpreis *et al.*, 1975; Kromer & Woinoff, 1980). Opiate binding has been shown in rat heart (Simantov *et al.*, 1978) and the rat vascular beds may also contain opiate receptors (Altura *et al.*, 1980). Therefore, the possibility that the cardiovascular responses to naloxone-precipitated opiate withdrawal may be, at least partly, due to a direct reaction of the cardiac tissues or blood vessels should be considered. However, this idea can now be excluded by the present findings that the hearts or tail arteries isolated from either naive or chronically morphine-treated rats reacted similarly to naloxone administration. Thus, the isolated hearts and tail arteries from chronically morphine-treated rats do not exhibit withdrawal reaction to a narcotic antagonist. The mechanism underlying the significant enhancement of force of contraction of isolated hearts by large doses of naloxone is not clear. It may be related to the removal by naloxone of the inhibitory effects of endogenous opioids in the cardiac tissues on noradrenergic neuroeffector transmission (Wong-Dusting & Rand, 1985). Further studies are needed.

Our present findings that various cardiovascular parameters decreased during naloxone-precipitated opiate withdrawal contradict those reported by others. The association of an elevated blood pressure and opiate withdrawal has been demonstrated in both man and experimental animals (Kolb & Himmelsbach, 1938; Wikler *et al.*, 1953; Marshall & Buccafusco, 1985). It is difficult to explain fully the opposite findings of the present investigation. However, the increase in blood pressure associated with opiate withdrawal was observed mainly in conscious animals or man (Kolb & Himmelsbach, 1938; Wikler *et al.*, 1953; Marshall & Buccafusco, 1985) and was believed to result from an exaggerated discharge of the sympathetic nervous system (Buccafusco, 1983). It is possible that the discrepancy between the findings of this investigation and those reported by others may be due to differences in the consciousness of the experimental animals, and to the presence or absence of the influence of psychomotor responses during opiate withdrawal. Labrecque & Domino (1974) reported that, in halothane-anaesthetized morphine-dependent cats, naloxone i.v. caused an initial increase and a subsequent decrease in blood pressure at 1–10 min and 75–105 min, respectively, without producing significant changes in heart rate. Furthermore, the possibility that the present results are due to the direct effects of pentobarbitone 60 mg kg^{-1} in morphine-dependent rats subjected to naloxone-precipitated opiate withdrawal cannot be excluded. Similar results may not be observed if other anaesthetics, or if different doses of pentobarbitone, are used. Therefore, further studies on chronic opiate-treated animals anaesthetized with different anaesthetics and with various doses of the same anaesthetic are required before a firm conclusion can be drawn.

In summary, the present study has shown that, under pentobarbitone anaesthesia, chronically morphine-treated rats exhibit cardiovascular depression during naloxone-precipitated opiate withdrawal in a treatment duration-dependent manner. The mechanisms remain unclear, but are unlikely to be due to cholinergic or noradrenergic hyperactivity nor the direct withdrawal reactions of the cardiac or vascular tissues.

The authors wish to thank Endo Lab. (N.Y., U.S.A.) for the gift of naloxone, Professor C.W. Ogle for his helpful criticism on the manuscript, and Mr Godfrey S.K. Man for his excellent technical assistances. Y.W. was a Special Studies Fellow supported by a China Medical Board Fellowship.

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(Received October 15, 1990
Accepted February 8, 1991)

Br. J. Pharmacol. (1991), **103**, 1407–1410 © Macmillan Press Ltd, 1991

N-nitro L-arginine causes coronary vasoconstriction and inhibits endothelium-dependent vasodilatation in anaesthetized greyhounds

[1]O.L. Woodman & *G.J. Dusting

Departments of Pharmacology and Physiology*, University of Melbourne, Parkville, Victoria, 3052, Australia

1 The effect of N-nitro-L-arginine (L-NNA), an inhibitor of nitric oxide biosynthesis, on large coronary artery diameter and coronary blood flow was examined in anaesthetized greyhounds. The effects of L-NNA on the coronary vascular responses to acetylcholine (ACh), glyceryl trinitrate (GTN) and 5-hydroxytryptamine (5-HT) were also assessed.

2 L-NNA (5 mg kg^{-1}), infused into the left circumflex coronary artery, increased systemic mean arterial pressure and decreased the external diameter of the artery. Infusion of L-NNA decreased coronary blood flow in 5 of the 7 dogs tested and increased mean coronary resistance but neither of these effects was statistically significant. There was no change in heart rate.

3 Intra-arterial injection of both ACh (0.01–0.05 μg kg^{-1}) and GTN (0.1–0.5 μg kg^{-1}) increased large coronary artery diameter and coronary blood flow. Coronary vascular responses to the endothelium-dependent vasodilator ACh were significantly reduced by L-NNA, whereas the responses to the endothelium-independent vasodilator GTN were not significantly affected.

4 5-HT (0.1 μg kg^{-1}, injected into the left circumflex coronary artery) decreased coronary artery diameter but increased coronary blood flow. After the administration of L-NNA the 5-HT-induced dilatation of the coronary resistance vessels was significantly attenuated whereas the constriction of the circumflex coronary artery was increased in 3 out of 3 dogs in which diameter could be measured, although the latter effect was not statistically significant.

5 These data indicate that L-NNA causes coronary and systemic vasoconstriction and selectively inhibits endothelium-dependent vasodilatation in the coronary circulation of the anaesthetized greyhound. Therefore endothelium-derived NO has an important role in the regulation of coronary vascular tone in the large arteries and the resistance vessels.

Keywords: Acetylcholine; coronary artery; glyceryl trinitrate, 5-hydroxytryptamine; vasoconstriction; vasodilatation; endothelium; N-nitro-L-arginine

Introduction

Vascular endothelial cells release a factor, closely related to nitric oxide (NO) that accounts for the vasodilator action of acetylcholine and other endogenous substances (Furchgott, 1984; Moncada *et al.*, 1989; Dusting *et al.*, 1989). It has recently been reported that a loss of endothelium-dependent coronary vasodilatation in man can be correlated with the presence of a number of risk factors for coronary artery disease (Vita *et al.*, 1990; Yasue *et al.*, 1990). Whilst this provides circumstantial evidence that endothelium-derived NO may play an important role in the regulation of the coronary circulation there have been few studies that have directly addressed this question. The finding that NO is synthesized in the endothelium from L-arginine (Palmer *et al.*, 1988) led to the discovery of L-arginine analogues which potently and specifically inhibit the synthesis of endothelium-derived NO both *in vitro* and *in vivo*. NO biosynthesis can be inhibited by N^G-monomethyl-L-arginine (L-NMMA, Palmer *et al.*, 1988) and N-nitro-L-arginine (L-NNA, Moore *et al.*, 1990; Dubbin *et al.*, 1990). Both of these agents increase arterial pressure in rats (Gardiner *et al.*, 1990; Zambetis *et al.*, 1990) and L-NMMA reduces forearm blood flow in human volunteers (Vallance *et al.*, 1989), constricts a variety of vascular beds in the conscious rat (Gardiner *et al.*, 1990) and causes coronary vasoconstriction in rabbit isolated hearts (Amezcua *et al.*, 1989). These studies suggest that endothelium-derived NO has an important role in the regulation of vascular tone and arterial pressure. The aim of this study is to examine the effect of L-NNA on coronary vascular tone and on vascular responses to acetylcholine (ACh), glyceryl trinitrate (GTN) and 5-hydroxytryptamine (5-HT) in the anaesthetized greyhound. Large coronary artery diameter and coronary blood flow were monitored in order to assess responses in both conduit and resistance vessels.

[1] Author for correspondence.

Methods

Greyhound dogs of either sex, weighing 25–38 kg were anaesthetized with thiopentone (25–30 mg kg^{-1}, i.v.) followed by α-chloralose (70 mg kg^{-1}, i.v., supplemented as necessary). The dogs were ventilated with room air plus additional oxygen as necessary to maintain arterial blood Po_2, Pco_2 and pH within the normal ranges (Po_2: 85–110 mmHg; Pco_2: 30–35 mmHg; pH: 7.25–7.35). Pressure in the thoracic aorta was measured by passing a catheter from the right femoral artery and connecting the catheter to a Druck pressure transducer. A continuous measurement of heart rate was obtained with a cardiotachometer coupler triggered by the ECG.

A thoracotomy was performed at the left fifth intercostal space and the pericardium was opened. A pair of 7 MHz piezoelectric transducers were sutured to opposing surfaces of the left circumflex coronary artery. The external diameter of the artery was measured with an ultrasonic transit-time dimension gauge (Triton Technology Inc.). Blood flow through the artery was measured with an electromagnetic flow probe (Statham) placed on the artery. A needle tipped catheter was placed in the artery proximal to the transducers for the administration of drugs. Care was taken during the placement of the transducers to limit dissection and damage of any visible nerves.

Experimental protocol

ACh (0.01–0.05 $\mu g kg^{-1}$), GTN (0.1–0.5 $\mu g kg^{-1}$) and 5-HT (0.1 $\mu g kg^{-1}$) were injected as a bolus of less than 0.5 ml directly into the left circumflex coronary artery. After control responses to these agents had been obtained L-NNA (5 $mg kg^{-1}$) was infused into the left circumflex coronary artery over a period of 30 to 40 min and 15 min was allowed after completing the infusion to permit the arterial pressure, coronary artery diameter and coronary blood flow to stabilize before re-examining the responses to ACh, GTN and 5-HT.

Drugs

Drugs used were: acetylcholine perchlorate (BDH); glyceryl trinitrate (Boots); 5-hydroxytryptamine creatinine sulphate (Sigma); N-nitro-L-arginine (Sigma). ACh was dissolved in saline; 5-HT and L-NNA were dissolved in distilled water. Further dilutions of all drugs were made in saline.

Statistics

Baseline values of the haemodynamic variables and the responses to 5-HT measured before and after infusion of L-NNA were compared by a Wilcoxon signed rank test. Statistical comparison of the responses to ACh and GTN recorded before and after treatment with L-NNA was achieved by a two-way analysis of variance the two factors being the dose of the agonist and the treatment. This test treated all doses as a group to reveal whether a treatment had any significant effect on a response.

Results

Effect of the intra-coronary infusion of N-nitro-L-arginine on baseline parameters

L-NNA (5 $mg kg^{-1}$) was slowly infused into the left circumflex coronary artery of seven anaesthetized greyhounds over a period of 30 to 40 min and the effects on baseline haemodynamic values measured are shown in Table 1. L-NNA significantly increased mean arterial pressure and decreased large coronary artery diameter. In two dogs the constriction of the large coronary artery was such that the baseline diameter was less than the minimum measurable level of 2 mm and these dogs have been excluded from any data concerning large artery diameter. There was a tendency for coronary blood flow to decrease and mean coronary resistance to increase but neither of these changes was statistically significant. There was no change in heart rate.

Table 1 Baseline levels of the haemodynamic variables measured in 7 anaesthetized greyhounds before and after infusion of N-nitro-L-arginine (L-NNA) (5 $mg kg^{-1}$) into the left circumflex coronary artery

	Control	*L-NNA*
Mean arterial pressure (mmHg)	114 ± 7	125 ± 7**
Mean external coronary artery diameter (mm)	3.0 ± 0.4	2.7 ± 0.4*
Mean coronary blood flow (ml min^{-1})	43 ± 8	35 ± 6
Mean coronary resistance (mmHg min ml^{-1})	3.2 ± 0.6	5.1 ± 1.7
Heart rate (beats min^{-1})	130 ± 8	123 ± 8

Values are presented as the mean ± s.e.mean of data obtained from seven animals. * $P < 0.05$; ** $P < 0.01$ compared to control, Wilcoxon's signed rank test.

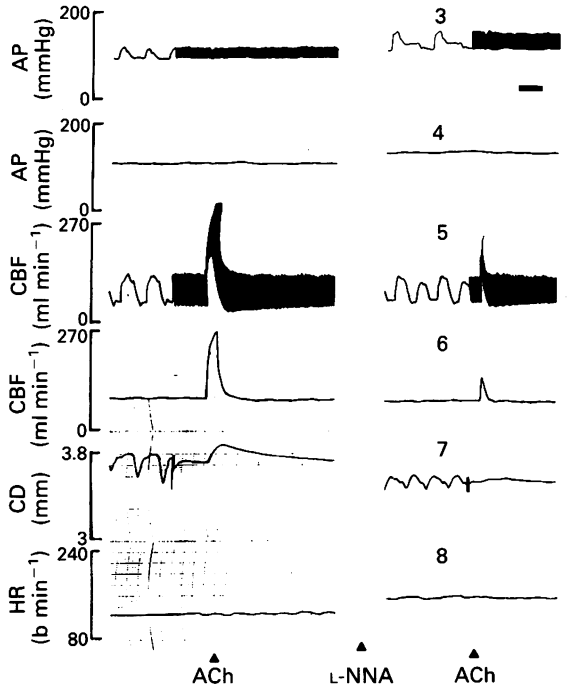

Figure 1 The effect of the intra-coronary injection of acetylcholine (ACh, 0.05 $\mu g kg^{-1}$) on arterial pressure (AP), left circumflex coronary blood flow (CBF), mean coronary artery external diameter (CD) and heart rate (HR) in an anaesthetized greyhound. Responses are shown under control conditions and following the intra-coronary infusion of N-nitro-L-arginine (L-NNA, 5 $mg kg^{-1}$). The time bar represents 0.4 s at fast chart speed and 1 min at slow chart speed.

Effect of N-nitro-L-arginine on coronary dilator responses to acetylcholine and glyceryl trinitrate

Intra-coronary bolus injections of ACh (0.01–0.05 $\mu g kg^{-1}$) and GTN (0.1–0.5 $\mu g kg^{-1}$) increased large coronary artery

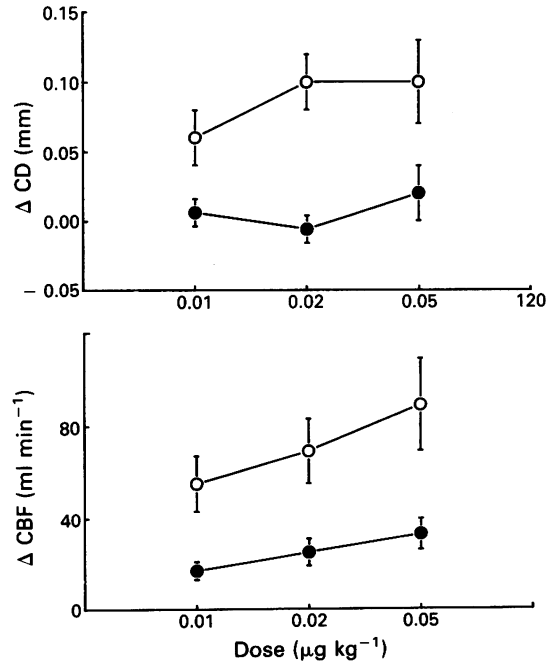

Figure 2 Increases in left circumflex coronary artery diameter (CD) and coronary blood flow (CBF) produced by the intra-coronary injection of acetylcholine are shown before (○) and after (●) treatment with N-nitro-L-arginine (L-NNA, 5 $mg kg^{-1}$, i.c.) in seven dogs. The values shown are the mean and vertical lines indicate the s.e.mean. Analysis of variance demonstrated that L-NNA treatment significantly ($P < 0.001$) attenuated the acetylcholine-induced dilatation of both the large coronary artery and the resistance vessels.

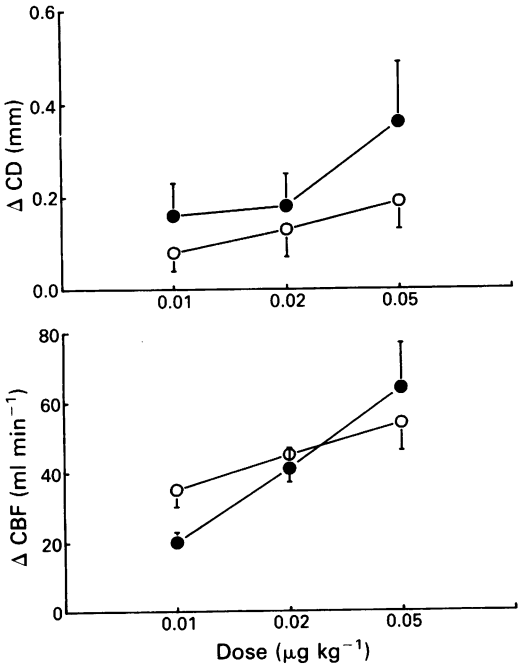

Figure 3 Increases in left circumflex coronary artery diameter (CD) and coronary blood flow (CBF) produced by the intra-coronary injection of glyceryl trinitrate are shown before (○) and after (●) treatment with N-nitro-L-arginine (L-NNA, 5 mg kg^{-1} i.c.) in seven dogs. The values shown are the mean and vertical lines indicate the s.e.mean. L-NNA had no significant effect on the dilator responses to glyceryl trinitrate.

diameter and coronary blood flow (Figures 1–3). Neither ACh nor GTN had any affect on mean arterial pressure or heart rate. An experiment demonstrating the response to ACh before and after L-NNA is shown in Figure 1. After infusion of L-NNA the injection of ACh had no significant effect on large coronary artery diameter and the resistance vessel vasodilator responses were significantly less than the control responses

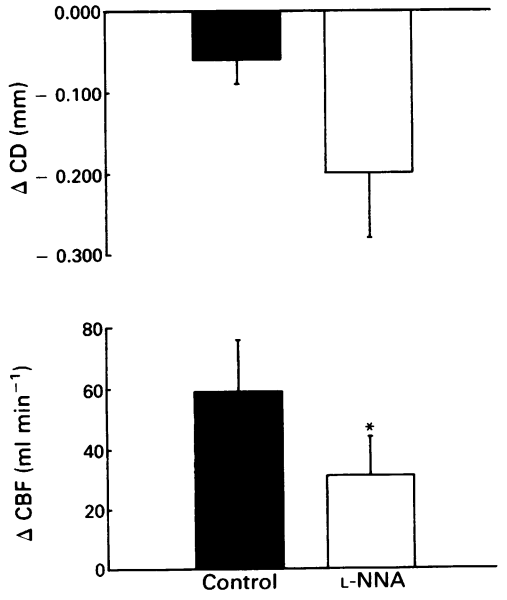

Figure 4 Decreases in left circumflex coronary artery diameter (CD) and increases in coronary blood flow (CBF) produced by the intra-coronary injection of 5-hydroxytryptamine (0.1 μg kg^{-1}) are shown before and after treatment with N-nitro-L-arginine (L-NNA, 5 mg kg^{-1}). The effect of 5-hydroxytryptamine on CBF is from 5 dogs but the effect on CD is from 3 dogs as in the 2 remaining dogs the artery constricted below the minimum level for detection of diameter. The values shown are the mean and the error bars indicate the s.e.mean. * $P < 0.05$, Wilcoxon's signed rank test.

(Figure 2). In contrast, GTN tended to cause greater increases in large coronary artery diameter in the presence of L-NNA although there was no significant difference between the two groups of responses (Figure 3). GTN-induced dilatation of the coronary resistance vessels was unaffected by the presence of L-NNA (Figure 3).

Effect of N-nitro-L-arginine on coronary responses to 5-hydroxytryptamine

In five dogs, intra-coronary artery injection of 5-HT (0.1 μg kg^{-1}) increased coronary blood flow but decreased large coronary artery diameter (Figure 4) without having any affect on mean arterial pressure or heart rate. Pretreatment with L-NNA significantly reduced the 5-HT-induced increase in coronary blood flow (Figure 4). The effect of 5-HT on large coronary artery diameter could only be measured in three of the dogs in the presence of L-NNA due to the reduction in baseline diameter. In each of these dogs the 5-HT-induced large artery constriction was potentiated in the presence of L-NNA but overall there was no statistically significant difference (Figure 4).

Discussion

L-NNA inhibits the biosynthesis of NO in endothelial cells (Moore *et al.*, 1990; Dubbin *et al.*, 1990) and increases arterial pressure in anaesthetized rats (Zambetis *et al.*, 1990). We have now shown that in the anaesthetized greyhound L-NNA increases arterial pressure and causes constriction of the left circumflex coronary artery despite the increase in distending pressure. Coronary blood flow declined and coronary resistance increased in five out of seven dogs treated with L-NNA, indicating constriction of resistance vessels. These effects are consistent with those of L-NMMA which increases arterial pressure in the rat (Whittle *et al.*, 1989; Gardiner *et al.*, 1990) and rabbit (Rees *et al.*, 1989), and constricts the carotid, renal, mesenteric and hindlimb vascular beds of conscious rats (Gardiner *et al.*, 1990). Our observations suggest that a basal release of NO from the arterial endothelium has an important dilator influence on the coronary vasculature.

In addition to its vasoconstrictor action, L-NNA inhibited the coronary vascular responses to the endothelium-dependent vasodilator ACh in both the large coronary artery and the coronary resistance vessels. This was not a non-specific effect resulting from the enhanced tone in those vessels after L-NNA treatment for the dilator responses to the endothelium-independent vasodilator GTN were unaffected by L-NNA. In fact GTN tended to cause greater dilatation of the large coronary arteries after treatment with L-NNA, perhaps due to the greater tone in that vessel and hence increased vasodilator reserve. The selective inhibitory action of L-NNA on endothelium dilatation further supports the hypothesis that the coronary vasoconstriction is due to inhibition of NO release from the endothelium.

L-NNA also attenuated increases in coronary blood flow induced by 5-HT. The coronary vascular response to 5-HT in the dog varies with vessel size. In this and in earlier studies (Brum *et al.*, 1984; Lamping *et al.*, 1985; Woodman, 1990) 5-HT has been shown to constrict the large coronary arteries but to dilate the resistance vessels. In isolated coronary arteries 5-HT causes the release of endothelium-derived relaxing factor (EDRF, Cocks & Angus, 1983; Cohen *et al.*, 1983) and removal of the endothelium in dogs enhances the constrictor response to 5-HT in the epicardial coronary arteries (Brum *et al.*, 1984; Lamping *et al.*, 1985), suggesting that in large coronary arteries release of endothelium-derived NO normally opposes the direct vasoconstrictor action of 5-HT. In our study the large artery constriction induced by 5-HT was increased after L-NNA, but the number of observations was limited by the marked constriction of the arteries to below the minimal detectable diameter. Importantly, L-NNA

significantly attenuated the increase in coronary blood flow produced by 5-HT indicating for the first time that the dilatation of the resistance vessels is endothelium-dependent.

In conclusion, our study demonstrates that L-NNA, an inhibitor of NO biosynthesis in endothelial cells, selectively inhibits endothelium-dependent vasodilatation in the coronary circulation of the dog. In addition L-NNA causes coronary vasoconstriction, particularly of the large coronary arteries, suggesting that endothelium-derived NO has an important role in the regulation of coronary vascular tone. It is known that endothelium-dependent vasodilatation is impaired by advanced atherosclerosis (Habib *et al.*, 1986; Verbeuren *et al.*, 1986; Bossaller *et al.*, 1987), and we have recently found that impairment of endothelial cell function precedes the development of intimal thickening in early atheroma-like lesions (Dusting *et al.*, 1990). Moreover, the loss of endothelium-dependent coronary vasodilatation in man correlates with the presence of risk factors for coronary artery disease (Vita *et al.*, 1990; Yasue *et al.*, 1990). Therefore our current findings support suggestions that endothelium-derived NO has an important role in the maintenance of coronary blood flow and that endothelial damage might provoke coronary vasospasm and myocardial ischaemia.

The authors wish to thank Joanne Hart and Tina Sozzi for expert technical assistance. This work was supported by a grant from the National Health and Medical Research Council of Australia.

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(Received June 27, 1990
Revised February 7, 1991
Accepted February 8, 1991)

Br. J. Pharmacol. (1991), **103**, 1411–1416 © Macmillan Press Ltd, 1991

Electrophysiological effects of the combination of mexiletine and flecainide in guinea-pig ventricular fibres

Eva Delpón, Carmen Valenzuela & Juan Tamargo

Department of Pharmacology, School of Medicine, Universidad Complutense, 28040 Madrid, Spain

1 The effects of flecainide alone, mexiletine alone and their combination at the Na^+ channel level were studied in guinea-pig papillary muscles. The maximum upstroke velocity (V_{max}) was used as an indirect index of the magnitude of the fast inward Na^+ current (I_{Na}).

2 In muscles driven at 0.02 Hz, neither mexiletine (10^{-5} M) nor flecainide (10^{-6} M) nor the combination of both drugs modified the action potential characteristics. Mexiletine, but not flecainide, increased the effective refractory period/action potential duration ratio; this enhancement was greater when flecainide was also present.

3 Mexiletine or flecainide alone produced a frequency-dependent V_{max} block. Although at 0.5 Hz the blockade induced by the combination of flecainide and mexiletine was similar to that produced by flecainide alone, in muscles driven at 1 and 2 Hz the combination increased the magnitude and the onset rate of the V_{max} block.

4 The time constant of recovery of V_{max} block was similar in the presence of flecainide or the combination mexiletine plus flecainide (τ_{re} = 16.4 ± 2.3 s and 16.7 ± 2.7 s, respectively), but the combination decreased the magnitude of the slow component of reactivation induced by flecainide (93.8 ± 1.5% versus 68.9 ± 1.7%). Moreover, the combination of both drugs was more effective in inhibiting the V_{max} of early test stimuli than either drug alone.

5 It is concluded that the combination of mexiletine and flecainide is synergistic at driving rates faster than 0.5 Hz without detracting from the characteristics of flecainide.

Keywords: Mexiletine; flecainide; V_{max}; ventricular muscle; heart

Introduction

Flecainide, a class Ic drug, is a highly potent antiarrhythmic agent which exhibits both very slow onset kinetics and recovery from V_{max} block (Campbell & Vaughan Williams, 1983; Campbell, 1983a,b). It has been found effective in treatment and prophylaxis of ventricular arrhythmias and certain supraventricular arrhythmias (Anderson *et al.*, 1981; Duff *et al.*, 1981; Campbell, 1983b; Neuss *et al.*, 1983; Hodges & Heel, 1985; Somberg & Tepper, 1986). Like other class Ic agents, flecainide exhibits not only very potent antiarrhythmic but unfortunately also proarrhythmic properties (CAST, 1989). Recently, Anno & Hondeghem (1989, 1990) reported that most of the frequency-dependent V_{max} block produced by flecainide is associated with the activated state of the Na^+ channels. Moreover, they concluded that flecainide also could bind very slowly to slow inactivated Na^+ channels, or could have promoted the development of ultra slow inactivation. Mexiletine is a class Ib antiarrhythmic drug (Campbell, 1983a) which has been proved to be useful in the treatment of ventricular tachyarrhythmias (Sandoe *et al.*, 1978; Podrid, 1986; Campbell, 1987; Morganroth, 1987). It binds preferentially to the inactivated state of the Na^+ channel (Yatani & Akaike, 1985) but also to the activated state (Hering *et al.*, 1983). However, it dissociated faster from the Na^+ channel receptor during diastole than does flecainide (Campbell, 1983a). Recently it has been proposed that one possible way to reduce the cardiodepressant effects of class I antiarrhythmic drugs would be to combine drugs from different subgroups, i.e. Ia, Ib, Ic (Hondeghem, 1987; Tamargo *et al.*, 1989). According to the modulated receptor hypothesis (Hondeghem & Katzung, 1977; 1984) antiarrhythmic drugs with very different kinetics block Na^+ channels binding to a common receptor site at the Na^+ channel. Thus, if it is assumed that two drugs bind to the same receptor site, the frequency-dependent effects of the combination would be less pronounced than those induced by the drug with slower kinetics (Hondeghem, 1987; Tamargo *et al.*, 1989; Valenzuela *et al.*, 1989). However, it would be also possible that although both drugs bind to the same receptor site, a fast (Ib) antiarrhythmic drug cannot displace a slow (Ic) drug from Na^+ channels when the drugs are given together. In this situation the combination would decrease the available I_{Na} at least at certain driving rates. Thus, the present work was undertaken to study in guinea-pig papillary muscles the electrophysiological effects of a combination of mexiletine (class Ib) plus flecainide (class Ic).

Methods

Guinea-pigs of either sex weighing 350–450 g were killed by a blow on the neck. The hearts were rapidly removed and placed in a dissection chamber where papillary muscles of 2–3 mm in length and less than 1 mm in diameter were excised from the right ventricle. The muscles were pinned to the bottom of a Lucite chamber and superfused continuously at a constant rate of 7 ml min^{-1} with Tyrode solution of the following composition (mM): NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaHCO_3$ 11, NaH_2PO_4 0.42, and glucose 5.5. Solutions were bubbled with 95% O_2:5% CO_2 and maintained at 34 ± 0.5°C (pH = 7.4). The preparations were initially driven at 1 Hz and equilibrated for at least 1 h while a stable impalement was obtained. Driving stimuli were rectangular pulses (2 ms in duration and twice diastolic threshold) delivered via a multipurpose programmable stimulator (Cibertec-CS220).

Transmembrane action potentials were conventionally recorded through glass microelectrodes filled with 3 M KCl (tip resistance of 8–15 MΩ). The microelectrode was connected via Ag-AgCl wire to high-input impedance capacity neutralizing amplifiers (WPI model 701). The maximum upstroke velocity (V_{max}) of the action potential was obtained by electronic differentiation (Valenzuela *et al.*, 1988; Delpón *et al.*, 1989). The differentiator used had an upper limit of linearity of 1000 V s^{-1} and variable input filters (3 Hz–260 kHz). The suitable frequency filter for minimizing noise without reducing the V_{max} was selected for each individual experiment. Another distortion of V_{max} can arise from the deformation of the foot of

the rising phase by the electrical stimulus. In order to diminish this artefact, stimulus intensity and duration were adjusted throughout each experiment to maintain a constant latency (1–2 ms) from the stimulus artefact to the initiation of the upstroke of the action potential (Valenzuela *et al.*, 1989). Both action potential and V_{max} were displayed on a storage oscilloscope (Tektronix 5104N) and photographed with a kymograph Grass camera (Model C4).

To study the influence of stimulation frequency on the depressant effects of mexiletine and flecainide on V_{max} block, papillary muscles were stimulated after a rest period of 3 min with trains of pulses of 40 s in duration at 0.5, 1 and 2 Hz. The preparations were then exposed to various solutions in the following sequence: (1) control Tyrode solution; (2) solution with mexiletine alone (30 min); (3) control solution (50 min); (4) solution with flecainide (30 min); (5) solution with mexiletine plus flecainide (30 min). The concentrations of mexiletine and flecainide tested were 10^{-5} M and 10^{-6} M, respectively, and were chosen because in previous studies they had been used to study onset and recovery kinetics (Courtney, 1981; Campbell & Vaughan Williams, 1983; Campbell, 1983a,b). In this way we were able to compare the individual effects of each drug alone with those induced by the combination. This experimental protocol demonstrated that both mexiletine and flecainide produced two types of V_{max} inhibition, a tonic and a frequency-dependent block. The tonic block was defined as the decrease of V_{max} of the first action potential preceded by a rest period, while the frequency-dependent block was defined by the decrease of the V_{max} observed during a train of stimuli.

Recovery of V_{max} from the frequency-dependent block was studied by applying a single test stimulus at various coupling intervals after a stimulation train for 7 s at 2 Hz (Valenzuela *et al.*, 1988; Delpón *et al.*, 1989). The intensity and duration of the test and conditioning stimuli were adjusted to obtain a constant latency from the stimulus artefact to the initiation of the upstroke of the action potential. The effective refractory period (ERP) was measured by introducing premature test-stimuli of twice threshold strength at different intervals from the preceding basic action potential (Tamargo, 1980). In another group of experiments the first 1000 ms of the recovery process of V_{max} were analyzed in muscles driven at a basic rate of 1 Hz by introducing a test stimulus (S_2) every 10th basic pulse (S_1). The test interval was defined as the interval between 90% repolarization of the conditioning action potential and the onset of the test action potential (Sánchez-Capula, 1985; Delpón *et al.*, 1990).

All experimental results were obtained from a single continuous impalement throughout the whole experiment.

Drugs

Flecainide hydrochloride (Laboratorios Dr. Esteve S.A.) and mexiletine (Boehringer Ingelheim S.A.) as a powder were initially dissolved in distilled deionized water. Further dilutions were carried out in Tyrode solution to obtain the desired concentrations.

Throughout the paper data are given as means ± s.e.mean and Student's *t* test was used to estimate the significance of differences from control values. For statistical comparison of more than two groups, a one-way analysis of variance was performed (Wallenstein *et al.*, 1980). A P value of less than 0.05 was considered as significant. More details on each procedure are given under Results.

Results

Effects on transmembrane action potentials

The electrophysiological effects of mexiletine alone (10^{-5} M), flecainide alone (10^{-6} M) and a combination of mexiletine plus flecainide were studied in 10 guinea-pig papillary muscles driven at the basal rate of 0.02 Hz. As is shown in Table 1 neither mexiletine nor flecainide alone nor the combination of both drugs had an effect on the resting membrane potential, action potential amplitude or the V_{max} values. Also the action potential duration measured at 50% and 90% of repolarization remained unchanged. However, whereas flecainide alone did not modify the ERP/APD_{90} ratio, mexiletine significantly increased this parameter ($P < 0.01$), and this enhancement of ERP/APD_{90} ratio was potentiated when both drugs were present.

Frequency-dependent blockade of V_{max}

Flecainide alone (10^{-6} M), mexiletine (10^{-5} M) or the combination of both drugs did not decrease the V_{max} of the first action potential preceded by a rest period and thus, the tonic block averaged 1.3 ± 0.5%, 1.7 ± 3.5% and 3.4 ± 1.2%, respectively. These results suggested that flecainide and mexiletine exhibited a low affinity for the rested state of the Na^+ channel. The influence of stimulation frequency on the depressant effect of mexiletine and flecainide alone and the combination of mexiletine plus flecainide on V_{max} was studied in papillary muscles driven by trains of stimuli at 0.5, 1 and 2 Hz for 40 s, separated from one another by a rest period of 3 min. As is shown in Table 2, under control conditions the V_{max} values were reduced by 1.7 ± 0.7%, 5.4 ± 2.8% and 7.0 ± 1.4% during stimulation trains at a rate of 0.5, 1 and 2 Hz, respectively. Mexiletine alone did not induce a significant frequency-dependent V_{max} block when trains of action potentials at 0.5 Hz or 1 Hz were applied, whereas at 2 Hz, mexiletine induced a frequency-dependent V_{max} block of 19.8 ± 2.4% ($P < 0.01$). As is shown in Table 2 and Figure 1, the frequency-dependent V_{max} block induced by flecainide was of the same order of magnitude as that induced by mexiletine when trains of action potentials at a rate of 2 Hz were applied (18.0 ± 2.2%, $P < 0.01$). In contrast, when trains of action potentials at a rate of 0.5 and 1 Hz were applied, flecainide induced a significant frequency-dependent V_{max} block and the steady-state V_{max} values were reduced by 5.4 ± 0.8% ($P < 0.01$) and 10.4 ± 1.4% ($P < 0.01$) respectively. When both mexiletine and flecainide were perfused, the frequency-dependent V_{max} block observed at 1 and 2 Hz was significantly

Table 1 Electrophysiological effects of mexiletine (10^{-5} M), flecainide (10^{-6} M) and the combination of both drugs in guinea-pig papillary muscles ($n = 10$)

	RMP (mV)	V_{max} ($V s^{-1}$)	*APA* (mV)	APD_{50} (ms)	APD_{90} (ms)	ERP/APD_{90}
Control	85.7 ± 1.5	202.5 ± 5.9	124.0 ± 0.6	211.1 ± 13.9	240.3 ± 14.4	1.02 ± 0.005
Mexiletine	85.1 ± 2.0	197.7 ± 4.0	123.7 ± 0.7	214.7 ± 13.6	245.8 ± 6.8	1.04 ± 0.005*
Control	85.1 ± 1.9	200.0 ± 3.1	123.8 ± 0.6	213.6 ± 13.7	243.4 ± 8.1	1.02 ± 0.006
Flecainide	83.8 ± 1.7	200.0 ± 2.9	123.8 ± 0.5	224.6 ± 17.1	256.4 ± 17.1	1.02 ± 0.006
Flecainide + mexiletine	84.5 ± 1.4	196.1 ± 2.9	123.4 ± 0.3	213.9 ± 35.4	248.6 ± 33.9	1.06 ± 0.004**

Values are mean ± s.e.mean ($n = 10$). * $P < 0.05$; ** $P < 0.001$.
APD: action potential duration. ERP: effective refractory period.

Table 2 Effects of mexiletine (10^{-5} M) alone, flecainide (10^{-6} M) alone and the combination of both drugs on the tonic and frequency-dependent V_{max} block in guinea-pig papillary muscles

		Frequency-dependent block (%)		
	Tonic block (%)	*0.5 Hz*	*1 Hz*	*2 Hz*
Control	0	1.7 ± 0.7	5.4 ± 2.8	7.0 ± 1.4
Mexiletine	1.3 ± 0.5	4.7 ± 1.0	9.1 ± 1.7	19.8 ± 2.4*
Flecainide	1.7 ± 3.5	5.4 ± 0.8*	10.4 ± 1.4*	18.0 ± 2.2*
Flecainide + mexiletine	3.4 ± 1.2	6.7 ± 0.7*	14.7 ± 1.3**	27.3 ± 1.4**

Values are mean ± s.e.mean (n = 10). * $P < 0.01$; ** $P < 0.001$.

more pronounced (14.7 ± 1.3% and 27.3 ± 1.4%, $P < 0.01$) than that induced by flecainide alone.

Figure 2 shows the quantitative analysis of the frequency-dependent V_{max} block produced by flecainide alone and flecainide plus mexiletine as a function of the interstimulus interval. The relationship fits well (correlation coefficients being 0.985 and 0.986, respectively) into the equation $y = a.x^{-b}$, where y is the % strength of frequency-dependent V_{max} block, a is a constant (dimension per time unit), x the interstimulus interval (s) and b a dimensionless constant (Kohlhardt & Seifert, 1985). This kind of relationship has been described previously for other antiarrhythmic drugs (Kohlhardt & Seifert, 1985; Valenzuela *et al.*, 1988). The a parameter calculated from the experimental values was augmented from 24.8 in the presence of flecainide alone to 41.2 when both drugs were present, which indicated that the V_{max} block induced by flecainide plus mexiletine will be more pronounced at higher frequencies.

The onset kinetics of the frequency-dependent V_{max} block can be defined in terms of an event-dependent process, that can be well-fitted by a single exponential curve (Courtney, 1980; Campbell, 1983a,b; Grant *et al.*, 1984). The onset rate constant per action potential [$K(AP^{-1})$] at which V_{max} decreased to a new steady-state level was calculated from the regression lines in semilogarithmic plots. As is shown in Table 3, the onset rate constant of V_{max} block was dependent on the stimulation frequency, being always higher at lower stimulation frequencies. In muscles driven at 0.5–2.0 Hz the onset kinetics of flecainide were fitted by a single exponential curve (K = 0.05 ± 0.004 AP^{-1} at 2 Hz, n = 10). At 0.5 and 1 Hz the onset kinetics of mexiletine was fitted by a single exponential curve, whereas at 2 Hz this process was better described by a biexponential curve (see Campbell, 1983b), the K values of the

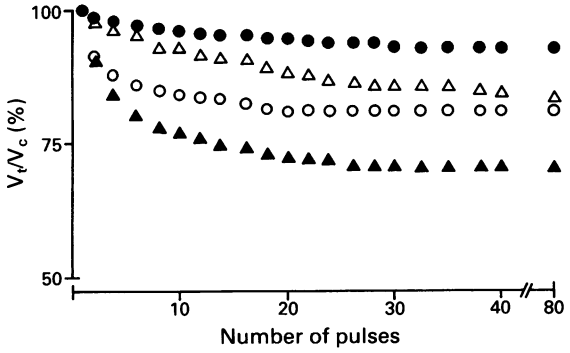

Figure 1 Frequency-dependent V_{max} block obtained under control conditions (●), in the presence of mexiletine alone (○, 10^{-5} M), flecainide alone (△, 10^{-6} M) and a combination of mexiletine and flecainide (▲) during stimulation trains at a frequency of 2 Hz after a rest period of 3 min. The percentage of V_{max} normalized by the value of the first action potential of the train [Vt/Vc (%), Vt = V_{max} of the action potentials of the train, Vc = V_{max} of the first action potential of the train] was plotted against the number of consecutive beats of the train.

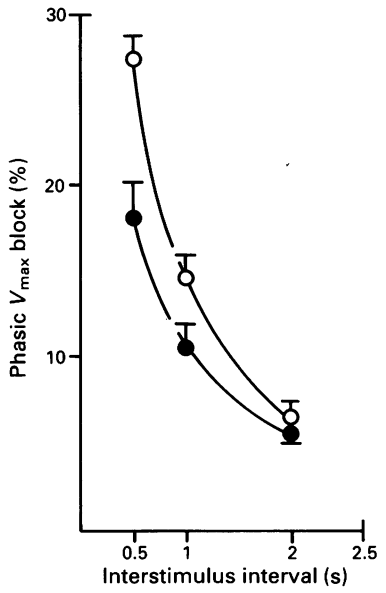

Figure 2 Dependence of frequency-dependent V_{max} block prior to (●) and after (○) mexiletine admixture to the flecainide-containing perfusate on the interstimulus interval. The curves have been drawn from $y = 24.8x^{-0.45}$ (r^2 = 0.985) in the presence of flecainide alone and from $y = 41.2x^{-0.39}$ (r^2 = 0.986) in the presence of flecainide plus mexiletine.

Table 3 Rate constants of development of frequency-dependent V_{max} block (K) in the presence of mexiletine alone (10^{-5} M), flecainide alone (10^{-6} M) and a combination of both drugs

	K (AP^{-1})		
	0.5	*1*	*2* (Hz)
Mexiletine	0.19 ± 0.02	0.17 ± 0.03	2.04 ± 0.30
	—	—	0.12 ± 0.01
Flecainide	0.14 ± 0.01	0.07 ± 0.005	0.05 ± 0.004
Flecainide + mexiletine	0.14 ± 0.01	0.10 ± 0.01	1.19 ± 0.20
	—	—	0.07 ± 0.20

Values are mean ± s.e.mean (n = 10).

fast and slow components being 2.04 ± 0.30 AP^{-1} and 0.12 ± 0.01 AP^{-1}, respectively. In the presence of mexiletine plus flecainide the onset kinetics at 0.5 and 1 Hz was also a monoexponential process, while at 2 Hz was better fitted by a biexponential curve, the K values being 1.19 ± 0.30 AP^{-1} and 0.07 ± 0.02 AP^{-1}, respectively. These results indicated that when compared to flecainide alone the combination of mexiletine plus flecainide increased the development kinetics of frequency-dependent V_{max} block.

Effect on recovery process of V_{max}

The effects of mexiletine alone, flecainide alone and a combination of both drugs were also studied on the recovery kinetics of frequency-dependent V_{max} block. In this group of experiments, muscles were driven every 3 min by a train of stimuli at a frequency of 2 Hz for 7 s and a test-stimuli was applied at variable coupling intervals.

In 7 papillary muscles the recovery process of V_{max} exhibits a time constant (τ_{re}) which averaged 24.5 ± 2.4 ms. In the presence of mexiletine alone the τ_{re} increased to 314.8 ± 51.6 ms, whereas flecainide alone (Figure 3) induced the slow component of recovery of V_{max} with a τ_{re} of 16.4 ± 2.3 s. The y-intercept of this slow phase of recovery can be taken as the fraction of Na^+ channels blocked by flecainide, which rose to a value of $93.8 \pm 1.5\%$. Figure 3 also shows that when both flecainide and mexiletine were present, two components in the reactivation process can be observed, a fast initial component followed by a second slow component of similar kinetics to that observed in the presence of flecainide alone ($\tau_{re} = 16.7 \pm 0.7$ s). However, in the presence of the combination, the fraction of sodium channels blocked (y-intercept of the slow component) significantly decreased to $68.9 \pm 1.7\%$ ($P < 0.001$). This result suggests that mexiletine decreased the fraction of sodium channels blocked by flecainide without changing the time constant of the recovery process.

In order to study the fast component of the recovery process, another experimental procedure was carried out. Preparations were driven at a basal rate of 1 Hz, and a test stimuli (S_2) was interpolated at different time intervals every 10th basic pulse (S_1). Figure 4 shows a typical experiment. Under control conditions the recovery process was completed within 30 ms. Mexiletine depressed the V_{max} of early test action potentials more than flecainide and lengthened the τ_{re} to

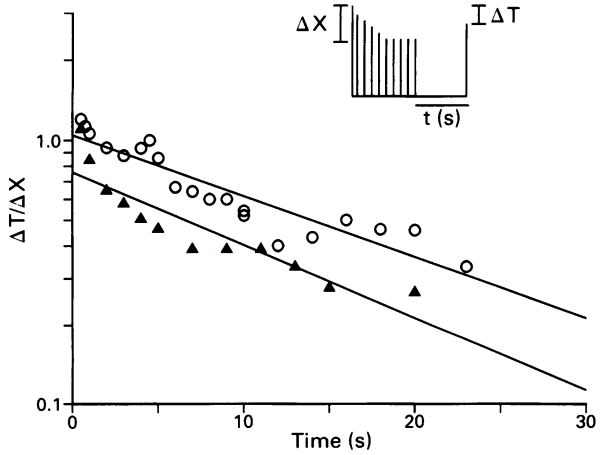

Figure 3 Effects of flecainide (○, 10^{-6} M) and flecainide plus mexiletine (▲, 10^{-5} M) on the recovery process of V_{max} block (mexiletine data were omitted for clarity) after trains of action potentials at 2 Hz. $\Delta T = V_{max}$ of the first action potential of the train minus V_{max} of the test response; $\Delta x = V_{max}$ of the first action potential minus steady-state value of V_{max} reached during the train; t = interval between the last action potential of the train and the test response. First-order regressions of the slow component of recovery were fitted by the method of least squares.

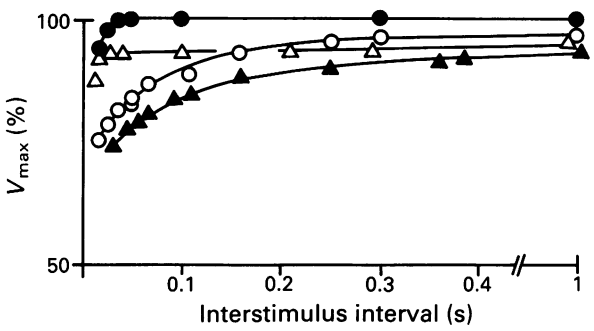

Figure 4 First 1000 ms of the time course of recovery of V_{max}. Data are plotted as percentage of the maximal value of V_{max} under control conditions. The recovery of V_{max} was determined in muscles driven at 1 Hz by applying test stimuli (S_2) every 10 basic stimuli (S_1). (●) Controls; (○) mexiletine, 10^{-5} M; (△) flecainide, 10^{-6} M; (▲) flecainide plus mexiletine.

206 ms. A further V_{max} inhibition of early test action potentials was observed with mexiletine plus flecainide, a finding which agrees with the results shown in Figure 2 where the combination increased the V_{max} block at frequencies of stimulation faster than 0.5 Hz. In the presence of flecainide alone or flecainide plus mexiletine the recovery process of V_{max} block exhibits two components, a fast initial followed by a slow component. In the presence of flecainide alone, the kinetics of the fast component were similar to those obtained under control conditions ($\tau_{re} = 36$ ms), whereas in the presence of the combination this initial component was almost similar to that obtained in the presence of mexiletine alone ($\tau_{re} = 243$ ms). Both in the presence of flecainide or of the combination this initial component was followed by the late slow component described in Figure 3.

Discussion

In the present paper the possible interactions between two antiarrhythmic drugs, mexiletine (class Ib) and flecainide (class Ic), in depressing the V_{max} of ventricular action potentials were studied in guinea-pig papillary muscles. The results obtained suggest that at high frequencies of stimulation this combination increased the frequency-dependent V_{max} block without modifying the time course of the slow component of reactivation produced by flecainide alone. However, the magnitude of the slow component of the reactivation of V_{max} block (y-intercept) in the presence of the combination of both drugs was less than that obtained in the presence of flecainide alone.

In this study the V_{max} values were used as an indirect estimative index of the magnitude of the fast inward Na^+ current (I_{Na}). Thus, even when V_{max} is a monotonic but not a linear index of the peak I_{Na} (Sheets *et al.*, 1988), there is little doubt that V_{max} is mainly generated by this current. Whether or not possible non-linearities between V_{max} and gNa affect the present results remains to be seen in reliable voltage clamp experiments under the same conditions of temperature and external Na concentrations as used in the present experiments.

Class I antiarrhythmic drugs are characterized by their ability to depress the V_{max} of the cardiac action potentials as a consequence of interaction of these drugs with Na^+ channels. On the basis of the modulated receptor hypothesis (Hondeghem & Katzung, 1977; 1984; Hondeghem, 1987; Tamargo *et al.*, 1989) class I antiarrhythmics bind to a receptor site located within or functionally associated with the Na^+ channel. In this model, the affinity of the receptor for the drugs is modulated by the state of the channel (resting-R, activated-A, inactivated-I). In addition, cardiac Na^+ channels also exhibited slow inactivation (SI) and ultra slow inac-

tivation (USI) (Saikawa & Carmeliet, 1982; Clarkson *et al.*, 1984). When these states are drug-associated they are termed RD, AD, ID, SID and USID, respectively.

The results obtained in the presence of individual drugs are in line with those previously described elsewhere (Courtney, 1981; Hohnloser *et al.*, 1982; Campbell, 1983a,b; Hering *et al.*, 1983; Varro *et al.*, 1985; Valenzuela *et al.*, 1989; Valenzuela & Sánchez-Chapula, 1989). Therefore while mexiletine produced a fast development of and fast recovery from V_{max} block, flecainide caused a very slow development and a very slow recovery from block. In fact, in the presence of mexiletine the recovery of V_{max} was completed in less than 800 ms after a conditioning action potential. This explains why mexiletine only produced frequency-dependent V_{max} block when trains of stimuli at 2 Hz were applied, but not at 0.5 or 1 Hz, while flecainide-induced V_{max} block became evident during repetitive stimulation at 0.5, 1 and 2 Hz. This is due to the fact that in the presence of flecainide the recovery kinetics from V_{max} block is a very slow process ($\tau_{re} = 16.4 \pm 2.3$ s), the diastolic interval during the trains not being long enough to bring about a complete reactivation of V_{max}. As a consequence, flecainide would produce an accumulation of blocked Na^+ channels during each successive action potential until a new steady-state is reached. Furthermore, the percentage of frequency-dependent V_{max} block produced by mexiletine plus flecainide significantly increased over that induced by flecainide alone as the rate of stimulation increased. Thus, while at 2 Hz the V_{max} block produced by the combination markedly increased over that induced by flecainide alone, this increment exponentially decreased at slower rates of stimulation, so that at 0.5 Hz both flecainide and the combination produced a similar amount of V_{max} block.

As can be predicted from the equation for the two-state model (Courtney, 1983) the onset rate of the frequency-dependent V_{max} block, expressed as a fractional decrease per action potential (K, AP^{-1}), was faster at slower stimulation frequencies at any drug concentrations tested. When 2 Hz-trains were applied, the development kinetics of V_{max} block induced by mexiletine consisted of a very fast initial process followed by a slower one ($K = 2.04 \pm 0.30$ AP^{-1} and 0.12 ± 0.01 AP^{-1}, respectively), whereas that induced by flecainide was a very slow process (0.05 ± 0.004 AP^{-1}). The development kinetics of flecainide plus mexiletine also exhibited two components ($K = 1.20 \pm 0.30$ AP^{-1} and 0.08 ± 0.02 AP^{-1}, respectively). The K values of the fast component were of similar magnitude both in the presence of mexiletine alone or in the presence of the combination, whereas the K values of the slow component obtained in the presence of the combination were similar to that obtained in the presence of flecainide alone. Thus, it can be concluded that the first component observed in the presence of the combination is probably due to the binding of mexiletine to the Na channels, while the slow one reflects the access of flecainide to its receptor site.

Recently, it has been described (Anno & Hondeghem, 1989; 1990) that in the presence of flecainide the recovery process of I_{Na} block showed two different components: (1) a very fast initial component which results from unblocking associated with activation (AD – A), and (2) a very slow component attributed to the transition from RD and SID to the R and SI states of the Na^+ channels (RD – R; SID – SI). These two components were observed both in the presence of flecainide alone and of the combination flecainide plus mexiletine. Therefore, the first fast component of V_{max} reactivation observed in the presence of flecainide alone was probably due to the unblocking of flecainide bound activated channels, whereas the component observed in the presence of the combination of mexiletine plus flecainide is probably due to the unblocking process of mexiletine. In fact, the time constant of this fast component was similar to that obtained in the presence of mexiletine alone. The interaction between mexiletine and flecainide could be understood in terms of the proposal of Clarkson & Hondeghem (1985), in which they adapted the modulated receptor hypothesis (Hondeghem & Katzung, 1977; 1984) to simulate the behaviour of V_{max} when two anti-arrhythmic drugs are combined. In these simulations the following assumptions were made: (1) in the absence of drugs, Na^+ channels can exist in three primary states (R, A, I); (2) each drug competes for a single common receptor associated with the Na^+ channel; and (3) drug-associated channels do not conduct and have the inactivation parameters offset by two different voltage shifts. Therefore, in the presence of the combination of two drugs, each one could compete for a limited number of receptor sites during each action potential. If both drugs have similar affinities for the different states of the Na^+ channel as well as similar rate constants of binding and dissociation, then the competition between them will be of little electrophysiological consequence. In contrast, when both drugs exhibit substantially different characteristics, as in the case of mexiletine and flecainide, then the competitive interaction becomes quite noticeable at least at certain driving rates. In fact, at stimulation frequencies higher than 0.5 Hz the V_{max} block induced by mexiletine plus flecainide increased over that induced by flecainide alone; this combination can be considered as synergistic according to what is predicted by the model of Clarkson & Hondeghem (1985).

It seems that there is an apparent contradiction in these results since the magnitude of the slow component of V_{max} reactivation was significantly less in the presence of the combination than in the presence of flecainide alone even when the time constant of this slow component was similar in both conditions. However, because mexiletine binds and unbinds faster from the Na^+ channels, it may decrease the number of channels available to be blocked by a drug with much slower binding kinetics as flecainide. Therefore, the slow component of reactivation, which represents the unblocking process of flecainide-bound channels, would be lowered in the presence of the combination. The synergistic effect observed at rapid frequencies of stimulation in the presence of the combination can also be explained by the additive effect of the slow binding kinetics of flecainide to the population of Na^+ channels available; that is, those which were not previously blocked by mexiletine.

Mexiletine and flecainide have been found effective in treating or preventing ventricular arrhythmias (Sandoe *et al.*, 1978; Anderson *et al.*, 1981; Duff *et al.*, 1981; Hodges & Heel, 1985; Somberg & Tepper, 1986; Podrid, 1986; Campbell, 1987). In the present study mexiletine produced a more marked depression of the V_{max} of early action potentials than flecainide alone, while the combination of both drugs produced more depression of early test action potentials than mexiletine alone. These results explain why the ERP/APD_{90} ratio significantly increased in the presence of mexiletine or the combination, but not in the presence of flecainide alone. Thus, this combination could be effective in preventing and/or treating ventricular tachyarrhythmias resistant to either drug alone. On the other hand, the present results also demonstrated that at frequencies higher than 0.5 Hz the combination of mexiletine plus flecainide produced a greater depression of V_{max} of the ventricular action potentials, thus suggesting that this combination may be accompanied by a higher depression of intraventricular conduction velocity and a higher incidence of proarrhythmic effects.

We thank Mrs Maria Tamargo for technical assistance. This work was supported by FISS and CICYT Grants.

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(Received April 3, 1990
Revised February 6, 1991
Accepted February 8, 1991)

Br. J. Pharmacol. (1991), **103**, 1417–1420 © Macmillan Press Ltd, 1991

Negative chronotropic effect of a novel class III antiarrhythmic drug, UK-68,798, devoid of β-blocking action on isolated guinea-pig atria

Tao Yang, Pål M. Tande & [1]Helge Refsum

Department of Medical Physiology, Institute of Medical Biology, University of Tromsø, N-9000 Tromsø, Norway

1 The chronotropic effects of a novel class III antiarrhythmic drug, UK-68,798, and the β-adrenoceptor blocker, propranolol, for comparison, were studied on spontaneously beating right atria isolated from guinea-pigs in the absence and presence of increasing concentrations of isoprenaline (10^{-10}–10^{-4} M).

2 UK-68,798 (10^{-9}–10^{-5} M) decreased spontaneous atrial rate by 6–21%. Propranolol (10^{-8}–10^{-6} M) also had a negative but significantly smaller chronotropic effect.

3 UK-68,798 dose-dependently reduced the maximal positive chronotropic effect induced by isoprenaline, but without significantly shifting the concentration-response curve for isoprenaline in a parallel fashion. A pD'_2 value of 5.88 was obtained. As expected, propranolol displayed a competitive inhibition with a pA_2 value of 8.21.

4 The results demonstrate a negative chronotropic effect of UK-68,798, which is not associated with a β-adrenoceptor blocking action. We suggest that the negative chronotropic effect is linked with potassium channel blockade and thereby the class III antiarrhythmic action of UK-68,798.

Keywords: Class III antiarrhythmic; automaticity; sinus node; non-competitive antagonism; I_K

Introduction

UK-68,798, i.e., N-[4-(2-{2-[4- (methanesulphonamide)phenoxy]-*N*-methylethylamino}ethyl)phenyl] methanesulphonamide, is a newly synthesized class III antiarrhythmic compound. It potently prolongs action potential duration and effective refractory period in isolated cardiac muscle and the intact heart, probably by selective inhibition of the delayed rectifying potassium current, I_K (Gwilt *et al.*, 1989a; Tande *et al.*, 1990b).

A negative chronotropic effect of UK-68,798 has also been noted both *in vitro* (Tande *et al.*, 1990b) and *in vivo* (Gwilt *et al.*, 1989b; Yang *et al.*, 1990). Other drugs with class III antiarrhythmic action like amiodarone (Kadoya *et al.*, 1985) and sotalol (Campbell, 1987) also inhibit sinus node function which may give bradycardia. It has been debated whether the negative chronotropic effect of amiodarone and sotalol could be due to their effect on repolarization alone, i.e. linked with the class III action, or other actions like antiadrenoceptor effects (Polster & Broekhuysen, 1975; Tande & Refsum, 1990a).

The present study was therefore designed to assess further the chronotropic effect of UK-69,798 and to investigate whether it has any β-adrenoceptor blocking action. Experiments were performed on guinea-pig right atria. Propranolol, a typical β-adrenoceptor blocker, was used for comparison.

Methods

Right atria with sinoatrial node tissue were quickly dissected from the hearts of female guinea-pigs ($n = 19$, weighing 200–300 g) and placed in a water-jacketed organ bath containing 20 ml Tyrode solution gassed with 95% O_2 plus 5% CO_2 kept at 32 ± 0.5°C. The solution had the following composition (mM): Na^+ 141.2, K^+ 5.3, Cl^- 129.4, Ca^{2+} 2.0, HCO_3^- 20, $H_2PO_4^-$ 1.2, Mg^{2+} 1.0, glucose 10. The pH was adjusted to 7.4. The atrial preparations were allowed to beat spontaneously throughout the experiments. To record spontaneous atrial rate (SAR), isometric contractions were measured with a force displacement transducer (Type FT03C, Grass Instruments Co, Quincy, Mass) connected to a dynograph recorder (Type 511A, Beckman Instruments Inc, Schiller Park, Ill). A preload of 500 mg was put on the preparations.

Experimental protocol

In order to study any possible β-blocking activity of UK-68,798, concentration-response (CR) curves for isoprenaline were obtained in 14 spontaneously beating right atria. Cumulative doses of isoprenaline (10^{-10} to 10^{-4} M) were introduced after an equilibration period of 60 min, each increment being added when the preceding one had produced its maximum effect. In this way, a control CR curve was obtained. Then, the preparation was repeatedly washed with the Tyrode solution for 1 h (five to six times), and the spontaneous frequency stabilized. In 7 experiments, 10^{-8} and 10^{-7} M UK-68,798 were added to the organ bath 20 min before repeating the CR curves for isoprenaline. In 5 of these experiments 10^{-9} M UK-68,798 was given 20 min before 10^{-8} M but without giving isoprenaline. In the other 7 experiments UK-68,798 at 10^{-6} and 10^{-5} M was given. For reference, the same procedure was repeated with propranolol (10^{-8}, 10^{-7} and 10^{-6} M) in 5 experiments. The direct effects on SAR of increasing concentrations of UK-68,798 (10^{-9}–10^{-5} M) or propranolol (10^{-8}–10^{-6} M) could thereby also be obtained before establishing the CR curves for isoprenaline.

Drug preparation

UK-68,798 was a gift from Pfizer Central Research (Sandwich, Kent, U.K.). (±)-Propranolol hydrochloride was obtained from Aldrich-Chemie (Steinheim, F.R.G.). Isoprenaline sulphate was obtained from NAF-Laboratory (Oslo, Norway). Stock solutions (10^{-3} M) of UK-68,798 (acidified to pH 3) and propranolol in distilled water were kept at −18°C. Dilutions were made in Tyrode solution during the course of each experiment.

Calculations and statistics

The effects on SAR of increasing concentrations of UK-68,798 or propranolol were calculated as percentages of change from baseline value obtained prior to addition of each concentration.

[1] Author for correspondence.

The concentration of isoprenaline eliciting half maximal response (EC_{50}) was obtained from graphical plots of the CR curve from each experiment. The EC_{50} value was expressed as pD_2 value ($pD_2 = -\log EC_{50}$, negative logarithm of molar concentration of agonist that caused half maximal response) (Van den Brink, 1977). For the competitive antagonism, pA_2 values were calculated from the following equation (Mackay, 1978):

$$pA_2 = \log(DR - 1) - \log[B] \quad (1)$$

where DR represents the agonist dose-ratio and [B] represents the molar concentration of the antagonist. The pA_2 value is defined as the negative logarithm of molar concentration of antagonist that caused a twofold shift of the CR curve for agonist. For the non-competitive antagonism, pD'_2 values were determined according to the following equation (Van den Brink, 1977):

$$pD'_2 = -\log[B] + \log(E_A/E_{AB}' - 1] \quad (2)$$

where [B] represents the molar concentration of antagonist, E_A represents the effect of agonist in the absence of antagonist and E_{AB}' represents the effect of agonist in the presence of antagonist. The pD'_2 value is equal to the negative logarithm of molar concentration of antagonist that caused half reduction of the CR curve for agonist.

Mean values ± s.e.mean are used to express the data. Two-tailed Student's t tests for either paired or unpaired observations were used to compare the results. $P < 0.05$ was considered statistically significant.

Results

Negative chronotropic effects of UK-68,798 and propranolol

The baseline SAR of the guinea-pig isolated right atria was 157 ± 5 beats min^{-1} ($n = 19$). Increasing concentrations of UK-68,798 (10^{-9}–10^{-5} M) decreased SAR by 6% ($n = 5$), 13%, 19% ($n = 7$), 20% and 21% ($n = 7$), respectively (Figure 1). Thus, even at a concentration of 10^{-8} M there was a significant negative chronotropic effect of UK-68,798 and at 10^{-7} M it seemed to have reached its maximum. In another group of 5 experiments, propranolol (10^{-8}–10^{-6} M) decreased SAR to a certain extent but significantly less than UK-68,798 at the same concentrations (Figure 1).

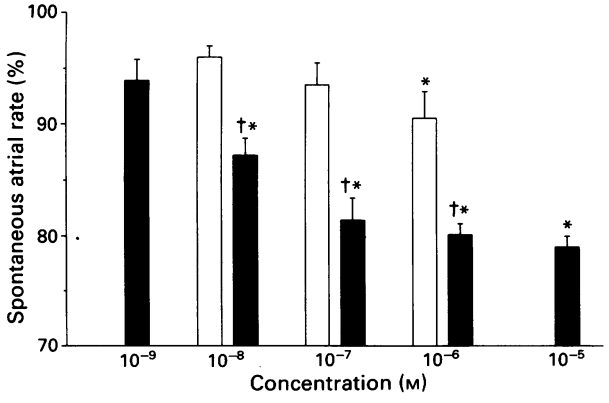

Figure 1 Effects of UK-68,798 (solid columns, 10^{-9} M, $n = 5$; 10^{-8}–10^{-7} M, $n = 7$; 10^{-6}–10^{-5} M, $n = 7$) and propranolol (open columns, 10^{-8}–10^{-6} M, $n = 5$) on spontaneous atrial rate of guinea-pig isolated rat atria 20 min after addition of each concentration. Mean values (s.e.mean shown by vertical bars) are calculated as percentages from baseline values. * $P < 0.05$ as compared with control values (paired t test based on real values); † $P < 0.05$ as compared with propranolol (unpaired t test based on the percentage changes).

Changes in the concentration-response curves for the isoprenaline-induced positive chronotropic response

Isoprenaline, used as β-adrenoceptor agonist, significantly increased SAR. Before addition of UK-68,798 or propranolol, the control pD_2 values of isoprenaline were 8.13 ± 0.08 ($n = 7$) and 8.18 ± 0.08 ($n = 7$) in the two UK-68,798 groups and 8.00 ± 0.12 ($n = 5$) in the propranolol group, respectively, in good agreement with the values reported by others (8.06–8.76) (O'Donnell & Wanstall, 1979; Polster et al., 1975). After addition of UK-68,798, the pD_2 value obtained after each concentration of UK-68,798 was not significantly different from the control value (Table 1). As shown in Figure 2, increasing concentrations of UK-68,798 did not significantly shift the CR curve for isoprenaline to a higher concentration-range in a parallel manner. However, the maximum response of the CR curve for isoprenaline was reduced dose-dependently in the presence of UK-68,798, which is characteristic for non-competitive antagonism. The pD'_2 value calculated for the various concentrations of UK-68,798 was 5.88 ± 0.59 ($n = 14$).

On the other hand, propranolol shifted the CR curve for isoprenaline to the right with no reduction in the maximum response (Figure 3), demonstrating competitive β-adrenoceptor antagonism. As shown in Table 1, the pD_2 value

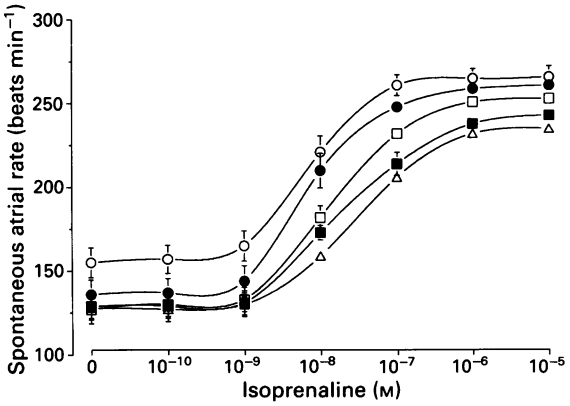

Figure 2 Cumulative concentration-response curves for the positive chronotropic effects of isoprenaline (10^{10}–10^{-5} M) on spontaneously beating guinea-pig right atria in the absence and presence of increasing concentrations of UK-68,798: (○) control ($n = 14$); (●) 10^{-8} M and (□) 10^{-7} M, ($n = 7$); (■) 10^{-6} M and (△) 10^{-5} M, ($n = 7$). Values shown are mean with s.e.mean indicated by vertical bars.

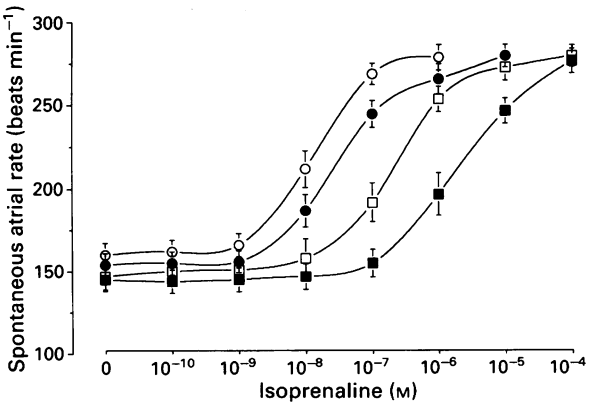

Figure 3 Cumulative concentration-response curves for the positive chronotropic effects of isoprenaline (10^{-10}–10^{-4} M) on spontaneously beating guinea-pig right atria in the absence and presence of increasing concentrations of propranolol: (○) control; (●) 10^{-8} M; (□) 10^{-7} M and (■) 10^{-6} M ($n = 5$). Values shown are mean with s.e.mean indicated by vertical bars.

Table 1 The effects of various concentrations of UK-68,798 and propranolol on pD_2, pA_2, pD'_2 and maximum chronotropic response (Iso_{max}) of guinea-pig isolated right atria to isoprenaline (10^{-10}–10^{-4} M)

Conc. (M)	n	pD_2	pA_2	pD'_2	Iso_{max} (%)
UK-68,798					
0	7	8.13 ± 0.08			100
10^{-8}	7	7.82 ± 0.11		7.24 ± 0.30	92 ± 3
10^{-7}	7	7.84 ± 0.15		6.38 ± 0.27	86 ± 4*
0	7	8.18 ± 0.08			100
10^{-6}	7	7.91 ± 0.08		5.33 ± 0.17	78 ± 7*
10^{-5}	7	7.89 ± 0.10		4.55 ± 0.15	71 ± 6*
Propranolol					
0	5	8.00 ± 0.12			100
10^{-8}	5	7.51 ± 0.12*	8.25 ± 0.17		99 ± 2
10^{-7}	5	6.82 ± 0.20*	8.31 ± 0.10		98 ± 1
10^{-6}	5	5.93 ± 0.12*	8.06 ± 0.22		97 ± 2

All values represent the mean ± s.e.mean of n experiments.
* $P < 0.05$ compared to control (0) value. The explanations for pD_2, pA_2 and pD'_2 are given in Methods.

for isoprenaline was significantly decreased. A pA_2 value of 8.21 ± 0.08 ($n = 5$) was obtained for propranolol.

Discussion

The spontaneously beating guinea-pig right atrial preparation has previously been shown to be a reliable method for studying chronotropic effects of cardiac drugs (Kline & Buckley, 1972). It has been demonstrated that guinea-pig atria, unlike cat atria, contain a single β-adrenoceptor type (β_1) and not a β-adrenoceptor mixture (β_1 and β_2) (O'Donnell & Wanstall, 1975). Therefore, guinea-pig atria should be a better tissue for assessing the potency of agonists and antagonists of β_1-adrenoceptors. Thus, in guinea-pig right atria with sinoatrial node tissue, the positive chronotropic effect induced by isoprenaline, a non-selective β-adrenoceptor agonist, is most likely to be mediated through activation of β_1-adrenoceptors (Spilkner *et al.*, 1974; O'Donnell & Wanstall, 1979).

In the present study increasing concentrations of UK-68,798 decreased spontaneous atrial rate. This is in good agreement with our previous results where we, in addition, demonstrated a parallel increase of sinus node recovery time. No effect on the corrected sinus node recovery time indicated that the spontaneous depolarization phase was not affected (Tande *et al.*, 1990b). A negative chronotropic effect of UK-68,798 has also been observed *in vivo* (Gwilt *et al.*, 1989b; Yang *et al.*, 1990). Similar negative chronotropic effects have been described for D- and L-sotalol on guinea-pig (Campbell, 1987) and rabbit (Kato *et al.*, 1986) sinoatrial node action potentials and attributed almost entirely to prolongation of repolarization. D-Sotalol also decreased heart rate in anaesthetized dogs pretreated with propranolol in correlation with the effect on the QT-time (Mortensen *et al.*, 1990).

UK-68,798 selectively prolongs action potential duration and effective refractory period in guinea-pig papillary muscle, an effect considered to result from inhibition of the delayed rectifying potassium current (I_K) (Gwilt *et al.*, 1989a; Tande *et al.*, 1990b). This is also supposed to be the mechanism behind the prolongation of the action potential by sotalol (Carmeliet, 1985). Studies have shown that a similar current, also named I_K, is also one of the ionic currents contributing to the pacemaker potentials of sinoatrial node cells (Noma & Irisawa, 1976; Yanagihara & Irisawa, 1980). Decay of I_K is believed to be a major controlling factor determining the rate of spontaneous depolarization (Noma & Irisawa, 1976). Furthermore, subsequent increase in I_K leads to a more rapid repolarization and contributes to the maximum diastolic potential (Brown *et al.*, 1979). Thus, a selective interaction with I_K of UK-68,798 could explain its negative chronotropic effect.

The effect of UK-68,798 on the CR curve for the isoprenaline-induced positive chronotropy indicates a non-competitive inhibition. This implies that UK-68,798 does not interact with the β-adrenoceptor agonist isoprenaline at the same sites on the cellular membrane. The reduction of the maximum response of the CR curve for isoprenaline by UK-68,798 might be explained by the suggestion that inhibition of I_K in pacemaker cells of the sinoatrial node is probably opposite to the mechanism by which isoprenaline induces its positive chronotropic effect. β-Adrenoceptor stimulation may partly accelerate both depolarization and repolarization of the pacemaker cells through increasing I_K (Brown & DiFrancesco, 1980).

Propranolol is a competitive β-adrenoceptor antagonist with membrane stabilizing properties. In isolated cardiac preparations, the membrane stabilizing activity of propranolol has been reported at 10^{-6} M or above (Doggrell & Hughes, 1986). Propranolol has also previously been shown to decrease spontaneous frequency of isolated atria (Nickerson & Collier, 1975). Thus, besides β-adrenoceptor blocking action of propranolol, which may be of significance *in vitro* due to liberation of endogenous catecholamines within atrial tissue (Tande & Refsum, 1988), its membrane stabilizing activity may partly be responsible for the reduced heart rate observed in isolated atrial preparations. In the present study, a parallel rightward shift in the cumulative CR curve for isoprenaline without depression of maximum response occurred in the presence of propranolol. This demonstrates how propranolol significantly antagonizes the accelerated heart rate induced by isoprenaline through competition at the same receptor sites, i.e. competitive β-adrenoceptor antagonism.

In conclusion, UK-68,798 exerts a negative chronotropic effect devoid of β-adrenoceptor blocking action. Class III antiarrhythmic action may thus be linked with negative chronotropy.

This study was supported by grants from the Norwegian Research Council for Science and the Humanities, and the Norwegian Council on Cardiovascular Diseases.

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(Received July 25, 1990
Accepted February 8, 1991)

Br. J. Pharmacol. (1991), **103**, 1421–1428 © Macmillan Press Ltd, 1991

Further characterization of the putative 5-HT receptor which mediates blockade of neurogenic plasma extravasation in rat dura mater

M. Gabriella Buzzi, [1]Michael A. Moskowitz, *Stephen J. Peroutka & Barkjang Byun

Stroke Research Laboratory, Neurosurgery and Neurology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, U.S.A. and *Department of Neurology, Stanford University School of Medicine, Stanford, CA 94305, U.S.A.

1 We describe the effects of pretreatment with 5-hydroxytryptamine (5-HT) receptor agonists and antagonists on neurogenically-mediated plasma protein extravasation ([^{125}I]-albumin) in rat dura mater and in extracranial tissues (temporalis muscle fascia, conjunctiva, eyelid and lip) induced by electrical stimulation of the right trigeminal ganglion.

2 Leakage of [^{125}I]-bovine serum albumin from blood vessels in dura mater following high intensity stimulation (1.2 mA, 5 ms, 5 Hz for 5 min) was significantly reduced by the intravenous administration of drugs active at 5-HT receptors with some selectivity for the 5-HT$_1$ receptor subtypes: 5-carboxamidotryptamine (5-CT) (threshold dose, 1 ng kg^{-1}); 5-benzyloxytryptamine (5-BT) (10, 30 or 100 μg kg^{-1}); 8-hydroxydipropylaminotetralin (8-OH-DPAT) (300 μg kg^{-1}); and as previously reported, sumatriptan (100 μg kg^{-1}), dihydroergotamine (DHE) (50 μg kg^{-1}), ergotamine tartrate (100 μg kg^{-1}) and chronically administered methysergide (1 mg kg^{-1}).

3 The putative 5-HT receptor antagonist, metergoline 100 μg kg^{-1}, inhibited partially the effect of sumatriptan in dura mater providing additional evidence for a 5-HT$_1$ receptor subtype-mediated mechanism, although it was not effective against 5-CT (1 ng kg^{-1}). Methiothepin (300 μg kg^{-1}) did not affect the response to sumatriptan. When administered at high concentrations (1 mg kg^{-1}) methiothepin and metergoline decreased plasma protein extravasation in rat dura mater.

4 Pretreatment with the 5-HT$_2$ receptor antagonists pizotifen, 300 μg kg^{-1}, or ketanserin, 300 μg kg^{-1}, or the 5-HT$_3$ receptor antagonists MDL 72222, 300 μg kg^{-1}, or ICS 205-930, 300 μg kg^{-1}, did not affect plasma protein leakage following electrical trigeminal stimulation. Blockade by sumatriptan of plasma protein extravasation was not inhibited by pizotifen (300 μg kg^{-1}) or MDL 72222 (300 μg kg^{-1}).

5 The 5-HT receptor(s) mediating this response were present only on intracranial tissues innervated by the trigeminal nerve; plasma protein extravasation in extracranial tissues was not blocked by pretreatment with the equivalent or higher concentrations of the above drugs following low intensity trigeminal stimulation (0.1 mA, 5 ms, 5 Hz).

6 The putative 5-HT receptor(s) mediating this response were not present on sympathetic fibres innervating dura mater since unilateral removal of the superior cervical ganglion did not prevent the development of plasma protein extravasation nor did it affect the blockade by sumatriptan 100 μg kg^{-1}.

7 The above pharmacological data suggest that intracranial vessels possess 5-HT receptor(s) which are coupled to inhibition of neurogenically-mediated plasma protein extravasation. These receptors cannot be detected on extracranial cephalic blood vessels innervated by the trigeminal nerve, although available evidence strongly suggests that the 5-HT receptors reside on perivascular trigeminal nerve fibres. The rank order of effective doses (threshold concentrations; 5-CT $\ll$ 5-BT < DHE < sumatriptan < 8-OHDPAT) is most consistent with a 5-HT$_{1B}$- or 5-HT$_{1D}$-mediated response, among the known 5-HT$_1$ family of receptors. However, the lack of effect of methiothepin against the actions of sumatriptan, or metergoline against the effects of 5-CT suggest important differences and the possibility that a previously unrecognized 5-HT receptor(s) is involved in this response.

Keywords: Neurogenic inflammation; 5-HT receptors; sumatriptan; dihydroergotamine; dura mater

Introduction

Neurogenic inflammation (vasodilatation and plasma protein extravasation) may be important to the pathophysiology of migraine headaches and to the action of antimigraine drugs (Chapman *et al.*, 1961; Dalessio, 1974; Moskowitz *et al.*, 1979; Fozard, 1982; Hardebo, 1984; Moskowitz, 1984; Saito *et al.*, 1988). Neurogenic plasma extravasation develops in dura mater and extracranial cephalic tissues following electrical trigeminal stimulation or systemic capsaicin administration and can be detected by leakage of albumin tracer (Markowitz *et al.*, 1987). Extravasation appears to be mediated in part by released neuropeptides (e.g., substance P, neurokinin A, and calcitonin gene-related peptide) from perivascular sensory fibres (Saria *et al.*, 1985). In the dura mater there is ultrastructural evidence for mast cell degranulation, platelet accumulation, and increase in the number of endothelial vesicles specifically in postcapillary venules following electrical stimulation (Buzzi *et al.*, 1990; Dimitriadou *et al.*, 1990). Protein leakage does not develop in the dura mater after pretreatment with clinically relevant doses of the antimigraine drugs sumatriptan, and the ergot alkaloids ergotamine tartrate, dihydroergotamine and chronic methysergide (Saito *et al.*, 1988; Buzzi & Moskowitz, 1990). Furthermore, the ultrastructural changes are blocked by sumatriptan and dihydroergotamine (Buzzi *et al.*, 1990).

The pharmacological data suggest that a 5-HT receptor subtype mediates this response. To define more clearly the

[1] Author for correspondence.

5-HT receptor which mediates the blockade of neurogenic plasma extravasation, we examined extravasation in the dura mater in the presence of drugs active at a variety of 5-HT subtypes. The possibility that the 5-HT receptor mediating this response resides on sympathetic fibres innervating blood vessels of the dura mater was also examined.

Methods

Electrical trigeminal stimulation and tissue dissection

Male Sprague-Dawley rats (150–200 g, Charles River Laboratories, Wilmington, Massachusetts, U.S.A.) were housed under diurnal lighting conditions and allowed food and water *ad libitum*. Anaesthetized rats (pentobarbitone, 60 mg kg^{-1}, i.p.) were placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA) and the skull was exposed by a midline incision. Symmetrical burr holes were drilled 2.5–2.8 mm laterally and 3.2 mm posteriorly to bregma for electrode placement. [^{125}I]-bovine serum albumin 70 μCi kg^{-1} was then injected into the left femoral vein as a bolus. No untoward effects of this protein were noted on blood pressure or body temperature in selected animals. After 5 min stainless steel bipolar electrodes, 5 mm shaft (Rhodes medical Instruments, Woodland Hills, CA) were lowered into the trigeminal ganglia to a depth of 9.2 mm from the dura mater. The incisor bar was set at −2.5 mm from the horizontal. To examine the extravasation response in dura mater, the right trigeminal ganglion was stimulated for either 5 min (1.2 mA, 5 ms duration, 5 Hz), or when indicated, for 3 min using identical parameters. To examine the response in facial tissues, the right trigeminal ganglion was stimulated for 1 or 2 min (0.1 mA, 5 ms, 5 Hz) (Microprocessor Stimulator Microstim III, Linear Iso III, World Precision Instruments, San Carlos, CA, U.S.A.; Hitachi Densi Oscilloscope, Tokyo). After the stimulation period, the animals were promptly perfused with saline via the left cardiac ventricle for 2 min at a constant pressure of 100 mmHg (approximately 100 ml) in order to remove completely iodinated albumin from blood vessels. The anaesthetized animals died during this procedure. The skull was then opened, the brain removed and the cranial cavity thoroughly rinsed with saline. Dura mater was dissected bilaterally as previously described (Markowitz *et al.*, 1987). The large dural sinuses, the region of dura through which the electrodes penetrated, and the dura covering the trigeminal ganglion were excluded from analysis. Fascia covering the temporalis muscle (approximately 3.5 mg wet weight), conjunctiva, eyelid and lip were dissected on both sides and levels of radioactivity determined (Micromedic Systems Inc., Huntsville, AL, U.S.A.).

Superior cervical ganglionectomy

In anaesthetized (pentobarbitone, 60 mg kg^{-1}) Sprague-Dawley rats (100–125 g) the cervical region was opened with an anterior midline incision. The underlying muscles were separated in order to expose the common carotid artery. The artery on the right side only was isolated with a 3-0 silk surgical suture and gently lifted aside at its bifurcation to visualize the ipsilateral superior cervical ganglion. Once the ganglion was exposed, it was totally removed with forceps. In a separate group of animals the common carotid artery was exposed and lifted as described above, and the superior cervical ganglion visualized but not removed (sham). Animals were allowed to recover from anaesthesia under a warming lamp, and then returned to the animal facilities where they were kept for twelve days before electrical stimulation of the right trigeminal ganglion was performed. Ptosis on the ipsilateral side of the above surgical procedure confirmed the success of the surgery.

Protocol

Drugs were administered i.v. 10 min before electrical trigeminal stimulation and 5 min before [^{125}I]-albumin to test their ability to block the extravasation response (i.e., to behave as agonists): sumatriptan 100 or 300 μg kg^{-1}; 8-OH-DPAT 100 or 300 μg kg^{-1}; 5-CT 0.1, 1, 10, 30, 100 and 300 ng kg^{-1}; metergoline 100, or 500 μg kg^{-1} or 1 mg kg^{-1}; methiothepin 0.3, 1 mg kg^{-1}; 5-BT 10, 30, 100 or 300 μg kg^{-1}; pizotifen 300 μg kg^{-1}; ketanserin 300 μg kg^{-1}; ICS 205-930 300 μg kg^{-1}; MDL 72222 300 μg kg^{-1}.

Some compounds were also tested as antagonists of sumatriptan. Metergoline 100 μg kg^{-1}, 5-BT 30 μg kg^{-1}, methiothepin 300 μg kg^{-1}, pizotifen 300 μg kg^{-1}, or MDL 72222 300 μg kg^{-1} were administered 20 min before electrical trigeminal stimulation and 10 min before sumatriptan (100 μg kg^{-1}). Metergoline (100 or 300 μg kg^{-1}) was also given 10 min before 5-CT (1 or 300 ng kg^{-1}). Animals were perfused immediately after stimulation and treated as described above.

Twelve days after the right superior cervical ganglionectomy or sham surgery, operated animals were subjected to electrical stimulation of the right trigeminal ganglion as described above. Sumatriptan 100 μg kg^{-1} or vehicle was administered 10 min before electrical stimulation.

Intra-arterial blood pressure recording

A 22 gauge catheter was placed in the left femoral artery, and mean arterial blood pressure (MABP) recorded (Winston Electronics Co., Millbrae, CA) in selected animals, starting 5 min before drug or vehicle administration and continuing until the end of the electrical trigeminal stimulation.

Data analysis

Three to five animals were studied in each group and each experiment was repeated at least once on separate days. Plasma [^{125}I]-bovine serum albumin extravasation is expressed as the ratio: c.p.m. mg^{-1} wet wt. (stimulated side)/ c.p.m. mg^{-1} wet wt. (unstimulated side). Results are shown as mean ± s.e.mean. Student's t test was used for statistical analysis between drug- and vehicle-treated groups. Blood pressure data were analyzed by ANOVA. Probability values (P) of less than 0.05 were considered significant.

Drugs

The following chemicals and drugs were used: [^{125}I]-bovine serum albumin (New England Nuclear, Boston, MA), capsaicin (Polyscience Inc., Wilmington, Pennsylvania), 5-HT (Sigma Chemical Co., St Louis, Missouri), sumatriptan [3-(2-dimethylaminoethy)-1H-indole-5-(N-methyl)methane sulphonamide], 5-carboxamidotryptamine (5-CT) (Glaxo, Ware, Hertfordshire, United Kingdom), 8-hydroxydipropylaminotetralin (8-OH-DPAT) (Research Biochemical Inc., Natick, Massachusetts), 5-benzyloxytryptamine (5-BT) (Aldrich), methiothepin maleate (Roche), metergoline (Farmitalia), ketanserin (Janssen), pizotifen, ICS 205-930 (Sandoz), 1αH,3α,5αH-tropan-3yl-3,5-dichlorobenzoate hydrochloride (MDL 72222) (Merrell Dow).

[^{125}I]-bovine serum albumin was diluted in saline; capsaicin was solubilized in saline:ethanol:Tween 80 8:1:1; 5-HT and sumatriptan were dissolved in saline 0.9%; 5-CT and MDL 72222 in distilled water; methiothepin, pizotifen, and ICS 205-930 in dimethylsulphoxide:saline 1:9; 5-BT in glacial acetic acid:distilled water 1:30; ketanserin and metergoline in dimethylsulphoxide. All the above were injected intravenously in a volume of 1 ml kg^{-1}, except metergoline and ketanserin which were given in a volume of 0.3 ml kg^{-1}. The same volume of vehicle was administered to the control group in each experiment.

Results

Mean arterial blood pressure

Baseline MABP ranged from 83 to 102 mmHg in pentobarbitone-anaesthetized animals. A 10–20 mmHg increase was observed during the electrical stimulation of the right trigeminal ganglion in all animals although it did not reach statistical significance in drug-treated animals. Baseline MABP after superior cervical ganglionectomy did not differ from baseline values in normal animals. Significant decreases in MABP were observed immediately following 5-CT, 8-OH-DPAT or ketanserin administration at the indicated doses. However MABP returned to baseline values within 2–8 min, and was at baseline prior to electrical trigeminal ganglion stimulation (Table 1).

Electrical trigeminal stimulation increases plasma protein extravasation in dura mater and extracranial tissues

High intensity stimulation (1.2 mA, 5 ms, 5 Hz, 5 min) In vehicle-treated animals, levels of iodinated albumin on the unstimulated side were 14 ± 2, 54 ± 9, and 36 ± 11 c.p.m. mg^{-1} wet wt. in dura mater, conjunctiva and temporalis muscle fascia, respectively. Unilateral electrical stimulation of the trigeminal ganglion increased leakage of iodinated albumin on the ipsilateral side to 26 ± 2 c.p.m. mg^{-1} wet wt. in dura mater, 293 ± 14 c.p.m. mg^{-1} wet wt. in conjunctiva, and 87 ± 19 c.p.m. mg^{-1} wet wt. in fascia. The ratio between stimulated and non-stimulated sides ranged from 1.4 ± 0.1 to 2.2 ± 0.2 in dura mater (70 experiments, $n = 400$). Larger ratios with greater variances were observed in extracranial tissues at this current intensity and ranged from 2.3 ± 0.3 to 4.8 ± 1.0 in conjunctiva (13 experiments, $n = 52$), and from 1.3 ± 0.1 to 2.8 ± 0.3 in fascia (9 experiments, $n = 36$) (Table 2).

Low intensity stimulation (0.1 mA, 5 ms, 5 Hz, 3 min) A significant increase in iodinated albumin was observed in extracranial tissues (conjunctiva, eyelid and lip) but not dura mater. The ratio between the two sides in 4 separate experiments ranged from 1.5 ± 0.1 to 1.9 ± 0.4 in conjunctiva, 1.2 ± 0.1 to 1.8 ± 0.2 in the eyelid, and 1.5 ± 0.2 to 2.0 ± 0.2 in lip in vehicle-treated animals. Because these ratios most closely approximated the response in dura mater at higher currents, 0.1 mA for 3 min was used in subsequent experiments to determine whether sumatriptan could block extravasation in extracranial tissues.

5-HT receptors and the neurogenic inflammatory response in dura mater

Plasma protein leakage in dura mater did not differ on the unstimulated side between vehicle- and drug-treated animals. The data (c.p.m. mg^{-1} wet wt.) contralateral to stimulation following 5-CT administration (1, 100 ng kg^{-1}) was 7.1 ± 0.4 ($n = 32$) versus 8.3 ± 0.6 (control, $n = 32$); 12.1 ± 1.1 (5-BT, 10–300 $\mu g\,kg^{-1}$, $n = 29$) versus 12.8 ± 0.9 ($n = 25$, control); 11.1 ± 1.2 (8-OH-DPAT 100,300 $\mu g\,kg^{-1}$, $n = 20$) versus 10.9 ± 1.2 ($n = 15$, control); 10.3 ± 1.0 (sumatriptan 30–300 $\mu g\,kg^{-1}$, $n = 28$) versus 10.2 ± 0.9 ($n = 21$; control). Data on the unstimulated side from conjunctiva and fascia also showed no differences between vehicle- and drug-treated animals (data not shown).

Putative 5-HT$_1$ receptor agonists

5-Carboxamidotryptamine 5-CT reduced the ratio of c.p.m. mg^{-1} wet wt. (stimulated side)/c.p.m. mg^{-1} wet wt. (unstimulated side) in doses as low as 1 ng kg^{-1} [1.9 ± 0.1 ($n = 20$) in vehicle-treated animals vs 1.3 ± 0.1 ($n = 22$), $P < 0.001$]. Ten, 30, 100, 300 ng kg^{-1} reduced the ratio by 85, 55, 50 and 67%, respectively. The lowest tested dose (0.1 ng kg^{-1}) decreased the ratio by 30% but this decrease did not reach statistical significance (Figure 1).

8-Hydroxydipropylaminotetralin 8-OH-DPAT 300 $\mu g\,kg^{-1}$ decreased the extravasation [1.7 ± 0.1 ($n = 15$) in vehicle-treated animals vs 1.4 ± 0.1 ($n = 20$), $P < 0.05$], whereas 100 $\mu g\,kg^{-1}$ did not.

5-Benzyloxytryptamine 5-BT 10, 30 or 100 $\mu g\,kg^{-1}$ administration decreased extravasation in dura mater by 42, 75,

Table 1 Mean arterial blood pressure responses (mmHg) to drug administration and/or electrical trigeminal stimulation in normal rats or following superior cervical ganglionectomy

		Time (min)			
Treatment (n)	*Baseline*	0[a]	5	10[b]	15
Vehicle (3)	96 ± 8	97 ± 4	98 ± 4	95 ± 4	110 ± 5*
5-CT (100 ng kg^{-1}) (4)	86 ± 2	92 ± 5	91 ± 4	92 ± 5	100 ± 5
8-OH-DPAT (300 $\mu g\,kg^{-1}$) (5)	87 ± 3	86 ± 3	94 ± 4	88 ± 5	98 ± 3
Ketanserin (300 $\mu g\,kg^{-1}$) (3)	88 ± 3	87 ± 2	93 ± 6	92 ± 4	97 ± 2
Methiothepin (1 mg kg^{-1}) (3)	97 ± 3	99 ± 1	94 ± 2	92 ± 3	108 ± 5
Metergoline (1 mg kg^{-1}) (3)	92 ± 5	91 ± 5	95 ± 1	91 ± 3	101 ± 8
5-BT (100 $\mu g\,kg^{-1}$) (3)	97 ± 4	99 ± 3	101 ± 2	101 ± 1	—
5-BT (30 $\mu g\,kg^{-1}$) (3)	95 ± 3	95 ± 3	97 ± 3	100 ± 3	—
Ganglionectomy (3)	96 ± 4	98 ± 2	98 ± 1	99 ± 3	110 ± 1*

Mean arterial blood pressure (MABP) was recorded via the left femoral artery in anaesthetized (pentobarbitone, 60 mg kg^{-1}, i.p.) rats placed in a stereotaxic frame with electrodes in the trigeminal ganglia. After steady baseline values were obtained, drugs or vehicle were administered i.v. at time 0 (a) at the indicated doses. Electrical stimulation of the trigeminal ganglion (1.2 mA, 5 ms, 5 Hz, 5 min) was begun at 10 min (b) and MABP recorded until the end of unilateral electrical stimulation. A slight increase was noted in all groups, which reached statistical significance in vehicle-treated animals and in sympathectomized animals. Transient (2–4 min) and significant decreases in MABP were observed following 5-CT, 8-OH-DPAT, or ketanserin administration (not shown). Five and 10 min after drug or vehicle injection however, MABP was not significantly different from baseline. In those groups receiving 5-BT, MABP was not recorded at 15 min. Data are expressed as mean ± s.e.mean. * $P < 0.05$ as compared to values at 10 min. For abbreviations, see text.

Table 2 Plasma protein extravasation in dura mater and extracranial tissues following pretreatment with drugs that bind to 5-HT receptors

Dura mater

Vehicle (*n*)	*Drug*	(*n*)	
1.6 ± 0.1 (9)	1.7 ± 0.2	(11)	Ketanserin (300 $\mu g\,kg^{-1}$)
1.9 ± 0.2 (6)	1.6 ± 0.1	(7)	Pizotifen (300 $\mu g\,kg^{-1}$)
1.7 ± 0.1 (4)	1.8 ± 0.1	(6)	ICS 205-930 (300 $\mu g\,kg^{-1}$)
1.9 ± 0.2 (10)	1.9 ± 0.2	(18)	MDL 72222 (300 $\mu g\,kg^{-1}$)

Conjunctiva

Vehicle (*n*)	*Drug*	(*n*)	
2.6 ± 0.4 (8)	3.9 ± 0.4	(8)	Methiothepin (1 $mg\,kg^{-1}$)
2.6 ± 0.3 (4)	2.2 ± 0.2	(4)	5-BT (300 $\mu g\,kg^{-1}$)
3.2 ± 0.6 (7)	2.5 ± 0.2	(8)	5-BT (100 $\mu g\,kg^{-1}$)
2.3 ± 0.3 (4)	2.7 ± 0.5	(5)	5-BT (30 $\mu g\,kg^{-1}$)
4.8 ± 1.0 (8)	3.8 ± 0.7	(9)	Ketanserin (300 $\mu g\,kg^{-1}$)
4.2 ± 1.7 (3)	5.0 ± 1.5	(5)	Pizotifen (300 $\mu g\,kg^{-1}$)
4.8 ± 0.6 (12)	6.4 ± 1.0	(11)	MDL 72222 (300 $\mu g\,kg^{-1}$)

(*n*)	*Conjunctiva*	*Eyelid*	*Lip*
Vehicle (14)	1.9 ± 0.1	1.6 ± 0.1	1.7 ± 0.2
Sumatriptan 100 $\mu g\,kg^{-1}$ (16)	1.7 ± 0.2	1.7 ± 0.2	1.5 ± 0.2
Vehicle (9)	1.6 ± 0.1	1.4 ± 0.1	1.6 ± 0.1
Sumatriptan 300 $\mu g\,kg^{-1}$ (8)	1.4 ± 0.2	1.3 ± 0.1	1.5 ± 0.1

Drugs or vehicle were administered i.v. at the indicated doses 10 min before electrical trigeminal stimulation (1.2 mA, 5 min) and 5 min before [^{125}I]-albumin. Animals were perfused and dura mater and conjunctiva dissected on each side as described in Methods. In other experiments involving the use of sumatriptan, animals were stimulated at a lower current intensity (0.1 mA, 3 min) and conjunctiva, eyelid and lip harvested on each side. Data represent the mean ± s.e.mean of the ratio:

$$\frac{\text{c.p.m.}\,mg^{-1}\text{ wet wt. (stimulated side)}}{\text{c.p.m.}\,mg^{-1}\text{ wet wt. (unstimulated side)}}.$$

The extravasation of plasma proteins was reduced within extracranial tissues at the lower current intensity, although sumatriptan did not attenuate this response.

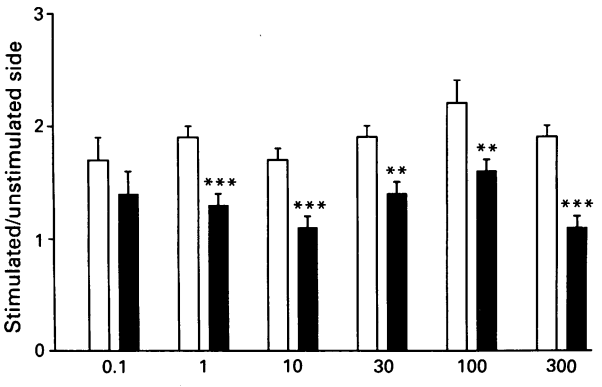

Figure 1 5-Carboxamidotryptamine (5-CT) significantly reduced plasma protein extravasation ([^{125}I]-albumin) in rat dura mater following electrical stimulation of the right trigeminal ganglion. Animals were injected with 0.1 (n = 9), 1 (n = 22), 10 (n = 7), 30 (n = 13), 100 (n = 10), or 300 (n = 12) $ng\,kg^{-1}$ i.v. of 5-CT 10 min before stimulation (1.2 mA, 5 Hz, 5 ms) and 5 min before [^{125}I]-albumin injection. After 5 min of stimulation, animals were perfused and dura dissected as described in Methods. 5-CT blocked plasma extravasation at a dose as low as 1 $ng\,kg^{-1}$. 5-CT did not change the level of radioactivity (c.p.m. mg^{-1} wet wt.) on the unstimulated side as compared to the vehicle-treated animals. The same number of vehicle-treated animals (open columns) was studied for each 5-CT-treated group (closed columns). Data represent mean (s.e.mean shown by vertical bars) of the ratio:

$$\frac{\text{c.p.m.}\,mg^{-1}\text{ wet wt. (stimulated side)}}{\text{c.p.m.}\,mg^{-1}\text{ wet wt. (unstimulated side)}}$$

** $P < 0.01$; *** $P < 0.001$ as compared to vehicle-treated group.

100%, respectively; 300 $\mu g\,kg^{-1}$ did not reach statistical significance (Figure 2). 5-BT did not exhibit any effect in conjunctiva (Table 2).

Sumatriptan High intensity stimulation: Pretreatment with sumatriptan (100 $\mu g\,kg^{-1}$) decreased the ratio in dura mater [1.8 ± 0.1 (n = 24) in vehicle-treated animals vs 1.1 ± 0.0 (n = 23) in sumatriptan-treated group, $P < 0.001$].

Low intensity stimulation: The administration of either 100 or 300 $\mu g\,kg^{-1}$ did not block the response to electrical stimulation in extracranial tissues. (Table 2).

Putative 5-HT$_1$ receptor antagonists Pretreatment with metergoline or methiothepin at high concentrations (1 $mg\,kg^{-1}$) significantly decreased the ratio in dura mater [1.9 ± 0.1 (n = 9; control) vs 1.2 ± 0.1 (n = 9) ($P < 0.001$) and 1.8 ± 0.1 (n = 7; control) vs 1.2 ± 0.1 (n = 7) ($P < 0.001$), respectively]. Metergoline (100, 500 $\mu g\,kg^{-1}$) or methiothepin (300 $\mu g\,kg^{-1}$) were without effect.

5-HT$_2$ and 5-HT$_3$ receptor antagonists Plasma protein extravasation in dura mater following trigeminal stimulation was not inhibited by pretreatment with ketanserin, pizotifen, MDL 72222, or ICS 205-930 (Table 2). Ketanserin, pizotifen or MDL 72222 pretreatment did not affect plasma extravasation in conjunctiva (Table 2).

Pretreatment with 5-HT antagonists prior to sumatriptan or 5-CT administration The effect of sumatriptan in dura mater was partially antagonized by metergoline, 100 $\mu g\,kg^{-1}$: vehicle-treated 1.8 ± 0.1 (n = 10); sumatriptan-treated 1.1 ± 0.1 (n = 9, $P < 0.001$ as compared to the vehicle group);

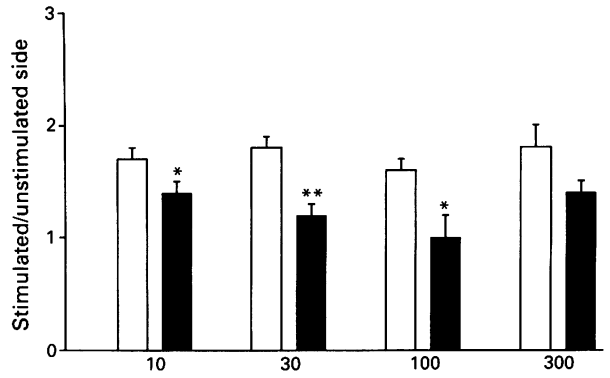

Figure 2 5-Benzyloxytryptamine (5-BT) significantly decreased plasma extravasation in dura mater. 5-BT 10 ($n = 4$), 30 ($n = 14$), 100 ($n = 7$), or 300 ($n = 4$) $\mu g\,kg^{-1}$ were administered i.v. 10 min before electrical stimulation of the trigeminal ganglion (1.2 mA, 5 ms, 5 Hz) and 5 min before iodinated albumin injection. After stimulation (5 min), animals were perfused and dura dissected as described in Methods. 5-BT did not change the level of radioactivity on the unstimulated side as compared to vehicle-treated animals. A significant decrease of the stimulated side/unstimulated side ratio was observed after pretreatment with 5-BT 100, 30, or 10 $\mu g\,kg^{-1}$. A small, not significant decrease was also observed after treatment with 300 $\mu g\,kg^{-1}$. The number of vehicle-treated animals (open columns) in each group differed by less than 10% from those given 5-BT (closed columns). Data are expressed as in Figure 1. * $P < 0.05$; ** $P < 0.01$ as compared to the vehicle group.

metergoline + sumatriptan 1.6 ± 0.1 ($n = 10$, $P < 0.05$ as compared to the sumatriptan group) (Figure 3).

Methiothepin 300 $\mu g\,kg^{-1}$ given 10 min before sumatriptan 100 $\mu g\,kg^{-1}$, did not antagonize the sumatriptan-induced blockade (Figure 3b). The administration of 5-BT 30 $\mu g\,kg^{-1}$ prior to sumatriptan added to the effect of sumatriptan: 2.2 ± 0.2 ($n = 3$; control), 1.4 ± 0.2 ($n = 3$; sumatriptan-treated group; $P < 0.05$ as compared to control), 1.0 ± 0.1 in sumatriptan + 5-BT-treated group ($n = 3$, $P < 0.05$ as compared to the vehicle group). Neither pizotifen (300 $\mu g\,kg^{-1}$) nor MDL 72222 (300 $\mu g\,kg^{-1}$) inhibited the effect of sumatriptan (Figure 3c,d).

The blockade achieved by 5-CT was not affected by metergoline pretreatment. When 5-CT was injected at 1 or 300 $ng\,kg^{-1}$, the ratio between the two sides decreased from 1.9 ± 0.1 (vehicle, $n = 16$) to 1.3 ± 0.1 ($n = 8$) or 1.2 ± 0.2 ($n = 4$), respectively, whereas after metergoline pretreatment at 100 $\mu g\,kg^{-1}$, the ratios were 1.2 ± 0.1 ($n = 8$) and 1.4 ± 0.1 ($n = 4$), respectively. When larger doses of metergoline (300 $\mu g\,kg^{-1}$) were administered prior to 5-CT at 1 or 300 $ng\,kg^{-1}$, the ratios between the two sides (1.0 ± 0.1, $n = 4$ and $1.3 + 0.1$, $n = 7$, respectively) did not differ from the response to 5-CT alone (1.3 ± 0.1, $n = 4$, and 1.2 ± 0.1, $n = 7$, respectively).

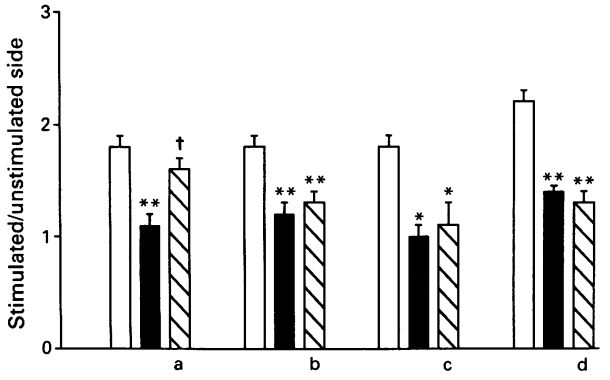

Figure 3 Metergoline, but not methiothepin, pizotifen or MDL 72222, antagonize blockade by sumatriptan of plasma protein extravasation in rat dura mater. (a) Metergoline (100 $\mu g\,kg^{-1}$) ($n = 10$), (b) methiothepin (300 $\mu g\,kg^{-1}$) ($n = 8$). (c) pizotifen (300 $\mu g\,kg^{-1}$) ($n = 4$) or (d) MDL 72222 (300 $\mu g\,kg^{-1}$) ($n = 4$) were administered i.v. 20 min before electrical trigeminal ganglion stimulation (1.2 mA, 5 min) and 10 min before sumatriptan (100 $\mu g\,kg^{-1}$, i.v.). Sumatriptan significantly reduced the stimulated side/unstimulated side ratio. Metergoline 100 $\mu g\,kg^{-1}$ partially inhibited this effect. The same number of animals were studied in vehicle- (open columns) and sumatriptan-treated (closed columns) groups; hatched columns: sumatriptan plus antagonist. Data are expressed as in Figure 1. * $P < 0.05$ and ** $P < 0.01$ as compared to vehicle-treated group. † $P < 0.05$ as compared to sumatriptan-treated group.

Sympathectomy and sumatriptan In rats previously subjected to unilateral superior cervical ganglionectomy, radioactivity (c.p.m. mg^{-1} wet wt.) on the unstimulated sides was similar to those in normal animals. However, a slight increase in the ratio was observed [2.4 ± 0.2 ($n = 16$) in superior cervical ganglionectomy vs 2.0 ± 0.1 ($n = 13$) in sham, $P < 0.05$]. Sumatriptan 100 $\mu g\,kg^{-1}$, decreased extravasation in both sympathectomized animals [2.4 ± 0.3 ($n = 10$) in vehicle-treated vs 1.4 ± 0.2 ($n = 8$), $P < 0.01$] and shams [1.9 ± 0.1 ($n = 8$) vs 1.4 ± 0.1 ($n = 8$), $P < 0.01$].

Discussion

5-HT$_{1B}$/5-HT$_{1D}$ receptor binding sites

In the mid 1980's, two distinct but analogous subtypes of 5-HT$_1$ receptor were identified in various species (Heuring *et al.*, 1986). 5-HT$_{1B}$ receptors are pharmacologically distinct from 5-HT$_{1D}$ receptors, yet appear to mediate similar functions in different species. 5-HT$_{1B}$ sites display < 10 nM affinity for 5-CT and moderate affinity for 5-BT, sumatriptan, methiothepin and metergoline (Table 3). The 5-HT$_{1B}$ receptors are species specific (Hoyer & Middlemiss, 1989). 5-HT$_{1B}$ binding sites have been demonstrated in rat and mouse brain and in a cell line derived from opossum kidney cells (Murphy & Bylund, 1988), but not in guinea-pig, cow, chicken, turtle, frog, or human tissues (Hoyer *et al.*, 1986; Heuring *et al.*, 1986). 5-HT$_{1B}$ receptors also appear to exist outside the central nervous system in rats and mice (see Middlemiss & Hudson, 1990).

In 1987, another distinct subtype of the 5-HT$_1$ receptor 'family' was identified in bovine brain membranes (Heuring & Peroutka, 1987). [^{3}H]-5-HT binding in bovine caudate was examined in the presence of 100 nM 8-OH-DPAT and 100 nM mesulergine, a condition designed to displace over 90% of specific binding to 5-HT$_{1A}$ and 5-HT$_{1C}$ receptor sites (Heuring & Peroutka, 1987). However, a significant amount of [^{3}H]-5-HT binding was still observed under such conditions. Moreover, the pharmacological characteristics of this site did not correlate with any known 5-HT receptor subtype. Therefore, this recognition site for [^{3}H]-5-HT was designated the '5-HT$_{1D}$' binding site (Heuring & Peroutka, 1987). It has been suggested that the 5-HT$_{1D}$ receptor is the receptor site responsible for the actions of ergot alkaloids and sumatriptan in migraine headaches (Hoyer *et al.*, 1989).

Among the 5-HT$_1$ receptor subtypes identified to date, the 5-HT$_{1B}$/5-HT$_{1D}$ subtypes appear most relevant to neurogenic plasma extravasation in dura mater. Based on the threshold concentrations, the potency order among the tested drugs was 5-CT > 5-BT/dihydroergotamine (Saito *et al.*, 1988)/sumatriptan > 8-OH-DPAT, reflecting the potency order of the affinities for the 5-HT$_{1B}$/5-HT$_{1D}$ receptors of these drugs (Table 3). (ID_{50} values were difficult to calculate in this model because of the narrow range between threshold and maximum response.) The 5-HT$_{1B}$/5-HT$_{1D}$ subtypes take on significance because the effect of sumatriptan can be blocked partially by metergoline (an antagonist with high affinity for the 5-HT$_{1B}$/5-HT$_{1D}$ recognition site) and because sumatriptan and 5-BT bind with higher affinity to 5-HT$_{1D}$ than 5-HT$_{1A}$ receptors (Peroutka *et al.*, 1990) and are relatively inactive at the 5-HT$_{1C}$ binding site. 5-HT$_2$ or 5-HT$_3$ receptor-mediated effects were ruled out because of the results following pretreatment with ketanserin, pizotifen, MDL 72222 and ICS 205-930,

Table 3 Drug potencies in inhibiting plasma protein extravasation in rat dura mater and drug affinities for 5-HT_{1B} and 5-HT_{1D} receptor binding sites in brain membranes

Drug	*Effective dose for inhibition of plasma extravasation* (nmol kg^{-1})	*Drug affinities* (nM) K_i (5-HT_{1B})	K_i (5-HT_{1D})
5-CT	0.003–0.94	5.0	0.75
5-BT	33	40	40
DHE	85**	3.0	19
Sumatriptan	240	440	17
8-OH-DPAT	910	6,000	700
Methiothepin	>750	43	36
Metergoline	>1,200	26	4.2
Ketanserin	>850*	1,900	>10,000
Pizotifen	>850*	>10,000	2,800
ICS 205-930	>850*	>10,000	>10,000
MDL 72222	>850*	>10,000	>10,000

The plasma protein extravasation data shown are derived from the current study. Radioligand binding affinities were derived from Hoyer *et al.*, 1985; Heuring & Peroutka, 1987; Offord *et al.*, 1988; Schlicker *et al.*, 1989; Waeber *et al.*, 1988; Peroutka *et al.*, 1990 and Peroutka (unpublished observations). * Higher doses than 300 $\mu g\,kg^{-1}$ were not tested. ** Taken from Saito *et al.*, 1988. For abbreviations, see text.

and because both sumatriptan and 5-BT exhibit only weak activity at 5-HT_2 and 5-HT_3 receptors. Similarly, 5-HT_{1B}/5-HT_{1D} receptors probably mediate the blockade of extravasation by less specific drugs such as methysergide.

On the other hand, our data suggest that an additional, as yet unidentified subtype(s) of the 5-HT_1 receptor might be more involved in the inhibition of plasma leakage. Important differences from the predicted effects of these drugs at a known 5-HT_1 receptor subtype(s) include: (1) the high potency (1 $ng\,kg^{-1}$, threshold) of the non-selective agonist 5-CT, (2) the inability of metergoline to block the effects of 5-CT (metergoline, 100 or 300 $\mu g\,kg^{-1}$, given 10 min prior to 5-CT, 1 or 300 $ng\,kg^{-1}$, did not inhibit the effects of 5-CT in dura mater), (3) the partial inhibition of the action of sumatriptan by metergoline, (4) the inability of methiothepin pretreatment to affect inhibition by sumatriptan of plasma leakage, (5) the ability of the antagonists to promote plasma extravasation when administered alone, and (6) the lack of effect of 5-HT (see below) in this model. The development of more selective drugs to discriminate and identify other 5-HT_1 receptor subtypes might help to resolve some of these questions, especially since much of the discrepant data involves the use of 5-HT_1 antagonists.

Receptor localization

Several lines of experimental evidence support the conclusion that 5-HT heteroreceptors are located on neuropeptide-containing unmyelinated C fibre and that drug blockade of plasma leakage within dura mater is mediated by a prejunctional mechanism. Firstly, sumatriptan (100 or 300 $\mu g\,kg^{-1}$, i.v.) and dihydroergotamine (50 $\mu g\,kg^{-1}$, i.v. or 1 $mg\,kg^{-1}$, i.p.) are inactive when tested against concentrations of substance P or neurokinin A which cause plasma leakage that most closely approximate the response following electrical trigeminal stimulation or capsaicin administration (Saito *et al.*, 1988; Buzzi & Moskowitz, 1990). Secondly, dihydroergotamine (50 $\mu g\,kg^{-1}$) and to a lesser extent sumatriptan (300 $\mu g\,kg^{-1}$) attenuate the increases in immunoreactive calcitonin gene-related peptide within sagittal sinus blood (e.g., venous effluent) during electrical stimulation of the trigeminal ganglion (Buzzi *et al.*, 1991). Thirdly, sumatriptan and dihydroergotamine pretreatment block platelet aggregation and endothelial vesicle formation within post-capillary venules and mast cell secretion and degranulation in the dura mater (Buzzi *et al.*, 1990). It is difficult to reconcile these data with activation of a postjunctional receptor either on vascular smooth muscle, endothelial cells or sympathetic fibres. In fact, sympathetic fibres were ruled out as a possible site by direct experimentation reported here. Prejunctional receptors coupled to inhibition of neuropeptide release could mediate all the above changes. The identified putative 5-HT_1 heteroreceptor in dura mater may be inhibiting calcium currents, as suggested by the observations that voltage-sensitive Ca^{2+} channels (dihydropyridine-sensitive) mediate substance P release from cultured rat primary sensory neurons (Perney *et al.*, 1986).

The natural ligand for the putative 5-HT_{1B}/5-HT_{1D} receptor identified on trigeminal perivascular fibres is most probably 5-HT. However, 5-HT itself is relatively inactive in the model studied here. In fact, pretreatment with 5-HT does not cause plasma extravasation after intravenous administration, except at very large concentrations (1 $\mu mol\,kg^{-1}$) (Markowitz *et al.*, 1987), nor does it block the extravasation response following electrical stimulation or capsaicin administration. In one experiment ($n = 3$ per group), the response to capsaicin (149% ± 0.5 versus 100% ± 0.5 vehicle) was unaffected by 5-HT pretreatment (0.1 $\mu mol\,kg^{-1}$; 150% ± 12).

The possibility that blockade of neurogenically-mediated extravasation is due to decreases in surface area and perfusion pressure secondary to vasoconstriction (e.g., postjunctional mechanism) deserves consideration. Sumatriptan constricts isolated canine saphenous vein (Humphrey *et al.*, 1988), canine and primate cerebral vessels (Connor *et al.*, 1989), canine carotid arterial circulation (Feniuk *et al.*, 1989). Ergotamine constricts isolated extracranial arteries (Muller-Schweinitzer & Weidmann, 1978). *In vivo*, methysergide selectively constricts the carotid vascular bed (Saxena, 1974). Sumatriptan (Perren *et al.*, 1989), ergotamine (Bom *et al.*, 1989a), 5-CT (Saxena & Verdouw, 1985) and 8-OH-DPAT (Bom *et al.*, 1989b) constrict arteriovenous shunts, although the effects of ergotamine may not be mediated by 5-HT_1-like or 5-HT_2 receptors (Bom *et al.*, 1989a). If closing arteriovenous shunts decreases vascular permeability following trigeminal stimulation, than leakage induced by intravenously administered neuropeptides (substance P or neurokinin A) should have been blocked as well. Constriction of arterioles (such as with phenylephrine) does not block neurogenic plasma extravasation nor does it reduce the increase in levels of calcitonin gene-related peptide in the sagittal sinus accompanying electrical stimulation (Saito *et al.*, 1988; Buzzi *et al.*, 1990). Constriction of extracranial cephalic blood vessels follows ergotamine administration but neurogenic plasma extravasation is not blocked in these tissues. Finally, it would be difficult to conceive how a vasoconstrictor drug action alone would explain the blockade of neuropeptide increases in sagittal sinus, platelet accumulation and mast cell degranulation accompanying trigeminal stimulation (Buzzi *et al.*, 1990; 1991).

Clinical relevance

Perivascular inflammation may play an important role in the pathogenesis of headache. Its development in cephalic tissues

has been documented in man (Sweet & Wepsic, 1974; Drummond *et al.*, 1983; Goadsby *et al.*, 1988) and experimental animals (Jancso-Gabor & Szolcsanyi, 1972; Couture & Cuello, 1984; Goadsby *et al.*, 1988). Both neurogenic plasma extravasation and headache can be blocked by aspirin and in selected cases, by indomethacin pretreatment (Buzzi *et al.*, 1989). A central role in migraine pathogenesis has been proposed for dural vessels (Penfield & McNaughton, 1940). Dural vessels are among the few pain-sensitive intracranial tissues (Ray & Wolff, 1940); they contain structures important to the development of neurogenic inflammation, namely fenestrated capillary venules surrounded by a network of neuropeptide-containing unmyelinated C-fibres and mast cells (McNaughton, 1938; Andres *et al.*, 1987). Platelet aggregation, mast cell degranulation, and fluid accumulation in the perivascular space of postcapillary venules attendant on electrical trigeminal stimulation may promote sensitization of polymodal nociceptors and sustain the process of sterile inflammation (Cline *et al.*, 1989). It is possible that the consequences of neurogenic inflammation such as local fluid accumulation, sensitization of polymodal nociceptors and release of proinflammatory agents underlie and sustain the pain of headache. The ability of putative 5-HT_1 receptor agonists which lack inherent analgesic properties, to prevent neurogenic plasma extravasation in the dura mater, not only suggests an action directly on primary afferent fibres, but in itself adds further weight to the notion that dural inflammation may be relevant to headache pathogenesis. Peripheral (dural) mechanisms are further emphasized by the observation that sumatriptan is hydrophilic and penetrates the blood brain barrier very poorly, if at all (P.P.A. Humphrey, personal communication). For the above reasons vasoconstriction must diminish in importance as a mechanism to explain the analgesic action of these drugs in headache patients. Rather, the data favour the importance of neurogenic inflammation in the pathogenesis of migraine, and the unmyelinated C-fibre as the site of the putative 5-HT receptor subtype.

These studies were supported by NS 21558 and NS 23560 from the National Institutes of Neurological Disease.

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(Received October 30, 1990
Revised February 6, 1991
Accepted February 12, 1991)

Br. J. Pharmacol. (1991), **103**, 1429–1434 © Macmillan Press Ltd, 1991

Response of the rat myometrium to phenylephrine in early pregnancy and the effects of 6-hydroxydopamine

Andrew Kaulenas, [1]Helena C. Parkington & Harold A. Coleman

Department of Physiology, Monash University, Clayton, Victoria, 3168, Australia

1 The contractile responses of the longitudinal and circular muscle layers of the rat uterus to the α_1-adrenoceptor agonist phenylephrine were measured on days 3–6 of gestation. There was a progressive increase in sensitivity to phenylephrine in both muscle layers between days 3 and 6 of gestation. Overall, this amounted to a 13 and 9 fold increase in sensitivity in longitudinal and circular muscles, respectively. In longitudinal muscle the slope of the Hill plot was 2 on day 3 of pregnancy and was decreased to 1 thereafter.

2 The sympathetic nerve terminals innervating the smooth muscle of the uterus were destroyed by administration of 6-hydroxydopamine ($2 \times 50\,mg\,kg^{-1}$) 4–7 days before testing with phenylephrine. Following this treatment there was a significant increase in sensitivity to phenylephrine on day 3 in both muscle layers. After day 4, the longitudinal muscle was less sensitive to phenylephrine.

3 In the longitudinal muscle there was a progressive increase in the contractile response to maximal concentrations of phenylephrine and to high potassium (100 mM) between days 3 and 6 of pregnancy. In the circular muscle the responsiveness to both phenylephrine and potassium remained unchanged between days 3 and 6 of gestation. 6-Hydroxydopamine had no effect on the maximal responses to phenylephrine or high potassium in either muscle layer.

4 In conclusion, denervation supersensitivity of uterine smooth muscle following injection of 6-hydroxydopamine is observed only on day 3 of pregnancy and appears to be replaced by subsensitivity by day 6. The decrease in the slope of the Hill plot in longitudinal muscle after day 3 may be explained by changes in events between activation of α_1-adrenoceptors and contraction.

Keywords: Denervation supersensitivity; myometrium; drug effects; blastocyst spacing; sympathectomy, chemical; hydroxydopamines; uterus, rat; sympathomimetics; blastocyst, peri-implantation phase

Introduction

It is likely that the contractile activity of uterine smooth muscle plays an important role in the spacing of blastocysts along the uterus in polytocous species (Böving, 1956; McLaren & Michie, 1959; Pusey *et al.*, 1980; Legrand *et al.*, 1987). The influence of the sympathetic innervation on the contractile activity of uterine smooth muscle around the time of implantation has been studied in rats (Roche *et al.*, 1985; Legrand *et al.*, 1987; 1989). Surgical destruction of the ovarian nerve had no effect on either blastocyst transport or spacing (Roche *et al.*, 1985). Injection of the α-adrenoceptor antagonists, prazosin and phenoxybenzamine, depressed electromyographical activity in the uterus of rats (Legrand *et al.*, 1989), disrupted transport and implantation, and even spacing of foetuses did not occur (Legrand *et al.*, 1987). The available evidence suggests that the cholinergic nerves provide most of the motor innervation of the uterus (Hollingsworth, 1974; Sato *et al.*, 1989). Prazosin interacts with the presynaptic control of some cholinergic nerves (Koss *et al.*, 1990; Thompson *et al.*, 1990) and this could explain the actions of this drug on the uterus. Alternatively, since blastocyst transport and spacing remained unaffected following injection of 6-hydroxydopamine to ablate the noradrenergic nerves in the reproductive tract of rats, the development of supersensitivity in the muscle of the uterus was proposed (Legrand *et al.*, 1987). It was postulated that, in such an event, low levels of catecholamines in the circulation might be sufficient to maintain normal uterine motility during this critical period of blastocyst transport, spacing and implantation (Legrand *et al.*, 1987).

In the present study, we set out to determine whether supersensitivity in response to α-adrenoceptor stimulation followed denervation with 6-hydroxydopamine in rat uterine smooth muscle in the peri-implantation period. The two muscle layers of the uterus were studied separately because they do not behave in the same way, even under identical hormonal conditions (Osa & Katase, 1975) and the population of adrenoceptors varies between the two layers (Kawarabayashi & Osa, 1976).

[1] Author for correspondence.

Methods

Sprague-Dawley rats were divided randomly into two groups:

Group 1

Pairs of virgin females were housed with one male and vaginal washings from the females were examined daily. The presence of spermatozoa in the vaginal smear was denoted as day 1 of pregnancy. Uterine activity was studied on one of days 3 to 6 of pregnancy since the blastocysts arrive in the uterus around mid-day on day 4 and become implanted within 24 h (Forcelledo *et al.*, 1981; Legrand *et al.*, 1987). These rats served as controls.

Group 2

Females were injected intraperitoneally with 6-hydroxydopamine $50\,mg\,kg^{-1}$ on two consecutive days and then mated as for group 1. Uterine activity was examined 4–7 days following the last injection to ensure that maximum supersensitivity had become established (Furness *et al.*, 1970).

The females were killed by decapitation. A segment from each uterine horn was removed immediately and frozen at −20°C for visualization of noradrenaline-containing nerve varicosities using fluorescence histochemistry.

Strips (7–12 mm long and 1.5–2 mm wide) of longitudinal and circular muscle were prepared. A truly circular strip of muscle is generally 2–4 mm in length at this stage of pregnancy, so a tight spiral strip was cut in order to obtain the

greater length. A silk thread was tied to each end of each strip. The two strips of tissue from each rat were mounted in tissue chambers. One end of each was fixed while the other end was connected to an isometric force transducer (FT03, Grass). The chambers were continuously perfused at 3 ml min^{-1} with oxygenated physiological solution containing (mM): NaCl 120, KCl 5, $CaCl_2$ 2.5, $MgSO_4$ 1, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11, gassed with 95% O_2, 5% CO_2 and maintained at 37°C. Both strips were stretched to approximately 0.2 g tension since this resulted in the production of >80% of maximal contraction in response to solution containing 100 mM potassium. Isometric tension was recorded on a chart recorder (Grass) and passed to a computer which digitized the signal for subsequent integration of the responses.

An equilibration period of about 1 h was allowed in order to stretch the preparations and for spontaneous activity to settle down to a constant level. For most experiments propranolol (10^{-6} M) was added to the perfusate during the final 30 min of the equilibration period and for the remainder of the experiment in order to block β-adrenoceptors.

Stimulation of α-adrenoceptors was achieved by perfusing the tissues with phenylephrine (10^{-8} to 10^{-4} M applied in random order) for 5 min with 15–20 min allowed for recovery before the next dose was administered. It was determined in pilot experiments that desensitization to phenylephrine did not occur under this regime. The response of the myometrium to phenylephrine is complex, particularly in the circular layer, with variations in the amplitude, frequency and duration of the phasic contractions. The response was assessed by integrating the area under the contraction curve which is a function of these three parameters. This was achieved by use of the computer software package pClamp (Axon Instruments). The integral of the spontaneous contractility during the 5 min immediately preceding was subtracted from the integral of the response to phenylephrine (Figure 1a). The result was normalized as a % of the maximum response to phenylephrine, generally 10^{-4} M. A dose-response curve was constructed from the data for each individual animal i.e. a sigmoid curve was fitted to the data with the computer software package, GraphPad (ISI Software) and an EC_{50} was calculated (Figure 1b). Data from 5–6 animals were examined for each day of pregnancy (days 3–6), and for each treatment, from which a mean EC_{50} was obtained. The same software was used to calculate the slope of the Hill plot for each set of data.

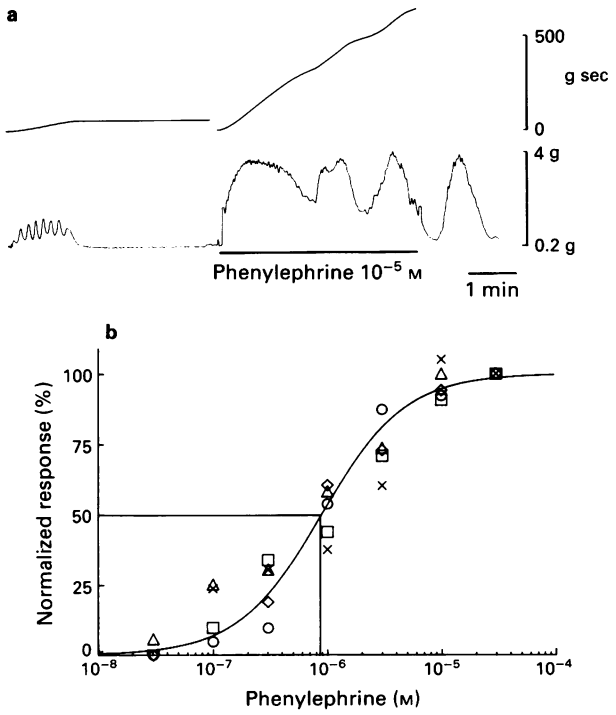

Figure 1 (a) Spontaneous activity was integrated for 5 min and was subtracted from the integrated response to applied phenylephrine. (b) The integral of the contractile responses from 5 control rats on day 4 of pregnancy were normalized and plotted against the concentration of phenylephrine on a logarithmic scale. A dose-response curve was plotted to the data from each animal and a logistic curve was plotted with its EC_{50} and slope being the mean EC_{50} and mean slope from all 5 animals.

In order to determine the capacity of the strips to contract in absolute terms, the cross-sectional area of each strip was calculated from the length and mass of muscle between the threads, measured at the end of each experiment. The contractile response to the maximal phenylephrine concentration was normalized per unit cross-sectional area of the muscle strips.

It has been observed that supersensitivity in many smooth muscle tissues may also extend to stimulatory agents other than the specific agonists under consideration, for example, to potassium (Westfall, 1981). Thus, the contractile responses to solutions containing 100 mM potassium (KCl replaced the isosmotic equivalent of NaCl in physiological solution) were also investigated in the present study.

Transverse sections (15 μm thick and 60–100 from each animal) of the frozen segments of uterus were stained with glyoxylic acid (de la Torre & Surgeon, 1976) and viewed under a fluorescence microscope to visualize noradrenaline-containing nerves. Hence, the presence of nerves in intact tissues or the effectiveness of denervation following injection of 6-hydroxydopamine was established for each preparation.

Data from different groups were tested by analyses of variance (ANOVA). The effects of 6-hydroxydopamine were analyzed by comparing EC_{50} values to phenylephrine. Similar tests were used to determine the significance of responsiveness to maximal concentrations of phenylephrine and 100 mM potassium. The mean and standard error is given and a significance level of 0.05 was used in testing.

(−)-Phenylephrine hydrochloride, 6-hydroxydopamine hydrobromide and (±)-propranolol hydrochloride were obtained from Sigma Chemical Company, U.S.A.

Results

Sensitivity to phenylephrine with gestational age

In preparations from control rats there was a progressive and significant increase in the sensitivity of the longitudinal muscle to phenylephrine over the four days of gestation under study (Figure 2). This is apparent from the 13 fold decrease in the value of the EC_{50} from 4.8×10^{-6} M on day 3 to 3.7×10^{-7} M on day 6 (see Table 1). The slope of the Hill plot was 2.06 ± 0.03 on day 3 and decreased significantly to 0.77 ± 0.19 on day 4 (Table 1). The change in sensitivity reflected an increase in sensitivity to the lower concentrations of phenylephrine (Figure 2).

In circular muscle strips there was a 9 fold shift of the dose-response curve to the left between day 3 (EC_{50}, 2.3×10^{-6} M) and day 6 (EC_{50}, 2.5×10^{-7} M) (Figure 2). The slope of the Hill plot did not change significantly over the four day period in this muscle layer (Table 1).

Sensitivity to phenylephrine following treatment with 6-hydroxydopamine

On day 3 of pregnancy there was a significant increase in the sensitivity to phenylephrine in longitudinal muscle obtained from animals that had been treated with 6-hydroxydopamine. The EC_{50} decreased from 4.8×10^{-6} M in control to 1.3×10^{-6} M following treatment (Figure 3). The slope of the Hill plot was significantly steeper in controls (2.06 ± 0.03 versus 0.61 ± 0.07) (Table 1). The increase in sensitivity appeared to be to the lower and not so much to the higher concentrations of phenylephrine. On day 4 of pregnancy the response to phenylephrine of preparations of longitudinal

Table 1 The logarithm of the EC_{50} values for phenylephrine (M) and the slope of the Hill plot in longitudinal and circular muscle strips obtained from control rats and following treatment with 6-hydroxydopamine (6-OHDA), on days 3 to 6 of gestation

	Day 3	*Day 4*	*Day 5*	*Day 6*
		Longitudinal muscle		
Control				
log EC_{50} ± s.e.	−5.32 ± 0.01	−5.81 ± 0.06	−6.24 ± 0.07	−6.43 ± 0.16†
Slope ± s.e.	2.06 ± 0.03	0.80 ± 0.08	0.88 ± 0.11	0.77 ± 0.19†
r^2	1.00	0.99	0.98	0.93
n	5	6	6	6
6-OHDA				
log EC_{50} ± s.e.	−5.90 ± 0.08*	−5.46 ± 0.07	−5.62 ± 0.09*	−5.48 ± 0.07*
Slope ± s.e.	0.61 ± 0.07*	0.75 ± 0.08	1.00 ± 0.17	0.73 ± 0.08
r^2	0.98	0.98	0.97	0.98
n	6	6	5	6
		Circular muscle		
Control				
log EC_{50} ± s.e.	−5.64 ± 0.06	−6.02 ± 0.03	−6.18 ± 0.04	−6.61 ± 0.07†
Slope ± s.e.	1.08 ± 0.16	1.00 ± 0.07	0.94 ± 0.07	0.71 ± 0.08†
r^2	0.98	0.99	0.99	0.98
n	6	6	6	6
6-OHDA				
log EC_{50} ± s.e.	−6.31 ± 0.10*	−5.94 ± 0.10	−6.14 ± 0.04	−6.26 ± 0.12
Slope ± s.e.	0.76 ± 0.13	0.65 ± 0.09*	0.77 ± 0.05	0.56 ± 0.08
r^2	0.96	0.97	0.99	0.96
n	5	6	6	5

* Different from control ($P < 0.05$).
† Effect of gestational age is significant ($P < 0.05$).

muscle from rats treated with 6-hydroxydopamine did not differ from those obtained from controls (Figure 3). Significant subsensitivity was observed on day 6 in tissues from treated rats (Figure 3).

The response to phenylephrine of circular muscle from animals treated with 6-hydroxydopamine was also significantly increased on day 3 of pregnancy (Figure 4) (EC_{50}

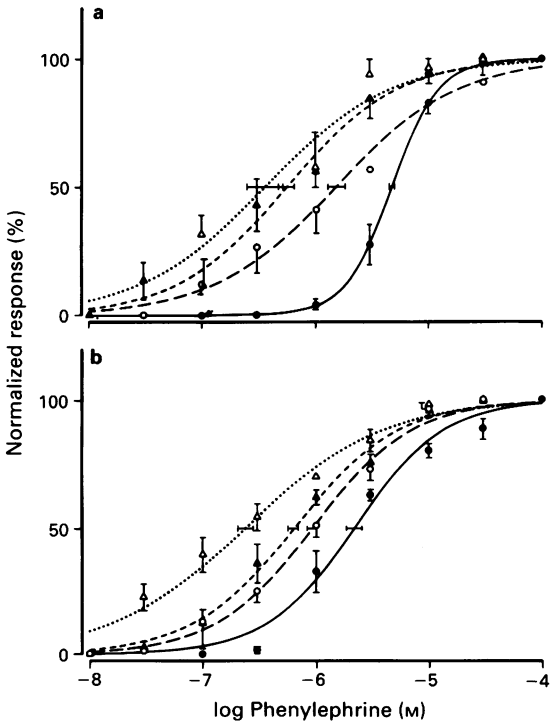

Figure 2 Dose-response curves to phenylphrine were obtained for longitudinal (a) and circular (b) muscles obtained from control rats on days 3 and 6 of gestation: day 3 (●—●); day 4 (○—○); day 5 (▲----▲); day 6 (△----△). The values (symbols with s.e. shown by vertical and horizontal bars) are shown for 5–6 animals. A curve was fitted to each set of data using a least squares fit and the EC_{50} (±s.e.) was calculated.

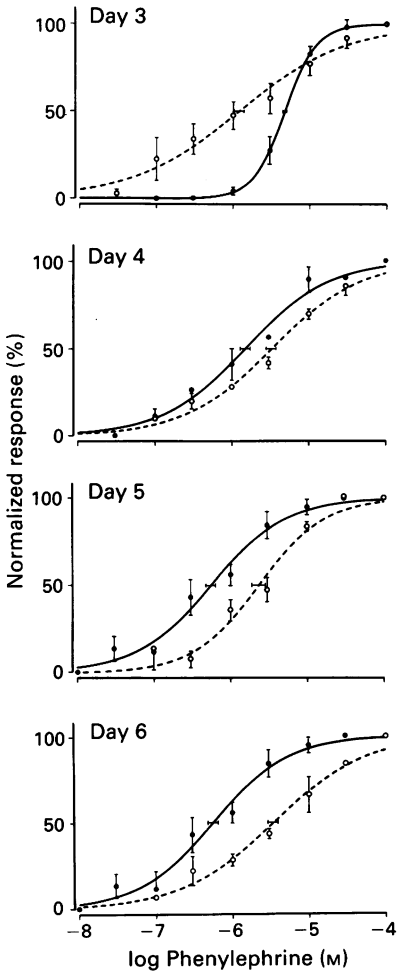

Figure 3 Dose-response curves to phenylephrine were obtained for strips of longitudinal muscle obtained from control rats (●—●) and following treatment with 6-hydroxydopamine (○----○). The values (symbols with s.e. shown by horizontal and vertical bars) are shown for 5–6 animals. A curve was fitted to each set of data using a least squares fit and the EC_{50} (± s.e.) was calculated.

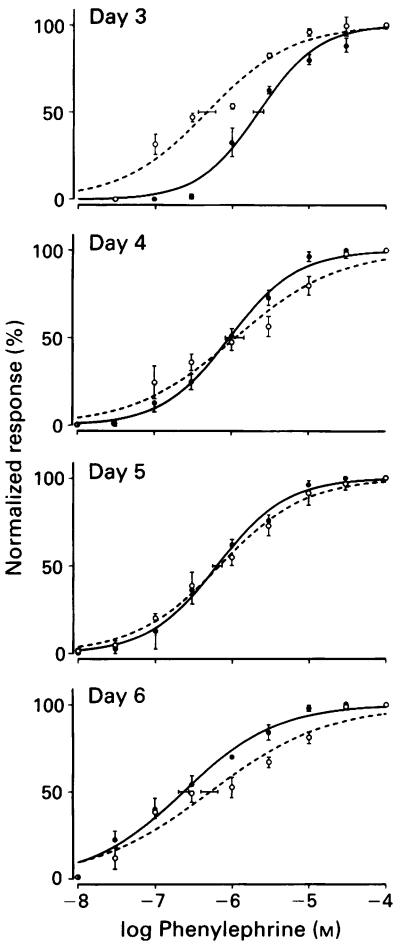

Figure 4 Dose-response curves to phenylephrine were obtained for strips of circular muscle obtained from control rats (●—●) and following treatment with 6-hydroxydopamine (○----○). The values (symbols with s.e. shown by horizontal and vertical bars) are shown for 5–6 animals. A curve was fitted to each set of data using a least squares fit and the EC_{50} (± s.e.) was calculated.

decreased from 2.3×10^{-6} in control to 4.9×10^{-7} M following treatment; see Table 1). However, there were no differences between the values of EC_{50} or the slope of the Hill plot in circular muscle on days 4 to 6 of pregnancy (Table 1).

Responsiveness to other contractile agents with gestational age

The contractile response of longitudinal muscle to the maximal concentration (10^{-4} M) of phenylephrine increased significantly between days 3 and 6 of gestation. There was a parallel increase in the contractile response to 100 mM potassium (Figure 5). The peak tension in response to 100 mM potassium was similar to the largest phasic contraction evoked by the maximal concentration of phenylephrine in every strip of longitudinal muscle. The tetanic contraction was sustained for the entire duration of exposure to high potassium in this muscle layer. The changes in the contractile responses with gestational age to both phenylephrine and potassium were unaffected following treatment with 6-hydroxydopamine (Figure 5).

In circular muscle there was no significant effect of gestational age on the contractile response to either 10^{-4} M phenylephrine or to 100 mM potassium in preparations from either control or treated rats (Figure 6). The contractile response of this layer to high potassium was not sustained but peaked within approximately 1 min and then declined rapidly, to a level that amounted to less than 0.3 g mm^{-2} within approx-

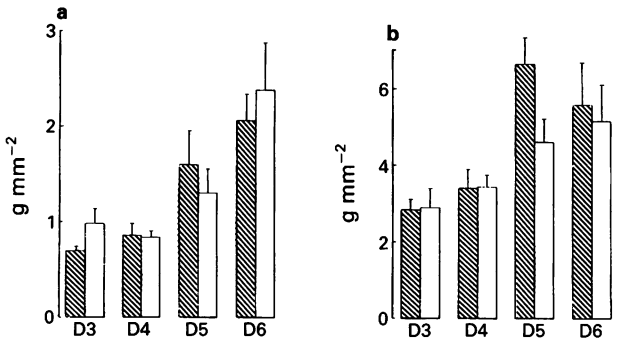

Figure 5 The contractile responses to 10^{-4} M phenylephrine (mean tension per min) (a) and to 100 mM potassium (peak tension) (b) of longitudinal muscle were obtained from control rats (hatched columns) and following treatment with 6-hydroxydopamine (open columns) on days 3 to 6 of gestation.

imately 2 min. The peak of the response to high potassium was often as great as the largest phasic response to the maximal concentration of phenylephrine, but this was not invariably the case.

Neither muscle layer responded to the α_2-adrenoceptor agonist, clonidine (10^{-9} to 10^{-4} M) at any stage of gestation studied.

Catecholamine fluorescence

In the control group the adrenergic innervation of the uterus was sparse. Most of the fluorescence that was observed appeared to be associated with blood vessels. Only a small amount of fluorescence was observed amongst the smooth muscle of the uterus. In tissues from animals that had been treated with 6-hydroxydopamine, there was a total lack of any fluorescence in all but two preparations and the contractile data from these were not included in the analysis.

Discussion

The results presented in this study demonstrate that supersensivity to α-adrenoceptor activation following treatment with 6-hydroxydopamine is evident only on day 3 of pregnancy. Since the blastocysts do not enter the uterus until day 4,

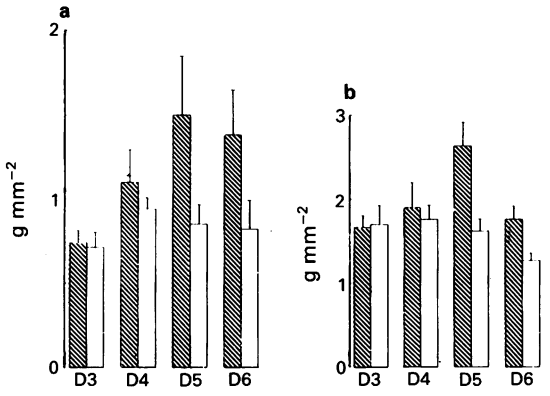

Figure 6 The contractile responses to 10^{-4} M phenylephrine (mean tension per min) (a) and to 100 mM potassium (peak tension) (b) of circular muscle were obtained from control rats (hatched columns) and following treatment with 6-hydroxydopamine (open columns) on days 3 to 6 of gestation.

supersensitivity is unlikely to account for the failure of the neurotoxin to disrupt the spacing of blastocysts. The lack of supersensitivity on days other than day 3 is hardly surprising in view of the sparsity of the catecholamine fluorescence amongst the smooth muscle in the rat uterus (Hollingsworth, 1974; Sato *et al.*, 1989; present study). The ability of the α-adrenoceptor antagonist, prazosin, to disrupt blastocyst spacing (Legrand *et al.*, 1987) may be via its conventional action on α-adrenoceptors since the sensitivity of both muscle layers to phenylephrine increased significantly on day 4 of pregnancy. This resulted from an increase in the ability of the muscle to respond to smaller concentrations of the α-adrenoceptor agonist, since its contractile capacity, either to phenylephrine or high potassium, was not enhanced on day 4. However, the contractions in response to stimulating the nerves in the rat uterus are markedly reduced by muscarinic blockers (Hollingsworth, 1974; Sato *et al.*, 1989) and not by the α-adrenoceptor blocker, phentolamine (Hollingsworth, 1974) suggesting that the principal motor innervation is via the release of acetylcholine. It is therefore more likely that the ability of prazosin to disrupt blastocyst spacing (Legrand *et al.*, 1987) may represent the ability of the drug to interfere with cholinergic neurotransmission (Koss *et al.*, 1990; Thompson *et al.*, 1990).

The contractile response of the longitudinal muscle to high potassium increased progressively over days 3 to 6 of pregnancy and a parallel increase was observed in the responses to maximal concentrations of phenylephrine. This could reflect an increase in the availability of calcium for contraction via an increase in the number of voltage-dependent calcium channels. It is also possible that the contractile capacity of the muscle may be increasing over this period. An increase in the myosin content of the tissue, an increase in calmodulin levels or an increase in myosin light chain kinase might all be expected to increase contractile capacity. Oestrogen stimulates an increase in the actomyosin content of the uterus (Csapo, 1950). Myosin light chain kinase and calmodulin levels in myometrium rise with progression of pregnancy in rabbits (Matsui *et al.*, 1983). Furthermore, these increases are hormone-dependent. Oestrogen increases the specific activity of the kinase and the level of calmodulin to a greater extent than progesterone.

The contractile response of circular muscle to potassium was transient while the response to phenylephrine persisted. It may be that the contractile response to α-adrenoceptor agonists relies more heavily on the release of calcium from intracellular stores in this muscle layer. It has been demonstrated that a number of different agonists including carbachol, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and noradrenaline acting on α-adrenoceptors can stimulate the production of inositol trisphosphate within the cytoplasm of rat myometrial cells (Ruzycky & Triggle, 1987). Inositol trisphosphate causes the release of calcium from the endoplasmic reticulum (Berridge, 1984). The hormonal status influenced this effect; progesterone-dominated tissues were capable of producing 30–40% higher levels of inositol trisphosphate than oestrogen dominated tissues. Alternatively, it may be that stimulation of α-adrenoceptors results in the transformation of calcium channels in the plasma membrane that are fundamentally voltage-dependent, but whose voltage-dependency is enhanced by the activation process. Such an occurrence has been described in the circular myometrium of guinea-pigs during early pregnancy (Coleman & Parkington, 1988). The results suggested that endogenous prostaglandin production by the endometrium is likely to maintain the channels in the excitable state *in vivo*. In this regard it is interesting to speculate that the effect of the innervation on uterine motility may be indirect, that is, to influence the production by the endometrium of agents that stimulate or inhibit uterine smooth muscle. Evidence exists which supports a role for prostaglandins in blastocyst implantation in rats (Kennedy, 1977). Rat blastocysts appear capable of influencing their rate of progress through the oviduct and uterus (Forcelledo *et al.*, 1981; Villalon *et al.*, 1982; Ortiz *et al.*, 1989) and this may be via an influence on the endometrium.

In conclusion, there is clear evidence that supersensitivity on day 3 of pregnancy can result from ablation of the adrenergic nerves, but it is unlikely to explain the failure of denervation to disrupt blastocyst transport and spacing. Indeed, it is likely that the α-component of the adrenergic innervation may not be the most important contributor to establishing the pattern of activity necessary for successful spacing. A complex pattern of contraction and relaxation may be required for successful spacing of blastocysts.

This work was supported by the National Health and Medical Research Council of Australia. We thank Ms Jill Poynton for producing the figures.

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(Received July 16, 1990
Revised February 6, 1991
Accepted February 12, 1991)

Br. J. Pharmacol. (1991), **103**, 1435–1440 © Macmillan Press Ltd, 1991

Interference of anti-inflammatory and anti-asthmatic drugs with neutrophil-mediated platelet activation: singularity of azelastine

[1]Patricia Renesto, Viviane Balloy, B. Boris Vargaftig & Michel Chignard

Unité de Pharmacologie Cellulaire, Unité associée Institut Pasteur/INSERM n° 285, 25 rue du Dr Roux, 75015 Paris, France

1 The capacity of various drugs (acetylsalicylic acid (ASA), ketoprofen, diclofenac, piroxicam, BW 755C, BW A4C, nedocromil sodium and azelastine) to inhibit human polymorphonuclear neutrophil (PMN)-mediated platelet activation was investigated. In this model, stimulated PMN release cathepsin G (Cat G), a serine proteinase which, in turn, induces platelet activation.

2 Among the different tested drugs, azelastine (100 μM for 1 min) was the only one able to prevent platelet aggregation. The cyclo-oxygenase inhibitors were all inactive, although used at effective concentrations as judged by inhibition of thromboxane B_2 (TxB_2) formation. Inhibition of platelet aggregation by azelastine was concentration-dependent, the range of active concentrations being of 20–70 μM. Release from platelets of 5-hydroxytryptamine was also inhibited at 30 μM and above, but never reached 100%.

3 The inhibition by azelastine is due to an effect on both cells. Indeed, β-glucuronidase release from activated PMN and platelet activation by purified Cat G were both affected.

4 However, used at high concentrations (>100 μM) azelastine was toxic since it released significant amounts of lactate dehydrogenase (LDH) from PMN and platelets.

5 These results show the capacity of azelastine, an anti-allergic and anti-asthmatic compound, to inhibit the cell-to-cell communication between PMN and platelets, an effect which may be relevant for its therapeutic efficacy or for a new application in diseases in which PMN and platelets are involved.

Keywords: Neutrophils; platelets; azelastine; inflammation; anti-asthmatic drugs; anti-inflammatory drugs.

Introduction

Agents such as N-formyl-Met-Leu-Phe (FMLP) or C5a induce the release from polymorphonuclear neutrophils (PMN) of cathepsin G (Cat G), a serine proteinase which induces, in turn, activation of nearby platelets (Chignard *et al.*, 1986; Selak *et al.*, 1988; Renesto *et al.*, 1990; Ferrer-Lopez *et al.*, 1990; Del Maschio *et al.*, 1990). This is reminiscent of different experimental acute inflammatory states evoked in laboratory animals. Thus, an intradermal injection of FMLP in the rabbit, triggers platelet deposition, a phenomenon which is absent in PMN-depleted but platelet-sufficient animals (Issekutz *et al.*, 1983). A PMN-dependent platelet deposition has also been observed during occlusion-reperfusion injury in the dog (Bednar *et al.*, 1985). In a PMN-mediated model of subendothelial immune complex glomerulonephritis, it has been shown that platelets mediate glomerular injury and proteinuria (Johnson *et al.*, 1988). In models of adult respiratory distress syndrome (ARDS), not only PMN are involved but also platelets participate (Tvedten *et al.*, 1985). In man, the participation of PMN (Warshawski *et al.*, 1986) and platelets (Heffner *et al.*, 1987) in lung injury observed during the ARDS is very likely. It has also been suggested that PMN contribute to immediate hypersensitivity (Baggiolini & Dewald, 1985; Weiss, 1989), and mediators released from these cells may be involved in allergic asthma (Durham *et al.*, 1984; Kay, 1987). Activated platelets are also potential contributors (Page, 1989). Moreover, they synthesize biologically active substances such as thromboxane A_2, 12-hydroxy-5,8,10,14-eicosatetraenoic acid (Marcus *et al.*, 1988), or platelet-activating factor (PAF) (Chignard *et al.*, 1979) which are relevant to allergic inflammation.

Thus, such a cell-to-cell communication may contribute to various inflammatory states, and its inhibition may be relevant for therapy. In this paper, we have investigated whether different anti-inflammatory drugs (ASA, ketoprofen, diclofenac, piroxicam, BW 755C and BW A4C) or anti-asthmatic and/or anti-allergic drugs (nedocromil sodium, azelastine) might inhibit PMN-mediated platelet activation. Azelastine, a new therapeutic agent with anti-asthmatic and anti-allergic properties used in the treatment of rhinitis (Meltzer *et al.*, 1988) and asthma (Spector *et al.*, 1987; Kemp *et al.*, 1987), was the only substance tested capable of preventing platelet aggregation and degranulation induced by stimulated PMN.

[1] Author for correspondence.

Methods

Preparation of human neutrophils

PMN were purified from blood of healthy donors collected on acid-citrate-dextrose (ACD) (1.5 ml for 10 ml of blood; composition, mM: trisodium citrate 85, citric acid 66, glucose 111) and heparin (20 i.u. ml^{-1}) as anticoagulant. Dextran (3% in saline) was added (0.5 volume for 1 volume of blood) and erythrocytes were allowed to sediment for 30 min. After centrifugation (350 *g*; 45 min) of the resulting supernatant through a Ficol-Paque gradient, the lysis of erythrocytes contaminating the pellet was performed with a specific 'lysis solution' (composition, mM: NH_4Cl 155, $KHCO_3$ 2.96, disodium EDTA 3.72). The resulting cell suspension was then gently inverted for 5 min and centrifuged (350 *g*; 10 min). After a wash in Hank's Balanced Salt Solution (HBSS), PMN were finally resuspended in HBSS in such a volume to obtain 10^7 PMN per ml. The resulting PMN were 98.3 ± 1.2% pure, as estimated with Türks stain, and 97.1 ± 2.6% viable as measured by the trypan blue exclusion method.

The concentrations of $CaCl_2$ and $MgCl_2$ were adjusted to 1.3 and 1.0 mM respectively, before each experiment.

Preparation of human washed platelets

Blood was collected as for PMN preparation. After a first centrifugation (180 *g*; 20 min), platelet rich plasma was collected and incubated with [^{14}C]-5-hydroxytryptamine ([^{14}C]-5-HT 0.05 μCi ml^{-1} final concentration) for 30 min at 37°C (during the rest of the process the preparation was kept at this temperature). The cell suspension was then centrifuged (1450 *g*; 15 min and the pellet was resuspended in a Tyrode buffer (composition, mM: NaCl 137.0, KCl 2.68, $NaHCO_3$

11.9, NaH_2PO_4 0.42, $CaCl_2$ 2.0, $MgCl_2$ 1.0, glucose 5.5, HEPES 5.0, BSA 0.35%; pH 7.4) supplemented with 10 nM prostacyclin and 50 i.u. ml^{-1} heparin. After a second centrifugation (1450g, 15 min) cells were washed again using the same medium but without heparin. Finally, the platelet concentration was adjusted to 4×10^8 cells per ml.

Aggregation and 5-hydroxytryptamine release determination

Platelet aggregation was studied with a Dual Chrono-Log Aggregometer (Chrono-Log-Corp., Hevertown, PA., U.S.A.). Platelets (250 μl) and PMN (250 μl) were incubated together for 5 min in siliconized test tubes, with stirring at 37°C, and in the presence of fibrinogen (0.77 mg ml^{-1}) and cytochalasin B (5 μg ml^{-1}). FMLP was then added, and the reaction was stopped 3 min later with 125 μl of a 'stopping solution' (composition, mM: EDTA 38.5, NaCl 68.8, formaldehyde 2.05%). Samples were rapidly centrifuged (13,000g; 2 min), and 400 μl of each supernatant were collected for [^{14}C]-5-HT release determination by aqueous counting scintillation (Counter 1212 Rackbeta, LKB-Wallac, Stockholm, Sweden). Aggregation was expressed as the percentage of change in light transmission, and 5-HT release as the percentage of total [^{14}C]-5-HT content of platelets.

β-Glucuronidase release determination

Aliquots of 1 ml of PMN (5×10^6 cells per ml) in HBSS-Tyrode (1:1) were preincubated for 4 min with cytochalasin B (5 μg ml^{-1}) and stirring at 37°C. Azelastine was then added, and 1 min later PMN were stimulated with 0.5 μM FMLP. The reaction was stopped after 3 min by placing the tubes on ice. Samples were then centrifuged (2,500g; 15 min), and 100 μl aliquots were collected and stored at −20°C in order to determine the amount of enzyme released in the supernatants. Pellets were resuspended in 1 ml of Triton X-100 0.2%, strongly vortexed, and incubated at 4°C overnight. Following centrifugation (2500g; 15 min) supernatants containing the enzyme which was retained in PMN were collected. Amounts of enzyme present in the two types of supernatants were determined spectrophotometrically, with 4-nitrophenyl-β-D-glucopyranosiduronic acid as substrate.

Release of β-glucuronidase was expressed as a percentage of the total β-glucuronidase content.

Lactate dehydrogenase determination

Aliquots of 2×10^8 platelets per ml or 5×10^6 PMN per ml (in HBSS-Tyrode 1:1) were placed in siliconized test tubes and after a 4 min preincubation period (as for aggregation procedure), azelastine was added at different concentrations. The reaction was stopped 4 min later with EDTA (5 mM final concentration). After centrifugation (2500g; 15 min), aliquots of the supernatants were stored at 4°C during the night and the pellets were resuspended in Triton X-100 0.1% in order to lyse the cells and to extract residual enzyme. Following an overnight incubation period, samples were centrifuged (2500g; 15 min) and supernatants collected. Enzyme concentration was determined with NADH as substrate. Release of lactate dehydrogenase (LDH) was expressed as percentage of the total LDH content.

Thromboxane B_2 and leukotriene B_4 measurements

Thromboxane B_2 (TxB_2) and leukotriene B_4 (LTB_4) were measured in supernatants of cells processed as for platelet aggregation, but without [^{14}C]-5-HT labelling. Briefly, for TxB_2 radioimmunoassay, samples were incubated with [^{125}I]-TxB_2 and anti-TxB_2 antibodies. Bound fractions were precipitated with polyethylene glycol 6000, and after centrifugation (2500g; 10 min) radioactivity of the pellet was counted in a gamma counter (Kontron Analytical MDA 312). For LTB_4 radioimmunoassay, [^{3}H]-LTB_4 and anti-LTB_4 antibodies were used. Addition of dextran-coated charcoal followed by a centrifugation (2500g; 10 min) allowed to separate unbound from bound fractions. Radioactivity in supernatants corresponding to the amount of radioactive free ligand was measured (Counter 1212 Rackbeta, LKB-Wallac, Stockholm, Sweden). Finally, TxB_2 and LTB_4 concentrations were determined by interpolation from standard curves.

Materials

Blood was obtained from the Centre National de Transfusion Sanguine, Paris, France. Dextran, PAF, cytochalasin B, FMLP, TxB_2, and prostacyclin were purchased from Sigma Chemical Corp., St. Louis, MO, U.S.A. The Ficoll-Paque was from Pharmacia, Uppsala, Sweden. The Aqueous Counting Scintillant (ACS II), [^{14}C]-5-HT, and [^{3}H]-LTB_4 assay reagent system were from Amersham International, Amersham, Buckinghamshire. The LDH kit was purchased from Boehringer, Mannheim, Germany. Heparin was from Choay, Paris, France, and BSA from Euromedex, Strasbourg, France. Polyethylene glycol 6000 and 4-β-D-glucopyrasidonuronic acid were from Merck, Darmstadt, Germany. Antibodies and radiolabelled ligand for RIA of TxB_2 were supplied by Prof. F. Dray, INSERM 207, Institut Pasteur, Paris, France. Human fibrinogen was obtained from Kabi, Stockholm, Sweden, and was treated with diisopropyl fluorophosphate in order to eliminate coagulant contaminants. Cat G was purified from human PMN following the method of Baugh & Travis (1976) modified by Martodam *et al.* (1979). ASA (Aspegic), was purchased from Synthélabo, Paris, France. Azelastine was a gift from Drs J.C. Venco, D. Perrisoud and J. Roux, Laboratoires Sarget, Merignac, France. Ketoprofen, diclofenac, and piroxicam were gifts from Dr B. Terlain, Rhône-Poulenc Santé, Vitry, France. N-(3-benzylcinnamyl) acetohydroxamic acid (BW A4C) and 3-amino-1-[*m*-(trifluoromethyl)-phenyl]-2-pyrazoline (BW 755C) were gifts from Dr S. Moncada, Wellcome Research Laboratories, Beckenham, Kent. Nedocromil sodium was a gift from Drs K. Rainey and A. Norris, Fisons plc, Loughborough, Leicestershire.

Statistical analysis

Each figure is the mean ± s.d. of the number of experiments indicated in parentheses. The Student's *t* test for unpaired samples was used to evaluate the significance. Results were significant (*) when $P < 0.05$.

Results

Effects of anti-inflammatory and anti-asthmatic drugs on the PMN-mediated platelet activation

As shown in Table 1, the cyclo-oxygenase inhibitors, ASA (100 μM for 5 min), ketoprofen, diclofenac and piroxicam (all 10 μM for 1 min) inhibited by approx. 30% PMN-induced release of 5-HT from platelets and failed to affect PMN-induced platelet aggregation ($P > 0.05$). TxB_2 measurements indicated that the anti-cyclo-oxygenase activity of these substances was effective in these experimental conditions. Acetohydroxamic acids, BW 755C and BW A4C (100 μM for 5 min) which possess anti-lipoxygenase and anti-cyclo-oxygenase properties (Randall *et al.*, 1980; Tateson *et al.*, 1988) were used at concentrations which inhibited LTB_4 and TxB_2 production, respectively. BW A4C also failed to modify platelet aggregation and 5-HT release, and BW 755C reduced marginally platelet aggregation, 5-HT release being reduced by 50%. The anti-asthmatic drug, nedocromil sodium (Eady, 1986), had no effect on the system. In fact, azelastine (100 μM for

Table 1 Effects of different drugs on platelet activation induced by FMLP-stimulated PMN

Drug	Concentration (μM)	Aggregation	5-HT release	LTB_4 synthesis	TxB_2 synthesis
ASA	100	97.5 ± 5.4	72.9 ± 12.3	77.8 ± 10.3	4.2 ± 5.3*
Ketoprofen	10	100.5 ± 4.7	64.0 ± 10.6	100.6 ± 31.1	2.8 ± 2.8*
Diclofenac	10	91.9 ± 7.0	73.4 ± 10.9	88.1 ± 16.9	0*
Piroxicam	10	96.9 ± 8.1	73.6 ± 13.9	98.0 ± 27.1	0.9 ± 1.9*
BW 755C	100	86.3 ± 9.0	44.0 ± 13.6*	1.7 ± 3.3*	10.5 ± 3.6*
BW A4C	100	110.3 ± 14.3	82.4 ± 16.8	2.6 ± 4.3*	0*
Nedocromil	100	104.8 ± 5.2	92.9 ± 1.3	ND	ND
Azelastine	100	0*	8.4 ± 5.2*	1.9 ± 2.8*	0.9 ± 1.5*

Drugs were added to the PMN-platelet cooperation system at the concentrations and for the time interval indicated. PMN were then challenged with 0.5 μM FMLP, and various parameters of cell activation were measured (for details see Methods). ND: not determined. The basal release of TXB_2 and LTB_4 by resting cells was 4.2 ± 0.2 ng ml^{-1} and 4.7 ± 12.0 pg ml^{-1}, and upon activation by FMLP these values were 45.8 ± 1.5 ng ml^{-1} and 555.5 ± 212.2 pg ml^{-1} respectively. Results are expressed in percentage of control values. Each value is the mean ± s.d. of 3 to 4 experiments.

1 min) was the only drug tested which prevented platelet aggregation induced by FMLP-stimulaed PMN.

Concentration-dependent inhibition by azelastine of PMN-mediated platelet aggregation and 5-hydroxytryptamine

Azelastine completely inhibited platelet aggregation, 5-HT release and synthesis of LTB_4 and TxB_2. Since the anti-lipoxygenase and anti-cyclo-oxygenase activities did not account for the inhibition of platelet aggregation by stimulated PMN (both acetohydroxamic compounds were ineffective), the capacity of azelastine to exert its inhibitory effect was further investigated.

Azelastine was added at increasing concentrations (5–300 μM) to a mixed cell suspension 1 min before stimulation by 0.5 μM FMLP (Figure 1). A strong platelet activation (37.7 ± 5.6% aggregation and 59.9 ± 19.2% 5-HT release; $n = 6$) was noted in absence of azelastine, whereas with 20 μM azelastine platelet aggregation was significantly inhibited ($P < 0.05$; $n = 4$; unpaired t-test) and suppressed at 70 μM. Release of 5-HT was also significantly inhibited by azelastine at 30 μM ($P < 0.05$; $n = 3$). However, inhibition never reached 100% and was less apparent for high concentrations of azelastine. Thus, in the presence of 300 μM azelastine the release was 16.2 ± 7% ($n = 4$) compared to 8.9 ± 4% ($n = 5$) with 70 μM.

Effect of azelastine on PMN and platelets used separately

In order to determine whether the target of azelastine was PMN or platelets, we studied its effects on both cells separately. First, we verified that inhibition was not restricted to FMLP, and accordingly was not accounted for by receptor antagonism. Indeed, platelet activation induced by C5a-stimulated PMN was also inhibited by azelastine (aggregation: 43.2 ± 2.1% vs 3.1 ± 0.3% with 100 μM azelastine; 5-HT release: 58.4 ± 6.5% vs 0.9 ± 0.6% with 100 μM azelastine; $n = 3$).

As illustrated in Figure 2, azelastine inhibited concentration-dependently the β-glucuronidase release from FMLP-activated PMN. The effective concentrations were 20–150 μM ($P < 0.05$). However, at concentrations above 100 μM, the enzyme release was increased.

Results presented in Figure 3 show that azelastine also inhibited platelet activation, irrespective of the agonist. Indeed, platelet aggregation and 5-HT release induced by PAF (Figure 3a), or by Cat G (Figure 3b), were suppressed in a concentration-dependent manner by azelastine. Other platelet agonists such as collagen or thrombin were also inhibited by azelastine, inhibition being surmountable by increasing the concentrations of the stimulating agents (data not shown). As observed for platelet aggregation, high concentrations of azelastine never totally suppressed 5-HT release, and indeed significant amounts of 5-HT were found in supernatants of

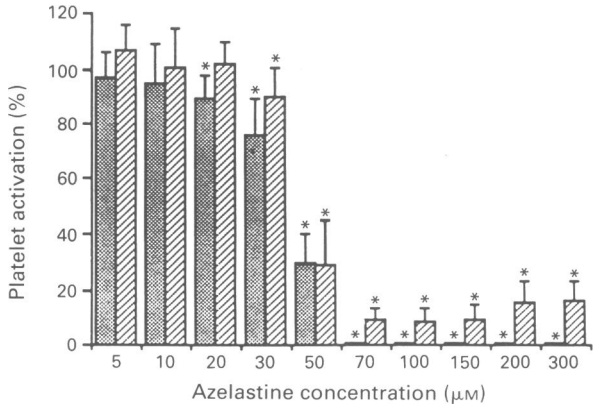

Figure 1 Inhibition by azelastine of platelet activation induced by FMLP-stimulated PMN: 5 × 10^6 PMN per ml and 2 × 10^8 platelets per ml were preincubated together for 4 min with cytochalasin B (5 μg ml^{-1}) and fibrinogen (0.77 mg ml^{-1}). Different azelastine concentrations were added, and 1 min later the cells were stimulated with 0.5 μM FMLP. Aggregation (stippled columns) and 5-hydroxytryptamine release (hatched columns) are expressed as percentage of activity in comparison with controls without azelastine. Each column is the mean of 4 distinct experiments; s.d. shown by vertical bars.

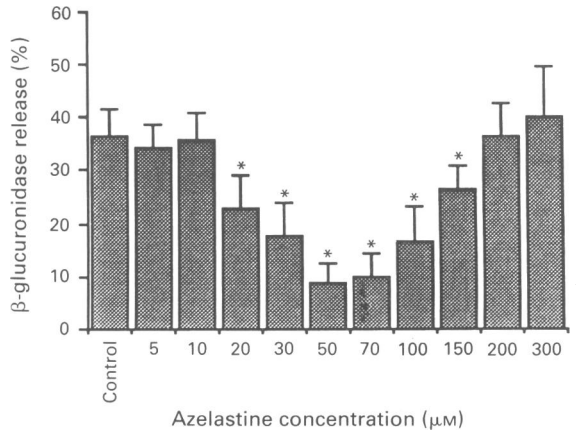

Figure 2 Interference of azelastine with β-glucuronidase release from PMN. 5 × 10^6 PMN per ml were preincubated at 37°C in the presence of cytochalasin B for 4 min before addition of different azelastine concentrations. PMN were stimulated 1 min later by 0.5 μM FMLP. The amounts of enzyme released were determined in supernatants and are expressed as a percentage of total enzyme recovered in the corresponding pellets treated with Triton X-100. Each column is the mean of 4 experiments; s.d. shown by vertical bars.

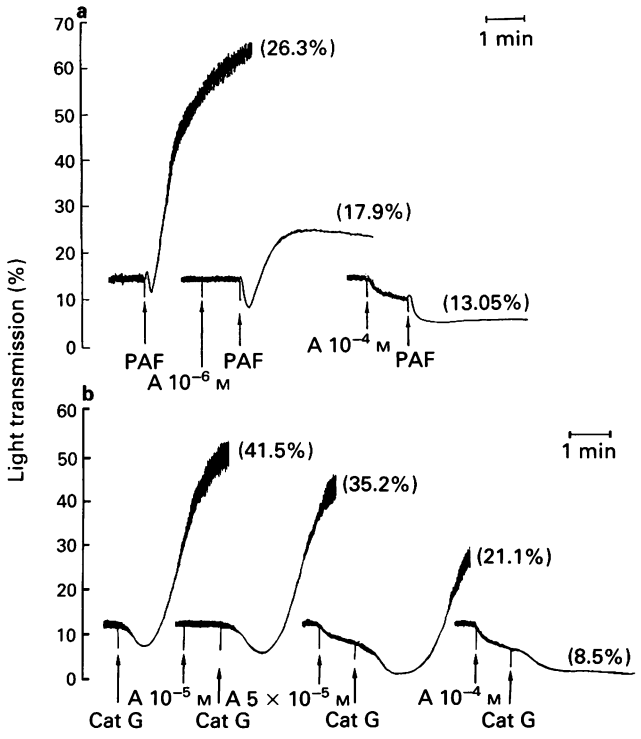

Figure 3 Inhibition by azelastine of platelet aggregation induced by PAF or cathepsin G. Platelets 2×10^8 per ml of HBSS-Tyrode (1:1) were incubated with fibrinogen at 37°C. Azelastine (A) was added, and 1 min later cells were stimulated either by 1 μM of PAF (a) or by 166 nM of Cat G (b). Values in parentheses represent the percentage of 5-HT released. Tracings are representative of 4 experiments.

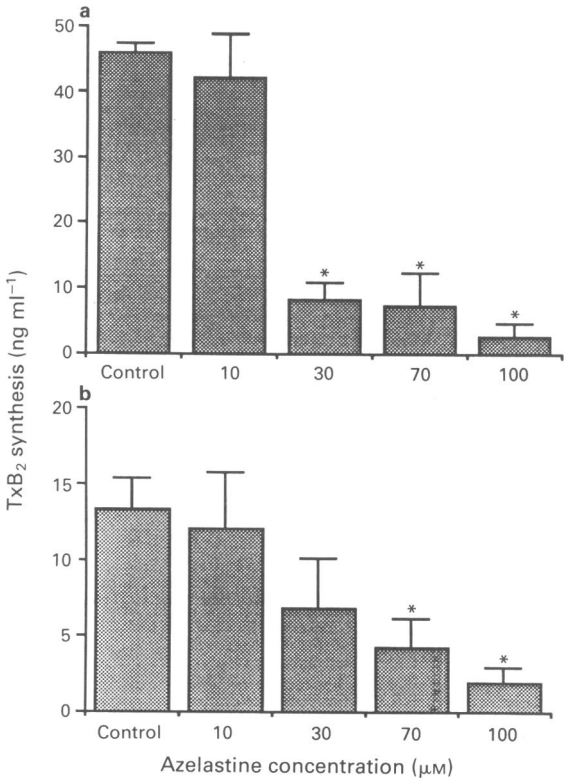

Figure 5 Inhibition by azelastine of thromboxane B_2 (TxB_2) production. (a) For details see Figure 4. (b) TxB_2 production was measured from supernatants of platelets pretreated with azelastine and stimulated with 166 nM of purified Cat G. Each column is the mean of 3 experiments; s.d. shown by vertical bars.

platelets which had not been stimulated but only incubated for 1 min with 70 μM or more azelastine. Thus, with 300 μM of the drug, the release was of 19.6 ± 9.1% ($n = 3$), which was comparable to the value observed in the PMN-platelets cooperation system (see Figure 1).

Effect of high azelastine concentrations on LDH release from PMN or platelets

Since it was likely that the effects observed for concentrations of azelastine above 100 μM (β-glucuronidase release from PMN, 5-HT release from platelets) resulted from cell permeation, lactic dehydrogenase (LDH) release was evaluated. LDH was indeed found in supernatants of PMN and platelets treated with high concentrations of azelastine, PMN being more affected than platelets since their treatment for 1 min with 100 μM azelastine induced a significant release of LDH ($P < 0.05$) whereas 200 μM were required to induce a similar effect on platelets. At 300 μM azelastine provoked a release of LDH from PMN which amounted to 63.5 ± 13.5% ($n = 3$). The same effect, although less pronounced, was observed with platelets: 36.6 ± 13.5% of LDH ($n = 3$).

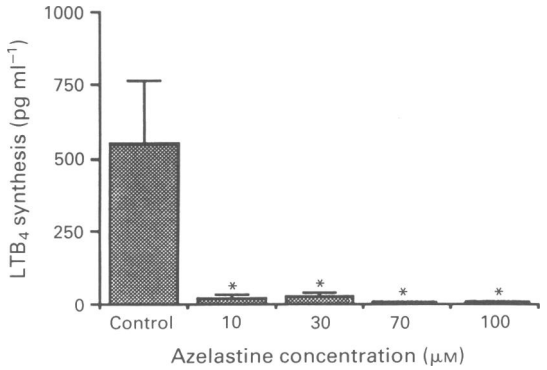

Figure 4 Inhibition by azelastine of leukotriene B_4 (LTB_4) production in the PMN-platelet cooperation model. LTB_4 production was evaluated by radioimmunoassay from supernatants of a mixed cell suspension (PMN and platelets) pretreated with different azelastine concentrations and then stimulated with 0.5 μM FMLP. Each column is the mean of 3 experiments; vertical bars show s.d.

Inhibition by azelastine of leukotriene B_4 and thromboxane B_2 production

Challenge of the mixed cell population with 0.5 μM FMLP led to the formation of LTB_4 (from PMN) and TxB_2 (from platelets) as shown previously (Renesto *et al.*, 1990). The results presented in Figure 4 show that LTB_4 production was strongly inhibited by azelastine. Thus, 10 μM azelastine reduced it to 2.2 ± 2.2% of the control values ($n = 3$). In addition, TxB_2 measurements performed in parallel showed an inhibition concentration-response curve (Figure 5a). The latter inhibition was a direct effect of azelastine on platelets since TxB_2 production from these cells stimulated by a submaximal concentration of Cat G was also effectively inhibited (Figure 5b).

Discussion

We have described here the effect of various drugs on PMN-mediated platelet activation. Such cell-to-cell communication, which involves the release from FMLP- or C5a-stimulated PMN of Cat G, a serine proteinase responsible for the subsequent platelet activation (Chignard *et al.*, 1986; Selak *et al.*, 1988; Renesto *et al.*, 1990; Ferrer-Lopez *et al.*, 1990; Del Maschio *et al.*, 1990) may be involved in different inflammatory processes, as mentioned in the introduction.

Among the substances tested, azelastine was the only one to inhibit PMN-induced platelet aggregation. In contrast, nedoc-

romil sodium, another anti-asthmatic drug (Eady, 1986) was inactive. ASA, ketoprofen, diclofenac and piroxicam, different anti-inflammatory substances with anti-cyclo-oxygenase activity, used at concentrations which suppressed TxB_2 formation by platelets, also failed to inhibit platelet aggregation. Furthermore, the two acetohydroxamic acids, BW 755C and BW A4C which have more anti-lipoxygenase than anti-cyclo-oxygenase properties (Randall *et al.*, 1980; Tateson *et al.*, 1988), did not affect platelet aggregation by more than 10% under conditions where LTB_4 and also TxB_2 synthesis were suppressed. This inhibition of TxB_2 formation resulted from the use of high concentrations (100 μM), of both drugs, even though BW A4C is described as a selective inhibitor of the 5-lipoxygenase activity (Tateson *et al.*, 1988). As indicated in Table 1, azelastine at 100 μM also inhibited the production of TxB_2 and LTB_4, but the use of cyclo-oxygenase and lipoxygenase inhibitors indicates clearly that these properties do not account for the inhibition of platelet aggregation and 5-HT release.

Inhibition of platelet activation by activated PMN may result from interference (i) with PMN, (ii) with platelets, or (iii) with Cat G, the mediator involved (Renesto *et al.*, 1990; Ferrer-Lopez *et al.*, 1990). Experiments with PMN or platelet suspensions used separately, allowed us to identify the target(s) of azelastine. PMN degranulation evaluated by β-glucuronidase release was affected, the lowest effective concentration of azelastine on PMN being the same as the lowest concentration effective in the PMN-platelet suspension system, i.e., 20 μM. Inhibition was not due to an antagonism at the FMLP receptor level, since activation of PMN by C5a was also prevented by azelastine. In fact, β-glucuronidase and Cat G are stored in the same azurophilic granules (Ford Bainton, 1988) and their release upon activation proceed in parallel (Renesto & Chignard, 1991 and unpublished data) on the one hand, and the degree of β-glucuronidase release is correlated with the level of platelet activation (Ferrer-Lopez *et al.*, 1990) on the other hand. Thus, it can be inferred that β-glucuronidase and Cat G release are equally affected by azelastine and that inhibition of the serine proteinase release accounts for the inhibition of platelet activation. The most effective concentration of azelastine was 50 μM but, surprisingly, higher concentrations were less potent and 300 μM did not reduce but rather enhanced β-glucuronidase release, suggesting that the drug was cytotoxic and induced PMN permeation. LDH measurements performed on the supernatants of PMN treated by this drug confirmed this hypothesis since β-glucuronidase release was always accompanied by LDH release when high concentrations of azelastine were used.

Azelastine also affected platelets since their aggregation by PAF, Cat G (Figures 3a and 3b), collagen or thrombin (not shown) was inhibited. However, unlike platelet aggregation, release of 5-HT was never totally blocked, as observed in the PMN-platelet suspension system. The fact that concentrations of azelastine above 70 μM provoked a less pronounced inhibition of 5-HT release from platelets strongly suggested a cytotoxic effect and indeed, as observed with PMN, addition of azelastine at high concentrations to platelets induced LDH release.

The evaluation of LTB_4 and TxB_2 production confirmed that the target of azelastine was not restricted to PMN but also involved platelets. Indeed, LTB_4 production from PMN was inhibited in the cell-to-cell communication system (Figure 4). In addition, this confirmed that inhibition of LTB_4 synthesis and of platelet aggregation were not correlated, since 10 μM azelastine suppressed the former but had no effect on the latter, as observed with BW 755C and BW A4C. In these experimental conditions, TxB_2 formation was not affected by 10 μM azelastine. This means that at this concentration, azelastine does not interfere with the platelet function (as confirmed with exogenously added Cat G, Figures 3b and 5b), nor with PMN stimulation, as evaluated by the inhibition of β-glucuronidase release (Figure 2), but is fully active against LTB_4 formation. This indicates a specific 5-lipoxygenase inhibitory activity, as shown by Nishihira *et al.* (1989). At concentrations above 70 μM, azelastine inhibited TxB_2 formation by Cat G-activated platelets dose-dependently (Figure 5b). This probably results from an effect unrelated to cyclo-oxygenase and/or thromboxane synthase inhibition, since aggregation was similarly affected by azelastine whereas the tested anti-inflammatory drugs with anti-cyclo-oxygenase properties suppressed TxB_2 formation without modifying aggregation.

The mechanism by which azelastine interferes with allergy is not established. It has been shown that it prevents the synthesis/release of mediators such as histamine (Chand *et al.*, 1985), leukotrienes (Chand *et al.*, 1989; Nishihira *et al.*, 1989), superoxide anions (Busse *et al.*, 1989; Taniguchi & Takanaka, 1989), arachidonic acid (Taniguchi & Takanaka, 1989), and that it antagonizes the effect of some of these mediators on target organs (Rafferty *et al.*, 1989). In addition, Nakamura *et al.* (1988) have shown that azelastine inhibits the release of Ca^{2+} from intracellular storage sites in guinea-pig peritoneal macrophages stimulated by PAF or by FMLP. Azelastine was also described as an inhibitor of leukocyte (Chand *et al.*, 1985; Taniguchi & Takanaka, 1989; Busse *et al.*, 1989), and platelet functions (Achterrath-Tuckermann *et al.*, 1988). Azelastine is effective in animals (Chand *et al.*, 1986; 1987; Achterrath-Tuckermann *et al.*, 1988), and has been claimed to reduce allergic rhinitis (Meltzer *et al.*, 1988) and asthma (Spector *et al.*, 1987; Kemp *et al.*, 1987). The present paper supports a specific target for this drug, i.e., the ability to inhibit PMN-mediated platelet activation, a property which is shared neither by cyclo-oxygenase and lipoxygenase inhibitors nor by nedocromil sodium. Interestingly, experiments performed on isolated lungs from sensitized guinea-pigs demonstrated that nedocromil sodium inhibits the establishment of hyperresponsiveness but is unable to prevent its manifestation once established (Pretolani *et al.*, 1990). By contrast and in parallel with the present results, azelastine is capable of inhibiting these same manifestations of hyperresponsiveness. In other words, azelastine suppressed the activation of inflammatory cells present in the lungs of sensitized animals (Pretolani *et al.*, unpublished data).

In the present study we have shown that azelastine interferes with the cell-to-cell communication between human PMN and platelets. This effect may be relevant for its therapeutic efficiency in asthma and may also suggest therapeutic values in pathologies in which PMN and platelets are involved.

The authors thank Dr A.J. Coyle for his advice in the preparation of the manuscript, and Laboratories Sarget for generous support.

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(Received December 14, 1990
Revised January 9, 1991
Accepted February 12, 1991)

Br. J. Pharmacol. (1991), **103**, 1441–1448 © Macmillan Press Ltd, 1991

Endothelial modulation and changes in endothelin pressor activity during hypoxia in the rat isolated perfused superior mesenteric arterial bed

Stephen A. Douglas, Simon James & [1]C. Robin Hiley

Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ

1 The isolated superior mesenteric arterial bed of the rat, perfused with Krebs-Henseleit solution containing 10 μM indomethacin, was used to study the effects of reducing dissolved O_2 tension on the pressor responses to endothelin-1, endothelin-3 and sarafotoxin S6b. The modulation of these responses by the endothelium was investigated by removing the intima with the detergent CHAPS and, for endothelin-1, by inhibiting nitric oxide production with N_ω-nitro-L-arginine methyl ester (L-NAME). Comparison was made with the effects of lowering O_2 tension on the pressor responses to noradrenaline and 5-hydroxytryptamine.

2 Lowering the perfusate O_2 tension from 551 ± 2 mmHg to 14.0 ± 0.5 mmHg did not change the ED_{50} for endothelin-1 but its maximal responses (R_{max}) were increased by 2.1 and 2.7 fold, respectively, in the presence and absence of endothelium. The R_{max} values for endothelin-3 were also greater in hypoxia either in the presence (by 2.3 fold) or absence of the endothelium (by 1.6 times) but those for sarafotoxin S6b were only enhanced significantly by hypoxia in the absence of the intima. Hypoxia reduced the potencies of endothelin-3 and sarafotoxin S6b whether or not endothelium was present.

3 Endothelial destruction, whether in hypoxic or oxygenated conditions, increased the R_{max} values for endothelin-1 and endothelin-3; at both O_2 tensions those for endothelin-3 increased more than those for endothelin-1. The ED_{50} for endothelin-1 was unchanged by destroying the endothelium but endothelin-3 was less potent in the absence of an endothelium than in its presence. Removal of the endothelium did not change the R_{max} for sarafotoxin S6b but increased its potency in both hypoxic and oxygenated tissues.

4 In hypoxia, and in the presence of both the endothelium and 100 μM L-NAME, the R_{max} for endothelin-1 was 1.6 times greater than that in hypoxia in the absence of L-NAME. Co-infusion of 100 μM L-arginine, but not of 100 μM D-arginine, with 100 μM L-NAME reversed this effect. The presence of L-NAME decreased the potency of endothelin-1.

5 Destroying the endothelium did not affect the R_{max} for noradrenaline in either oxygenated conditions or hypoxia. Changing O_2 tension when the endothelium was intact had no effect on the R_{max} but it was 11% greater in oxygenated, than in hypoxic, endothelium denuded preparations. Endothelial destruction decreased the potency of noradrenaline in hypoxia but increased it in oxygenated tissues. In hypoxia, L-NAME had no effect on the ED_{50} relative to control preparations with endothelium but the R_{max} was 30% greater.

6 5-Hydroxytryptamine gave very small pressor responses in the presence of endothelium in both oxygenated and hypoxic tissues but the R_{max} was 1.7 times greater in hypoxia. L-NAME increased the R_{max} by 9.8 times in oxygenated preparations and 6.3 fold in hypoxia. The ED_{50} values were the same in all conditions.

7 It is concluded that, although hypoxia generally increased the R_{max} for the endothelin/sarafotoxin peptides, the changes could not be explained by a simple increase in receptor number since hypoxia decreased the potency of endothelin-3 and sarafotoxin S6b. Thus alterations in receptor binding or activation properties, or both, also occurred. The changes associated with hypoxia were not common to all vasoconstrictor agonists since, in the absence of endothelial function, hypoxia did not affect the R_{max} values for either noradrenaline or 5-hydroxytryptamine. Also, the pressor responses to the peptides and both the amines can be modulated by the endothelium in hypoxia as well as in oxygenated conditions.

Keywords: Endothelin-1; endothelin-3; sarafotoxin S6b; hypoxia; vasoconstriction; endothelium; 5-hydroxytryptamine; L-arginine; (−)-noradrenaline; N_ω-nitro-L-arginine methyl ester

Introduction

Amongst the first indications that the endothelium could produce a vasoconstrictor factor was the observation that, *in vitro*, hypoxia-induced contraction of pig pulmonary artery strips was reduced in the absence of the endothelium (Holden & McCall, 1984). Subsequently Rubanyi & Vanhoutte (1985) showed that the endothelium of dog coronary arteries released a diffusible, non-eicosanoid, vasocontractant when subjected to anoxia. The isolation of endothelin from cultured aortic endothelial cells of the pig (Yanagisawa *et al.*, 1988) led to suggestions that this peptide might be the factor mediating hypoxic vasoconstriction. This proposition was called into doubt, largely because the contractile responses induced by endothelin-1 and anoxia had different time courses and because it was difficult to wash out responses to endothelin, unlike those to anoxia (Vanhoutte *et al.*, 1989). However, exposure to hypoxia for 24 h stimulated endothelin release from cultured bovine endothelial cells (Hieda & Gomez-Sanchez, 1990) and increasing amounts of immunoreactive endothelin-1 were released from rat resistance vessels perfused for 30–150 min *in vitro* with lowered O_2 tension (Rakugi *et al.*, 1990). Thus there remains the possibility that endothelin may play a role in physiological responses to hypoxia.

Even though endothelin may not be a primary mediator of cardiovascular responses to hypoxia or anoxia, the effects of these conditions on the responses to endothelin and on its receptors have been examined. Liu *et al.* (1990b) found that

[1] Author for correspondence.

reoxygenation, but not hypoxic perfusion itself, increased the density of [^{125}I]-endothelin-1 binding sites and decreased its binding affinity in membranes from rat Langendorff heart preparations. In addition, endothelin-1 was more potent as a systemic pressor agent in pithed rats respired at a lower respiratory volume than in controls and, in the *in situ* blood-perfused mesentery of this preparation, the peptide was twice as potent as a vasoconstrictor when O_2 levels were lowered by reducing ventilation volume (MacLean *et al.*, 1989). The systemic pressor response to an analogue, [^{1,3,11,15}Ala]endothelin-1, was also potentiated in pithed rats when ventilation volume was reduced (MacLean & Hiley, 1989); thus the effect is not restricted to endothelin-1. With pithed rats it was not possible to determine whether the changed responses were due to the change in O_2 tension or to the accompanying alterations in CO_2 levels and pH. Also, endothelin-1 was the only peptide studied in the mesenteric bed. Thus the present study was carried out in the rat mesenteric bed *in vitro*, in order that the level of O_2 could be varied independently of pH and CO_2 concentration, and two other peptides of the endothelin/sarafotoxin family, endothelin-3 and sarafotoxin S6b, have been included as well as endothelin-1. The effects of hypoxia on the pressor actions of these peptides were compared with changes taking place in the responses to noradrenaline and 5-hydroxytryptamine in order to see whether or not all vasoconstrictor agents might be affected similarly.

Another feature of the endothelin/sarafotoxin peptides is that their effects, like those of many vasoconstrictor agents, are modulated by the endothelium (Randall *et al.*, 1989; Warner *et al.*, 1989), apparently by the release of nitric oxide (Douglas & Hiley, 1990). It has been reported that the effectiveness of endothelium-dependent relaxation is changed when O_2 tension is very low. Originally, De Mey & Vanhoutte (1983) reported that endothelium-dependent relaxation to acetylcholine in dog femoral artery was reduced in conditions of near anoxia but, more recently, other studies have suggested that hypoxia may stimulate the release of endothelium-derived relaxing factor (EDRF) (Pohl & Busse, 1989). Thus it was of interest to study the modulation by the endothelium of the responses to the three peptides, endothelin-1, endothelin-3 and sarafotoxin S6b, under hypoxic conditions. This was done either by removing the endothelium with a detergent, CHAPS (3 - [(3 - cholamidopropyl) - dimethylammonio] - 1 - propane - sulphonate), or by inhibiting nitric oxide synthesis with N_ω-nitro-L-arginine methyl ester (L-NAME; Moore *et al.*, 1990).

Methods

The isolated perfused superior mesenteric arterial bed of the rat

Adult male Wistar rats (250–400 g; Bantin & Kingman, Hull) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}: Sagatal; RMB Animal Health Ltd, Dagenham, Essex) and heparinized (100 u kg^{-1}; Paines & Byrne, Greenford, Middlesex) with a single i.p. injection. The perfusion of the rat mesenteric arterial bed was carried out essentially according to the protocol of McGregor (1965). Briefly, an abdominal incision was made to allow the superior mesenteric artery to be identified and cleared of adherent tissue. The artery was ligated, close to the abdominal aorta, cannulated with polythene PP50 tubing (Portex, Hythe, Kent) and then the mesenteric arterial bed was perfused at 37 ± 1°C and 2 ml min^{-1} with Krebs-Henseleit solution. The perfused mesentery was dissected away from the gastrointestinal tract and placed on a warmed Perspex block maintained at 37 ± 1°C. Nescofilm (Nippon Shoji Kaishi Ltd, Osaka, Japan) was placed over the tissue to avoid surface damage and to minimize access for atmospheric O_2. The perfusion pressure in the mesentery was recorded from a T-piece, placed in the perfusion circuit close to the point at which the cannula was inserted into the artery, using a Bell & Howell Type 4-422-0001 pressure transducer coupled to a Grass Model 79D polygraph. Zero pressure was set at zero flow and so, since the flow rate was constant, changes in perfusion pressure were directly proportional to changes in vascular resistance.

The Krebs-Henseleit solution had the following composition (mM): NaCl 118.0, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, $CaCl_2$ 2.5 and D-glucose 5.5. This was bubbled with 95% O_2:5% CO_2 for oxygenated preparations or with 95% N_2:5% CO_2 for hypoxic tissues. Unless stated to the contrary, the perfusion fluid contained 10 μM indomethacin. All preparations were perfused for the first 20 min with oxygenated Krebs-Henseleit solution and then those which were to be studied in hypoxia were perfused with a nitrogenated solution; administration of agonists was started about 1 h later when the O_2 tension was <25 mmHg.

The partial pressure of O_2 in the perfusate was monitored with a Strathkelvin Instruments (Glasgow) type 1302 O_2 electrode placed in a heated (37 ± 1°C) type MC 100 microcell; these were placed in the perfusion circuit just before the T-piece leading to the pressure transducer. A continuous recording of O_2 tension was obtained by taking the output from a Strathkelvin Instruments Model 781 O_2 meter to the polygraph. In order to minimize entry of atmospheric O_2 into the perfusate, all tubing through which it flowed was either polythene (Portex) or neoprene (Watson-Marlow, Falmouth, Cornwall). The O_2 partial pressures in the microcell when the perfusate was bubbled with 95% O_2:5% CO_2 were 551 ± 2 mmHg (n = 219) and 14.0 ± 0.5 mmHg (n = 206) when it was bubbled with 95% N_2:5% CO_2; these values were those obtained at the mid-point of the determination of the dose-response relationship.

Agonists were administered into the perfusion circuit in volumes of 100 μl or less except for the highest dose of endothelin-3 which required injection of 400 μl. After the first dose, the peptides were given 5 min after the tissue perfusion pressure had returned to the basal value following the previous dose. Doses of noradrenaline or 5-hydroxytryptamine were administered 3 min apart.

When required, the endothelium was removed by perfusion of the bed with 0.3% (w/v) CHAPS in distilled water for 120–150 s (Hiley *et al.*, 1987). The tissue was then allowed 45 min to re-equilibrate. In all preparations, before construction of a dose-pressor response curve, the presence or absence of a functional endothelium was assessed by determining whether or not acetylcholine opposed noradrenaline-induced vasoconstriction (Hiley *et al.*, 1987).

The role of nitric oxide in modulating the pressor actions of endothelin-1, noradrenaline and 5-hydroxytryptamine was studied by adding 100 μM (final concentration) L-NAME to the perfusate. Finally, assessment was made of the effects on the pressor responses to endothelin-1 of the co-infusion of either 100 μM D- or L-arginine with 100 μM L-NAME.

Statistical and data analysis

Values are given as the mean ± s.e.mean. The values of n show the number of preparations in which the individual doses used to construct a dose-response curve were examined; since some doses of an agonist were used on all the preparations, the greatest value of n shows the total number of preparations employed. Comparison of means was by Student's unpaired t test.

Dose-response curves were analysed by fitting the logistic equation:

$$R = \frac{R_{max} \times D^{n_H}}{ED_{50}{}^{n_H} + D^{n_H}}$$

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

response. A modified Marquardt procedure, implemented in Harwell routine VB01A on an IBM 3084Q mainframe computer, was used to carry out the curve fitting (Aceves *et al.*, 1985).

Drugs and peptides

All solutions were made up on the day of the experiment. (−)-Noradrenaline bitartrate (Koch-Light, Haverhill, Suffolk) was dissolved in distilled water containing 1 mg ml^{-1} ascorbic acid (Fisons, Loughborough, Leicestershire) to give a stock solution of 5 mg ml^{-1}. 5-Hydroxytryptamine creatinine sulphate (Koch-Light) was dissolved in distilled water also to give a solution of 5 mg ml^{-1}. Dilutions of both stock solutions were made in 0.9% NaCl (w/v). L-NAME, acetylcholine bromide and D- and L-arginine hydrochloride (Sigma Chemical Company, Poole, Dorset) were dissolved in saline. Indomethacin (Sigma) was dissolved in 5% (w/v) sodium bicarbonate to give a 1 mg ml^{-1} solution and then diluted to the required concentration in the Krebs-Henseleit solution. CHAPS was obtained from Sigma and dissolved in distilled water.

Endothelin-1, endothelin-3 and sarafotoxin S6b (Peptide Institute, Osaka, Japan) were dissolved in distilled water to give 100 μM stock solutions. The concentrations of endothelin-1 and sarafotoxin S6b were checked by absorbance spectrophotometry at 280 nm using a molar extinction coefficient of 7250 for a 10 mm path length. For endothelin-3 the extinction coefficient used was 10050 at 279 nm.

Results

Effect of hypoxia and endothelial destruction with CHAPS on the pressor actions of endothelin-1, endothelin-3 and sarafotoxin S6b

Removal of the endothelium, by perfusion of the superior mesenteric bed with 0.3% CHAPS, increased the maximal responses to endothelin-1 in both oxygenated (Figure 1a) and hypoxic tissues (Figure 1b). The greatest R_{max} was found in preparations without endothelium perfused under hypoxic conditions; it was 2.7 times greater than that in hypoxic tissues with endothelium. The ratio of the maxima obtained in oxygenated conditions in the presence and absence of the endothelium was similar at 2.1 fold (Table 1). Hypoxia enhanced the maximal response to endothelin-1 whether or not the endothelium was present (Figure 1); hypoxic preparations without endothelium had an R_{max} 2.0 fold greater than the corresponding oxygenated tissues and in preparations with an intact intima the ratio was 1.6. There were no significant differences between any of the ED_{50} values for endothelin-1 (Table 1).

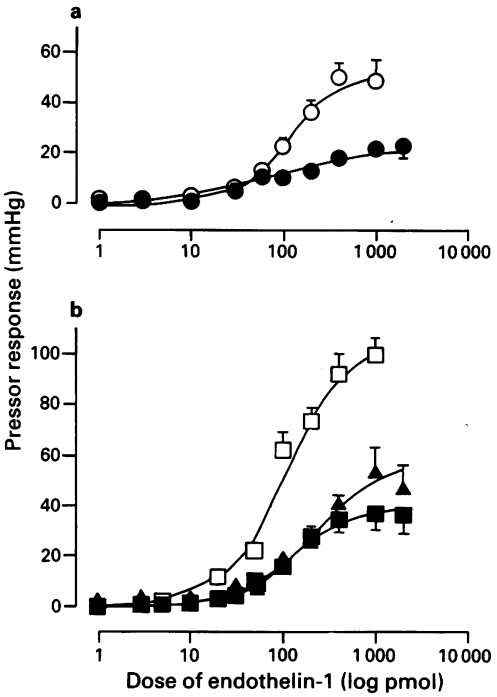

Figure 1 Log dose-response curves to endothelin-1 in oxygenated (a) and hypoxic (b) conditions. (●, $n = 7$–22; ■, $n = 4$–17) show the responses obtained in the presence of endothelium and (○, $n = 17$–28; □, $n = 3$–5) those obtained after destruction of endothelial cells by perfusion with 0.3% CHAPS. In all these cases, 10 μM indomethacin was present in the perfusion medium. (▲, $n = 5$–10) shows the responses obtained in the presence of the endothelium but in the absence of indomethacin. The points are the mean and the vertical bars show the s.e.mean; where a bar is not shown the error lies within the symbol. The lines show the curve of best fit obtained from the fitting procedure.

When hypoxic preparations with endothelium were perfused with Krebs-Henseleit solution which did not contain indomethacin (Figure 1b), endothelin-1 was significantly less potent than it was under the same conditions when 10 μM indomethacin was present (Table 1). Also, the R_{max} for endothelin-1 was significantly higher in the absence of the cyclo-oxygenase inhibitor than in its presence but the slope function was unchanged (Table 2).

A pattern similar to that with endothelin-1 was obtained with endothelin-3 (Figure 2) although the magnitudes of the changes associated with endothelial destruction were somewhat greater; the ratio of the R_{max} values obtained in hypoxic tissues in the presence and absence of the endothelium was 5.9 and it was 8.5 in oxygenated preparations (Table 1). Thus, as

Table 1 Effects of removing the endothelium with 0.3% CHAPS and of lowering O_2 tension in the perfusate on the pressor responses to endothelin/sarafotoxin peptides in rat superior mesenteric arterial bed

	With endothelium				*Without endothelium*			
Peptide	R_{max} (mmHg)	ED_{50} (nmol)	n_H	n	R_{max} (mmHg)	ED_{50} (nmol)	n_H	n
Endothelin-1								
Oxygenated	24.7 ± 3.3	0.138 ± 0.059	0.9 ± 0.1	7–22	52.3 ± 2.6***	0.114 ± 0.012	1.7 ± 0.2**	17–28
Hypoxic	39.5 ± 2.5††	0.124 ± 0.015	1.4 ± 0.1†††	4–17	107 ± 9***/†††	0.110 ± 0.024	1.3 ± 0.1	3–5
Endothelin-3								
Oxygenated	9.8 ± 1.1	0.92 ± 0.32	0.8 ± 0.1	4–7	83.6 ± 6.3***	4.2 ± 1.0*	0.9 ± 0.1	2–12
Hypoxic	22.7 ± 2.4†††	2.4 ± 0.4††	1.8 ± 0.2†††	3–6	133 ± 19***/††	12.1 ± 3.3*/††	1.0 ± 0.1**	2–9
Sarafotoxin S6b								
Oxygenated	117 ± 10	0.471 ± 0.126	0.9 ± 0.1	4–7	96.4 ± 4.1	0.114 ± 0.016	1.4 ± 0.1**	2–6
Hypoxic	137 ± 7	1.08 ± 0.21†	1.2 ± 0.1†	2–9	128 ± 2†††	0.156 ± 0.007**/†	2.1 ± 0.1***/†††	2–7

Statistical significance: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ for the difference between preparations at the same O_2 tension with and without endothelium; † $P < 0.05$; †† $P < 0.01$; ††† $P < 0.001$ for the difference between hypoxic preparations and the corresponding (i.e. with or without endothelium) oxygenated preparation.

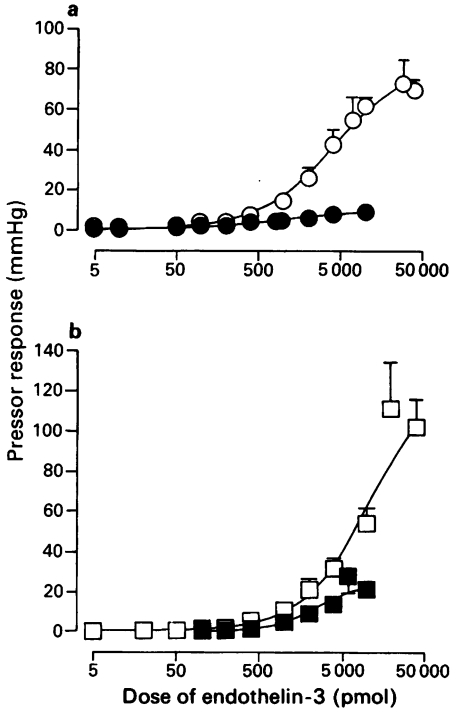

Figure 2 Effects of hypoxia on the log dose-response curves to endothelin-3 in the rat isolated superior mesenteric arterial bed. In (a) are shown the responses obtained in oxygenated conditions in the presence (●, $n = 4$–7) and absence of endothelium (○, $n = 2$–12); (b) shows the results obtained during hypoxia in untreated preparations (■, $n = 3$–6) and those perfused with CHAPS (□, $n = 2$–9). The curves are the lines of best fit obtained from fitting to a logistic equation. The points show the mean and the vertical bars show the s.e.mean; where a bar is not shown the error lies within the symbol.

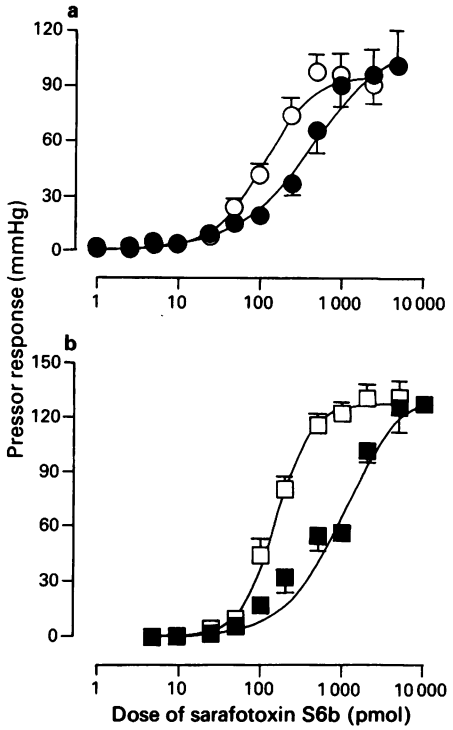

Figure 3 Log dose-response curves for sarafotoxin S6b in the rat isolated superior mesenteric bed in oxygenated (a) and hypoxic (b) conditions. (●, $n = 4$–7; ■, $n = 2$–9) show the responses obtained in the presence of endothelium and (○, $n = 2$–6; □, $n = 2$–7) those obtained after its destruction. The points are the mean and the vertical bars show the s.e.mean; if a bar is not shown the error lies within the symbol. The curves drawn are those which were obtained from the curve fitting procedure.

for endothelin-1, endothelial cell destruction significantly enhanced the R_{max} of endothelin-3 under both oxygenated and hypoxic conditions. Additionally, reducing the partial pressure of O_2 in the perfusate also resulted in increases in the R_{max} for endothelin-3 of 2.3 fold in the presence of a functional endothelium and 1.6 fold in its absence (Table 1). The results with endothelin-3 differed from those with endothelin-1 in that both endothelial cell destruction and hypoxia caused significant decreases in the potency of the peptide (Table 1).

In contrast to the two endothelins, endothelial destruction had no effect on the R_{max} values for sarafotoxin S6b (Figure 3). However, in the absence of endothelium, lowering O_2 tension increased the R_{max} (by a ratio of 1.3) but there was no significant effect of hypoxia on the maximum responses in tissues with endothelium. In the absence of the endothelium the R_{max} values for sarafotoxin S6b were not significantly different from those for endothelin-3 obtained under the same O_2 tensions. In both hypoxic and oxygenated tissues, sarafotoxin S6b was more potent in the absence of endothelium than in its presence whereas, regardless of whether the intima was present or not, reduction of the partial pressure of O_2 significantly decreased the potency of the peptide (Table 1).

Effect of L-NAME on the pressor responses to endothelin-1 in hypoxia and its reversal with L-arginine

Figure 4 shows that inclusion of 100 μM L-NAME in the perfusion fluid enhanced the R_{max} to endothelin-1 as compared to the control obtained in the presence of endothelium in hypoxic conditions (Table 2). This effect was reversed by co-infusion of L-arginine with L-NAME but not by co-infusion of 100 μM D-arginine and L-NAME (Figure 4; Table 2).

On the other hand, Table 2 shows that, when compared to the control responses to endothelin-1 during hypoxia, inclusion of L-NAME in the perfusate reduced the potency of the peptide regardless of whether L- or D-arginine was present or not. During co-administration of L-NAME and D-arginine, the ED_{50} was not significantly different from those obtained in the presence of either L-NAME alone or L-NAME and L-arginine.

Table 2 Effects on the pressor responses to endothelin-1 of removing indomethacin from, or adding N_ω-nitro-L-arginine methyl ester (L-NAME) to, the perfusion medium in hypoxia

	R_{max} (mmHg)	ED_{50} (pmol)	n_H	n
Control	39.5 ± 2.5	124 ± 15	1.4 ± 0.1	4–17
No indomethacin	58.2 ± 6.8**	223 ± 49*	1.3 ± 0.1	5–10
L-NAME alone	64.1 ± 2.3**	186 ± 15**	1.6 ± 0.1	4–12
L-NAME + L-arginine	43.2 ± 4.1††	210 ± 30**	1.6 ± 0.1	4–10
L-NAME + D-arginine	72.3 ± 7.6§§/**	261 ± 81*	1.0 ± 0.1*/†††/§§§	7–9

The control data are taken from Table 1. Indomethacin (10 μM) was present in the perfusate throughout except for the 'No indomethacin' group. The concentrations of L-NAME, L-arginine and D-arginine in the perfusate were all 100 μM. Statistical significance: * $P < 0.05$; ** $P < 0.01$ from the control; †† $P < 0.01$; ††† $P < 0.001$ from L-NAME alone; §§ $P < 0.01$; §§§ $P < 0.001$ from L-NAME + L-arginine.

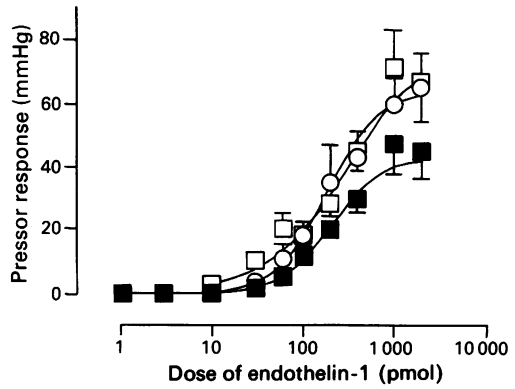

Figure 4 Effects of N_ω-nitro-L-arginine methyl ester (L-NAME) on the log dose-response curves to endothelin-1 in hypoxic conditions and their reversal with L-, but not with D-, arginine: (○, $n = 4$–12) results obtained in the presence of 100 μM L-NAME alone; (■, $n = 4$–10) results obtained in the presence of both L-NAME and 100 μM L-arginine; and (□, $n = 7$–9) results obtained in the presence of L-NAME and 100 μM D-arginine. The curves show the lines obtained by fitting to the logistic equation. The points show the mean and the vertical bars show the s.e.mean; where a bar is not shown the error lies within the symbol.

Effect of hypoxia, L-NAME and endothelial destruction on the pressor actions of noradrenaline

Figure 5 and Table 3 show that removing the endothelium had no significant effect on the maximal responses to noradrenaline in either hypoxic or oxygenated tissues nor did changing the degree of oxygenation when the endothelium was intact. However, in oxygenated preparations treated with CHAPS, the R_{max} was significantly greater than that in the corresponding hypoxic preparations. Removal of the endothelium in either oxygenated (Figure 5a) or hypoxic (Figure 5b) preparations significantly changed the ED_{50} for noradrenaline, but in opposite directions. In oxygenated tissues, noradrenaline was more potent as a pressor agent in preparations without endothelium than in intact tissues (Table 3). On the other hand, in hypoxic preparations, treatment with CHAPS resulted in a 2 fold significantly lower potency for noradrenaline (Table 3). Regardless of whether or not the endothelium was intact, hypoxia significantly decreased the potency of noradrenaline.

Noradrenaline also produced dose-dependent increases in perfusion pressure in hypoxic tissues exposed to 100 μM L-NAME. L-NAME had no effect on the potency of noradrenaline when compared to hypoxic preparations with an intact endothelium (unlike CHAPS treatment), but inhibition of nitric oxide synthesis significantly enhanced the R_{max} (Table 3) in comparison to control observations (an effect not seen with CHAPS).

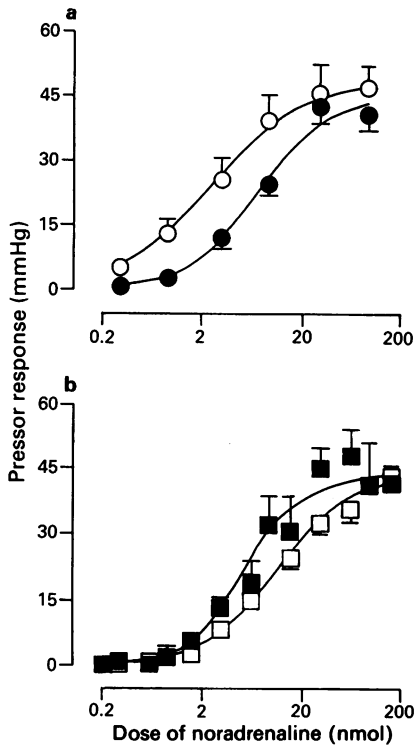

Figure 5 Log dose-response curves for the pressor responses to noradrenaline in oxygenated conditions (a) and hypoxia (b) both in the presence (closed symbols) and absence (open symbols) of the endothelium. Values of n are: (●) 15; (○) 8; (■) 3–10; and (□) 4. The points represent the mean and the vertical bars show the s.e.mean where it does not lie within the symbol. The curves drawn are the lines of best fit obtained from the fitting procedure.

Effect of hypoxia or L-NAME on the pressor actions of 5-hydroxytryptamine

Figure 6 shows that, in the presence of endothelium, 5-hydroxytryptamine gave very small pressor responses when administered to either oxygenated or hypoxic tissues; nevertheless, the R_{max} was significantly greater in hypoxia (Table 3). The values for ED_{50} in oxygenated and hypoxic conditions were not significantly different.

Table 3 Effects on the pressor responses in rat superior mesenteric arterial bed to noradrenaline or 5-hydroxytryptamine of removing the endothelium with CHAPS or of adding 100 μM N_ω-nitro-L-arginine methyl ester (L-NAME) to the perfusion medium in hypoxia and in oxygenated tissues

	R_{max} (mmHg)	ED_{50} (nmol)	n_H	n
(A) *Noradrenaline*				
Oxygenated + endothelium	45.0 ± 3.2	7.4 ± 1.3	1.3 ± 0.1	15
Oxygenated − endothelium	48.5 ± 1.2	2.6 ± 0.2**	1.0 ± 0.1	8
Hypoxia + endothelium	43.2 ± 3.2	5.6 ± 1.4†	1.6 ± 0.2	3–10
Hypoxia − endothelium	43.7 ± 1.9†	11.3 ± 1.1*/†	1.2 ± 0.1	4
Hypoxia + L-NAME	56.2 ± 4.4§§	5.1 ± 1.4	1.1 ± 0.4	6
(B) *5-Hydroxytryptamine*				
Oxygenated + endothelium	2.6 ± 0.5	0.48 ± 0.13	1.8 ± 0.5	6
Oxygenated + L-NAME	25.4 ± 3.4§§§	0.33 ± 0.14	1.0 ± 0.4	4–6
Hypoxia + endothelium	4.5 ± 0.3†	0.27 ± 0.13	1.0 ± 0.3	6
Hypoxia + L-NAME	28.5 ± 3.2§§§	0.17 ± 0.07	1.5 ± 0.4	6

All preparations perfused with L-NAME had an intact endothelium. Statistical significance of differences: * $P < 0.05$; ** $P < 0.01$ from preparations perfused under the same degree of oxygenation but in the presence of endothelium; † $P < 0.05$ from the corresponding (i.e. with or without endothelium) oxygenated preparations; §§ $P < 0.01$; §§§ $P < 0.01$ from the corresponding (i.e. hypoxic or oxygenated) preparations with endothelium.

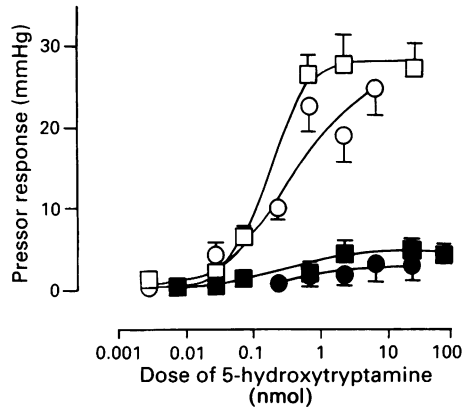

Figure 6 Effects of inhibiting the production of nitric oxide with N_ω-nitro-L-arginine methyl ester (L-NAME) on the responses to 5-hydroxytryptamine under both oxygenated and hypoxic conditions. Circular symbols show the responses obtained under oxygenated conditions in the absence (●, $n = 6$) and presence (○, $n = 4$–6) of 100 μM L-NAME and square symbols represent the results obtained in hypoxic conditions in the absence (■, $n = 6$) and presence (□, $n = 6$) of 100 μM L-NAME. The points are the mean and the vertical bars show the s.e.mean; where a bar is not shown the error lies within the symbol. The lines show the curve of best fit obtained from the fitting procedure.

Inhibition of nitric oxide production by including 100 μM L-NAME in the perfusate significantly increased the maximal responses to 5-hydroxytryptamine in both oxygenated conditions and hypoxia (Table 3; Figure 6); again neither the maximum responses nor the ED_{50} values at the two degrees of oxygenation were significantly different.

Discussion

Reducing the dissolved O_2 tension in the perfusate clearly changed the dose-response relationships for the pressor responses to endothelin-1, endothelin-3 and sarafotoxin S6b. In the absence of endothelium, the R_{max} for each of the peptides was increased and, in its presence, there were significant increases for both endothelins, but not for sarafotoxin S6b (although the numerical mean was greater in hypoxia than in oxygenated conditions, there was no significant difference). Since the experiments with 5-hydroxytryptamine and noradrenaline show that, in the absence of a functional endothelium, hypoxia only increased the maximal pressor responses to the endothelins and sarafotoxin S6b, the cellular change responsible for this enhancement must occur upstream of the mechanisms for contractile activation common to the peptides and the amines.

The potency of endothelin-1 was unaffected by the degree of oxygenation but hypoxia significantly decreased the potency of endothelin-3 and sarafotoxin S6b, irrespective of whether or not the endothelium was intact. Again these effects are not common to the peptides and both the amines since reducing O_2 partial pressure had no effect on the potency of 5-hydroxytryptamine although it reduced the potency of noradrenaline relative to the corresponding oxygenated preparations.

An increase in the R_{max} for endothelin-1 in hypoxia, without a change in ED_{50}, can be explained by a relatively simple mechanism such as an increase in the number of functional receptors for the peptides. Such a hypothesis would accord, to a certain degree, with the findings of Liu *et al.* (1990a,b) who demonstrated that the maximal binding capacity for [^{125}I]-endothelin-1 in rat cardiac membranes increased significantly after periods of global cardiac ischaemia longer than 30 min; the increase was apparently the result of externalization of binding sites (Liu *et al.*, 1989). Hypoxic perfusion for up to 60 min did not change either the number of binding sites or their affinity but subsequent reoxygenation for 15 min significantly increased maximal binding and decreased the affinity of the binding sites for the ligand (Liu *et al.*, 1990b). Another study showed that ischaemia (that is cessation of perfusate flow through the Langendorff preparation with the heart temperature being maintained at 37°C) increased cell surface receptor number without changing affinity until ischaemic periods in excess of 60 min were employed when, again, the affinity of [^{125}I]-endothelin-1 for its binding sites decreased by about 25% (Liu *et al.*, 1990a).

These results obtained in the heart with endothelin-1 show that affinity may decrease as a result of manipulation of O_2 tension. Changes in binding affinity might be expected to lead to changes in the potency of the agonist and, although this did not happen during hypoxia in the mesenteric bed with endothelin-1, decreases in potency were observed for endothelin-3 and sarafotoxin S6b. Thus the increased maximal pressor responses could result from an increase in functional receptor number but, for endothelin-3 and sarafotoxin S6b, a decrease in affinity for the receptors might also have occurred. Although in the absence of specific antagonists the identity of receptor sites cannot be established for certain, it seems likely that all 3 peptides are acting at the same receptors in the mesenteric bed since a high dose of endothelin-1 (1 nmol) desensitizes responses to subsequent doses (approximately equal to the ED_{50}) of itself, endothelin-3 or sarafotoxin S6b but not of noradrenaline (McStay, Hiley & Douglas, unpublished observations). Hence, several changes must occur in hypoxia which, together, increase the reactivity of the vascular smooth muscle to the peptides but decrease its sensitivity to endothelin-3 and sarafotoxin S6b. This difference between the peptides corresponds with endothelin-3 and sarafotoxin S6b being full agonists whilst endothelin-1 is a partial agonist (see below) and they must therefore have different modes of interaction with the receptor.

The presence of the endothelium results in a more complex system to analyse. Here, one mechanism for the increased maximal responses to the peptides when O_2 tension is reduced is that, since hypoxia increases the permeability of cultured endothelial cell layers (Ogawa *et al.*, 1990), they might gain access more readily to the underlying smooth muscle. This, however, is unlikely as we have previously found that, in this preparation, there is no evidence for the endothelium acting as a barrier to diffusion of these peptides in oxygenated conditions (Douglas & Hiley, 1990).

Endothelin-1, endothelin-3 and sarafotoxin S6b cause endothelium-dependent relaxations in the rat isolated superior mesenteric bed which are not due to eicosanoid production (Randall *et al.*, 1989; Douglas & Hiley, 1990). Thus, it might be argued that the enhanced maximal responses to endothelin-1 and endothelin-3 observed in hypoxic preparations with an intact endothelium could be due to a decrease in either the basal or the agonist-induced release of EDRF such as has been suggested to occur in hypoxia by Furchgott & Zawadzki (1980) and Rodman *et al.* (1990). This explanation is unlikely as endothelial cell destruction enhanced to similar extents the pressor actions of these peptides in both oxygenated and hypoxic preparations. Also, vasodilatation in certain vascular beds is a prominent, *in vivo*, systemic response to hypoxia (Marshall & Metcalfe, 1990) and it has been postulated that this hypoxia-induced relaxation is the result of the release of EDRF (Pearce *et al.*, 1989; 1990).

Although the level of O_2 in the perfusate supplied to the tissues in our experiments was very low, some O_2 was still present and there might have been some leaking into the tissue from the atmosphere. It is not possible, therefore, to be sure of the exact O_2 tension in the tissue but clearly it was lowered sufficiently to cause significant changes in the dose-response relationships for the peptides but not to alter the ability of the endothelium to produce EDRF. Thus, the various studies show that EDRF activity is preserved during prolonged periods of hypoxia *in vivo* and *in vitro* and suggest that total anoxia may be required before its activity can be inhibited. Indeed, Richards *et al.* (1991) showed that cultured

porcine aortic endothelial cells produced EDRF during a 30 min exposure to an O_2 level in the bathing Krebs solution of as low as 2.8%, a level slightly above that found in the microcell in our experiments.

EDRF is thought to be nitric oxide which is synthesized from the terminal guanidino nitrogen of L-arginine and O_2 (Palmer *et al.*, 1988; Schmidt *et al.*, 1988; Kwon *et al.*, 1990). The results obtained with endothelin-1 show that L-NAME inhibited nitric oxide synthase in hypoxia in a fashion which was stereoselectively reversed with L-arginine but not D-arginine. In normally oxygenated tissues, inhibition by L-NAME that is reversible by L-arginine but not D-arginine has been reported in a variety of different tissues; for instance L-NAME inhibits non-adrenergic, non-cholinergic (NANC)-induced relaxations in the rat anococcygeus muscle and this effect can be selectively reversed by L-arginine (Hobbs & Gibson, 1990). Since administration of L-NAME to hypoxic tissues augmented the maximal responses to endothelin-1, noradrenaline and 5-hydroxytryptamine it would appear that nitric oxide synthesis and release continue even during periods of prolonged hypoxia and that nitric oxide activity forms the basis for endothelium-dependent modulation of pressor responses during hypoxia. Also, the half life of nitric oxide is enhanced when O_2 tension is reduced (Kelm & Schrader, 1990) and thus the actions of endogenous nitric oxide might be expected to be enhanced in hypoxia.

In hypoxia, inhibition of nitric oxide synthesis in endothelium-intact preparations with L-NAME increased the R_{max} for noradrenaline relative to that obtained in hypoxic tissues without endothelium. Rat aortic smooth muscle maintained in an organ bath contains a nitric oxide synthase which uses L-arginine as a substrate and is inhibited by N^G-monomethyl-L-arginine and L-iminoethyl-L-ornithine (Rees *et al.*, 1990). Thus the discrepancy between the effects of CHAPS and L-NAME on noradrenaline pressor responses in hypoxia could be due to L-NAME affecting nitric oxide production in smooth muscle as well as in the endothelium.

If all 3 peptides were acting on a single receptor type to cause vasoconstriction, then it may be seen that modulation by the endothelium affected a full agonist, sarafotoxin S6b, as well as a partial agonist, endothelin-1. As expected when the actions of a pressor agonist are opposed by those of a vasodilator (in this case EDRF), endothelial destruction increased the potency of sarafotoxin S6b whereas for endothelin-1 there was at least a doubling in the maximal response (although it is still a partial agonist relative to sarafotoxin S6b after removal of the endothelium).

Endothelin-3 presents a slightly different pattern. It appears to be a partial agonist relative to sarafotoxin S6b in the presence of the endothelium but is a full agonist after endothelial cell destruction in both hypoxic and oxygenated tissues. This is the result that would be expected if it were only just a full agonist in this preparation. However, it was less potent in endothelium-denuded tissues than in the corresponding intact preparations regardless of the O_2 tension in the perfusate, an effect observed previously in normally oxygenated tissues (Douglas & Hiley, 1990). This decrease in the sensitivity of smooth muscle to endothelin-3 cannot be simply explained by the removal of an opposing vasodilator factor and shows that the endothelium might also modulate the binding or activation properties of the receptor, or both. Nevertheless, changing the dissolved O_2 tension does not modify the effects of removing the endothelium.

In oxygenated conditions, the pressor actions of endothelin-1 are not modulated by the release of eicosanoids in either the isolated mesentery or the *in situ* blood perfused hindquarters of the rat (Randall *et al.*, 1989). In contrast, the vasoconstrictor actions of endothelin-1 were sensitive to the presence or absence of indomethacin in the *in situ* blood-perfused mesentery and the present study suggests that eicosanoids may modify the actions of endothelin-1 in the isolated mesentery when O_2 tension is reduced; omission of indomethacin from the perfusate increased the R_{max} for this peptide whilst, at the same time, its potency was reduced. Endothelin-1 releases vasoconstrictor eicosanoids from the vasculature; one or other of prostaglandin H_2 (PGH_2), $PGF_{2\alpha}$, thromboxane A_2 (TXA_2) and TXB_2 have been implicated in mediating endothelin-1 induced contractions in a variety of vascular beds (Shirahase *et al.*, 1990; Armstead *et al.*, 1990; Reynolds & Mok, 1990). It is possible that, in oxygenated tissues, the effects of vasoconstrictor arachidonate metabolites are counterbalanced by the release of vasodilator eicosanoids such as PGI_2 or PGE_2 from the mesenteric vessels (Rakugi *et al.*, 1989; Tabuchi *et al.*, 1989). Other vasoactive agents might be released in response to hypoxia; for example, human umbilical vein endothelial cells respond to changes in O_2 tension by modulating expression of the mRNA for platelet-derived growth factor (Kourembanas *et al.*, 1990). Also, in bovine aortic endothelial cells, prostacyclin synthase activity is enhanced during short periods (30 min) of hypoxia but prolonged periods (5 h) of severely reduced O_2 levels significantly reduce its activity (Modat *et al.*, 1990); thus vasoconstrictor eicosanoids may only become important in contributing to the pressor actions of endothelin-1 in the mesentery when the partial pressure of O_2 in the perfusate is reduced and PGI_2 can no longer counteract the actions of these vasoconstrictors.

In conclusion, the results indicate that the effects of hypoxia, although generally consisting of increased maximal responses to the endothelin/sarafotoxin peptides, cannot be explained simply in terms of changes of endothelin/sarafotoxin receptor numbers. The changes in potency of sarafotoxin S6b and endothelin-3 show that alterations in receptor binding or activation properties, or both, also occurred. Finally, it appears that even during periods of reduced O_2 tension, all five of the agonists studied have their pressor activities modulated by the release of EDRF/nitric oxide from the mesenteric vasculature.

S.A.D. is a Medical Research Council Research Student. We are grateful to the Marion Merrell Dow Research Institute, Strasbourg for generous gifts of some of the peptides used in the study. This project was supported by a project grant from the Medical Research Council.

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(Received November 2, 1990
Revised February 11, 1991
Accepted February 12, 1991)

Br. J. Pharmacol. (1991), **103**, 1449–1452 © Macmillan Press Ltd, 1991

Prejunctional modulatory action of neuropeptide Y on responses due to antidromic activation of peripheral terminals of capsaicin-sensitive sensory nerves in the isolated guinea-pig ileum

Miyako Takaki & Sosogu Nakayama

Department of Physiology, Okayama University Medical School, Okayama 700, Japan

1 The effect of neuropeptide Y (NPY) on motor responses produced by activation of capsaicin-sensitive primary afferents in the guinea-pig isolated ileum was determined by use of capsaicin itself and electrical mesenteric nerve stimulation as stimuli.

2 NPY inhibited or suppressed the cholinergic contractile response produced by electrical mesenteric nerve stimulation while leaving the contractile response to a threshold concentration of capsaicin.

3 NPY had no effect on motor responses produced by a submaximal concentration of substance P, the putative endogenous mediator of the 'efferent' function of sensory fibres in this preparation.

4 It is concluded that NPY exerted a prejunctional inhibitory action on transmitter release from peripheral endings of capsaicin-sensitive nerves at interneuronal synapses.

Keywords: Neuropeptide Y; capsaicin; sensory nerves; antidromic activation; ileal motility

Introduction

Capsaicin-sensitive primary sensory neurones synthesize several neuropeptides, e.g. tachykinins and calcitonin gene-related peptide (CGRP). The latter are transported to both central and peripheral nerve endings at which level the peptides are released to produce their sensory and 'efferent' function, respectively (Szolcsanyi, 1984; Maggi & Meli, 1988; Holzer, 1988). Pharmacological evidence indicates that these sensory nerve endings are endowed with various receptors for transmitters and autacoids (e.g. opioids, γ-aminobutyric acid (GABA), and prostaglandins), the activation of which produces changes in excitability, thereby regulating the amount of released transmitter (Szolcsanyi & Bartho, 1978; Yaksh *et al.*, 1980; Grbovic & Radmanovic, 1983; Takaki *et al.*, 1986; Bartho *et al.*, 1987; Go & Yaksh, 1987; Saria *et al.*, 1988a,b; Maggi *et al.*, 1988a,b; 1989a; Giuliani *et al.*, 1989a,b). These modulatory influences can also be observed in peripheral tissues, indicating that the 'efferent' function of these primary sensory neurones can be regulated by other components of the autonomic nervous system. Neuropeptide Y (NPY) is a 36 amino acid peptide, widely distributed in the mammalian peripheral nervous system; it has been shown to be present in sympathetic nerves where it is co-localized with noradrenaline and released by electrical nerve stimulation (Lundberg *et al.*, 1982; 1983; 1984; Fried *et al.*, 1985; Stjarne *et al.*, 1986). A dense plexus of nerve fibres containing NPY-like immunoreactivity is present in the heart, respiratory and digestive tracts of various species (Furness *et al.*, 1983; Lundberg *et al.*, 1983; Sundler *et al.*, 1983; Uddman *et al.*, 1984; Sternini & Brecha, 1985; Potter, 1988). In addition to sympathetic NPY fibres of extramural origin, the gastrointestinal tract also contains an abundance of nerve cells and fibres containing immunoreactive NPY-like material that are devoid of noradrenaline and originate in intramural ganglia (Furness *et al.*, 1983; Sundler *et al.*, 1983). Among other effects, NPY has been shown to exert a prejunctional inhibitory influence on transmitter release from both cholinergic and noradrenergic nerves in the guinea-pig heart (Lundberg *et al.*, 1984; Potter, 1987). Recently, a prejunctional modulatory action of NPY on peripheral terminals of capsaicin-sensitive sensory nerves in the guinea-pig isolated left atria, bronchi and trachea has been reported (Giuliani *et al.*, 1989b; Grundemar *et al.*, 1990).

Here we present data indicating that NPY exerts a potent inhibitory action on motor responses produced in the guinea-pig ileum when the sensory fibres of the mesenteric nerve are activated by electrical antidromic stimulation but not by capsaicin. The present data suggest that the intrinsic neurones of the intestine could influence the excitability of extrinsic sensory nerves, at least with regard to their 'efferent' function.

Methods

Adult guinea-pigs of either sex were stunned and exsanguinated. The ileum, except the terminal 20 cm, was used for experiments. A 2.5 cm length of ileum with (or without) the attached mesenteric vessels and nerves was excised and suspended under a tension of 0.5 g in an organ bath containing 15 ml of Krebs solution at 36°C, and aerated with 5% CO_2 in O_2. The composition of the modified Krebs solution was (mM): NaCl 120.9, KCl 5.9, $NaHCO_3$ 14.4, NaH_2PO_4 1.1, $CaCl_2$ 1.9, $MgCl_2$ 1.2 and glucose 11.5. The preparations were left to settle in the organ bath for 1.5–2 h. The tissues were treated with guanethidine (2 μM) and hexamethonium (50–200 μM) before and during the experiments. The mesenteric vessels and nerves were placed between a pair of annular platinum electrodes for mesenteric nerve (MN) stimulation. Field stimulation was effected via two platinum ring-electrodes placed around the segment. The MN stimulation parameters were: 20 Hz, 0.5 ms, supramaximal currents; stimulation was given for 30 s at 5.5 min intervals. The continuous field stimulation parameters through the experiment were: 0.1 Hz, 0.5 ms, supramaximal currents. The mechanical activity of the longitudinal muscle was recorded through an isotonic transducer.

The drugs used were: atropine sulphate (Dainippon Seiyaku), capsaicin (Sigma), guanethidine sulphate (Tokyo-Kasei), hexamethonium bromide (Sigma), neuropeptide Y (Human) (NPY; Peptide Institute), substance P (Peptide Institute). Capsaicin (10 mM) was dissolved in 100% dimethylsulphoxide (DMSO); when used, this stock solution was diluted one-hundred fold with distilled water. DMSO at 0.1% had no effect of its own. An available vial of neuropeptide Y contains 0.02 μmol of NPY. Thus, 0.1 μM NPY was the maximal concentration available for the present experiments.

All values are presented as the means ± s.e.mean. The statistical significance of differences between means was estimated with Student's t test for unpaired data. A P value of <0.05 was considered statistically significant.

Results

NPY (0.1 μM) produced either a small, transient relaxation or had no effect on spontaneous ileal motility (Figures 1a and 2a). In the presence of guanethidine (2 μM) and hexamethonium (50–200 μM), supramaximal mesenteric nerve (MN) stimulation (20 Hz, 0.5 ms) consistently caused a contraction, i.e., a capsaicin-sensitive contractile response, which was irreversibly abolished by capsaicin (1 μM) as shown in Figures 1b and 2b. This MN-evoked response has been characterized previously as being dependent upon activation of capsaicin-sensitive sensory nerves which release a tachykinin-like transmitter responsible for the cholinergic contractile effect (Szolcsanyi & Bartho, 1978; Takaki *et al.*, 1987). In the present study, it was reconfirmed that the contractions evoked by MN stimulation were abolished by atropine (1 μM). Contractions evoked by MN stimulation were abolished in the presence of high magnesium (12.5 mM):low calcium (1.25 mM), as shown in Figure 3a ($n = 7$), or high magnesium (12.5 mM): very low calcium (0.25 mM) ($n = 5$) Krebs solution. In normal medium, as shown in Figure 3b, the first application of capsaicin (1 μM) caused a strong contraction, which is known to be due to activation of capsaicin-sensitive sensory nerves that release a tachykinin-like transmitter, such as substance P (Holzer, 1988), and irreversibly abolished the contractile response to MN stimulation. No contraction was elicited by a second application of capsaicin (1 μM) ($n = 7$); this event is thought to be a specific marker for the action of capsaicin on sensory nerves (Maggi & Meli, 1988). Capsaicin caused only a small contraction in the presence of high magnesium (12.5 mM):low calcium (1.25 mM) medium (Figure 3a) and did not cause any contraction in the presence of high magnesium (12.5 mM): very low calcium (0.25 mM) medium for 60 min, respectively. However, in both cases, after replacement with the normal medium, a second application of capsaicin had no effect. An example is shown in Figure 3a. In contrast, after treatment of the high magnesium (12.5 mM):low (1.25 mM) or very low calcium (0.25 mM) medium (without capsaicin) for 60 min, a first application of capsaicin (1 μM) caused a strong contraction after replacement with the normal medium ($n = 4$). Thus, a first application of capsaicin was still effective in these high magnesium:low or very low calcium medium.

NPY (0.01 μM) did not affect the capsaicin-sensitive contractile response to MN stimulation. The response was inhibited to 69.6 ± 5.9% ($n = 8$) at 0.05 μM and at 0.1 μM was inhibited to 59.9 ± 3.6% ($n = 7$) of controls (Figure 4), 3–18 min after application of NPY. Sufficient NPY was not available to use concentrations higher than 0.1 μM in the present experiments. However, no significant difference between effects of 0.05 μM and 0.1 μM was observed. Thus, 0.1 μM NPY seems to be close to the maximal effective dose. A typical example is shown in Figure 1a. During the presence of NPY for 30 min, the inhibition of the response to MN stimulation persisted; a decline in the effects of NPY to 90% and 82.5% of the maximal effect was observed at least 11 and 14 min after an application in 2 out of 7 cases. The inhibitory effect of NPY was fully reversible 30 min later, after thorough washing of the preparation (Figure 2a).

On the other hand, NPY (0.1 μM) caused a transient slight but statistically significant inhibition (13.3 ± 4.8%, $n = 4$, after 1 min) on the twitch responses induced by continuous field stimulation (0.1 Hz, 0.5 ms), but after 5 min the responses recovered completely (Figure 5). These twitch responses were

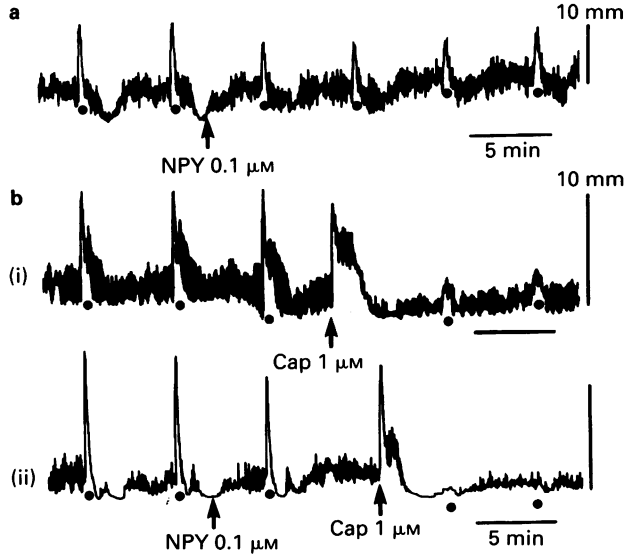

Figure 1 A typical tracing showing the inhibitory effect of neuropeptide Y (NPY, 0.1 μM; at the arrow) on the cholinergic contractile response to mesenteric nerve (MN) stimulation (at the dots) in the guinea-pig isolated ileum (guanethidine pretreated, hexamethonium 50 μM in the bath) in (a). In (b), neuropeptide Y (NPY 0.1 μM; at the first arrow) did not reduce the contractile response induced by capsaicin *per se* (Cap, 1 μM; at the second arrow) in (ii) to compare with a control response in (i) (guanethidine pretreated, hexamethonium 200 μM in the bath); (i) and (ii) were from different preparations of the same animal. Capsaicin abolished the response to MN stimulation (at the dots) as described previously (Szolcsanyi & Bartho, 1978; Takaki *et al.*, 1987).

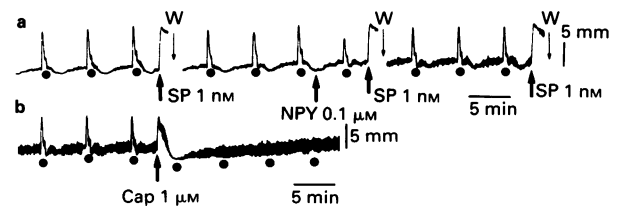

Figure 2 A typical tracing showing no inhibitory effect of neuropeptide Y (NPY, 0.1 μM, at the second arrow) on the contractile response induced by exogenous substance P (SP; 1 nM, at the first, third and fourth arrow) in (a). In the middle trace, after confirming the inhibitory effect of NPY on the contractile response to mesenteric nerve (MN) stimulation (at the dots), SP was applied. In (b), capsaicin (Cap, 1 μM) abolished the contractile response to MN stimulation; (a) and (b) were from the same preparation. W, washing for 30 min.

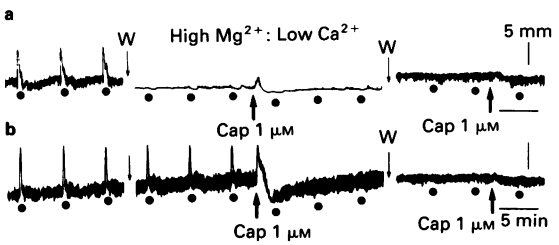

Figure 3 Effect of high magnesium (12.5 mM):low calcium (1.25 mM) Krebs solution on the capsaicin-sensitive contractile response to MN stimulation (at the dots) and the contractile effects of capsaicin *per se*. In (a), the left trace showing the control response to MN stimulation, the middle trace showing that the responses to MN stimulation were abolished in the presence of high magnesium:low calcium medium for 60 min, but a small contraction was produced by a first application of capsaicin (Cap, 1 μM; at the arrow), and the right trace showing that the responses to MN stimulation did not recover after 1 h washing with normal Krebs solution and a second application of capsaicin (Cap, 1 μM; at the arrow) did not cause any effect. In (b), in normal Krebs solution, a first application of capsaicin (Cap, 1 μM; at the arrow) caused a strong contraction and irreversibly abolished the responses to MN stimulation and a second application of capsaicin (Cap, 1 μM; at the arrow) did not cause any effect after 1 h washing. W, washing.

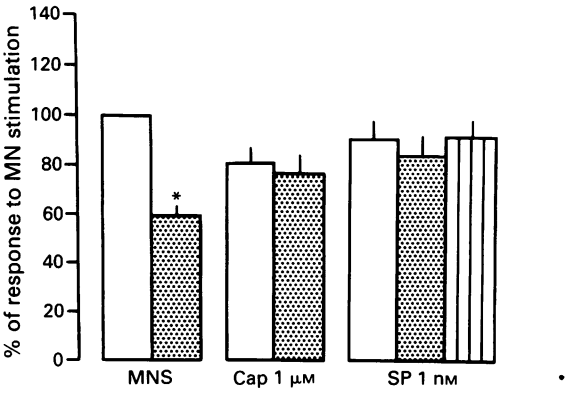

Figure 4 Comparison of the capsaicin-sensitive contractile response to mesenteric nerve stimulation (MNS, 20 Hz, 0.5 ms, supramaximal currents for 30 s), capsaicin (Cap) or exogenously administered substance P (SP) in the guinea-pig isolated ileum (guanethidine pretreated, hexamethonium 50–200 μM throughout). Open columns, control; stippled columns, 3–18 min after addition of neuropeptide Y (0.1 μM); striped column, after thorough washing for 30 min. Significantly different from controls, * $P < 0.001$.

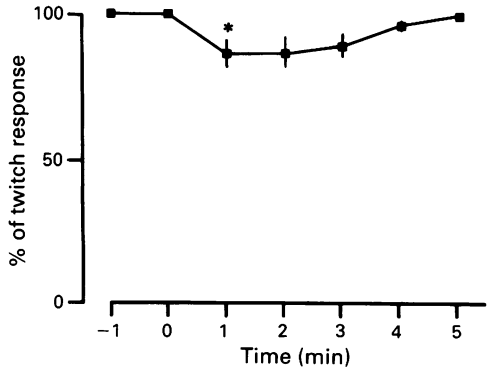

Figure 5 Effect of neuropeptide Y (NPY) on the twitch responses to continuous electrical field stimulation at 0.1 Hz. NPY (0.1 μM) was applied at 0 min. Points indicate mean values of 4 determinations; vertical line indicates s.e.mean.
Significantly different from the pre-drug responses, * $P < 0.05$.

unaffected by hexamethonium (50–200 μM) but abolished by atropine (1 μM).

In contrast, NPY (0.05 μM and 0.1 μM) had no inhibitory effect on contractile responses due to activation of capsaicin-sensitive sensory nerves by application of capsaicin (1 μM, $n = 6$) as shown in Figure 1b, or contractile responses to exogenous substance P (1 nM, $n = 7$) as shown in Figure 2, at concentrations matched to produce a contractile response similar to that produced by MN stimulation (Figure 4). The concentration of capsaicin was just above threshold and at concentrations higher than 3 μM, capsaicin exerted a non-specific action on the smooth muscle. The concentration of substance P, which caused about 45% of the amplitude of the maximal contraction, was submaximal (1% of the maximal effective concentration). At lower concentrations than 1 nM, substance P did not produce contractions of constant amplitude. Under the present experimental conditions, maximal contractile effects were obtained with 10 μM capsaicin, which included a non-specific action on the intestinal muscles, and 0.1 μM substance P.

Discussion

Under appropriate experimental conditions, it is possible to elicit cholinergic motor responses produced by antidromic activation of capsaicin-sensitive afferents in the guinea-pig isolated ileum (Szolcsanyi & Bartho, 1978; Takaki *et al.*, 1987). These responses are highly reproducible and appear particularly suitable for studying the modulatory influence of substances on the excitability of the sensory nerves. The present findings indicate (i) that NPY exerted a potent inhibitory effect on motor responses produced when the capsaicin-sensitive sensory nerves were antidromically activated by electrical MN stimulation but not when they were activated by capsaicin and (ii) that NPY inhibited just transiently the twitch contractions mediated via intrinsic cholinergic motor neurones which were activated by electrical field stimulation.

As NPY did not affect responses to substance P, the putative endogenous mediator of the contractile responses produced by activation of capsaicin-sensitive nerves, present data strongly suggest a prejunctional action of NPY. This conclusion is consistent with the well known neuromodulatory action of NPY on autonomic nerves (Lundberg *et al.*, 1984; Wahlestedt *et al.*, 1986; Potter, 1988) and capsaicin-sensitive sensory nerves in the guinea-pig isolated left atria, bronchi and trachea (Giuliani *et al.*, 1989b; Grundemar *et al.*, 1990). It has been previously shown by Maggi *et al.* (1988b,c; 1989a) that the activation of peripheral endings of capsaicin-sensitive sensory nerves by electrical field stimulation (antidromic stimulation) and capsaicin differ in their sensitivity to a potent blocker of N- and L-type voltage-sensitive calcium channels, ω-conotoxin fraction GVIA. In particular, ω-conotoxin suppressed the responses produced by electrical field stimulation while leaving the responses to capsaicin unaffected. In the present study, high magnesium (12.5 mM):low (1.25 mM) or very low calcium (0.25 mM) medium, which is known to block voltage-sensitive calcium channels and inhibit transmitter release, abolished the contractile response (capsaicin-sensitive) to MN stimulation but did not block the specific effect of capsaicin on sensory nerves. These findings support the proposal by Giuliani *et al.* (1989a,b) that the responses produced by these two stimuli are dependent on electrical antidromic stimulation and independent of (capsaicin) activation of ω-conotoxin-sensitive voltage-dependent calcium channels. A selective inhibition of sensory nerve excitability when they are activated by electrical field stimulation but not when they are activated by capsaicin has been reported previously for opioids (Bartho *et al.*, 1987) and for the neuropeptide, galanin (Giuliani *et al.*, 1989a), and for NPY (Giuliani *et al.*, 1989b; Grundemar *et al.*, 1990) in the guinea-pig bronchi, trachea, ear and left atria. This may reflect a peculiar mode of action of capsaicin which is actually capable of concentrating extracellular calcium within sensory neurones (Wood *et al.*, 1988) in such a way that even micromolar calcium concentrations in the medium are sufficient to sustain a full release of sensory neuropeptides from peripheral endings of capsaicin-sensitive nerves (Maggi *et al.*, 1988d; 1989b).

NPY has been shown to inhibit calcium currents in rat dorsal root ganglia and abolish the depolarization-coupled (high K medium) release of substance P (Ewald *et al.*, 1988; Walker *et al.*, 1988). As NPY selectively inhibits the responses produced via the ω-conotoxin-sensitive mode of activation of sensory fibres (Maggi *et al.*, 1988b), the present findings agree with those of Walker *et al.* (1988) suggesting inhibition of voltage-sensitive calcium channels as a likely mechanism for the prejunctional inhibitory action of NPY on excitability of primary sensory neurones.

The NPY containing nerve terminals found elsewhere in the gut wall are those of intrinsic non-noradrenergic neurones (Furness *et al.*, 1983; Sundler *et al.*, 1983). In addition to capsaicin-sensitive sensory nerves, vagal parasympathetic fibres were found to be contained in the mesenteric nerves (Hirst & McKirdy, 1974) which when stimulated, can release NPY from intramural non-noradrenergic neurones. The released NPY could act in turn on sensory nerve endings at interneuronal synapses within the myenteric plexus.

The present findings suggest a possible mode of interaction between enteric and sensory nerves at interneuronal synapses, that is, a form of prejunctional inhibition of sensory nerve excitability by NPY.

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(Received May 15, 1990
Revised December 10, 1990
Accepted February 13, 1991)

Br. J. Pharmacol. (1991), **103**, 1453–1457 © Macmillan Press Ltd, 1991

Effects of propafenone on ^{45}Ca movements and contractile responses in vascular smooth muscle

Rosalía Carrón, Francisco Pérez-Vizcaino, Eva Delpón & [1]Juan Tamargo

Department of Pharmacology, School of Medicine, Universidad Complutense, 28040-Madrid, Spain

1 In rat isolated aorta the class Ic antiarrhythmic drug, propafenone, dose-dependently inhibited the contractile responses induced by high K (80 mM) and noradrenaline (NA, 10^{-5} M), the IC_{50}s being $2.5 \pm 0.7 \times 10^{-6}$ M and $8.7 \pm 0.8 \times 10^{-6}$ M, respectively. These inhibitory actions were also observed with propafenone added after the induced contractions.

2 Contractile responses induced by addition of Ca to 0 Ca high-K solution were also inhibited by propafenone ($IC_{50} = 2.5 \pm 0.8 \times 10^{-6}$ M). Moreover, propafenone inhibited the contractile responses elicited by NA in strips incubated in 0 Ca ($IC_{50} = 1.9 \pm 0.9 \times 10^{-6}$ M).

3 Propafenone also inhibited ($IC_{50} = 1.2 \pm 0.4 \times 10^{-5}$ M) the development of spontaneous mechanical activity in portal vein segments.

4 Propafenone, 5×10^{-6} M and 10^{-5} M, inhibited ^{45}Ca uptake stimulated by high K or NA without altering ^{45}Ca uptake in resting strips.

5 These results indicated that in rat isolated aortae and portal veins propafenone inhibited Ca entry through voltage-operated channels and NA-induced Ca uptake as well as Ca release from intracellular stores. As a consequence it would reduce the concentration of intracellular free Ca available at the contractile apparatus for vascular smooth muscle contraction.

Keywords: Propafenone; rat aorta; ^{45}Ca movements

Introduction

Propafenone hydrochloride is a class Ic antiarrhythmic drug effective both in experimental and clinical ventricular and supraventricular tachyarrhythmias (Seipel & Breidhardt, 1980; Connolly *et al.*, 1983; Harron & Brogden, 1987; Somberg *et al.*, 1988; Funk-Brentano *et al.*, 1990). In isolated cardiac ventricular muscle propafenone exhibited a high affinity for the inactivated and activated states of the Na channel and dissociated very slowly from the Na channel receptor site (Kohlhardt & Seifert, 1983; Kohlhardt, 1984; Valenzuela *et al.*, 1989). Moreover, propafenone also inhibited the slow contractions and action potentials elicited by isoprenaline or histamine in ventricular muscle fibres partially depolarized with high K (Ledda *et al.*, 1981; Dukes & Vaughan Williams, 1984; Tamargo & Delgado, 1985; Delgado *et al.*, 1985). These results suggest that propafenone may also inhibit the slow inward Ca current in cardiac tissues. In fact, in voltage-clamp experiments it reduced the slow inward Ca current of the rabbit sino-atrial node (Satoh & Hashimoto, 1984) and cat ventricular muscle fibres (Kohlhardt, 1977).

In addition, propafenone inhibited contractile responses induced by high K depolarization in bovine coronary arteries (Hapke & Prigge, 1976) and rat aorta (Philipsborn *et al.*, 1984), produced a relaxant effect in tracheal muscle strips and guinea-pig isolated colon (Hapke & Prigge, 1976; Kukovetz *et al.*, 1977) and inhibited the tetraethylammonium (TEA)-induced action potentials in vascular smooth muscle of canine coronary arteries (Harder & Bellardinelli, 1980). These results may be explained through an inhibition of Ca entry into vascular and non-vascular smooth muscle fibres. However, the possibility that the calcium antagonist properties of propafenone may be responsible for the relaxation of vascular and non-vascular smooth muscle has not been studied yet (Funk-Brentano *et al.*, 1990). Therefore, the present study was undertaken to examine the effects of propafenone on contractile responses and ^{45}Ca uptake in rat isolated aortic strips and portal veins.

[1] Author for correspondence.

Methods

Experimental procedure

Sprague-Dawley rats of either sex (200–250 g) were killed by a blow on the head. The thoracic aorta were dissected and placed immediately in a modified Krebs-Henseleit solution (KS) of the following composition (mM): NaCl 118, KCl 4.75, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 1.8, KH_2PO_4 1.2 and glucose 11. After excess of fat and connective tissue were removed, the aortae were cut into helical strips of about 2.0 mm width and 20 mm in length. Aortic strips and vein segments were mounted vertically under 1 g of tension in organ baths containing 25 ml of KS as previously described (Barrigón & Tamargo, 1986). The tissue bath was maintained at 34°C and bubbled with 95% O_2–5% CO_2 gas mixture. Contractile responses were recorded isometrically through a force-displacement transducer (Grass FT03) connected to a Grass polygraph (Tamargo & Tejerina, 1989). Each preparation was allowed to equilibrate for at least 2 h before the start of experiments and for this period the incubation medium was changed every 15 min.

After equilibration the following experiments were performed: (1) In some experiments aortic strips were exposed to single submaximal concentrations of KCl (80 mM) and noradrenaline (NA, 10^{-5} M). An initial 25 min control contraction was obtained in each experiment with the appropriate stimulatory agent and strips were then rested for a minimum of 30 min. Control contractile responses for each agonist were obtained at the beginning of the experiment until two successive responses were almost identical in height. This was followed by exposure to propafenone for a 30 min period in both cases before addition of KCl or NA and during the 25 min re-exposure to stimulatory agents. Only one agonist was used

in each experiment. The results of these experiments are expressed as a percentage of the maximal control agonist-induced contractile responses. (2) To determine whether propafenone could relax an existing contraction, aortic strips were contracted by single submaximal concentrations of NA or KCl. When the contractile response to either agonist was maximum, propafenone was added in progressively increasing cumulative concentrations (10^{-7} M–10^{-4} M). Strips were allowed to reach a new steady-state tension before each successive concentration of the relaxant drug. The results are expressed as a percentage of the maximal control agonist-induced responses. (3) In additional experiments dose-response curves to $CaCl_2$ (1–5 mM) were obtained in aortic strips incubated in Ca-free KS containing 2 mM EGTA for 2 h and then in Ca-free high-K (80 mM) solution for 5 min before addition of drugs (Barrigón et al., 1984). The results are expressed as a percentage of the maximal contractile response induced by 5 mM $CaCl_2$. (4) To study the effects of propafenone on a membrane store of Ca mobilized by NA, the experimental protocol employed was similar to that previously described by Ebeigbe & Aloamaka (1985). Strips incubated in Ca-free KS were stimulated by successive additions of 10^{-5} M NA every 30 min ('Ca-washing'). When the contractile response to NA was suppressed the strips were exposed for 5 min to 2.7 mM Ca KS (Ca-loading') and then exposed to Ca-washing KS for 2–3 min, followed by 10^{-5} M NA stimulation. The above protocol was repeated in the presence of propafenone added at the end of the 'Ca-loading' period. Control experiments were performed using the same four experimental protocols but omitting propafenone.

Measurements of ^{45}Ca movements

Techniques employed to estimate ^{45}Ca movements in aortic strips were as previously described by Godfraind (1983) and Barrigón et al. (1984). To determine ^{45}Ca uptake, aortic strips were bisected and in each experiment half of the aorta served as the control and the other half as the experimental strip. Both halves were equilibrated for 1 h under 1 g of tension in KS of the following composition (mM): NaCl 122, KCl 5, $NaHCO_3$ 25, $CaCl_2$ 1.25, $MgSO_4$ 1.2 and glucose 11.5. This solution was adjusted to pH 7.4 and gassed with 95% O_2-5% CO_2. After equilibration, experimental strips were treated with propafenone. Then both experimental and control strips were incubated for 5 min in KS containing ^{45}Ca (specific activity 2 μCi ml^{-1}; Amersham International, Buckinghamshire) either containing or not containing propafenone and then for another 5 min in the same solution with or without the addition of 80 mM KCl or 10^{-5} M NA. At this time strips were washed for 5 min at 0°C to remove extracellular bound Ca in a lanthanum solution (mM): NaCl 122, KCl 5.9, $MgCl_2$ 1.25, glucose 11, $LaCl_3$ 50 and Tris maleate 15 (pH 6.8). Afterwards strips were removed, blotted and weighed. The strips were then placed in scintillation vials and 0.5 ml of Protosol (Dupont) added and the strips digested overnight at 60°C. Radioactivity was assayed in a liquid scintillation counter (LKB Wallac 1211 Rack) as previously described (Barrigón & Tamargo, 1986).

Drugs

The following drugs were used: propafenone (Knoll-Made, S.A.) and noradrenaline bitartrate (Sigma, London). Propafenone as a powder was initially dissolved in distilled deionized water and further dilutions were made in KS. Ascorbic acid (10^{-4} M) was added to each solution of NA, made up freshly every day. Throughout the paper values are expressed as the mean ± s.e.mean and statistical analysis was performed with Student's t test. Differences between control and experimental values were considered significant when $P < 0.05$. Dose-response slopes were analyzed to give the concentration of propafenone producing a 50% inhibition of the maximal contractile response (IC_{50}) by linear regression analysis (method of least squares).

Results

Effects on resting tension and spontaneous myogenic activity

At concentrations between 10^{-7} M and 10^{-4} M, propafenone had no effect on base-line tension in aortic strips. However, at this range of concentrations propafenone inhibited the amplitude but not the frequency of the spontaneous myogenic activity in portal veins. Thus, at 10^{-5} M propafenone inhibited the amplitude of the spontaneous contractions by 44.6 ± 9.9% ($n = 4$), whereas at 5×10^{-5} M it completely suppressed the spontaneous activity, the IC_{50} for the inhibition being $1.2 \pm 0.4 \times 10^{-5}$ M.

Effect on contractions induced by KCl and noradrenaline

The inhibitory effect of propafenone on contractile responses induced by KCl (80 mM) and NA (10^{-5} M) were quantified and compared in isolated aortic strips. Both agonists produced submaximal contractile responses of 435.4 ± 79.7 mg and 289.6 ± 44.9, respectively ($n = 8$), these values representing 65.0 ± 5.2% and 95.0 ± 2.0% of the maximal responses induced by 140 mM KCl and 10^{-4} M NA, respectively. In untreated strips the contractile responses induced by successive applications of both stimulatory agents did not change by more than 6.8 ± 3.5%. The effects of propafenone, 10^{-7} M–5×10^{-4} M, on the contractile responses induced by KCl- and NA are shown in Figure 1a. At concentrations $\geq 10^{-6}$ M, propafenone produced a concentration-dependent inhibition of the contractile response induced by both stimulatory agents. However, whereas 10^{-4} M propafenone suppressed the contractile response induced by NA, this concentration left unaltered about 12.0 ± 2.0% of the response induced by high K. This contractile response was completely suppressed by 4×10^{-4} M propafenone. The IC_{50} values for propafenone depression of high-K and NA-induced responses were $2.5 \pm 0.7 \times 10^{-6}$ M and $8.7 \pm 0.8 \times 10^{-6}$ M, respectively, which indicates that propafenone was slightly more potent ($P < 0.05$) in inhibiting the contractile responses induced by high K than those induced by NA. The inhibitory effects of propafenone were reversed by washing with drug-free KS.

Figure 1b shows the relaxant ability of cumulatively increasing concentrations of propafenone on aortic strips previously contracted with either 80 mM KCl or 10^{-5} M NA. In untreated aortic strips contractile responses induced by both stimulatory agents remained stable for more than 3 h. Cumulative increases in the concentration of propafenone (10^{-6} M–10^{-4} M) to strips previously contracted resulted in a dose-dependent relaxation. However, whereas in the presence

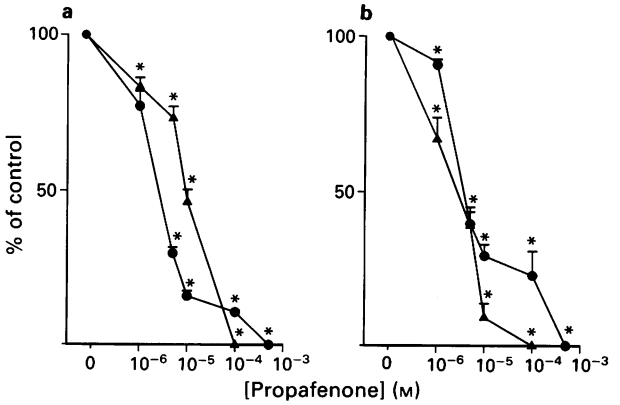

Figure 1 Effect of propafenone, 10^{-6} M–10^{-4} M, on the contractile responses induced by high K (●, 80 mM) and noradrenaline (▲, 10^{-5} M) in rat aortae when added either before (a) or after the induced contractions (b). Ordinate scale: relaxation (% of control values). Abscissa scale: propafenone concentration (M). Each point represents the mean of 8 experiments; vertical bars show s.e.means. The asterisks denote significant differences (* $P < 0.05$) from control values.

of 10^{-4} M propafenone the responses to NA were completely suppressed, this concentration inhibited by 74.5 ± 5.1% the contractions elicited by high K. Thus, the IC_{50}s for inhibition of the contractions induced by high K and NA were significantly different ($6.7 \pm 0.7 \times 10^{-6}$ M and $2.0 \pm 0.6 \times 10^{-6}$ M, respectively. $P < 0.05$).

Effects on Ca-induced contractions

The inhibitory effects of propafenone on the $[Ca]_o$-dependent contractile responses are shown in Figure 2. Following the incubation for 2 h in a Ca-free high-K (80 mM) KS, the addition of Ca to the bathing media (1–5 mM) caused a concentration-dependent contraction of the aortic strips. The maximum contractile response induced by 5 mM $CaCl_2$ was 210.3 ± 45.9 mg, not significantly different from that induced by 10^{-5} M NA (289.6 ± 44.9. $n = 8$). Propafenone, 10^{-6} M and 5×10^{-6} M not only produced a progressive decrease in the amplitude of Ca-induced contractions of K-depolarized aortic strips but also caused a progressive shift of Ca concentration-response curves downward and to the right. Regression analysis of the inhibition yielded an IC_{50} of $2.5 \pm 0.9 \times 10^{-6}$ M.

In another group of experiments the effects of propafenone were studied on the contractile responses induced by NA in strips which were incubated in Ca-free KS, then in 'Ca-loading' KS for 5 min and finally in Ca-free KS for 2–3 min. At this time stimulation with NA (10^{-5} M) resulted in a phasic contraction of an amplitude of 131.0 ± 38.0 mg ($n = 6$), presumed to reflect Ca mobilization from a membrane-bound store (Karaki *et al.*, 1979). In 6 experiments propafenone, 10^{-6} M and 5×10^{-6} M, when applied after 'Ca-loading' inhibited by 29.7 ± 3.3% ($P < 0.001$) and 81.0 ± 1.7%, ($P < 0.001$), respectively, the magnitude of the NA-induced phasic contraction, the IC_{50} value being $1.9 \pm 0.9 \times 10^{-6}$ M.

Effects on ^{45}Ca uptake

From the previous experiments two concentrations of propafenone (5×10^{-6} M and 10^{-5} M) were selected to study the effects of the drug on the La-resistant ^{45}Ca uptake in resting, non-stimulated, as well as in aortic strips stimulated by 80 mM KCl and 10^{-5} M NA. Propafenone, 10^{-5} M, had no significant effects on ^{45}Ca uptake in resting, non-stimulated aortic strips (85.4 ± 3.5 vs. 76.2 ± 5.2 μmol kg^{-1} aorta; $n = 12$; $P > 0.05$). Addition of high K and NA increased ^{45}Ca uptake to 231.6 ± 12.4 ($n = 12$; $P < 0.001$) and 161.6 ± 10.4 μmol kg^{-1} aorta ($n = 12$; $P < 0.001$), respectively. At 5×10^{-6} M and 10^{-5} M propafenone significantly decreased ^{45}Ca uptake stimulated either by KCl to 166.3 ± 10.1 ($n = 12$; $P < 0.05$) and 133.5 ± 11.8 μmol kg^{-1} aorta ($n = 12$; $P < 0.001$) and by NA to 119.0 ± 11.3 ($n = 12$; $P < 0.01$) and 91.2 ± 12.1 μmol kg^{-1} aorta ($n = 12$; $P < 0.001$).

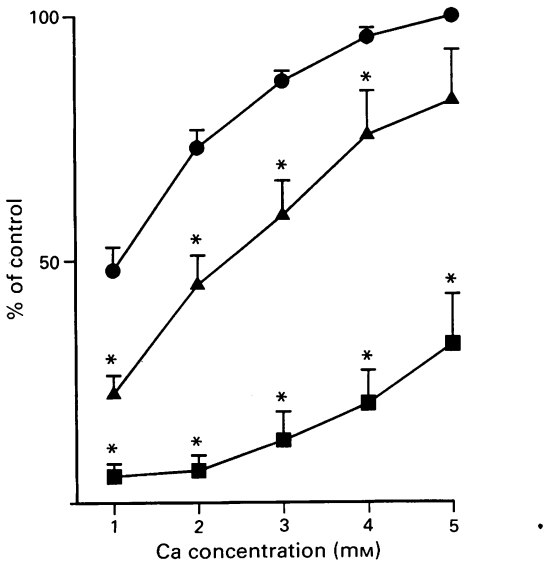

Figure 2 Effects of propafenone on the restoration of the isometric contractions of aortic strips by addition of calcium (1–5 mM) to Ca-free high K (80 mM) medium. Ordinate scale: percentage of the maximum control contractions obtained with the highest concentration of Ca in each experiment. Abscissa scale: calcium concentration (mM). Each point represents the mean of 6 experiments; vertical bars show s.e.means. (●) Controls; (▲) after propafenone, 10^{-6} M and (■) 5×10^{-6} M. The asterisks denote significant differences between control responses and those after propafenone (* $P < 0.05$).

Discussion

The results of this work demonstrated that in rat isolated aortic strips and portal veins propafenone, a class Ic anti-arrhythmic drug, inhibited: (1) the contractile responses induced by high K or by increases of extracellular Ca in K depolarized aortic strips, (2) NA-induced contractions both in a Ca-containing or 0 Ca medium, (3) ^{45}Ca uptake in aortic strips stimulated by high K and NA, and (4) the spontaneous myogenic contractions recorded in portal veins. These results confirmed and extended previous experimental evidence showing that propafenone exhibited spasmolytic effects in vascular (Hapke & Prigge, 1976; Harder & Bellardinelli, 1980; Philipsborn *et al.*, 1984) and non-vascular smooth muscle fibres (Hapke & Prigge, 1976; Kukovetz *et al.*, 1977).

In rat isolated aorta NA- and high K-induced contractions have been resolved into a phasic and a tonic component (Hudgins & Weiss, 1968; Godfraind & Kaba, 1969). An increase in Ca entry through receptor-operated channels (ROCs) seems to determine the tonic component of NA-induced contractions (Hudgins & Weiss, 1968; Deth & Van Breemen, 1974; Bolton, 1979), whereas both contractile responses induced by high K or by increasing Ca concentration in K-depolarized aortae are thought to be entirely dependent upon the influx of extracellular Ca through voltage-sensitive channels (VSCs) (Hudgins & Weiss, 1968; Godfraind & Kaba, 1969; Bolton, 1979). Propafenone inhibited in a concentration-dependent manner the contractile responses induced by high K depolarization or by Ca which have been used to provide a simple means of studying drugs with possible Ca entry blocking properties (Haeusler, 1972; Godfraind *et al.*, 1986) as well as the tonic component of NA-induced responses. Moreover, in the present experiments propafenone decreased high K- as well as NA-induced contractions which indicated that it may inhibit Ca entry both through VSCs and ROCs. Another two groups of experiments seem to agree with this hypothesis. Firstly, propafenone inhibited the spontaneous myogenic activity in rat portal veins, the IC_{50} value being quite similar to that obtained in K-depolarized aortic strips. Since myogenic activity has been attributed to depolarization of the membrane which activated VSCs (Sigurdsson *et al.*, 1975), the inhibitory effect of propafenone may be explained through its ability to decrease Ca entry through VSCs. Secondly, at the same range of concentrations at which propafenone inhibited the amplitude of the tonic component of high K- and NA-induced contractions, it also inhibited ^{45}Ca uptake stimulated by both agonists. In fact, the percentage of inhibition for high K- and NA-induced contractions and ^{45}Ca uptake were almost superimposed, thus suggesting that propafenone-induced inhibition can be related to the blockade of Ca entry through VSCs and ROCs, respectively. However, if at the high concentrations used in this study NA depolarized the membrane potential of aortic smooth muscle fibres, the inhibitory effects of propafenone on NA-induced contractions and ^{45}Ca uptake could be attributed to inhibition of Ca entry through both ROCs and VSCs.

It has been reported that propafenone inhibited Ca entry through VSCs in cardiac muscle (Kohlhardt, 1977; Ledda *et al.*, 1981; Satoh & Hashimoto, 1984; Tamargo & Delgado,

1985; Delgado *et al.*, 1985) and in fact in the present experiments propafenone inhibited Ca entry induced both by NA and high K. This lack of specificity is in contrast to what has been described with Ca antagonists, which in vascular smooth muscle fibres inhibited high K-induced contractions to a much greater extent than NA-induced contractions (Cauvin *et al.*, 1983; Tamargo, 1984; Godfraind *et al.*, 1986). However, like Ca antagonists (Cauvin *et al.*, 1983; Godfraind *et al.*, 1986) propafenone had no effect on ^{45}Ca uptake in resting aortic strips, which suggests that Ca entry through passive membrane leak channels seems to be insensitive to the drug.

Furthermore, propafenone also inhibited the phasic component of NA-induced contractions which, since they persisted in 0 Ca medium have been attributed to the release of Ca from intracellular stores (Hudgins & Weiss, 1968; Godfraind & Kaba, 1969; Deth & Van Breemen, 1977). This finding suggested that propafenone in addition may also inhibit the release of Ca from an agonist releasable intracellular store. These phasic contractions have been attributed to mobilization of Ca from a membrane-bound store (Karaki *et al.*, 1979; Ebeigbe & Alaomaka, 1985). In the present study, propafenone when applied after 'Ca-loading' inhibited NA-induced phasic contractions elicited in Ca-free medium which suggested that the inhibitory effect of propafenone on NA-induced contractions can be at least partly related to a decrease in the release of Ca from an intracellular store. Another possibility is that in addition, propafenone enhances Ca extrusion from vascular smooth muscle fibres, but this possibility was not analyzed in the present experiments.

The clinical relevance of the present results remains to be determined. In fact, propafenone is highly bound to plasma and cardiac proteins (Harron & Brogden, 1987) and is extensively hydroxylated to 5-hydroxypropafenone, a metabolite which presents similar properties to those of propafenone (Philipsborn *et al.*, 1984; Valenzuela *et al.*, 1987; 1988). Furthermore, a reduction in aortic blood pressure is not a constant finding either in experimental animals (Hapke & Prigge, 1976; Karagueuzian *et al.*, 1984) or in clinical studies (Connolly *et al.*, 1983; Harron & Brogden, 1987) even when a slight reduction in mean arterial pressure has also been described in some clinical studies (Bachour & Hochrein, 1977; Seipel & Breidhardt, 1980). A possible explanation for the low incidence of hypotension may be related to the negative inotropic effects of propafenone which may increase systemic vascular resistances, thus overriding the expected direct vasodilator and cardiodepressant effects of the drug. Therefore, caution should be exercised in the extrapolation of the present *in vitro* results to clinical situations.

In conclusion, the present data demonstrated that in rat isolated aortae and portal veins, propafenone inhibited Ca entry through voltage- and receptor-operated channels as well as Ca release from intracellular stores. As a consequence it would reduce the concentration of intracellular free Ca available at the contractile apparatus for vascular smooth muscle contraction.

We thank Knoll-Made for the generous gift of propafenone. This work was supported by a CICYT (PM88-0009) and FIS Grants.

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(Received June 14, 1990
Revised January 14, 1991
Accepted January 18, 1991)

Br. J. Pharmacol. (1991), **103**, 1458–1462 © Macmillan Press Ltd, 1991

IgE-dependent activation of alveolar macrophages augments neurally mediated contraction of small airways

J. Tamaoki, N. Sakai, T. Kanemura, I. Yamawaki & [1]T. Takizawa

Pulmonary Division, First Department of Medicine, Tokyo Women's Medical College, Tokyo 162, Japan

1 We studied the effect of immunologically activated pulmonary alveolar macrophages (PAM) on functions of canine bronchiolar smooth muscle under isometric conditions *in vitro*.

2 PAM, stimulated with monoclonal anti-dinitrophenyl (DNP) IgE antibody and DNP-human serum albumin (DNP-HSA), augmented the contractile responses of bronchioles to electrical field stimulation, whereas PAM treated with IgE antibody alone had no effect.

3 In contrast, the contractile responses to exogenously administered acetylcholine were not influenced by immunologically activated PAM.

4 The PAM-induced increase in the contractile responses to field stimulation was inhibited by pretreatment of PAM with indomethacin and by addition of the thromboxane A_2 (TxA_2) receptor antagonist SQ 29548.

5 The release of TxA_2 from PAM was increased by anti-DNP IgE and DNP-HSA, an effect that was prevented by indomethacin.

6 These results suggest that PAM may play a role in the development of antigen-induced hyperreactivity of small airways through an IgE-dependent release of TxA_2 which potentiates prejunctionally the parasympathetic component of bronchiolar smooth muscle tone.

Keywords: Asthma; allergy; cholinergic nerve; thromboxane A_2; airway hyperreactivity

Introduction

Pulmonary alveolar macrophages (PAM) reside predominantly in the alveoli and small airways on top of epithelial cells, but are also present at the air surface interface of relatively large airways (Brain *et al.*, 1984). In normal human beings as well as patients with asthma, PAM have been shown to constitute more than 90% of the cells recovered from bronchoalveolar lavage and approximately 40% of the cells recovered from lavage of isolated bronchial segments (Eschenbacher & Gravelyn, 1987; Joseph *et al.*, 1983). Because of this strategic location and their pronounced phagocytic capabilities, PAM play an important role not only in the first line of the lung defence mechanism but also in the induction of allergic reactions by encountering inhaled particles and allergens (Fels & Cohn, 1986).

Among several cells contributing individually and in concert to the development and maintenance of airway hyperresponsiveness that is a characteristic feature of asthma (Boushey *et al.*, 1980), interest has recently been focused on PAM as one of the potential candidates for the following reasons: (1) PAM possess functional Fc receptors for binding of IgE (Spiegelberg, 1984); (2) activation of PAM by IgE and specific allergens, or IgE and anti-IgE, elicits the synthesis and release of a wide variety of biochemically different substances (Rankin *et al.*, 1982; Balter *et al.*, 1988); and (3) some of these mediators have a putative role in asthmatic syndromes by modulating humoral and cellular components of airway inflammation (Henderson, 1987).

On the other hand, it has been shown that augmentation of parasympathetic components regulating airway calibre may be involved in airway hyperresponsiveness produced by allergen challenge (Gold *et al.*, 1972; Mitchell *et al.*, 1986). Therefore, we have used canine isolated bronchiolar segments, to determine whether the immunological activation of PAM alters contractility of small airways in response to cholinergic stimulation.

[1] Author for correspondence.

Methods

Preparation of pulmonary alveolar macrophages

Mongrel dogs of either sex (22–31 kg) were anaesthetized with intravenous sodium pentobarbitone (30 mg kg^{-1}) and bronchoalveolar lavage was carried out with 20 × 50 ml cold phosphate-buffered saline. The lavage fluid was filtered through 4 layers of gauze and centrifuged for 20 min at 400 *g*. The cell pellets were washed three times with Ca^{2+}-Mg^{2+}-free Hanks' balanced salt solution. PAM were separated from epithelial cells by density-gradient centrifugation (Shaw & Anderson, 1984) and resuspended in Krebs-Henseleit (KH) solution of the following composition (mM): NaCl 118, KCl 5.9, $CaCl_2$ 2.5, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25.5 and glucose 5.6 to produce a cell count of 5×10^7 ml^{-1}. Approximately 95% of the cells in this preparation were PAM as determined morphologically by Wright-Giemsa staining, and viability was greater than 90% as assessed by Trypan blue exclusion.

Preparation of bronchiolar smooth muscle

After the completion of bronchoalveolar lavage, both lungs were rapidly removed and immersed in oxygenated KH solution. Bronchioles (tenth to thirteenth generation) with an outer diameter of 1.0–2.0 mm were dissected from the lung and cut helically (45°-pitch) to obtain thin bronchiolar strips (2 mm wide and 20 mm long).

Tissue strips were then mounted in glass chambers filled with 4 ml of KH solution maintained at 37°C and at pH 7.4, bubbled constantly with a mixture of 95% O_2:5% CO_2, and were connected to strain gauges (TB-652T; Nihon Kohden, Tokyo, Japan) for continuous recording of isometric tension (pen recorder WT-685G; Nihon Kohden). Each organ bath was fitted with two rectangular platinum electrodes (4 × 30 mm) placed alongside the tissue for transmural electrical field stimulation (biphasic pulse; pulse width, 0.5 ms; supramaximal voltage, 20 V for 20 s). After 2 h of equilibration, changing the bathing solution every 15 min, resting tension was adjusted so that contractile responses to field stimulation

were maximal, from preliminary studies maximal responses occurred at 500 mg resting tension. The β-adrenoceptor antagonist propranolol (10^{-6} M) was present in the chamber throughout the experiments to avoid a β-adrenergic component of neurally mediated contraction of canine airway smooth muscle.

IgE challenge

After incubating suspensions of PAM with affinity-purified mouse monoclonal hybridoma anti-dinitrophenyl (DNP) IgE antibody (final IgE concentration 10 μg ml^{-1}, Sigma, St. Louis, Missouri, U.S.A.) for 20 min at 37°C, PAM (10^7 cells ml^{-1}) were added to organ chambers containing canine bronchiolar smooth muscle, and the chambers were then continuously perfused with DNP-human serum albumin (DNP-HSA, 100 ng ml^{-1}, Sigma). This procedure did not alter resting tension of the tissues.

To assess whether IgE immune complexes affect contractile responses of bronchiolar smooth muscle to field stimulation, we obtained responses at increasing impulse frequencies (0.5 to 50 Hz) before addition of PAM and again at 5 min after starting the DNP-HSA perfusion. Control experiments were carried out to examine the effect of PAM incubated with anti-DNP IgE antibody alone on the responses to field stimulation. Frequency-response relationships were linear and regression analysis used to assess the frequency that produced 50% of the maximal contraction (ES_{50}).

The effect of varying density of PAM was assessed by measuring contractions produced by field stimulation (5 Hz) before and 5 min after addition of PAM (0.3–30 $\times$ 10^6 cells ml^{-1}) and after 5 min of DNP-HSA perfusion. The number of PAM required to produce a half-maximal effect was also determined.

To determine whether the site of action of stimulated PAM on bronchiolar smooth muscle was prejunctional or postjunctional, the effect of IgE challenge on the contractile responses to acetylcholine (10^{-9} to 10^{-3} M) was assessed with the same time sequence for the measurements of the responses to field stimulation. Acetylcholine was added cumulatively either at 5 min intervals or 2 min after a steady-state response was achieved.

The possible contributions of arachidonic acid metabolites released from PAM were investigated by adding the following compounds to PAM suspensions 15 min before anti-DNP IgE: the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA, 10^{-5} M), the cyclo-oxygenase inhibitor indomethacin (3×10^{-6} M) and the thromboxane (Tx) A_2 receptor antagonist (Ogletree *et al.*, 1985) SQ 29548 (10^{-8} to 10^{-5} M). PAM (10^{-7} cells ml^{-1}) treated as described were then stimulated with anti-DNP IgE antibody and DNP-HSA and the effect on the contractile responses of bronchioles to field stimulation (5 Hz) assessed in the usual way.

Release of eicosanoids

Among arachidonic acid metabolites released from PAM, TxA_2 and prostaglandin $(PG)F_{2\alpha}$ are known to potentiate the electrical field stimulation-induced contraction of canine airway (Leff *et al.*, 1985; Tamaoki *et al.*, 1987a). Thus, to determine whether IgE immune complexes stimulated the release of these eicosanoids from PAM, aliquots (100 μl) taken from the medium before and 5 min after IgE challenge were analysed in duplicate for TxB_2, a stable metabolite of TxA_2 and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) by radioimmunoassay (New England Nuclear, Boston, MA, U.S.A.) as described previously (Tamaoki *et al.*, 1987b).

Drugs

The following drugs were used: (±)-propranolol hydrochloride, acetylcholine chloride, NDGA, indomethacin (Sigma), and [1S-[1α,2β(5Z),3β,4α]]-7-[3-[[2-[(phenylamino) carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid (SQ 29548, a gift from The Squibb Institute for Medical Research, Princeton, NJ, U.S.A.).

Statistics

Results are expressed as means ± s.e. Statistical analysis was performed by analysis of variance or the Newman-Keuls multiple comparison test, and a P value of less than 0.05 was considered significant.

Results

PAM stimulated with anti-DNP IgE antibody and DNP-HSA had no effect on the resting tone of canine bronchiolar smooth muscle, but potentiated the contractile responses to field stimulation at all frequencies (Figure 1). Thus, the frequency-response curves were displaced to the left, so that the ES_{50} value decreased from 1.9 ± 0.5 to 0.7 ± 0.2 Hz ($P < 0.01$, $n = 8$). On the other hand, PAM treated with anti-DNP IgE antibody alone did not significantly alter the contractile responses of bronchiolar smooth muscle to field stimulation. The potentiation of bronchiolar contraction was dependent on the number of PAM added, the maximal increase from the baseline response being 34.0 ± 5.5% ($P < 0.001$, $n = 9$) and 50% of this maximum occurred 1.8×10^6 cells ml^{-1} (Figure 2). Addition of IgE antibody alone to PAM at a concentration of up to 3×10^7 cells ml^{-1} was without effect.

To determine the site and mechanism of action of immunologically stimulated PAM in the vagal motor pathway, the effect of PAM on the contractile responses to exogenously

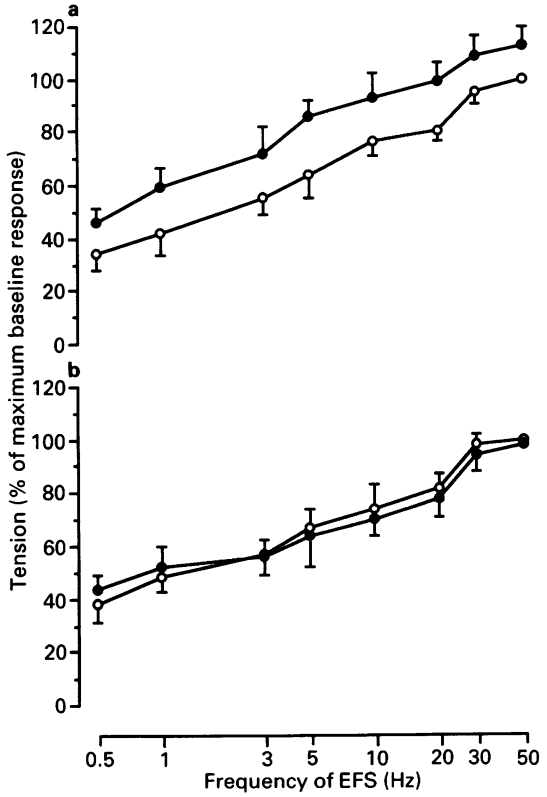

Figure 1 Effect of pulmonary alveolar macrophages (PAM) on contractile responses of canine bronchiolar smooth muscle to electrical field stimulation (EFS) at increasing impulse frequency. After obtaining baseline responses to EFS (○), PAM (10^{-7} cell ml^{-1}) stimulated with monoclonal anti-DNP IgE antibody and then DNP-HSA (PAM + challenge, a) or those incubated with anti-DNP IgE antibody alone (PAM + Ab alone, b) were added, and the measurements were repeated (●). Data are expressed as percentage of maximum baseline responses. Each point represents mean of $n = 8$; vertical bars show s.e.mean.

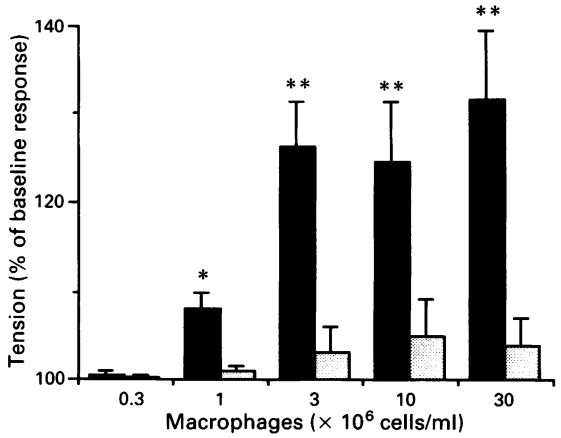

Figure 2 Dose-dependent effect of pulmonary alveolar macrophages stimulated with anti-DNP IgE antibody and then DNP-HSA (solid columns) or those incubated with anti-DNP IgE antibody alone (stippled columns) on contractile responses of bronchioles to electrical field stimulation at 5 Hz. Values are expressed as a percentage of baseline responses obtained before macrophage administration. Data are means of $n = 9$ for each column; vertical bars show s.e.mean. * $P < 0.05$; ** $P < 0.01$, significantly different from baseline responses.

administered acetylcholine was examined. In contrast to the effect on the contractions induced by field stimulation, acetylcholine-induced contractions of bronchioles were not affected by either PAM stimulated with IgE or PAM incubated with IgE antibody alone (Figure 3).

The increase in the contractile responses to PAM stimulated with IgE challenge was not influenced by pretreatment

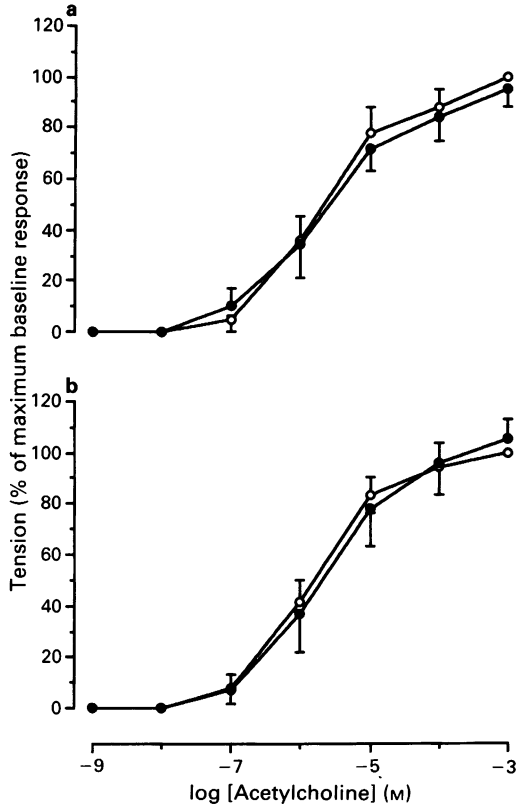

Figure 3 Effect of pulmonary alveolar macrophages (PAM) on contractile responses of canine bronchiolar smooth muscle to acetylcholine. After obtaining baseline responses to acetylcholine (○), PAM (10^7 cells ml^{-1}) stimulated with anti-DNP IgE antibody and then DNP-HSA (PAM + challenge, a) or those incubated with anti-DNP IgE antibody alone (PAM + Ab alone, b) were added, and the measurements were repeated (●). Data are expressed as percentage of maximum baseline responses. Each point represents mean of $n = 8$; vertical bars show s.e.mean.

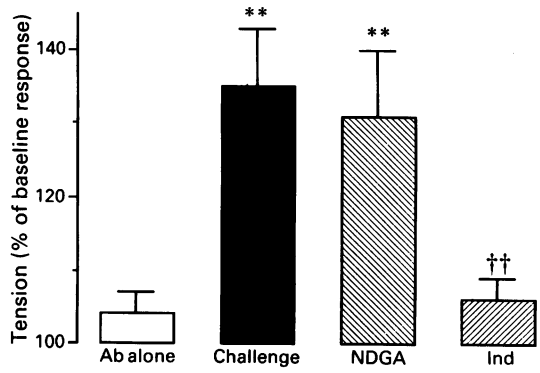

Figure 4 Effect of pretreatment of pulmonary alveolar macrophages (PAM) with 10^{-5} M nordihydroguaiaretic acid (NDGA) or 3×10^{-6} M indomethacin (Ind) on the increase in contractile responses of bronchioles to electrical field stimulation (5 Hz) produced by PAM (10^7 cells ml^{-1}) stimulated with anti-DNP IgE antibody and DNP-HSA (Challenge). Values are expressed as percentage of baseline responses obtained before administration of PAM. Data are means of $n = 7$; vertical bars show s.e.mean. ** $P < 0.01$, significantly different from responses to Ab alone. †† $P < 0.01$, significantly different from values for challenge.

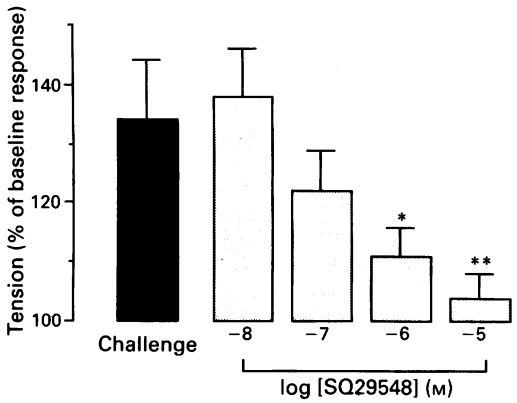

Figure 5 Dose-dependent effect of the thromboxane receptor antagonist SQ 29548 on the increase in contractile responses of bronchioles to electrical field stimulation (5 Hz) produced by pulmonary alveolar macrophages (PAM, 10^7 cells ml^{-1}) stimulated with anti-DNP IgE antibody and DNP-HSA (■). Values are expressed as percentage of baseline responses obtained before administration of PAM. Data are means of $n = 8$ for each column; vertical bars show s.e.mean. * $P < 0.05$; ** $P < 0.01$, significantly different from values for challenge.

of PAM with NDGA but it was greatly inhibited by pretreatment with indomethacin (Figure 4). Moreover, addition of the TxA_2 receptor antagonist SQ 29548 to the organ bath attenuated the effect of stimulated PAM on bronchiolar smooth muscle in a dose-dependent fashion (Figure 5) but did not alter either the resting tension of the responses to field stimulation. The PAM-induced potentiation of the bronchiolar contractions was abolished by SQ 29548 10^{-5} M ($P < 0.01$, $n = 8$).

Incubation of PAM with anti-DNP IgE, followed by incubation with DNP-HSA, increased the release of cyclooxygenase products from 12 ± 9 to 120 ± 44 pg/10^6 cells min^{-1} for $PGF_{2\alpha}$ ($P < 0.01$, $n = 9$) and 36 ± 10 to 460 ± 85 pg/10^6 cells min^{-1} for TxB_2 ($P < 0.001$, $n = 10$). Pretreatment of PAM with indomethacin greatly reduced the stimulated release of these eicosanoids whereas NDGA was without effect (Table 1).

Discussion

Our *in vitro* studies demonstrate that immunologically stimulated PAM potentiate the neurally mediated contraction of small airways in dogs. The exposure of isolated smooth

Table 1 Release of cyclo-oxygenase products from canine alveolar macrophages

	Eicosanoid release ($pg/10^6$ cells min^{-1})				
	PAM alone	*Ab*	*Challenge*	*Challenge + NDGA*	*Challenge + indomethacin*
$PGF_{2\alpha}$	12 ± 9	15 ± 8	120 ± 44**	95 ± 28**	10 ± 8††
TxB_2	36 ± 10	50 ± 12	460 ± 85***	422 ± 94***	22 ± 6†††

Values are means ± s.e. for 8–10 experiments. PAM, pulmonary alveolar macrophages; Ab, IgE antibody; NDGA, nordihydroguaiaretic acid; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; TxB_2, thromboxane B_2.
** $P < 0.01$, *** $P < 0.001$, significantly different from corresponding values for PAM alone.
†† $P < 0.01$, ††† $P < 0.001$, significantly different from corresponding values for IgE-challenge.

muscle from canine bronchioles to PAM incubated with monoclonal anti-DNA IgE antibody and the subsequent application of DNP-HSA caused an increase in the contractile response to field stimulation, whereas PAM incubated with IgE antibody alone had no effect. These findings suggest that the hyperresponsiveness of bronchiolar smooth muscle appears to be associated with release of mediators from PAM induced by IgE immune complexes.

In contrast to the effect on responses to field stimulation, stimulated PAM did not alter the contractile responses to exogenously administered acetylcholine. In the presence of propranolol to prevent the β-adrenergic effect of noradrenaline released from sympathetic nerve endings, transmural electrical stimulation, as performed in this study, elicits contraction of canine airway smooth muscle via stimulation of postganglionic vagal nerve fibres with the release of acetylcholine, whereas exogenous acetylcholine is known to act directly on smooth muscle cells (Russell, 1978; Walters *et al.*, 1984). Therefore, the increased responsiveness to field stimulation cannot be attributed to the interaction between PAM-derived products and released acetylcholine, or concomitant changes in smooth muscle function. Rather, the action of mediator(s) released by an IgE-dependent mechanism from PAM is presynaptic.

It has been recognized that PAM possess IgE Fc receptors (Spiegelberg, 1984), which are referred to as type 2 and differ both structurally and functionally from type 1 IgE Fc receptors on mast cells and basophils. Although this type of receptor on PAM is a low-affinity receptor, the number of IgE Fc receptor-positive PAM is known to increase in patients with atopic asthma compared to normal subjects (Melewicz *et al.*, 1982). In addition, upon stimulation with IgE and antigen, PAM can release a wide variety of substances that have a putative role in asthma. In the present study, pretreatment of PAM with indomethacin greatly reduced the PAM-induced increase in the contractile responses of bronchioles to EFS, suggesting that cyclo-oxygenase products of arachidonic acid may play a major role in the stimulatory effect of PAM on small airways. Because, among cyclo-oxygenase products released from PAM, TxA_2 and $PGF_{2\alpha}$ are known to potentiate cholinergic neurotransmission in the canine airway (Leff *et al.*, 1985; Tamaoki *et al.*, 1987a), we measured these substances and found that the release of each eicosanoid from PAM was enhanced by anti-DNP IgE antibody and DNP-HSA, and was abolished by indomethacin treatment. Furthermore, we found that the effect of stimulated PAM on the EFS-induced contraction was inhibited by SQ 29548, a specific antagonist at the TxA_2 receptor (Ogletree *et al.*, 1985), in a dose-dependent manner. Therefore, the potentiation of cholinergic neurotransmission in small airways induced by PAM may be accounted for by TxA_2 released from PAM.

In addition to its direct bronchoconstrictor effect, TxA_2 has been known to have neuromodulatory action on canine airway smooth muscle. We and others have previously shown that the TxA_2-mimetic 11α,9α-epoxymethano-PGH_2 (U46619) (Coleman *et al.*, 1981), at a concentration insufficient to cause muscle contraction, increased the contractile responses to field stimulation but not responses to an exogenous cholinoceptor agonist (Munoz *et al.*, 1986). In the present study, stimulated PAM at cell densities up to 3×10^7 cells ml^{-1} did not cause spontaneous contraction of bronchioles, suggesting either that TxA_2 levels in the chamber were subthreshold or bronchodilator prostaglandins such as PGE_2 released concomitantly from PAM overcome TxA_2-induced contraction. The site of action of TxA_2 was prejunctional in the vagal motor pathway, but we did not directly measure the release of acetylcholine. However, Serio & Daniel (1988), using a sucrose gap method, obtained evidence to suggest that TxA_2 does not enhance the initial amount of acetylcholine released *per se*, but prolongs the duration of release or improves propagation of impulses through the muscle.

In conclusion, our present study raises the possibility that PAM play a role in the development of antigen-induced hyperreactivity of small airways through an IgE-dependent release of TxA_2, which prejunctionally augments the parasympathetic component of bronchiolar smooth muscle tone. There is increasing evidence that TxA_2 may be an important mediator in the pathogenesis of airway hyperresponsiveness in animal models (O'Byrne *et al.*, 1985; Chung *et al.*, 1986), but the role of this cyclo-oxygenase product in causing asthma in human subjects is much less clear. Previous studies with thromboxane synthetase inhibitors (Fujimura *et al.*, 1986) and with thromboxane receptor antagonist (Finnerty & Holgate, 1990) have provided conflicting results. Thus, further research needs to be done to extend our observations to human asthma.

The authors thank Masayuki Shino and Yoshimi Sugimura for their technical assistance. We are grateful to The Squibb Institute for Medical Research for providing us with SQ 29548. This work was supported in part by Grant for Scientific Research No. 01770527 from the Ministry of Education, Science and Culture, Japan.

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(Received December 11, 1990
Revised January 15, 1991
Accepted January 22, 1991)

Br. J. Pharmacol. (1991), **103**, 1463–1469 © Macmillan Press Ltd, 1991

Receptors mediating tachykinin-induced contractile responses in guinea-pig trachea

[1]S.J. Ireland, F. Bailey, A. Cook, R.M. Hagan, C.C. Jordan & M.L. Stephens-Smith

Department of Neuropharmacology, Glaxo Group Research Ltd., Park Road, Ware, Hertfordshire, SG12 0DP

1 The classification of tachykinin receptors in the guinea-pig trachea has been investigated. This was of interest because, from previous studies, it was not clear whether the guinea-pig trachea contains either a mixture of NK_1 and NK_2 receptors or, alternatively, a single type of novel tachykinin receptor.

2 In the present study, the guinea-pig trachea was contracted by tachykinin agonists selective for NK_1 receptors (substance P methylester (SPOMe) and GR73632) or NK_2 receptors (GR64349) but not NK_3 receptors (senktide).

3 Against SPOMe and GR73632, the NK_1 antagonist, GR71251, behaved as a reversible competitive antagonist having apparent affinity (pK_B 7.05 vs SPOMe) consistent with action at NK_1 receptors. GR71251 (3 μM) did not antagonize responses to GR64349.

4 The NK_2 antagonists L-659,877 and Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH_2 (R396) antagonized GR64349 although only R396 appeared to behave competitively (pK_B 5.73). Neither L-659,877 (30 μM) nor R396 (30 μM) blocked responses to SPOMe.

5 For L-659,877 and R396, comparison was made between activity in guinea-pig trachea and in preparations known to contain tachykinin receptors predominantly of the NK_2 type. In the rabbit trachea, both L-659,877 and R396 had effects similar to those in guinea-pig trachea. In contrast, in the rat colon muscularis mucosae, both L-659,877 and R396 appeared to behave competitively with pK_B values against GR64349 of 7.83 and 6.90 respectively.

6 It is concluded that in guinea-pig trachea, contractile responses can be induced by activation of both NK_1 and NK_2 receptors. The present data are discussed with reference to the proposed existence of subtypes of the NK_2 receptor.

Keywords: Tachykinin receptors; receptor subtypes; guinea-pig trachea; rabbit trachea; rabbit aorta; rat colon muscularis mucosae

Introduction

Recently, considerable interest has been shown in the possible involvement of tachykinins in the pathogenesis of asthma. Activation of tachykinin receptors in the airways could lead to bronchoconstriction, hyperreactivity and inappropriate secretion of mucus (see for example Saria *et al.*, 1985; Barnes, 1986; 1989; Martling *et al.*, 1987; Crimi & Mistretta, 1989; Joos, 1989; Rogers *et al.*, 1989). Elucidation of the precise role (if any) of tachykinins in asthma is likely to be facilitated by an understanding of the tachykinin receptors present in airways.

In the present study, an investigation has been made of the tachykinin receptors mediating contraction of the guinea-pig isolated trachea. This tissue was of interest since, in previous studies, two distinct hypotheses had been formulated: the guinea-pig trachea was proposed to contain either a mixture of NK_1 and NK_2 receptors (Ireland *et al.*, 1988) or alternatively, a single type of novel tachykinin receptor termed 'NK_4' (McKnight *et al.*, 1987; Maguire *et al.*, 1989). This is of particular significance since it has been suggested that this latter novel receptor may exist also in the central nervous system (see Dourish *et al.*, 1988a,b).

Certain NK_1 or NK_2 antagonists are reported to be inactive in the guinea-pig trachea (see Maguire *et al.*, 1989), consistent with the notion that the tachykinin receptors in this tissue may indeed be novel. However, the question of whether they belong to one or more than one type is still open. The present study was performed in an attempt to answer this latter point, essential before meaningful receptor classification can be undertaken. Use was made of recently described agonists and antagonists selective for either NK_1 or NK_2 receptors.

During the course of the study, it was found that on the guinea-pig trachea, the NK_2 antagonists tested were only weakly active against the NK_2 selective agonist GR64349 (Hagan *et al.*, 1989). It was considered that this could indicate activity of GR64349 at non-NK_2 receptors. Therefore, comparison was made of the activity of the NK_2 antagonists against both GR64349 and the naturally-occurring agonist neurokinin A (NKA) in preparations shown to contain tachykinin receptors predominantly of the NK_2 type: rabbit trachea, endothelium-denuded rabbit thoracic aorta and rat colon muscularis mucosae.

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

Methods

Animals

Male guinea-pigs (300–600 g, Dunkin-Hartley strain, Porcellus), male rabbits (2–3 kg, New Zealand White strain, Froxfield) and male rats (250–500 g, AHA strain, Glaxo) were used. Guinea-pigs and rats were killed by concussion followed by exsanguination, whilst rabbits were killed with a captive-bolt gun; required tissues were excised immediately.

Guinea-pig trachea and rabbit trachea

Guinea-pig trachea was prepared according to the method of Constantine (1965). Briefly, each excised trachea was mounted on a glass rod (approximately 2 mm diameter) and cut to yield a spiral strip which was divided longitudinally to provide up to four individual preparations. Rabbit trachea was also mounted on the rod but was cut into rings 3–5 mm wide.

No attempt was made to assess whether the epithelium was intact in these preparations. However, in a preliminary study,

[1] Author for correspondence.

examination of guinea-pig trachea preparations that had been stained with hematoxylin and eosin and embedded in paraffin suggested that the epithelium was likely to be present although not necessarily intact.

Rabbit aorta

Each rabbit thoracic aorta was threaded over a Perspex rod (approximate diameter 2 mm) to remove the endothelium (see Furchgott, 1983) and then cut to yield rings approximately 2–3 mm wide.

Rat colon muscularis mucosae

Rat colon muscularis mucosae (RCMM) were prepared as described by Bailey & Jordan (1984).

Use of isolated tissues

Tissue preparations were mounted in glass organ baths of approximately 3 ml capacity (trachea or RCMM) or 5 ml capacity (aorta), filled with physiological medium, gassed with CO_2 (5%) in O_2 and maintained at 37 ± 1°C. To reduce adsorption of peptides, all glassware was pre-treated with dichlorodimethylsilane (Aldrich).

Contraction of guinea-pig trachea, rabbit trachea or rabbit aorta was recorded isometrically via Grass FT.03 transducers, with a basal tension of approximately 0.02N (rabbit aorta) or 0.01N (other tissues). Contraction of RCMM preparations was recorded isotonically via Chemlab CS-IT100 transducers, pre-loaded with approximately 0.005N.

Experimental design

In tracheal preparations, concentration-contraction response curves to agonists were constructed by cumulative addition; in aorta and RCMM, non-cumulative addition of serially-increasing concentrations was used. On each preparation, at least two concentration-response curves were constructed to the chosen agonist. Tissues were used in the study only if two consecutive curves were found to have EC_{50} values consistent to within a factor of 2 and maxima reproducible to within about $\pm 15\%$.

Routinely, antagonists were pre-equilibrated with the tissue for 15 min. Adequacy of this period was verified in separate experiments by comparison of results obtained after 15 min or 60 min pre-equilibration. Each preparation was exposed to one concentration of antagonist only. In control preparations not exposed to antagonist, EC_{50} values for agonist concentration-response curves were reproducible to within 2 fold. No measurements were made of reversibility of effect on wash-out of antagonist.

Data analysis

For agonists, EC_{50} values and maxima of concentration-response curves were estimated by the curve-fitting programme "Allfit" (De Lean *et al.*, 1977). EC_{50} values are quoted as the mean ($\pm$ s.e.mean) of single determinations made on at least four preparations of a given tissue, each obtained from a separate animal.

Antagonist-induced parallel displacement of agonist concentration-response curves was quantified as the ratio of equi-active molar concentrations. These were estimated graphically at the level of the half-maximal response. Estimates of $\log_{10}$(concentration-ratio $-$ 1) were plotted against $\log_{10}$(concentration of antagonist). Provided that such plots were linear and had gradients not significantly different from unity, the apparent affinity (pK_B) of the antagonist was estimated as the mean ($\pm$ s.e.mean) of the individual values:

$$pK_B = \log(\text{concentration-ratio} - 1) - \log(\text{molar concentration of antagonist})$$

For each concentration of antagonist, data were obtained from a minimum of four separate preparations of the chosen tissue.

Solutions

Modified Tyrode solution was used for experiments on guinea-pig trachea, rabbit trachea and RCMM. It had the following composition (in mM): NaCl 137, KCl 2.8, $NaHCO_3$ 11.9, $MgCl_2$ 2.1, NaH_2PO_4 0.32, $CaCl_2$ 1.8 and glucose 5.6. For rabbit aorta preparations, modified Krebs-Henseleit medium was used. The composition was (in mM): NaCl 118, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.18, $MgSO_4$ 1.18, $CaCl_2$ 2.5 and glucose 11. Media were prepared in glass-distilled water and A.R.-grade reagents were used (BDH). The following drugs (each at 1 μM) were added routinely to prevent possible indirect effects of peptides: atropine sulphate (Sigma), indomethacin (Sigma), mepyramine maleate (May & Baker) and methysergide hydrogen maleate (Sandoz). For trachea preparations only, the medium contained the peptidase inhibitors phosphoramidon (1 μM; Peninsula) and bestatin (100 μM; Peninsula) since in guinea-pig airways, peptidase inhibitors have a marked effect on the activities of a number of tachykinin agonists (see Devillier *et al.*, 1988; Stephens-Smith *et al.*, 1988; Djokic *et al.*, 1989; Frossard *et al.*, 1989; Maggi *et al.*, 1990a).

Drugs

Substance P (SP), substance P methylester (SPOMe), neurokinin A (NKA), eledoisin and senktide were purchased from Peninsula or Cambridge Research Biochemicals; L-659,877 (cyclo(Gln-Trp-Phe-Gly-Leu-Met)) from Cambridge Research Biochemicals. Other compounds were synthesized in the Department of Medicinal Chemistry, Glaxo Group Research Ltd., Greenford. These were GR71251 ([D-Pro^9[spiro-γ-lactam]Leu^{10},Trp^{11}]$SP_{(1-11)}$); GR64349 ([Lys^3,Gly^8-**R**-γ-lactam-Leu^9]$NKA_{(3-10)}$); GR73632 (δaminovaleryl[Pro^9,N-MeLeu^{10}]$SP_{(7-11)}$); δaminovaleryl[L-Pro^9]$SP_{(7-11)}$; δaminovaleryl[D-Pro^9]$SP_{(7-11)}$; [pGlu^6,Gly^9-**R**-γ-lactam-Leu^{10}]$SP_{(6-11)}$ (Cascieri *et al.*, 1986) and Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH_2 (Maggi *et al.*, 1990b; R396). The identity and concentration of peptides were confirmed as described by Brown *et al.* (1986).

Concentrated solutions (5–10 mM) of each compound were divided into aliquots and stored frozen (-20°C) under nitrogen in plastic tubes. Aliquots were diluted immediately before use. Solutions of L-659,877, R396, eledoisin and senktide were prepared in dimethysulphoxide (DMSO; BDH); other compounds were dissolved in 0.01 M acetic acid (BDH). Stock solutions of all compounds, with the exception of L-659,877, could be diluted with physiological solution without causing visible precipitation. For L-659,877, it was necessary to include DMSO (up to 1.0%) to prevent precipitation. In all cases, the concentration of solvent was limited such that it had no effect on agonist-induced responses.

Results

On all preparations, the effects of antagonists (GR71251, R396 or L-659,877) were not increased by pre-incubation for 60 min rather than the standard 15 min. This was taken to indicate that complete equilibration had been achieved within 15 min. For analysis, data for both pre-incubation periods have been combined.

Guinea-pig trachea

On the guinea-pig trachea, contractile responses were induced by low concentrations of a number of tachykinin agonists (see Table 1). Of particular note were the high activities of the NK_1-selective agonists SPOMe and GR73632 and of the

Table 1 Mean EC_{50} values for tachykinin agonist-induced contractile responses

Compound	*Mean EC_{50} (nM)* Guinea-pig trachea	Guinea-pig ileum (NK_1)	RCMM (NK_2)	Rat portal vein (NK_3)
SPOMe	38.0 ± 10.0	7.7 ± 1.7	>100,000	>30,000
GR73632	4.9 ± 0.5	4.0 ± 0.5	961 ± 175	>15,000
δAva[L-Pro^9]$SP_{(7-11)}$	5.4 ± 0.5	13.4 ± 2.4	13,000	>10,000
GR64349	2.9 ± 0.2	4237 ± 1295	3.7 ± 0.6	117 ± 446
δAva[D-Pro^9]$SP_{(7-11)}$	12.8 ± 1.4	830 ± 410	92.0 ± 24.2	>30,000
SP	98.4 ± 3.0	5.6 ± 0.7	167 ± 20	7500 ± 1200
Neurokinin A	2.4 ± 1.0	13.7 ± 2.1	1.7 ± 0.3	563 ± 99
[pGlu^6,Gly^9-**R**-γ-lactam-Leu^{10}]$SP_{(6-11)}$	3.9 ± 0.3	30.3 ± 4.2	68.5 ± 11.9	435 ± 45
Eledoisin	25.0 ± 9.7	12.4 ± 2.2	13.0 ± 2.4	ND
Senktide	>10,000	>30,000	>30,000	11.8 ± 0.9

Values are mean ± s.e.mean.
On each preparation, concentration-response curves for active agonists were approximately parallel to those obtained with the chosen standard [pGlu^6,Gly^9-**R**-γ-lactam-Leu^{10}]$SP_{(6-11)}$ on guinea-pig trachea, substance P (SP) on guinea-pig ileum, neurokinin A (NKA) on rat colon muscularis mucosae (RCMM) or senktide on rat portal vein); maxima were similar. An EC_{50} value preceded by > indicates that at the concentration indicated, the agonist caused a response smaller than half the maximum to the standard. Each value is the mean (± s.e.mean) of single determinations in at least four separate preparations each obtained from a different animal. Data for guinea-pig ileum, RCMM and rat portal vein are from Ireland *et al.* (1988) or Hagan *et al.* (1990).
Abbreviations: SPOMe: substance P methylester; δAva; δaminovaleryl; ND: not determined.

NK_2-selective agonist GR64349. In addition, there was only a small difference between the activities of the L-proline and D-proline isomers of δaminovaleryl [Pro^9]$SP_{(7-11)}$ (Table 1). Of the compounds tested, only senktide, which showed the greatest selectivity for NK_3 receptors, was inactive (Table 1).

GR71251 (0.1–30 μM) behaved as a reversible competitive antagonist of contractile responses induced by SPOMe (Figure 1). Apparent affinity, expressed as a pK_B value, was 7.05 ± 0.06 ($n = 26$). When tested at a single concentration (3 μM), GR71251 antagonized, by an approximately equal extent, responses induced by the NK_1-selective agonists SPOMe and GR73632 but had negligible effect against a number of other tachykinins including the NK_2-selective agonist GR64349 (Table 2).

The NK_2 antagonist R396 (10–50 μM) caused parallel rightward displacement of the concentration-response curve to GR64349 (Figure 2). The Schild plot constructed from the antagonism data approximated to a straight line with gradient 0.96 (95% confidence limits 0.64–1.27, Figure 3). The mean pK_B was 5.73 ± 0.04 ($n = 19$). R396 (30 μM) had no significant effect against contraction of the guinea-pig trachea induced by SPOMe (mean concentration-ratio (95% confidence limits) 1.2 (1.0–1.4), $n = 6$).

Like R396, L-659,877 (0.3–10 μM) caused parallel rightward displacement of the concentration-response curve to GR64349. However, this effect was not clearly concentration-dependent: the Schild plot had a gradient of 0.28 (0.08–0.49)

Table 2 Effect of GR71251 (3 μM) on tachykinin-induced contraction of the guinea-pig isolated trachea

Tachykinin agonist	*GR71251 (3 μM)* Mean concentration-ratio
Substance P methylester	38.1 (15.7–92.8)
GR73632	42.3 (23.7–75.5)
GR64349	2.4 (1.5–3.8)
δAva[D-Pro^9]$SP_{(1-11)}$	3.3 (1.8–6.1)
Neurokinin A	1.8 (0.9–3.4)
[pGlu^6,Gly^9-R-γ-lactam-Leu^{10}]$SP_{(6-11)}$	3.6 (2.7–5.2)
Eledoisin	6.2 (3.7–10.3)

Each result is the mean (95% confidence limits) of single determinations in at least four individual tissues, each obtained from a separate guinea-pig.
Abbreviations: δAva: δaminovaleryl; SP: substance P.

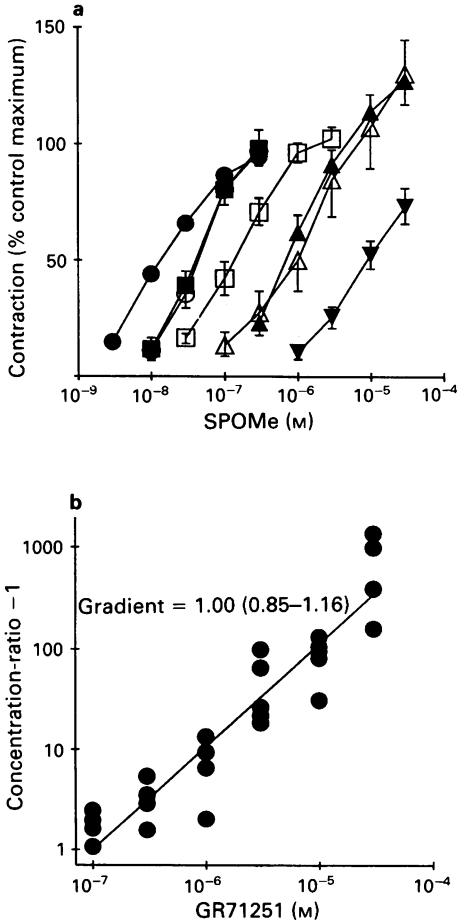

Figure 1 Antagonism by GR71251 of contractile responses induced by substance P methylester (SPOMe) in guinea-pig isolated trachea. In (a) symbols indicate control responses (●) or the presence of GR71251 at 0.1 (○), 0.3 (■), 1.0 (□), 3.0 (▲), 10.0 (△) or 30.0 μM (▼). Each point is the mean with the vertical line indicating the s.e.mean of single determinations in at least four separate tissues. (b) Schild plot of [concentration-ratio − 1] against [concentration of GR71251]. Each point represents the result obtained in a separate tissue. The gradient (95% confidence limits) of the best-fit straight line was determined by linear regression analysis.

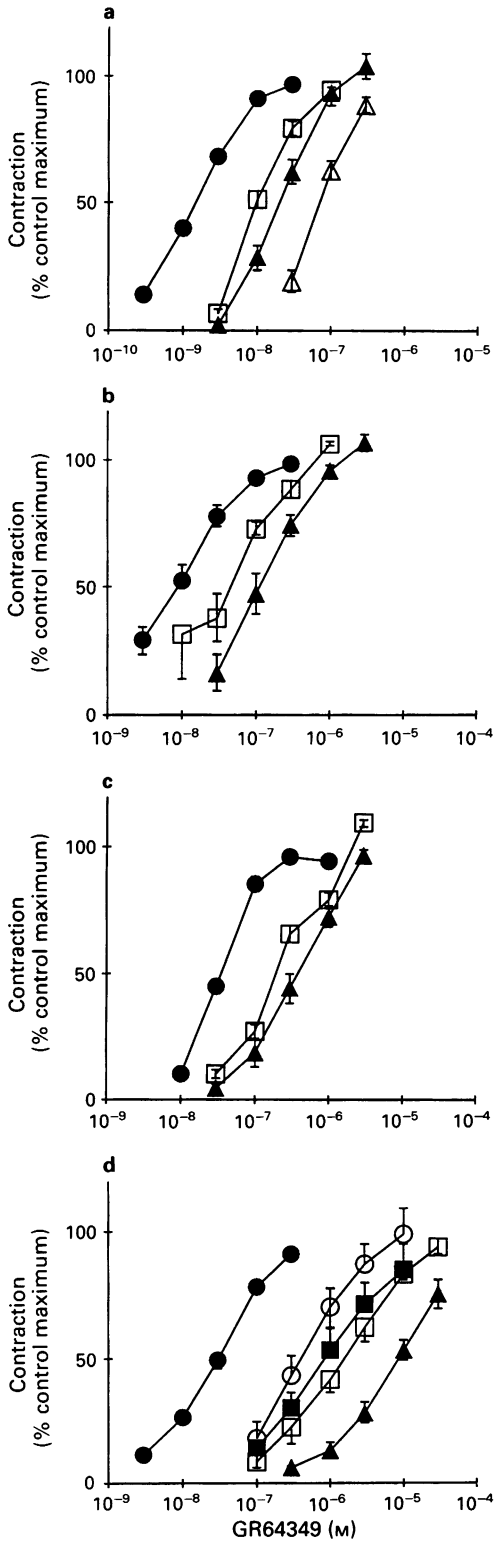

Figure 2 Antagonism by Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH_2 (R396) of contractile responses induced by GR64349 in (a) guinea-pig trachea, (b) rabbit trachea, (c) rabbit thoracic aorta or (d) rat colon muscularis mucosae. Symbols indicate control responses (●) or the presence of antagonist at 1.0 (○), 3.0 (■), 10.0 (□), 30.0 (▲) or 50.0 μM (△). Each point is the mean, with vertical lines indicating the s.e.mean, of single determinations in at least four separate preparations.

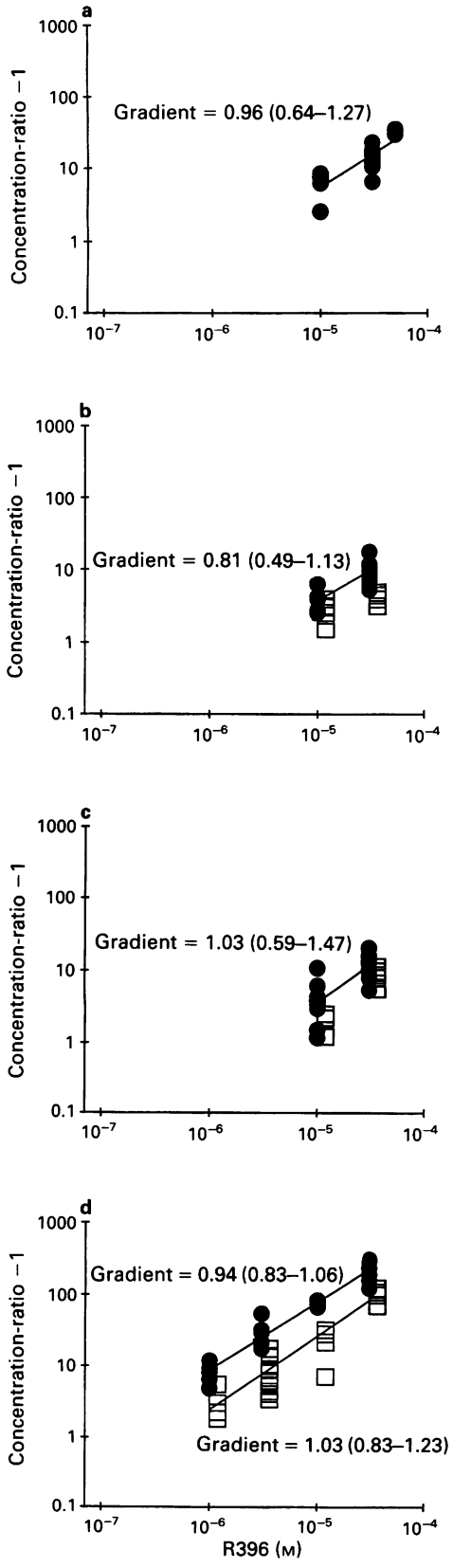

Figure 3 Schild plots of [concentration-ratio − 1] against [concentration of antagonist] for antagonism by Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH_2 (R396) of tachykinin agonist induced contraction in (a) guinea-pig trachea, (b) rabbit trachea, (c) rabbit thoracic aorta or (d) rat colon muscularis mucosae. Each point represents data obtained from a separate preparation. Results were obtained with GR64349 (●) or neurokinin A (□) as agonist. Note that to avoid overlap, data obtained with neurokinin A are shown displaced 1.2 fold to the right. For each type of preparation, the gradient of the best-fit straight line (95% confidence limits) was determined by linear regression analysis.

(Figures 4, 5). At a very high concentration, L-659,877 (30 μM) appeared to reduce the amplitude of the maximum response to GR64349 (Figure 4) but had no effect against contractile responses induced by SPOMe (mean concentration-ratio 1.0 (0.4–2.5), $n = 4$).

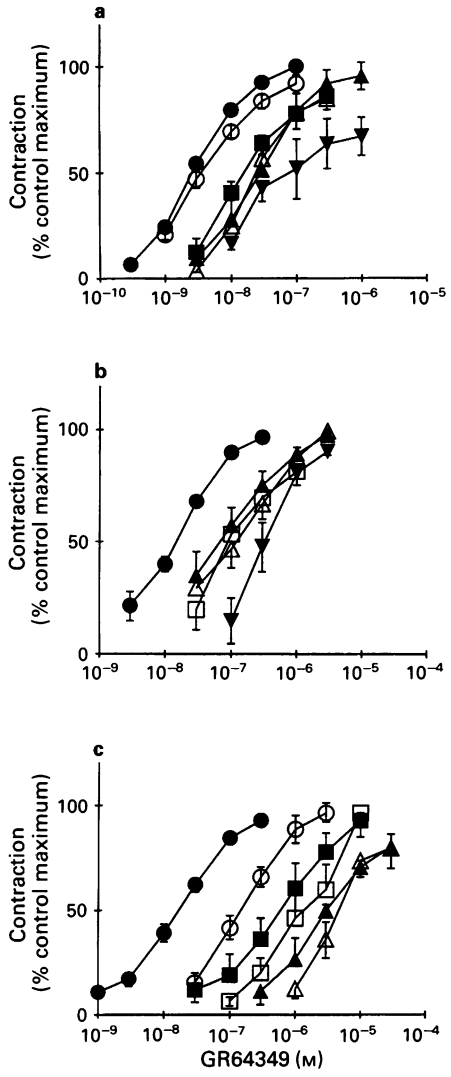

Figure 4 Antagonism by L-659,877 of contractile responses induced by GR64349 in (a) guinea-pig trachea, (b) rabbit trachea or (c) rat colon muscularis mucosae. Symbols indicate control responses (●) or the presence of antagonist at 0.1 (○), 0.3 (■), 1.0 (□), 3.0 (▲), 10.0 (△) or 30.0 μM (▼). Each point is the mean, with vertical lines indicating the s.e.mean, of single determinations in at least four separate preparations.

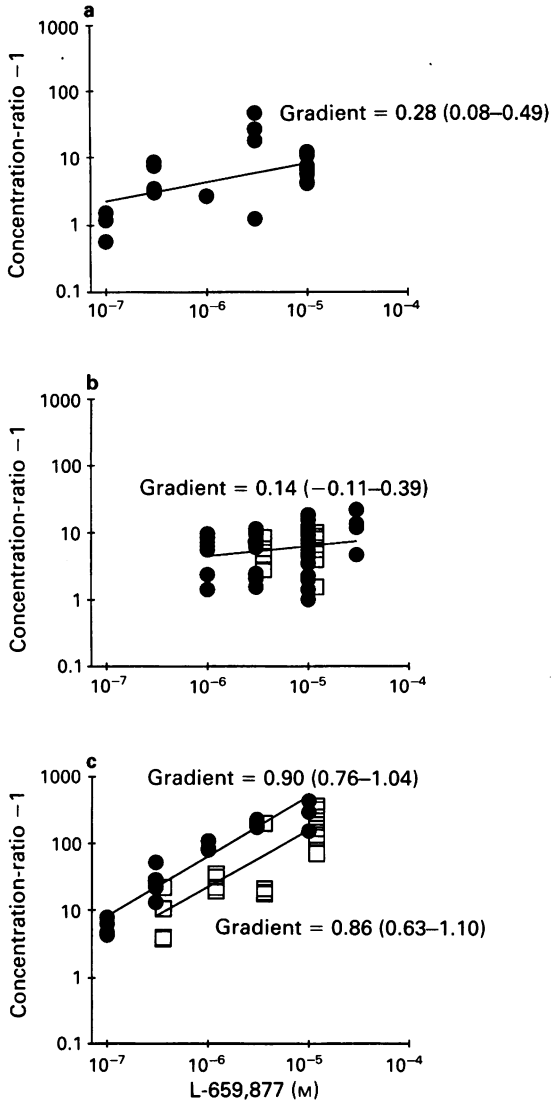

Figure 5 Schild plots of [concentration-ratio − 1] against [concentration of antagonist] for antagonism by L-659,877 of tachykinin agonist-induced contraction in (a) guinea-pig trachea, (b) rabbit trachea or (c) rat colon muscularis mucosae. Each point represents data from a separate preparation. Results were obtained with GR64349 (●) or neurokinin A (□) as agonist. Note that to avoid overlap, data obtained with neurokinin A are shown displaced 1.2 fold to the right. For each type of preparation, the gradient of the best-fit straight line (95% confidence limits) was determined by linear regression analysis.

Rabbit aorta, rabbit trachea and rat colon muscularis mucosae (RCMM)

Rabbit aorta preparations were contracted by the NK_2-selective agonist GR64349 but not the NK_1-selective agonists SPOMe or GR73632 nor by the NK_3-selective agonist senktide. In addition, when pre-contracted with phenylephrine (0.3 μM), no relaxation was observed on addition of SPOMe (1.0 μM) or GR64349 (1.0 μM) (cf. Zawadzki *et al.*, 1981). Therefore, the pharmacology of this preparation resembles that of the endothelium-denuded rabbit pulmonary artery which appears to respond to stimulation of tachykinin receptors of the NK_2 type only (D'Orleans-Juste *et al.*, 1985) as do

Table 3 Estimates of apparent affinity for NK_2 antagonists

		Apparent affinity (mean pK_B)			
Antagonist	*Agonist*	Guinea-pig trachea	Rabbit trachea	Rabbit aorta	RCMM
R396	GR64349	5.73 ± 0.04	5.51 ± 0.03	5.58 ± 0.05	6.90 ± 0.03
R396	NKA	ND	5.28 ± 0.05	5.40 ± 0.04	6.40 ± 0.04
L-659,877	GR64349	NC	NC	ND	7.83 ± 0.05
L-659,877	NKA	ND	NC	ND	7.29 ± 0.07

Data were calculated for the experiments illustrated in Figures 3 and 5. Estimates of apparent affinity (pK_B) are the mean ± s.e.mean of single determinations in at least 11 separate preparations.
Abbreviations: RCMM: rat colon muscularis mucosae; NKA; neurokinin A; ND: could not be determined (see text); NC: could not be calculated (gradient of Schild plot less than unity; $P < 0.05$).

the rabbit trachea (see Cook et al., 1990) and the RCMM (see Bailey et al., 1986; Burcher et al., 1986).

On the rabbit trachea, rabbit aorta and RCMM, R396 (10–30 μM) behaved as a reversible competitive antagonist of contractile responses induced by either GR64349 or NKA (Figures 2, 3). Shifts of the concentration-response curves tended to appear greater when GR64349 rather than NKA was used as agonist, although the magnitude of such agonist-dependence was small (see Figure 3, Table 3). However, irrespective of the agonist used, R396 had markedly greater activity in the RCMM than in the rabbit tissues (Table 3).

On the rabbit trachea, L-659,877 (1–30 μM) caused parallel rightward displacements of concentration-response curves to GR64349 or NKA although the magnitude of displacement was not clearly concentration-dependent (Figures 4, 5). In contrast, in RCMM, L-659,877 (0.3–10 μM) appeared to behave competitively (Figures 4, 5); estimates of apparent affinity are given in Table 3. The rabbit aorta appeared more sensitive than the other tissue preparations to the effects of the solvent (DMSO) necessary to prevent precipitation of L-659,877. For this reason, the highest concentration of L-659,877 that could be tested on rabbit aorta was 3 μM (in 0.3% DMSO (v/v)): this caused a mean rightward displacement of the concentration-response curves to GR64349 of 3.94 (95% confidence limits 3.49–4.46; $n = 5$). As with R396, on the RCMM, there was a tendency for L-659,877 to appear more active against GR64349 than NKA although the differences were small (Figure 5, Table 3).

Discussion

On the guinea-pig isolated trachea, tachykinin agonists were found to induce contractile responses: of the compounds tested, only the NK_3-selective agonist senktide (Wormser et al., 1986) was found to be inactive. This latter result suggests that in the smooth muscle of the trachea, few NK_3 receptors are coupled to this response. It was not possible to draw firm conclusions from the activities of the other agonists: data were not consistent with the presence of a single population of either NK_1 or NK_2 receptors. Thus, low concentrations of either NK_1-selective agonists (SPOME, GR73632 or δaminovaleryl[L-Pro^9]SP$_{(7-11)}$) or NK_2-selective agonists (GR64349 or δaminovaleryl[D-Pro^9]SP$_{(7-11)}$) were active. Although these data are consistent with the presence of a mixture of NK_1 and NK_2 receptors, an alternative explanation, that the guinea-pig trachea contains a single type of novel tachykinin receptor (Maguire et al., 1989) was also considered.

To evaluate these alternative hypotheses, examination was made of the effects of selective NK_1 or NK_2 antagonists against responses induced by SPOMe or GR64349, these being, respectively, the most NK_1- or NK_2-selective of the agonists examined. GR71251 behaved as a reversible competitive antagonist of SPOMe-induced contraction with apparent affinity (pK_B 7.05) comparable with that exhibited at NK_1 receptors in other tissues (7.72, guinea-pig ileum; 7.06, rabbit aorta; Hagan et al., 1990), but quite different from that measured at NK_2 receptors (pK_B 4.8; Ward et al., 1990). The relatively small differences in apparent affinity of GR71251 in the three NK_1 receptor containing preparations remains to be explained, as does the observation that in guinea-pig trachea, another NK_1 antagonist, L-668,169, is inactive against SPOMe (Maguire et al., 1989) despite having a pA_2 of 7.0 against NK_1 receptor-mediated contraction of the guinea-pig ileum (McKnight et al., 1988).

In the present study on the guinea-pig trachea, GR71251 was an effective antagonist of responses induced by the NK_1-selective agonist GR73632, with apparent affinity comparable to that shown against SPOMe. In contrast, GR71251 was markedly less active against other tachykinin agonists, including GR64349, all of which have appreciable activity at NK_2 receptors (see Tables 1, 2). These data are inconsistent with the hypothesis that in the guinea-pig trachea, responses to tachykinin agonists are mediated by a single type of receptor. Rather, they are consistent with the presence of an NK_1 receptor plus another receptor which, classified according to the effects of the agonists used, resembles an NK_2 receptor. Further characterization of this latter receptor was attempted with two NK_2 antagonists: L-659,877 (Williams et al., 1988) and Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH_2 (Maggi et al., 1990b; R396); GR64349 was used as agonist.

L-659,877 was a weak antagonist of GR64349-induced contraction and its effects were not clearly concentration-dependent. In contrast, R396 did behave as a reversible competitive antagonist of GR64349 although its apparent affinity was low (pK_B 5.73). Interestingly this value is close to that obtained for R395 at NK_2 receptors in rabbit pulmonary artery (pK_B 5.42 vs NKA) but much lower than at NK_2 receptors in hamster trachea (pK_B 7.63 vs NKA) (Maggi et al., 1990b). However, before concluding that the present data contribute to evidence for subtypes of the NK_2 receptor, it was considered important to exclude alternative explanations. Of particular concern was the possibility that the low activity of L-659,877 or R396 resulted from GR64349 having activity at some type of receptor which would not be activated by a naturally-occurring agonist such as NKA. This question was addressed by measuring the effects of the two antagonists in tissue preparations which appear to contain tachykinin receptors of the NK_2 type only and in which activity against GR64349 and NKA could be compared without the complication of needing concomitant NK_1 receptor blockade. The preparations used were rabbit trachea (see Cook et al., 1990), endothelium-denuded rabbit thoracic aorta (see Results) and the rat colon muscularis mucosae (RCMM; see Bailey et al., 1986; Burcher et al., 1986).

In each of these preparations, the effects of the antagonists were not increased by using NKA as agonist rather than GR64349. Indeed, the converse tended to occur. Tachykinin receptor antagonists have been found to exhibit agonist-dependence in other tissue preparations which seem to contain only one type of tachykinin receptor (see Bailey, 1985; Bailey et al., 1986; Hall & Morton, 1991) but definitive explanation of such behaviour is awaited. In the rabbit trachea, both L-659,877 and R396 had effects qualitatively and quantitatively similar to those in guinea-pig trachea. In the rabbit aorta, R396 antagonized responses to GR64349 or NKA with apparent affinities comparable to those in rabbit trachea, guinea-pig trachea and also rabbit pulmonary artery (Maggi et al., 1990b). In contrast, in RCMM, R396 had apparent affinity (pK_B 6.90) intermediate between that observed on the guinea-pig or rabbit tissues and that reported at NK_2 receptors in hamster trachea (pK_B 7.63; Maggi et al., 1990b). The RCMM too was the only preparation of those examined in which L-659,877 caused clearly concentration-dependent antagonism of responses to GR64349 or NKA and had activity (pK_B 7.83 vs GR64349) comparable to that at NK_2 receptors in rat vas deferens (pA_2 8.0; McKnight et al., 1988). The behaviour of L-659,877 remains to be explained. In the rabbit aorta, it was not possible to test a range of concentrations of L-659,877 due to its low aqueous solubility and the apparent sensitivity of this preparation to organic solvent (DMSO). Nevertheless, L-659,877 (3 μM) was clearly less active in the rabbit aorta than in the RCMM.

The present results show that the effects of NK_2 antagonists can be tissue-dependent and, as such, confirm the conclusion of Maggi et al. (1990b). Such tissue-dependence was shown not to be a consequence of incomplete equilibration of the antagonist and appeared independent of the agonist used. Further, it is considered unlikely that it resulted from different rates of inactivation of the antagonist since tissue preparations likely to metabolize tachykinins were treated with peptidase inhibitors (see also Maggi et al., 1990b).

In conclusion, the results obtained in the present study suggest that in the guinea-pig trachea, tachykinin-induced

contraction can be mediated via a mixture of NK_1 and NK_2 receptors. They are not consistent with the notion that such contraction is mediated via a single population of a novel type of tachykinin receptor. The present data support the possible existence of subtypes of the NK_2 receptor: in terms of the effects of the NK_2 antagonists examined, the NK_2 receptors in guinea-pig trachea resemble those in rabbit trachea, rabbit thoracic aorta and rabbit pulmonary artery but seem distinct from those in hamster trachea and RCMM.

We thank Mr T.J.N. Mills and Mr J.H.M. Simon for excellent technical assistance and Ms H. Adams, Mr S.P. Clegg, Mr M.J. Deal, Mr G. Ewan, Dr A.B. McElroy, Dr P. Seale, Dr P. Smith and Dr P. Ward for synthesis of peptides.

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(Received October 22, 1990
Revised January 4, 1991
Accepted January 25, 1991)

Br. J. Pharmacol. (1991), **103**, 1470–1474 © Macmillan Press Ltd, 1991

Epithelium-dependent potentiation of anaphylactic contractions by β-endorphin in tracheae isolated from actively sensitized guinea-pigs

[1,] *A.J.M. Van Oosterhout, [2,] *†L. Celeda, *K.C. Delsman, †D. de Wied & *F.P. Nijkamp

*Department of Pharmacology, Faculty of Pharmacy, University of Utrecht, Catharijnesingel 60, 3511 GH Utrecht, The Netherlands and †Rudolf Magnus Institute, Medical Faculty, University of Utrecht, Vondellaan 6, 3521 GD Utrecht, The Netherlands

1 It has been shown that opioid peptides modulate airway function. In the present study, the effect of β-endorphin on antigen-induced contractions of isolated tracheal rings from actively sensitized guinea-pigs has been studied.

2 β-Endorphin had a concentration-dependent bimodal effect on anaphylactic contractions of the trachea. Low concentrations of β-endorphin (10^{-10} and 10^{-8} M) significantly potentiated anaphylactic contractions, whereas higher concentrations (10^{-7} and 10^{-6} M) significantly suppressed anaphylactic contractions of guinea-pig trachea.

3 β-Endorphin in concentrations of 10^{-8} M and 10^{-7} M did not affect the responsiveness of the tracheal rings to histamine or leukotriene D_4. This indicates that β-endorphin does not influence the responsiveness of tracheal smooth muscle to anaphylactic mediators.

4 In the presence of the non-selective opioid receptor antagonist naloxone, 10^{-8} M β-endorphin still potentiated the anaphylactic contractions of the trachea. In addition, an equimolar concentration of des-Tyr[1]-β-endorphin, a fragment of β-endorphin without opioid-like activity, also potentiated anaphylactic contractions. The potentiation of anaphylactic contraction by 10^{-8} M β-endorphin is not therefore mediated by classical opioid-receptors.

5 In the presence of naloxone, 10^{-7} M, β-endorphin did not suppress anaphylactic contractions of the trachea. Thus, the suppression of anaphylactic contraction is mediated via a classical opioid-receptor.

6 In epithelium-denuded tracheae, both 10^{-8} and 10^{-7} M β-endorphin suppressed the anaphylactic contractions, whereas 10^{-8} and 10^{-7} M des-Tyr[1]-β-endorphin did not affect anaphylactic contractions. It is concluded that the potentiation of the anaphylactic contraction in intact trachea is epithelium-dependent whereas the suppression of the anaphylactic contraction is epithelium-independent.

Keywords: β-Endorphin; naloxone; anaphylaxis; trachea; mast cell; epithelium

Introduction

Opioid peptides play a role in the modulation of airway function. It has been shown that opioid peptides inhibit non-adrenergic, non-cholinergic neural control of mucus secretion (Rogers & Barnes, 1989a,b), inhibit cough and reflex bronchoconstriction (Karlsson *et al.*, 1990) and inhibit non-adrenergic, non-cholinergic-induced bronchoconstriction (Barnes *et al.*, 1990). Besides beneficial effects, opioid peptides may also have harmful effects on airway function. Popa & Rients (1989) demonstrated that exercise-induced bronchoconstriction in asthmatic patients could be partially prevented by the opiate receptor antagonist naloxone. Furthermore, opiate antagonists have been found to protect animals against anaphylactic shock (Amir, 1982).

Opioid peptides may affect airway function after release of these peptides from the anterior pituitary into the blood or after local release from nerves (Fischer, 1988; Olson *et al.*, 1989; Shimosegawa *et al.*, 1989; Barnes *et al.*, 1990). In the present study, we investigated the effect of the opioid peptide β-endorphin on ovalbumin-induced contractions in tracheae isolated from actively sensitized guinea-pigs. Effects of β-endorphin on cell functions are either mediated by binding to classical opioid receptors or via a non-opioid receptor-mediated mechanism (De Wied & Jolles, 1982; Fischer, 1988; Sibinga & Goldstein, 1988). Binding of β-endorphin to classical opioid receptors requires an intact NH_2-terminal tyrosine and is sensitive to blockade by naloxone (De Wied & Jolles, 1982; Sibinga & Goldstein, 1988). To investigate whether the effects of β-endorphin on anaphylactic contractions of guinea-pig trachea are mediated by classical opioid receptors or via a non-opioid receptor-mediated mechanism, the non-opioid derivative, des-Tyr[1]-β-endorphin and the non-selective opioid receptor antagonist, naloxone, have been used. Furthermore, the effect of β-endorphin and des-Tyr[1]-β-endorphin on anaphylactic contractions in epithelium-denuded tracheae was examined. Some preliminary results of this study have been presented at the European Histamine Research Society (1989, Breda, The Netherlands).

Methods

Male guinea-pigs weighing 300–350 g (Olac Ltd., Bicester, England) were sensitized by intraperitoneal injection of ovalbumin (2 mg) in aluminium hydroxide adjuvant (Van Oosterhout & Nijkamp, 1986). Three weeks after sensitization the animals were killed by an i.p. injection of 180 mg pentobarbitone sodium (Nembutal), the tracheae removed and dissected free of connective tissue and blood vessels. In one series of experiments, epithelium denudation of the tracheae was performed with a cotton swab. The effectiveness of this procedure was examined by light microscopy (hematoxylin and eosin staining). One trachea was divided in 4 to 6 parts each of three cartilage rings. The tracheal rings were mounted in organ baths filled with Krebs bicarbonate buffer (37°C) of the following composition (mmol^{-1}): NaCl 118.1, KCl 4.7, $CaCl_2.2H_2O$ 2.5, $MgSO_4.7H_2O$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and glucose 8.3. The organ baths were siliconized to prevent adherence of the peptides to the glass. Krebs solution was

[1] Author for correspondence.
[2] Present address: Ministry of Agriculture, Tesnov 16, Prague 1, Czechoslovakia.

continuously gassed with a 5% CO_2 and 95% O_2 gas mixture. Changes in tension were measured by an isometric transducer (Harvard Bioscience) and displayed on a two-channel pen recorder (Servogor, type 220). Before the experiment, tracheal rings were equilibrated for at least 60 min under a resting load of 2 g. β-Endorphin (10^{-12} M to 10^{-6} M) or des-Tyr^1-β-endorphin (10^{-8} M) was introduced into the organ bath 1 min before the first ovalbumin or histamine addition. Naloxone (10^{-6} M) was administered 5 min prior to the addition of β-endorphin. A single (10^{-3} mg ml^{-1}) or cumulative concentration(s) of ovalbumin (10^{-8} to 10^{-1} mg ml^{-1}), histamine (10^{-9} to 3×10^{-4} M) or leukotriene D_4 (10^{-11} to 10^{-7} M) was added to the organ bath in the absence or presence of drugs. Different parts of one trachea were alternately used in each group to avoid possible differences between proximal and distal parts. Results are expressed as a percentage of the maximal response of the tracheal ring to ovalbumin in the absence of drugs. Results of histamine and leukotriene D_4 concentration-response curves are expressed as absolute value's. Levels of significance were calculated by the paired t test. A value of $P < 0.05$ was considered to be significant. Responses are presented as mean ± s.e.mean.

Materials

Nembutal, containing pentobarbitone sodium 60 mg ml^{-1} was from Abbot Laboratories, North Chicago, IL, U.S.A. Histamine phosphate was obtained from the Onderlinge Pharmaceutische Groothandel, Utrecht, The Netherlands. Leukotriene D_4 was a gift from J. Rokach (Merck-Frosst, Point Clair-Dorval, Canada). β-Endorphin and des-Tyr^1-β-endorphin were kindly donated by Organon International B.V., Oss, The Netherlands. Naloxone was obtained from DuPont Pharmaceuticals, Wilmington, Delaware, U.S.A.

Results

Addition of β-endorphin in concentrations of 10^{-12} M to 10^{-6} M to the organ bath did not affect initial tension of the tracheae (results not shown). The mean anaphylactic contraction of tracheal rings induced by ovalbumin 10^{-3} mg ml^{-1} in the absence of β-endorphin was 1.78 ± 0.24 g ($n = 7$). β-Endorphin had a concentration-dependent effect on ovalbumin-induced contractions. At 10^{-12} M, β-endorphin did not affect these contractions, whereas at 10^{-10} and 10^{-8} M β-endorphin significantly potentiated anaphylactic contractions in tracheal rings induced by 10^{-3} mg ml^{-1} ovalbumin as compared to the contractions in the absence of β-endorphin (Figure 1). The potentiation of contractions induced by 10^{-3} mg ml^{-1} ovalbumin amounted to 27% and 22% in the presence of respectively 10^{-10} and 10^{-8} M β-endorphin, as compared to contractions in the absence of β-endorphin. Higher concentrations of β-endorphin (10^{-7} and 10^{-6} M) significantly suppressed ovalbumin induced contractions of tracheal rings as compared to responses in the absence of β-endorphin (Figure 1).

In Figure 2, the contractions of the tracheal rings to cumulative concentrations of ovalbumin are presented. The maximal response of tracheal rings to ovalbumin (10^{-1} mg ml^{-1}) in the absence of drugs amounted to 1.55 ± 0.21 g ($n = 6$). At 10^{-8} M, β-endorphin significantly enhanced anaphylactic contractions induced by 10^{-4} to 10^{-1} mg ml^{-1} ovalbumin, but not contractions induced by 10^{-2} mg ml^{-1} ($P = 0.15$). The potentiation varied between 69% at 10^{-4} mg ml^{-1} and 39% at 10^{-1} mg ml^{-1} ovalbumin. In the presence of the non-selective opioid receptor antagonist naloxone (10^{-6} M), 10^{-8} M β-endorphin significantly enhanced the contractions of the tracheal rings induced by 10^{-3}, 10^{-2} and 10^{-1} mg ml^{-1} ovalbumin by 115%, 82% and 64%, respectively. Naloxone, 10^{-6} M, itself however, significantly suppressed the anaphylactic contractions of tracheal rings induced by 10^{-4} to 10^{-1} mg ml^{-1} ovalbumin by about 30% as compared to contractions in the absence of naloxone.

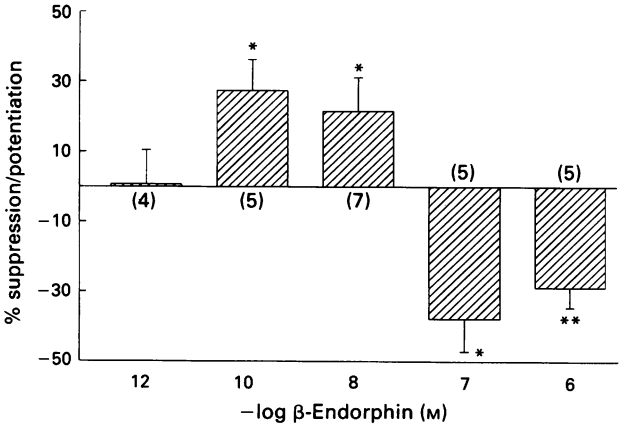

Figure 1 The effect of different concentrations of β-endorphin on ovalbumin (10^{-3} mg ml^{-1})-induced anaphylactic contractions in tracheal rings isolated from actively sensitized guinea-pigs. The results are expressed as the percentage potentiation or suppression as compared to the contractions of tracheal rings in the absence of β-endorphin ($n = 7$). The number of experiments is indicated in parentheses. *$P < 0.05$ and **$P < 0.01$ as compared to contractions of tracheal rings in the absence of β-endorphin.

Additionally, an equimolar concentration of des-Tyr^1-β-endorphin, the non-opioid fragment of β-endorphin, also significantly potentiated anaphylactic contractions induced by 10^{-3}, 10^{-2} and 10^{-1} mg ml^{-1} ovalbumin by about 40% (Table 1). In this experiment the maximal contraction of tracheae to ovalbumin in the absence of drugs was 0.83 ± 0.09 g ($n = 4$).

The effect of 10^{-7} M β-endorphin on cumulative ovalbumin-induced contractions is presented in Figure 3. The mean anaphylactic contraction of tracheae in the absence of drugs was 1.98 ± 0.24 g ($n = 8$). Suppression of ovalbumin-induced contractions in the presence of 10^{-7} M β-endorphin was significant at 10^{-2} and 10^{-1} mg ml^{-1} ovalbumin and was 28% and 24% respectively. Naloxone itself significantly decreased anaphylactic contractions of the tracheal rings induced by 10^{-1} mg ml^{-1} ovalbumin by 30%. In the presence of naloxone (10^{-6} M), 10^{-7} M β-endorphin did not further suppress ovalbumin-induced contractions. The effects of β-endorphin

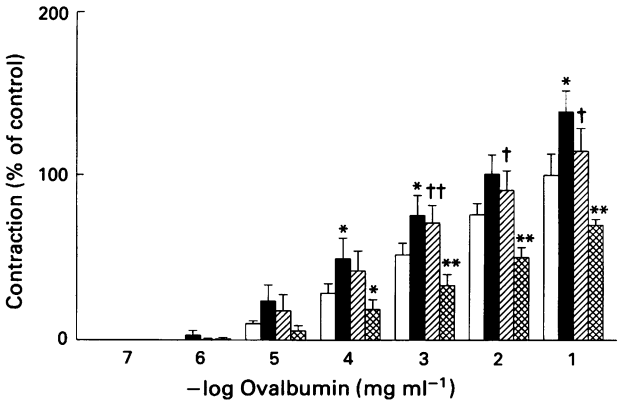

Figure 2 The effect of 10^{-8} M β-endorphin in the absence (filled columns) or presence (hatched columns) of 10^{-6} M naloxone on the anaphylactic contractions in tracheal rings isolated from actively sensitized guinea-pigs induced by cumulative concentrations of ovalbumin. The results ($n = 6$) are expressed as a percentage of the maximum ovalbumin-induced (10^{-1} mg ml^{-1}) contraction in the absence of β-endorphin and naloxone (open columns). *$P < 0.05$ and **$P < 0.01$ as compared to contractions of tracheal rings in the absence of β-endorphin and naloxone. †$P < 0.05$ and ††$P < 0.01$ as compared to contractions of tracheal rings in the absence of β-endorphin and in the presence of naloxone (cross-hatched columns).

Table 1 The effect of 10^{-8} M des-Tyr^1-β-endorphin on the anaphylactic contractions of tracheal rings induced by cumulative concentrations of ovalbumin

Ovalbumin (mg ml^{-1})	*Control*	*des-Tyr^1-β-Endorphin*
10^{-5}	1 ± 1	1 ± 1
10^{-4}	9 ± 2	13 ± 3
10^{-3}	36 ± 10	53 ± 8*
10^{-2}	66 ± 11	94 ± 14**
10^{-1}	100 ± 11	141 ± 7*

The results ($n = 4$) are expressed as a percentage of the maximum ovalbumin-induced (10^{-1} mg ml^{-1}) contraction in the absence of des-Tyr-β-endorphin (control).
*$P < 0.05$ and **$P < 0.01$ as compared to control.

and des-Tyr^1-β-endorphin (10^{-8} and 10^{-7} M) on ovalbumin-induced contractions of epithelium-denuded tracheae are summarized in Table 2. Ovalbumin-induced (10^{-1} mg ml^{-1}) contractions of tracheae in the absence of drugs (control) were 1.37 ± 0.18 g ($n = 7$) and 1.36 ± 0.11 g ($n = 5$) in experiments with β-endorphin and des-Tyr^1-β-endorphin respectively. In epithelium-denuded tracheal rings, β-endorphin (10^{-8} and 10^{-7} M) significantly suppressed anaphylactic contractions by about 20–25% (Table 2). des-Tyr^1-β-endorphin (10^{-8} and 10^{-7} M) did not affect ovalbumin-induced contractions in epithelium denuded tracheae.

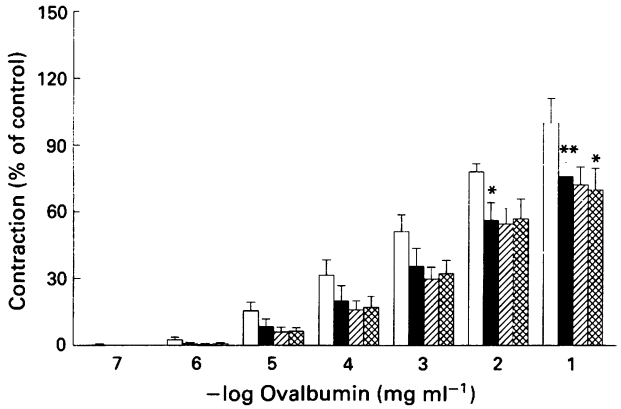

Figure 3 The effect of 10^{-7} M β-endorphin in the absence (filled columns) or presence (hatched columns) of 10^{-6} M naloxone on the anaphylactic contractions in tracheal rings isolated from actively sensitized guinea-pigs induced by cumulative concentrations of ovalbumin. The results ($n = 8$) are expressed as a percentage of the maximum ovalbumin-induced (10^{-1} mg ml^{-1}) contraction in the absence of β-endorphin and naloxone (open columns). *$P < 0.05$ and **$P < 0.01$ as compared to contractions of tracheal rings in the absence of β-endorphin and naloxone. Cross-hatched columns represent effects of naloxone alone.

Table 2 The effect of β-endorphin (β-End) and des-Tyr^1-β-endorphin (dTβ-End, 10^{-7} and 10^{-8} M) on the anaphylactic contractions of epithelium-denuded tracheal rings induced by 10^{-1} mg ml^{-1} of ovalbumin

Treatment	n	*Contraction* (% of control)
Control	7	100 ± 6
10^{-7} M β-End	7	78 ± 14*
10^{-8} M β-End	7	77 ± 3*
Control	5	100 ± 6
10^{-7} M dTβ-End	5	110 ± 10
10^{-8} M dTβ-End	5	102 ± 7

The results are expressed as a percentage of the ovalbumin-induced contraction in the absence of drugs (control).
*$P < 0.01$ as compared to control.

Table 3 The effect of β-endorphin (β-End, 10^{-8} and 10^{-7} M) on histamine- and leukotriene D_4-induced contraction of guinea-pig tracheal rings

Treatment	n	*Maximal response* (g)	EC_{50}
Histamine			($\times 10^{-6}$ M)
Control	4	1.76 ± 0.17	15.69 ± 2.27
10^{-8} M β-End	4	1.79 ± 0.11	15.75 ± 1.91
Control	3	2.13 ± 0.24	18.03 ± 3.25
10^{-7} M β-End	3	2.20 ± 0.25	17.33 ± 1.69
Leukotriene D_4:			($\times 10^{-8}$ M)
Control	4	0.55 ± 0.10	1.95 ± 0.70
10^{-8} M β-End	4	0.58 ± 0.06	1.82 ± 0.26
10^{-7} M β-End	4	0.64 ± 0.10	1.88 ± 0.45

The maximal response and the EC_{50} values of the histamine concentration-response curves are given. Leukotriene D_4 concentration-response curves were established with 10^{-7} M being the highest concentration. This concentration induces a near maximal contraction. The EC_{50} values for leukotriene D_4 are the concentrations that induce 50% of the contraction by 10^{-7} M. The results are expressed as the mean value of 3 or 4 experiments (n) performed in duplicate.

β-Endorphin (10^{-8} and 10^{-7} M) had no effect on the sensitivity or reactivity of the tracheal rings to histamine or leukotriene D_4 (Table 3).

Discussion

β-Endorphin, in the concentrations tested, while not affecting the tension of the tracheae when used alone, had a concentration-dependent bimodal influence on ovalbumin-induced contractions. At low concentrations (less than 10^{-7} M), β-endorphin potentiated anaphylactic contractions of the tracheae whereas higher concentrations suppressed contractions. Effects of β-endorphin on cell functions are either mediated by binding to classical opioid receptors or via a non-opioid receptor-mediated mechanism (De Wied & Jolles, 1982; Fischer, 1988; Sibinga & Goldstein, 1988). Binding of β-endorphin to classical opioid receptors requires an intact NH_2-terminal tyrosine and is sensitive to blockade by naloxone (De Wied & Jolles, 1982; Fischer, 1988; Sibinga & Goldstein, 1988). In our experiments, the non-selective opioid receptor antagonist naloxone did not inhibit the potentiation of the anaphylactic contraction induced by 10^{-8} M β-endorphin. Additionally, the non-opioid fragment des-Tyr^1-β-endorphin, derived from β-endorphin, appeared equally potent in enhancing anaphylactic contractions of the tracheae. Thus, potentiation of anaphylactic contractions by β-endorphin (10^{-8} M) is not mediated by classical opioid receptors. Furthermore, 10^{-8} M β-endorphin also seems to inhibit anaphylactic contractions through opioid receptors since the potentiation of the anaphylactic contraction with this concentration of β-endorphin is enhanced in the presence of naloxone. The suppression of the anaphylactic contraction by β-endorphin becomes predominant at 10^{-7} M β-endorphin and can be antagonized by naloxone. It can be concluded that β-endorphin potentiates anaphylactic contractions via a non-opioid receptor-mediated mechanism and at the same time suppresses anaphylactic contractions via classical opioid receptors.

In the presence of naloxone, to prevent the suppressive influence of β-endorphin, the anaphylactic contractions of the tracheae were not enhanced by 10^{-7} M β-endorphin. Thus, the potentiation of anaphylactic contractions with β-endorphin disappears at concentrations higher than 10^{-8} M. This indicates a bell-shaped concentration-response relationship for the potentiation of the anaphylatic contractions with β-endorphin. This agrees with our unpublished observations that 10^{-7} M des-Tyr^1-β-endorphin does not enhance anaphylactic contractions of guinea-pig isolated tracheae. A bell-

shaped concentration-response relationship for the effects of β-endorphin on immune cells has been described by several other groups (Sibinga & Goldstein, 1988). The reason for this phenomenon remains unclear.

Our data show that naloxone itself is also able to suppress anaphylactic contractions of the tracheae. The inhibition of anaphylactic contractions with naloxone does not necessarily imply that endogenous opioid peptides are involved, because naloxone, in high concentrations ($>10^{-4}$ M), has apparent opioid receptor agonistic activity and can modify the effects of a variety of non-opioid drugs (Badawy *et al.*, 1983; Swaynok *et al.*, 1979). Although we cannot rule out similar effects of naloxone in our experiments, it is unlikely since 10^{-6} M naloxone was used.

The bimodal influence of β-endorphin on the anaphylactic contractions in our study, may be due to an interaction with mast cells, smooth muscle cells or other cells present in tracheal rings. We demonstrated that 10^{-8} and 10^{-7} M β-endorphin do not affect the sensitivity and reactivity of tracheal rings to histamine or leukotriene D_4. Thus, an effect of β-endorphin on the responsiveness of smooth muscle to anaphylactic mediators seems unlikely. However, epithelial cells are involved in the β-endorphin-induced potentiation of anaphylactic contractions. This suggests that β-endorphin induces the production of a factor by epithelial cells that influences anaphylactic contractions, possibly through an interaction with mast cells. At present, nothing is known about epithelial-derived factors that can potentiate anaphylactic reactions. Another explanation for the epithelium-dependent potentiation of anaphylactic contractions could be that β-endorphin is cleaved into fragments by peptidases. One of these fragments may then be responsible for the potentiation of anaphylactic contractions. Aminopeptidases, endopeptidases and enkephalinases can degrade β-endorphin into fragments such as α- and γ-endorphin and the des-Tyr[1] fragments of these endorphins (De Wied & Jolles, 1982). Interestingly, the cell membrane-bound enzyme enkephalinase also called neutral endopeptidase is largely present in epithelial cells of the airways of many species, including guinea-pigs (Dusser *et al.*, 1989; Erdos & Skidgel, 1989). Besides epithelial cells, other cells in the airways also contain the enzyme enkephalinase such as nerves, smooth muscle cells and glands (Dusser *et al.*, 1989), which makes this possibility less likely.

It has been shown that opioid peptides like β-endorphin can affect mediator release of peritoneal mast cells (Mediratta *et al.*, 1988; Sydbom, 1988). However, these data cannot be directly extrapolated to the airways due to tissue-related mast cell heterogeneity (Shanahan *et al.*, 1984; Bienenstock, 1988). In rodents, the mast cells present in the airways are of the mucosal type (Bienenstock, 1988; Kitamura, 1989). Shanahan *et al.* (1984) compared endorphin-induced histamine release from peritoneal and mucosal mast cells in the rat and demonstrated that only peritoneal mast cells responded to endorphins. Recently, Miadonna *et al.* (1988) demonstrated that opioid peptides neither elicited histamine release from human lung mast cells nor influenced IgE-mediated histamine release. In our experiments, tracheal mast cell mediator release was not studied. Therefore, no conclusion can be drawn as to whether β-endorphin directly or indirectly affects mast cell mediator release.

The opioid peptide β-endorphin is released from the anterior pituitary into the blood depending on autonomous rhythms and psychophysiological state (Fischer, 1988; Sibinga & Goldstein, 1988). The level of circulating β-endorphin is increased after stress, exercise, or endotoxin injection (Fischer, 1988; Sibinga & Goldstein, 1988). Opioid peptides may play a role in the pathophysiology of asthma. Firstly, Popa & Rients (1989) recently demonstrated that exercise-induced bronchoconstriction in asthmatic patients could be partially prevented by the opiate-receptor antagonist naloxone. Secondly, opioid peptides can be produced by immune cells such as lymphocytes and monocytes or macrophages (Smith *et al.*, 1985; Lolait *et al.*, 1986; Sibinga & Goldstein, 1988). Furthermore, it has recently been demonstrated that preproenkephalin mRNA is present in a mast cell line (Martin *et al.*, 1987). These data may point to a local production of opioid peptides in airway inflammation, which is common in asthma.

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(Received November 12, 1990
Accepted January 24, 1991)

Br. J. Pharmacol. (1991), **103**, 1475–1483 © Macmillan Press Ltd, 1991

A comparison of the effects of angiotensin II and Bay K 8644 on responses to noradrenaline mediated via postjunctional α_1- and α_2-adrenoceptors in rabbit isolated blood vessels

[1]W.R. Dunn, C.J. Daly, J.C. McGrath & [2]V.G. Wilson

Autonomic Physiology Unit, Institute of Physiology, University of Glasgow, Glasgow G12 8QQ

1 The effects of angiotensin II (AII) and Bay K 8644 on responses to noradrenaline (NA) mediated via postjunctional α_1- and/or α_2-adrenoceptors have been compared in three isolated venous preparations from the rabbit, the lateral saphenous vein, the left renal vein and the ear vein.

2 A similar action of AII and Bay K 8644 was observed only in the lateral saphenous vein; each potentiated responses to NA after isolation of a homogeneous population of postjunctional α_2- adrenoceptors. However, even in this preparation the mechanism of action for these agents was not identical. The sensitivity of KCl-induced contraction to changes in extracellular calcium ions (reflecting activation of voltage-dependent Ca^{2+} channels) was enhanced by Bay K 8644 but reduced by AII.

3 AII produced a selective facilitation of responses mediated via postjunctional α_2-adrenoceptors. In the lateral saphenous vein it reduced the effectiveness of prazosin and facilitated responses after isolation of α_2-adrenoceptors with phenoxybenzamine and rauwolscine. It directly enhanced responses to NA in the ear vein, where only α_2-adrenoceptors are involved. In contrast, AII did not influence responses mediated via postjunctional α_1-adrenoceptors in the left renal vein (even after the receptor reserve had been removed with phenoxybenzamine) nor the 'rauwolscine-resistant' component of responses to NA in the saphenous vein.

4 Bay K 8644 enhanced contractile responses to NA mediated both via α_2-adrenoceptors, in the lateral saphenous vein, and via α_1-adrenoceptors in the left renal vein. Thus, unlike angiotensin II, no preferential effect was apparent.

5 Bay K 8644 was inactive against responses to NA in the rabbit isolated ear vein. Since the sustained component of responses to NA in this preparation is dependent upon the influx of extracellular Ca^{2+}, these observations suggest that the influx of Ca^{2+} stimulated by NA is mediated via receptor-operated (1,4-dihydropyridine-resistant) Ca^{2+} channels.

Keywords: Noradrenaline; angiotensin II; Bay K 8644; postjunctional α_1-adrenoceptors; postjunctional α_2-adrenoceptors

Introduction

Both postjunctional α_1- and α_2-adrenoceptor-mediated pressor responses can be clearly demonstrated in whole animals (McGrath *et al.*, 1989). Since their discovery it has been suggested that a number of factors can differentially modulate responses to α-adrenoceptor agonists, such as blood pH and blood gases (McGrath *et al.*, 1982; Grant *et al.*, 1985), calcium channel blockers (CCBs) (van Meel *et al.*, 1981) or angiotensin converting enzyme (ACE) inhibitors (de Jonge *et al.*, 1982; Timmermans *et al.*, 1982). It was initially suggested that α_2-adrenoceptor-mediated responses were more susceptible to the action of CCBs and ACE inhibitors than were equivalent sized α_1-adrenoceptor-mediated responses (van Meel *et al.*, 1981; de Jonge *et al.*, 1982; Timmermans *et al.*, 1982), although there is now evidence that both CCBs and ACE inhibitors preferentially inhibit the late phase of the pressor response to a bolus injection of an agonist, irrespective of subtype specificity (O'Brien *et al.*, 1985; McGrath & O'Brien, 1987). The study of modulatory influences on responses mediated via different adrenoceptor subtypes in models such as the pithed rat is limited, however, by a number of factors. These include the biphasic nature of responses to the bolus injection of an agonist, the non-'equilibrium' nature of such responses and by the effects of modulatory drugs, such as ACE inhibitors, on basal blood pressure.

While postjunctional α_1-adrenoceptors are easily demonstrated in isolated blood vessels, there are relatively few examples of α_2-adrenoceptor-mediated contractions *in vitro* (e.g. Constantine *et al.*, 1982; Daly *et al.*, 1988a). Therefore, comparative studies of the effects of angiotensin II (AII) and calcium channel facilitators on responses mediated via postjunctional α_1- and α_2-adrenoceptors in isolated blood vessels have not previously been attempted. However, in the rabbit lateral saphenous vein, a preparation containing both α-adrenoceptor subtypes (Daly *et al.*, 1988b), AII renders responses to B-HT 920 resistant to the α_1-adrenoceptor antagonist prazosin, suggesting a selective enhancement of α_2-adrenoceptor-mediated contractions (Schumman & Lues, 1983). Furthermore, two recent studies suggest a similarity of action for AII and Bay K 8644 in isolated vascular preparations: Sulpizio & Hieble (1987) have shown that Bay K 8644 introduces responses mediated via postjunctional α_2-adrenoceptors in the canine saphenous artery, while Dunn *et al.* (1989) have observed a similar action for AII in the corresponding arterial preparation from the rabbit. Thus, the facilitatory effect of AII on α_2-adrenoceptor-mediated responses might be attributable to an action on dihydropyridine-sensitive Ca^{2+}-channels.

In the present study we have examined the influence of AII and Bay K 8644 on responses to noradrenaline (NA) mediated via either α_1- or α_2-adrenoceptors in three isolated venous preparations from the rabbit: (1) the rabbit isolated lateral saphenous vein, a preparation containing both α-adrenoceptor subtypes, before and after isolation of a homogeneous population of postjunctional α_2-adrenoceptors (see Daly *et al.*, 1988c); (2) the rabbit ear vein, which contains almost exclusively α_2-adrenoceptors (Daly *et al.*, 1988a) and (3) the rabbit renal vein, a preparation containing a homogeneous population of α_1-adrenoceptors (Daly *et al.*, 1988d).

[1] Present address: Department of Pharmacology, University of Vermont, Given Medical Building, Burlington, Vermont, 05405, U.S.A.

[2] Author for correspondence at present address: Department of Physiology and Pharmacology, Medical School, Queens Medical Centre, Clifton Boulevard, Nottingham, NG7 2UH.

Methods

White albino rabbits of either sex weighing 2.3–3.0 kg were killed by stunning followed by exsanguination. Segments of the lateral saphenous vein, left renal vein or ear vein were cleaned of fat and connective tissue *in situ* and placed in an ice-cold Krebs bicarbonate saline. When necessary, preparations were cleaned further with the aid of a dissecting microscope. Segments (3–5 mm in length) were taken from each vein and suspended between two 0.2 mm thick wire supports as described by Hooker *et al.* (1976). The upper support was connected by cotton to a Grass FT03 isometric transducer while the lower support was connected to a glass tissue holder. The preparations were then mounted in 30 ml isolated organ baths under an initial resting tension of 2 g wt. (lateral saphenous vein), 0.5 g wt. (left renal vein), 0.3 g wt. (ear vein) respectively and allowed to relax. Each preparation was bathed in Krebs solution maintained at 37°C and gassed with 95% O_2 plus 5% CO_2.

After a 60 min equilibration period, during which a steady resting tension was achieved, each preparation was exposed to 3 μM NA and allowed to contract for 10 min. Following complete washout, an additional 1 h equilibration period was allowed before starting the experiment. This procedure was found to minimize changes in the sensitivity of the preparation to further addition of agonists and is similar to the method of Ruffolo *et al.* (1979). Basal tension following the sighting response remained stable for the rest of the experiment at 0.3–0.4 g wt. (lateral saphenous vein), 0.15–0.2 g wt. (left renal vein) and 0.07–0.1 g wt. (ear vein); values previously found to be optimal for maximum responsiveness (Daly *et al.*, 1988d). Isometric contractions were recorded on a Linseis 6052 pen recorder. In all experiments cumulative concentration-response curves (CCRCs) to agonists were constructed by increasing the concentration of agonist in the organ bath by approximately 3 fold increments. In the majority of preparations, contractile responses to NA were not sustained and so addition of the next concentration was made as close to the peak as possible. Following attainment of the maximal control contraction, preparations were washed until complete relaxation was effected and 30–60 min later one of the following experimental protocols was adopted:

(1) The lateral saphenous vein, left renal vein or ear vein were exposed to either AII or Bay K 8644 15 min before the start of a second CCRC to NA.

(2) The left renal vein was exposed to phenoxybenzamine (1 nM) for a period of 30 min. The tissues were subsequently washed at least 4 times and 30 min later a CCRC to NA was constructed. After washout and a further 60 min equilibration period another CCRC to NA was constructed. In some experiments AII was added 15 min prior to the final NA CCRC.

(3) In the lateral saphenous vein, preparations were exposed to various concentrations of prazosin or rauwolscine for a minimum period of 45 min and the NA CCRC repeated. In some tissues, AII (0.05 μM) or Bay K 8644 (0.03 μM) was added to the organ bath 15 min prior to the onset of the second CCRC to NA, in the continued presence of antagonist.

(4) A population of postjunctional α_2-adrenoceptors was isolated in the lateral saphenous vein as previously described (Daly *et al.*, 1988c). Briefly, tissues were exposed to rauwolscine (1 μM) 5 min prior to phenoxybenzamine (0.3 μM) which was left in contact with the tissues for a further 30 min. The tissues were then washed a minimum of seven times over a 45 min period. During the first two washes rauwolscine (1 μM) was present to maximize protection of α_2-adrenoceptor. After a further 30 min period a CCRC was obtained to NA. The residual response remaining after the protection protocol is resistant to prazosin but susceptible to rauwolscine (Daly *et al.*, 1988c). After attainment of the maximum response to NA the preparations were washed until complete relaxation was effected. A further period of 60 min was allowed before construction of another CCRC to NA. In some experiments AII or Bay K 8644 was added 15 min prior to the onset of the final NA CCRC. In others saralasin (0.1 μM) was added 10 min prior to AII.

(5) Some experiments were performed on the lateral saphenous vein to examine the effects of AII and Bay K 8644 on the influx of extracellular Ca^{2+} into the cell stimulated either by depolarization or by postjunctional α_2-adrenoceptors. CCRCs to Ca^{2+} were constructed following exposure to nominally Ca^{2+}-free Krebs solution with either 65 mM KCl (exchanged with NaCl on an equimolar basis) or 30 μM NA (after isolation of postjunctional α_2-adrenoceptors) added 10 min prior to the start of the Ca^{2+} CCRC. Following attainment of the control maximum response, tissues were washed in nominally Ca^{2+}-free Krebs solution (containing 23 μM EDTA) until complete relaxation was affected and allowed to bathe in this solution. A second Ca^{2+} CCRC (in the presence of high K^+ or NA) was obtained 45–60 min later in the presence of AII or Bay K 8644.

All responses are expressed as a percentage (mean ± s.e.mean) of the maximum response of the first concentration-response curve to NA, or to Ca^{2+} re-addition, as appropriate, obtained in each tissue. Differences between means were considered statistically significantly different if $P < 0.05$ for either paired or unpaired observations (Student's t test).

The composition of the modified Krebs-Henseleit saline was (mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4:7H_2O$ 1.2, $NaHCO_3$ 24.9, KH_2PO_4 1.2 and glucose 11.1. Na_2EDTA 23 μM was included in all experiments to prevent oxidative degradation of NA. Propranolol 1 μM and cocaine 10 μM were included to inhibit β-adrenoceptors and uptake$_1$, respectively. The following compounds were used: (−)-noradrenaline bitartrate (Sigma), phenoxybenzamine HCl (Smith, Kline & French), rauwolscine HCl (Roth), angiotensin II amide (Ciba), Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate; Bayer), (±)-propranolol HCl (Sigma), cocaine HCl (Macarthys), saralasin ([Sar^1, Ala^8]-angiotensin II) (Sigma), and prazosin HCl (Pfizer). With the exception of Bay K 8644 and phenoxybenzamine, all drugs were dissolved in distilled water and added to the organ bath in volumes of 0.3 ml or less. Stock solutions of phenoxybenzamine (1 mM) were prepared in 20% absolute ethanol in distilled water and a drop of 1 N HCl was added to remove turbidity. Stock solutions of Bay K 8644 were prepared in 20% absolute ethanol. Further dilutions of both drugs were made in distilled water.

Results

Lateral saphenous vein

Responses to noradrenaline NA (1 nM–10 μM) produced concentration-dependent contractions of the lateral saphenous vein (Figure 1a). Successive concentration-response curves were associated with an increase in the maximum response (< 10% of the 1st maximum), without a change in the pD_2 of the agonist. AII (0.005–0.5 μM) produced concentration-dependent contractions in the lateral saphenous vein, the maximum response of which ranged between 25–40% of the maximum response to NA. Responses to high concentrations of AII (0.05–0.5 μM), consisted of an initial rapid response which returned to baseline after 6–8 min. Lower concentrations produced contractions which were slower in onset, longer lasting and returned to baseline after 12–15 min. Baseline tension remained stable after the transient contraction produced by AII, for at least the duration of the experiment.

AII (0.05–0.5 μM) had no effect on responses to NA (Figure 1a). In marked contrast, 30 nM Bay K 8644, which did not produce a contractile response, increased both the sensitivity

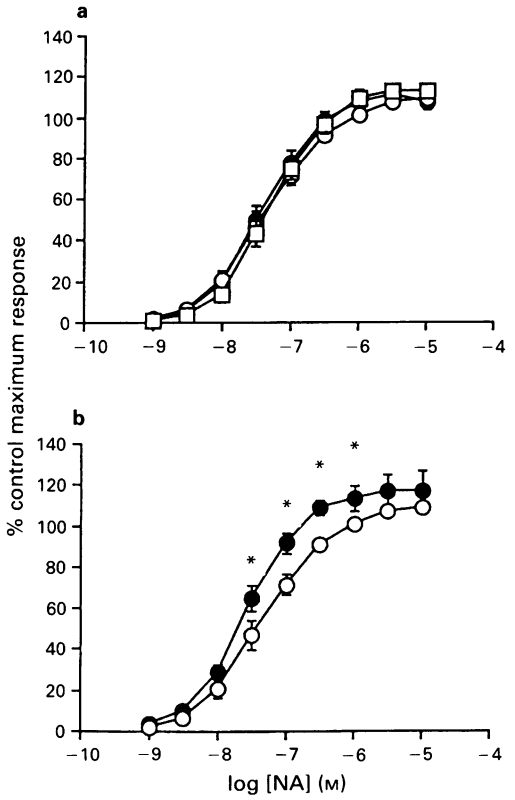

Figure 1 The effects of (a) angiotensin II and (b) Bay K 8644 on noradrenaline (NA)-induced contractile responses of the rabbit isolated saphenous vein. (a) The effect of noradrenaline in the absence (○) and in the presence of 0.05 μM (●) and 0.5 μM (□) angiotensin II. (b) The effect of noradrenaline in the absence (○) and in the presence of 0.03 μM Bay K 8644 (●). All responses have been expressed as a percentage of the maximum response in the first concentration-response curve. Each point represents the mean of $n = 6$–10; s.e.mean shown by vertical bars. * Indicates a significant difference ($P < 0.05$) between responses to noradrenaline in presence and absence of the ancillary agent.

and the maximum response of the preparation to NA (Figure 1b, Table 1). High concentrations of Bay K 8644 (>0.3 μM) induced spontaneous contractions in the lateral saphenous vein.

α-Adrenoceptor antagonist potency In a previous study in the rabbit isolated saphenous vein, it was found that the presence of AII rendered contractile responses to the 'selective' α_2-adrenoceptor agonist, BHT-920, more resistant to prazosin, suggesting a selective facilitation of α_2-adrenoceptor-mediated responses (Schümann & Lues, 1983).

In the present study, the inhibition of NA CCRCs produced by 0.01 μM prazosin was not significantly affected by 0.05 μM AII (Figure 2a), but that produced by 0.1 μM and 1 μM prazosin, particularly at low NA concentrations, was significantly less in the presence of the peptide (Figures 2b and c). The log concentration-ratio for NA produced by 0.1 μM prazosin (measured at the 25% level of the maximum response), was less in the presence of 0.05 μM AII but this did not reach significance (Table 2). Similarly, although the pA_2 value for prazosin (8.19: 8.67–7.72, 95% confidence limits) was reduced in the presence of 0.05 μM AII (7.75: 7.84–7.26, 95% confidence limits), this did not reach significance. Furthermore, the slopes of the Schild plot for prazosin in either the presence (0.81: 0.68–0.93, 95% confidence limits) or absence (0.63: 0.51–0.72, 95% confidence limits) of AII were significantly different from unity, but not from each other.

In marked contrast to the findings in the presence of prazosin, AII (0.05 μM) was without effect on the inhibition produced by the selective α_2-adrenoceptor antagonist rauwolscine: neither the rauwolscine-sensitive nor the rauwolscine-resistant components were affected (Figure 3, Table 1). Bay K 8644 (0.03 μM), significantly reduced the inhibitory potency of prazosin against NA contractions (Table 2), such that 1 μM prazosin failed to produce any greater inhibition than that observed with 0.1 μM prazosin (Figures 4a and b). Thus, in the presence of Bay K 8644 it was no longer possible to determine a pA_2 value for prazosin against NA.

After α_2-adrenoceptor isolation After isolation of postjunctional α_2-adrenoceptors, both AII and Bay K 8644 markedly enhanced responses to NA (Figure 5, Table 1). This was associated with an increase in both the sensitivity and

Table 1 pD_2 values for noradrenaline (NA) (± s.e. of the mean) and E_{max} values (control maximum after correction for time related changes, $E_{max} = 1$) in the rabbit isolated lateral saphenous vein (LSV), lateral saphenous vein after receptor protection with rauwolscine (1 μM) and phenoxybenzamine (0.3 μM) (LSVRP), left renal vein (LRV), left renal vein post treatment with phenoxybenzamine (1 nM) (LRVP) and the ear vein (EV)

		pD_2 value		E_{max}
Preparation	n	*Control*	AII	AII
LSV	10	7.31 ± 0.08	7.40 ± 0.10	1.05 ± 0.05
LSVRP	13	6.83 ± 0.06	7.18 ± 0.08***	1.18 ± 0.03***
LRV	5	6.12 ± 0.19	6.12 ± 0.11	1.02 ± 0.04
LRVP	5	5.76 ± 0.16	5.85 ± 0.17	0.93 ± 0.05
EV	10	7.62 ± 0.07	7.80 ± 0.09*	1.03 ± 0.07
	n	*Control*	*Bay K 8644*	*Bay K 8644*
LSV	6	7.28 ± 0.13	7.53 ± 0.10*	1.22 ± 0.05**
LSVRP	6	6.81 ± 0.16	7.23 ± 0.20***	1.25 ± 0.05***
LRV	7	6.06 ± 0.08	6.24 ± 0.10*	1.30 ± 0.03***
EV	5	7.82 ± 0.09	7.79 ± 0.05	1.09 ± 0.11

Differences between control and treated preparations were considered statistically significant if $P < 0.05$ for either paired or unpaired observations (Student's t test) and are denoted by: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 2 Log agonist concentration-ratio values for noradrenaline (NA) measured at the 25%, 50% and 75% level of the maximum response for NA in the presence of prazosin (0.1 μM) or rauwolscine (0.05 μM) and the effects of angiotensin II (AII, 0.05 μM) and Bay K 8644 (0.03 μM) thereon

Antagonist	n	25%	50%	75%
Prazosin (0.1 μM)	12	0.81 ± 0.07	0.69 ± 0.07	0.64 ± 0.10
Prazosin (0.1 μM) with AII (0.05 μM)	7	0.60 ± 0.09	0.57 ± 0.08	0.59 ± 0.09
Prazosin (0.1 μM) with Bay K 8644 (0.03 μM)	5	0.49 ± 0.08*	0.35 ± 0.09*	0.33 ± 0.08*
Rauwolscine (0.5 μM)	5	1.47 ± 0.14	1.70 ± 0.12	1.85 ± 0.09
Rauwolscine (0.5 μM) with AII (0.05 μM)	5	1.38 ± 0.18	1.73 ± 0.08	1.86 ± 0.07

* Denotes a significant difference in the value for prazosin (0.1 μM) calculated at the 25%, 50% and 75% levels of the maximum response to NA in the presence of Bay K 8644 compared to control. $P < 0.05$ Student's t test.

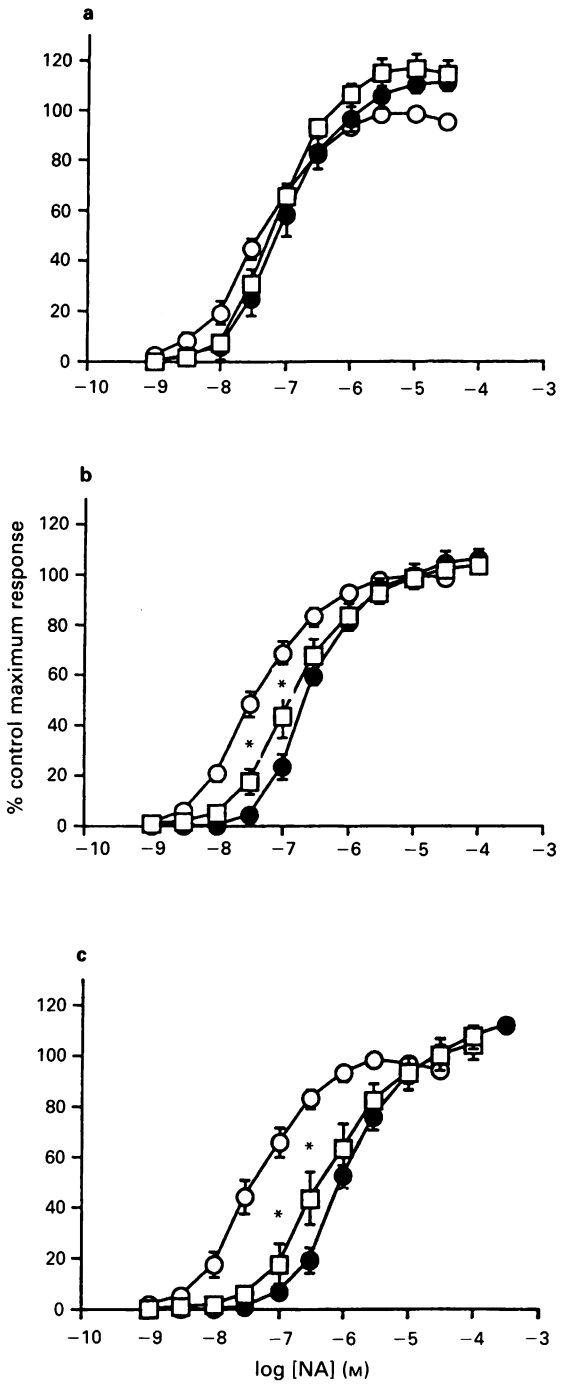

Figure 2 The effects (a) 0.01 μM, (b) 0.1 μM and (c) 1 μM prazosin against noradrenaline (NA)-induced contractions of the rabbit isolated saphenous vein in the absence (●) and in the presence of (□) 0.05 μM angiotensin II. The control concentration-response curve to NA prior to exposure to prazosin and angiotensin II is represented by (○). Each point represents the mean of 5–6 observations; vertical bars show s.e.mean. * Indicates a significance difference ($P < 0.05$) between responses to noradrenaline remaining after prazosin in presence and absence of angiotensin II.

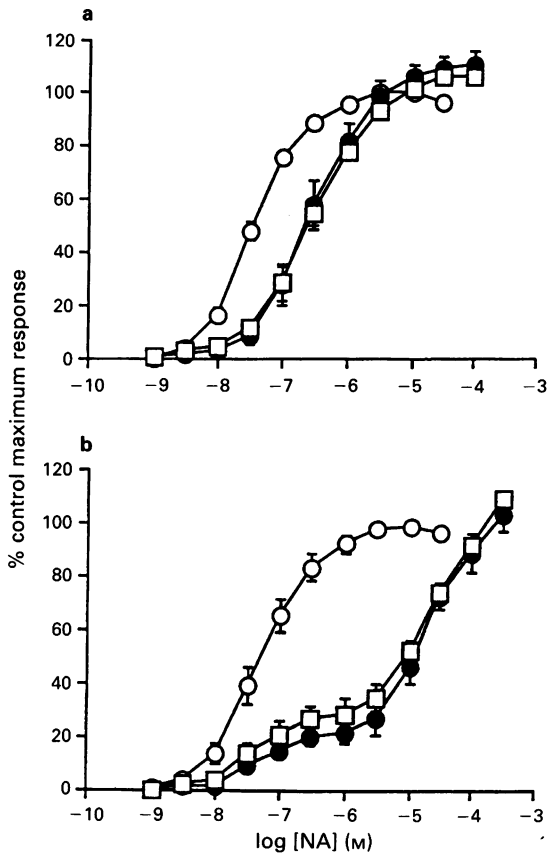

Figure 3 The effects of (a) 0.05 μM and (b) 2.5 μM rauwolscine against noradrenaline (NA)-induced contractions of the rabbit isolated saphenous vein in the absence (●) and presence (□) of 0.05 μM angiotensin II. The control concentration-response curve to NA prior to exposure to rauwolscine and angiotensin II is represented by (○). Each point represents the mean of 5–6 observations; vertical bars show s.e.mean.

maximum response of the preparation to NA. It is interesting to note that this facilitatory action of AII was observed with both 0.05 μM and 5 nM AII, the latter concentration producing a small contraction in only 2 of 6 preparations. The potentiation of α_2-adrenoceptor-mediated responses produced by AII was not observed if the angiotensin receptor antagonist, saralasin (0.1 μM) was included.

Sensitivity to Ca^{2+}; comparison of direct depolarization and α_2-adrenoceptor activation The superficial similarity of the action of AII and Bay K 8644 in relation to the α_2-adrenoceptor function in the lateral saphenous vein (both agents enhanced responses) prompted a study of their effects on the influx of extracellular Ca^{2+} associated with either direct depolarization (KCl) or activation of postjunctional α_2-adrenoceptors by NA.

After prolonged exposure to 'nominally Ca^{2+}-free' Krebs-Henseleit saline, no response was observed to the addition of either 0.03 μM Bay K 8644 or 0.05 μM AII, nor to the subsequent addition of extracellular Ca^{2+} ions (0.01 mM–2.5 mM). In marked contrast, the exchange of 'nominally Ca^{2+}-free' Krebs-Henseleit for 65 mM KCl 'nominally Ca^{2+}-free' Krebs-Henseleit solution (with equimolar replacement of NaCl) was associated with a small contraction in most preparations (equivalent to 5–10% of the maximum response observed upon readdition of 2.5 mM Ca^{2+}). Subsequent addition of Ca^{2+} ions (0.01 mM–2.5 mM) was associated with concentration-related contractions (Figure 6a). Consecutive Ca^{2+} CCRCs in the presence of 65 mM KCl were superimposable (Figure 6a).

The addition of AII (0.05 μM) prior to the second Ca^{2+} CCRC was associated with a significant rightward displacement of the Ca^{2+} CCRC (pD_2 for Ca^{2+} = 3.49 ± 0.07 and 3.29 ± 0.07, $n = 9$; $P < 0.05$. Figure 6c) without altering the maximum response. In contrast, Bay K 8644 (0.03 μM) failed to alter the sensitivity of the depolarized preparation to Ca^{2+} (pD_2 = 3.47 ± 0.06 and 3.46 ± 0.06, $n = 8$) but produced a small but significant increase in the contractile response to Ca^{2+} readdition (Figure 6c).

After isolation of postjunctional α_2-adrenoceptors and exposure to 'nominally Ca^{2+}-free Krebs solution', NA (30 μM) produced a small transient contraction (7.7 ± 1.9% of the maximum response to readdition of Ca^{2+} ions, $n = 9$), which

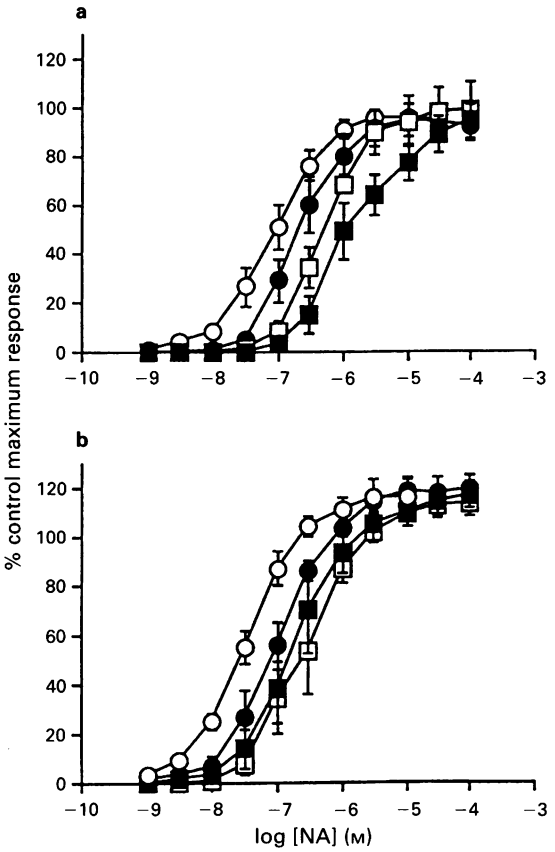

Figure 4 The effects of 0.01 μM (●), 0.1 μM (□) and 1 μM (■) prazosin against noradrenaline (NA)-induced contractions of the rabbit isolated saphenous vein in (a) the absence and (b) the presence of 0.03 μM Bay K 8644. The control concentration-response curve to NA prior to exposure to prazosin is represented by (○), and all responses have been expressed as a percentage of the first concentration-response curve in the absence of both prazosin and Bay K 8644. Each point represents the mean of 5–6 observations; vertical bars show s.e.mean.

returned to baseline within 10 min. Consecutive CCRCs to the re-addition of Ca^{2+} under these conditions was associated with a small reduction in the maximum attainable response, but no change in sensitivity (Figure 6b). AII (0.05 μM) produced a small increase in the maximum response to the re-addition of Ca^{2+}, although this was not associated with a change in sensitivity of the preparation to Ca^{2+} (Figure 6d). In contrast Bay K 8644 increased both the sensitivity and the maximum response to the re-addition of Ca^{2+}, upon stimulation of postjunctional α_2-adrenoceptors with NA (Figure 6d).

Ear vein

NA produced concentration-dependent contractions in the isolated ear vein, a preparation containing predominantly postjunctional α_2-adrenoceptors (Daly *et al.*, 1988a). Consecutive CCRCs to NA were not reproducible with time: there was an approximately 25% increase in the maximum response, but no change in sensitivity (pD_2 for NA, 7.82 ± 0.08 and 7.83 ± 0.08 for curves 1 and 2 respectively, $n = 11$). AII 0.05 μM, produced a transient contraction equivalent to 46.3 ± 8.3% of the NA maximum response ($n = 11$) and, after correcting for time-related changes, produced a small, but significant, increase in the sensitivity of the preparation to NA (pD_2 for NA 7.62 ± 0.07 and 7.84 ± 0.08 in the absence and presence of 0.05 μM AII, $P < 0.05$ for paired Student's t test, $n = 10$). In contrast, the addition of Bay K 8644 (0.03 μM) was not associated with a contractile response and failed to alter responses to NA.

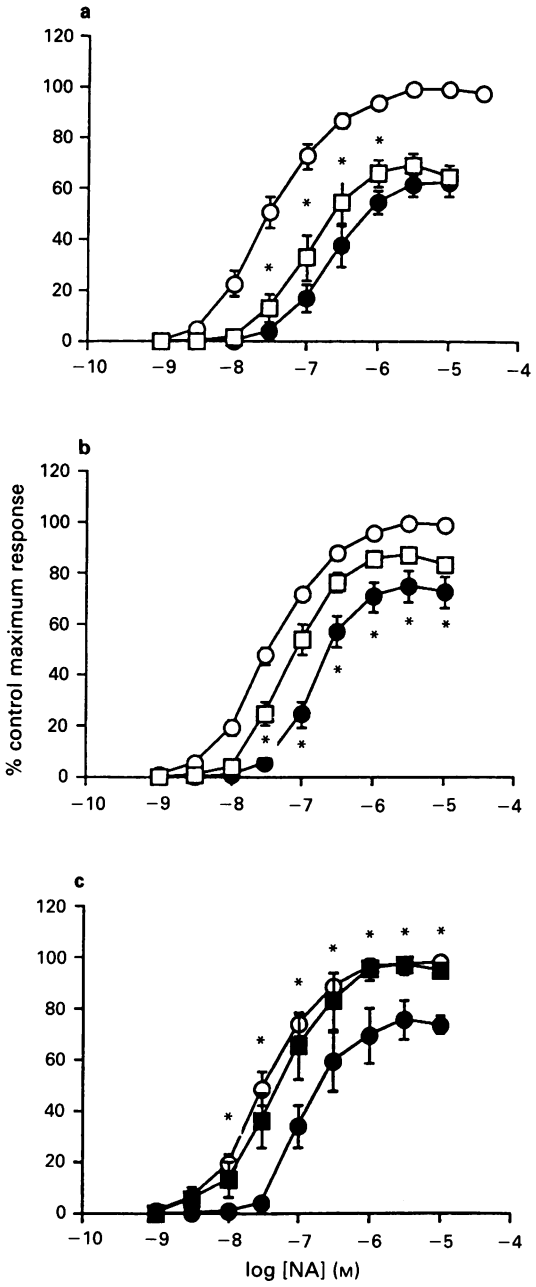

Figure 5 The effects of angiotensin II and Bay K 8644 on noradrenaline (NA)-induced contractile responses of the rabbit isolated saphenous vein after isolation of postjunctional α_2-adrenoceptors. (a) Noradrenaline-induced contractions in the (●) absence and in the (□) presence of 5 nM angiotensin II. (b) Noradrenaline-induced contraction in the (●) absence and in the (□) presence of 0.05 μM angiotensin II. (c) Noradrenaline-induced contractions in the (●) absence and in the (■) presence of 0.03 μM Bay K 8644. All responses have been expressed as a percentage of the maximum response in the first concentration-response curve (○) prior to the receptor protection procedure. Each point represents the mean of $n = 6$–13; vertical bars show s.e.mean. * Indicates a significant difference ($P < 0.05$) between responses to noradrenaline in presence and absence of the ancillary agent.

Left renal vein

The rabbit isolated left renal vein contains a homogeneous population of postjunctional α_1-adrenoceptors (Daly *et al.*, 1988d). Consecutive CCRCs to NA in this preparation were reproducible. AII (0.05 μM) produced a transient contraction in the left renal vein, which was equivalent to 31.9 ± 8.7% of the NA maximum response ($n = 11$). This concentration of AII was without effect on responses to NA (Figure 7a). In

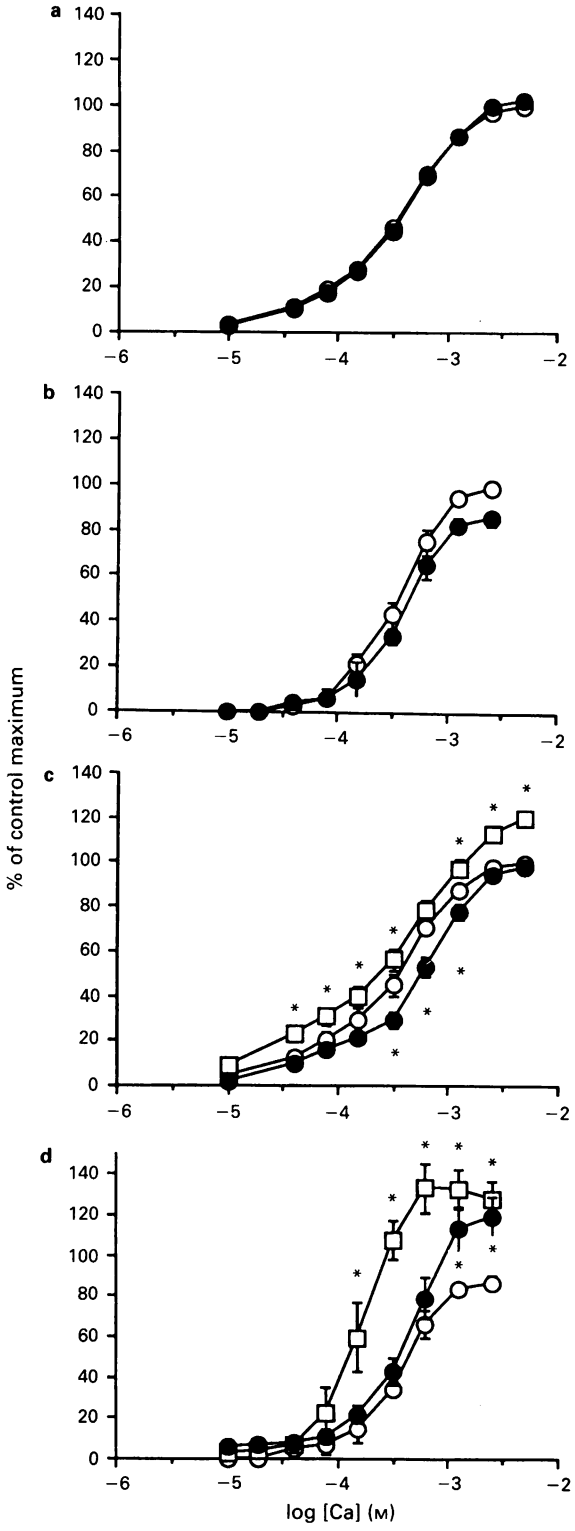

Figure 6 The effect of angiotensin II and Bay K 8644 on Ca^{2+} concentration-response curves in the rabbit isolated saphenous vein following depolarization or α_2-adrenoceptor stimulation. (a,b) The reproducibility of Ca^{2+} concentration-responses curves following exposure to nominally Ca^{2+}-free Krebs-Henseleit saline and then (a) 65 mM KCl Krebs-Henseleit saline or (b) α_2-adrenoceptor stimulation with 30 μM noradrenaline (NA): (○) 1st concentration-response curve and (●) 2nd concentration-responses curve. (c,d) The effects of (●) 0.05 μM angiotensin II and (□) 0.03 μM Bay K 8644 on contractile responses elicited by the re-addition of Ca^{2+}, following exposure to 'nominally Ca^{2+}-free' Krebs-Henseleit saline, in the presence of (c) 65 mM KCl Krebs-Henseleit saline and (d) 30 μM NA after isolation of postjunctional α_2-adrenoceptors. Results are expressed as a % of the maximum response in the control curve for Ca^{2+} readdition. Each point represents the mean of $n = 6$–8; vertical bars show s.e.mean. * Indicates a significant difference ($P < 0.05$) between responses to noradrenaline in presence and absence of the ancillary agent.

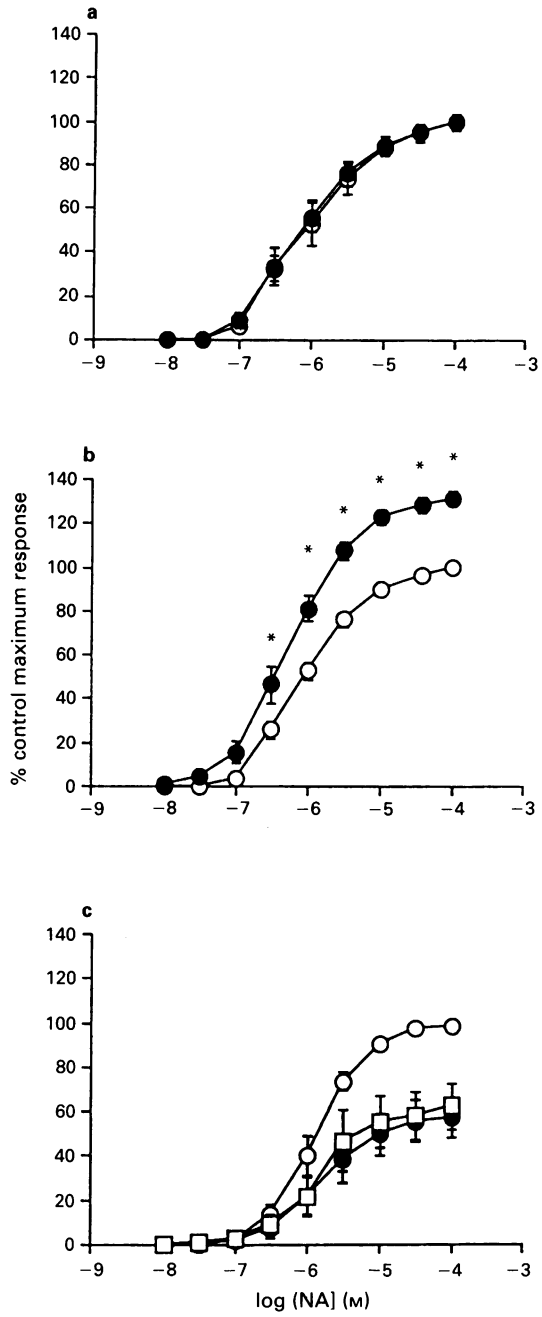

Figure 7 The effects of angiotensin II and Bay K 8644 on noradrenaline (NA)-induced contractions of the rabbit isolated left renal vein. (a) Responses to noradrenaline in the (●) presence and (○) absence of 0.05 μM angiotensin II. (b) Responses to noradrenaline in the (●) presence and (○) absence of 0.3 μM Bay K 8644. (c) Responses to noradrenaline, following 30 min exposure to 1 nM phenoxybenzamine and subsequent washout, in the (●) presence and (□) absence of 0.05 μM angiotensin II. Responses to noradrenaline prior to exposure to phenoxybenzamine are represented by (○). Each point represents the mean of $n = 5$–7; vertical bars show s.e.mean. * Indicates a significant difference ($P < 0.05$) between responses to noradrenaline in presence and absence of the ancillary agent.

contrast, Bay K 8644 (0.03 μM), which again had no effect on resting baseline tension, increased the sensitivity and the maximum response of the preparation to NA (Figure 7b).

It has previously been shown that reducing the receptor reserve in the rabbit femoral artery increased the facilitatory effect of AII on responses to NA mediated via α_1-adrenoceptors (Purdy & Weber, 1988). Therefore, the left renal vein was exposed to phenoxybenzamine (1 nM) for 30 min to reduce the receptor reserve. This produced a reduction of 42.0 $\pm$ 9.3% (range 17–75%, $n = 6$) in the maximum response

to NA (Figure 7c), although there was no change in the sensitivity of the preparation to NA (pD_2 for NA, 5.91 ± 0.04 and 5.92 ± 0.11, before and after treatment with phenoxybenzamine respectively, $n = 6$). In two further preparations, phenoxybenzamine abolished all responses and in another it failed to reduce the maximum response. These results have been excluded from the analysis. Even under these conditions, AII (0.05 μM) had no effect on the residual response remaining after phenoxybenzamine (Figure 7c).

Discussion

Comparison of the actions of angiotensin II and Bay K 8644

ACE inhibitors and Ca^{2+} channel blockers (CCB) have qualitatively similar effects on α-adrenoceptor-mediated pressor responses in the pithed rat (van Meel *et al.*, 1981; De Jonge *et al.*, 1981; 1982; O'Brien *et al.*, 1985). In complementary experiments on isolated blood vessels, the Ca^{2+} channel facilitator Bay K 8644 and AII have been shown to 'unmask' responses mediated via postjunctional α_2-adrenoceptors in the canine and rabbit saphenous arteries, respectively; effects produced in the absence of a sustained elevation of vascular tone (Sulpizio & Hieble, 1987; Dunn *et al.*, 1989). Furthermore, in the rabbit isolated saphenous vein the presence of both AII and Bay K 8644 was associated with a reduction in the inhibitory potency of the α_1-adrenoceptor antagonist, prazosin (present study). Taken together, these observations suggest that both agents may influence α-adrenoceptor-mediated responses via a common mechanism involving dihydropyridine-sensitive Ca^{2+} channels.

However, a comparison of the effects of AII and Bay K 8644 in three isolated venous preparations from the rabbit revealed several differences. With the exception of the ear vein, Bay K 8644 produced a small but significant enhancement of responses to NA in all preparations tested irrespective of the receptor involved. On the other hand, AII potentiated only responses mediated by α_2-adrenoceptors responses in the rabbit isolated ear vein and lateral saphenous vein (the latter only manifest after irreversible inactivation of post-junctional α_1-adrenoceptors).

Although both agents produce qualitatively similar effects against α_2-adrenoceptor function in the rabbit isolated saphenous vein, the essential difference in the mode of action of these two agents is underlined by studies on voltage-operated Ca^{2+} channels; the presumed site of action of Bay K 8644 (Schramm *et al.* 1983; Su *et al.*, 1984). Bay K 8644 enhanced Ca^{2+}-induced contractions in the presence of a depolarizing concentration of KCl, without altering the sensitivity of the voltage-operated Ca^{2+} channel. The unexpectedly small effect may be a consequence of the degree of depolarization produced by 65 mM KCl, i.e. the response cannot be further facilitated. In marked contrast, AII inhibited the Ca^{2+}-induced contractions mediated via voltage-operated channels, an effect associated with a small reduction in channel sensitivity to Ca^{2+}. The basis for this effect is not clear, but the possibility that AII produces a vasodilator prostaglandin is worthy of investigation.

Since α_2-adrenoceptor contractions in the rabbit isolated saphenous vein are entirely dependent upon the entry of extracellular Ca^{2+} (Daly *et al.*, 1990), and only part of this is via dihydropyridine-sensitive Ca^{2+} channels (Dunn *et al.*, 1991b), it seems likely that the similar facilitatory effects of Bay K 8644 and AII against α_2-adrenoceptor-mediated contractions arise from discrete effects on dihydropyridine-sensitive and dihydropyridine-resistant Ca^{2+} channels, respectively. The precise relationship between α-adrenoceptor subtype, voltage-operated Ca^{2+} channels and dihydropyridine-sensitive Ca^{2+} channels in the rabbit isolated saphenous vein awaits detailed electrophysiological studies.

Effects of angiotensin II on responses mediated via postjunctional α_1- and α_2-adrenoceptors

A number of observations in the present study support a preferential interaction between AII and responses mediated via postjunctional α_2-adrenoceptors rather than α_1-adrenoceptors. First, AII produced a modest reduction in the potency of prazosin against responses to NA in the lateral saphenous vein. These results are essentially similar to those obtained by Schümann & Lues (1983) in the same preparation, who demonstrated that responses to BHT-920, which under normal circumstances were markedly inhibited by prazosin, became much more resistant to the α_1-adrenoceptor antagonist in the presence of AII. Secondly, AII produced a small but significant enhancement of responses to NA in the rabbit isolated ear vein, a preparation containing an almost homogeneous population of postjunctional α_2-adrenoceptors (Daly *et al.*, 1988). Thirdly, AII failed to modify the antagonism of NA contractions produced by rauwolscine in the rabbit isolated saphenous vein; the 'rauwolscine-resistant' component (previously shown to be prazosin-sensitive; Daly *et al.*, 1988b) is similar in the absence and presence of AII (Figure 3b). Finally, in the left renal vein, a preparation containing a homogeneous population of postjunctional α_1-adrenoceptors (Daly *et al.*, 1988d), AII was without effect on responses to NA.

It has been suggested that the presence of a large 'receptor reserve' for α-adrenoceptors can limit functional antagonism produced by either Ca^{2+} channel blockers (Nichols & Ruffolo, 1988) or a reduction in temperature (Flavahan & Vanhoutte, 1986). Since the corollary of this is that scope for facilitation is similarly reduced in a system with a large receptor reserve, we also investigated the effect of AII on α-adrenoceptor function in the renal vein after exposure to phenoxybenzamine. Phenoxybenzamine produced a reduction in the maximum response to NA, without altering the sensitivity of the preparation, but this procedure failed to uncover a facilitatory effect of AII.

These observations fail to generalize those of Purdy & Weber (1988) who were able to demonstrate a facilitation of 'α_1-adrenoceptor'-mediated responses in rabbit isolated femoral artery, this effect being particularly evident following exposure to the irreversible antagonist, benextramine. However, two observations suggest that postjunctional α_2-adrenoceptors may be present in the rabbit isolated femoral artery and, as such, provide an alternative basis for the observed potentiation. First, AII uncovers previously unseen responses to the selective α_2-adrenoceptor agonist UK-14304 in the rabbit femoral artery (authors, unpublished observations). Secondly, Purdy & Weber (1988) noted that following irreversible inactivation of α-adrenoceptors with benextramine and subsequent exposure to AII, responses of the rabbit isolated femoral artery to NA exhibited slightly greater resistance to prazosin. With respect to this latter observation, there are clear parallels with findings in the rabbit isolated saphenous vein (present study) and the rabbit isolated distal saphenous artery (Dunn *et al.*, 1991a).

Further support for a preferential interaction between AII and postjunctional α_2-adrenoceptors is provided by several reports that predate the subclassification of postjunctional α-adrenoceptors. For example, AII was found to potentiate responses to NA in a number of isolated vascular preparations such as perfused mesenteric arteries from a number of species (Malik & Nasjletti, 1976; Chiba & Tsukada, 1986; Panisset & Bourdois, 1968), human digital arteries (Moulds & Worland, 1980) and in canine hindpaw (Zimmerman & Kraft, 1979). It is noteworthy that a re-examination of the α-adrenoceptor population in many of these preparations has revealed the presence of the α_2-subtype as in human digital

arteries (Glusa & Mardwardt, 1983; Stevens & Moulds, 1985); feline mesenteric vascular beds (Lippton et al., 1987) and canine saphenous artery (Sulpizio and Hieble, 1987).

It should be noted, however, that while the above evidence favours a preferential interaction between α_2-adrenoceptors and AII, it does preclude the possibility that AII can also facilitate α_1-adrenoceptor responses. This is underlined by the report of Day & Moore (1976) which showed a non-specific facilitatory action for AII on responses to various contractile agents, including NA, in rabbit isolated aorta. Similarly, Chiba & Tsukada (1986) also observed an increase in response of the canine mesenteric artery to KCl, as well as NA, in the presence of AII. Since ouabain produced similar effects, these authors concluded that AII modulated responses to vasoactive agents by producing a small depolarization of the cell membrane, thereby reducing the threshold for contraction. However, a non-specific sensitization of vascular smooth muscle is unlikely to account for the effects of AII in the present study since, when the full receptor complement was present in the saphenous vein, responses to bradykinin (unpublished observations) and NA were unaffected by AII. It seems more likely that AII is producing a particular pattern of effects on the signalling process which selectively synergizes with the coupling involved with many α_2-adrenoceptor-activated processes. There is no reason to suppose that this is either ubiquitous, unique to AII or selective only to α_2-adrenoceptors but it does seem to be widespread in blood vessels.

Finally, the facilitatory effect of AII on α_2-adrenoceptor responses in the rabbit isolated saphenous vein was blocked by the AII receptor antagonist, saralasin (Zimmerman & Kraft, 1979), indicating an effect mediated via specific AII receptors. Interestingly, despite the fact that saralasin (0.01 μM) virtually abolished contractile responses to AII, a 10 fold higher concentration of the antagonist was required to reverse completely the facilitatory action of AII. These results represent a dissociation between the concentrations of AII required for a facilitatory action on responses to NA and a direct solo action on vascular smooth muscle, such that the former can occur with subcontractile concentrations of AII. In accord with this idea, 5 nM AII also produced a consistent facilitation of postjunctional α_2-adrenoceptor-mediated responses, yet it produced a very small contraction in only 2 of 6 preparations.

Effects of Bay K 8644 on responses mediated via postjunctional α_1- and α_2-adrenoceptors

The sustained component of responses to NA in rabbit isolated lateral saphenous vein and left renal vein has been shown to be largely dependent on the influx of extracellular Ca^{2+} (Daly et al., 1990). No evidence was obtained in these preparations to suggest that Bay K 8644 differentially influenced responses mediated via either postjunctional α_1- or α_2-adrenoceptors. Both could be enhanced. For example, Bay K 8644 produced an enhancement of responses mediated via α_2-adrenoceptors in the lateral saphenous vein. This was suggested by the reduction in potency of prazosin against responses to NA in the presence of Bay K 8644 and was demonstrated directly after isolation of a homogeneous population of postjunctional α_2-adrenoceptors. Bay K 8644 also potentiated responses to NA mediated via postjunctional α_1-adrenoceptors in the left renal vein.

In marked contrast, however, Bay K 8644 was without effect on responses to NA mediated via postjunctional α_2-adrenoceptors in the isolated ear vein. Since the sustained component of responses to NA in this preparation are also largely dependent upon extracellular Ca^{2+} (Daly et al., 1990), responses to NA probably reflect the entry of Ca^{2+} via 1,4-dihydropyridine-resistant, receptor-operated channels. In agreement with this hypothesis, nifedipine is ineffective against responses to NA in the ear vein (Dunn et al., 1991b).

The few other comparative studies which have been attempted *in vitro* further outline the dangers inherent in generalizing the susceptibility of responses mediated via a particular receptor to the blocking action of agents which interfere with one aspect of stimulus-response coupling. For example, while Bay K 8644 can enhance postjunctional α_2-adrenoceptor-mediated responses in a number of vascular preparations such as the rat saphenous vein (Cheung, 1985), feline mesenteric bed (Lippton et al., 1987) or canine saphenous artery (Sulpizio & Hieble, 1987), responses to NA, in the presence of prazosin (α_2-adrenoceptor-mediated), are unaffected by Bay K 8644 in the human hand vein (Arner et al., 1988). In addition, postjunctional α_1-adrenoceptor-mediated responses are facilitated by Bay K 8644 and reduced by nimodipine in human hand veins (Arner et al., 1988), but in contrast are resistant to the action of Bay K 8644 in the canine saphenous vein (Eskinder & Gross, 1987).

Therefore a sufficient number of exceptions can be demonstrated in isolated vascular preparations, to prevent the direct association between α-adrenoceptor subtype and susceptibility to 1,4-dihydropyridine derivatives, which has been postulated *in vivo*. The differential effect of CCBs in whole animals is more likely to reflect a limitation of measuring only the change in the peak response to the bolus injection of an agonist. For example, CCBs have no differential effect on responses mediated via either postjunctional α_1- or α_2-adrenoceptors, if the area under the curve is used as a measure of the whole response to a given agonist (McGrath & O'Brien, 1987). Likewise, these agents reduce the size of the responses to infusions, which give a more sustained type response, of agonists selective for either subtype (McGrath & O'Brien, 1987; Lefevre-Borg et al., 1988).

In summary, in three isolated venous preparations from the rabbit, a comparison of the actions of AII and Bay K 8644 revealed marked differences in their effects on responses to NA. AII appears to produce a preferential facilitation of responses mediated via postjunctional α_2-adrenoceptors. In contrast, Bay K 8644 facilitated responses to NA in two of the three preparations (with the exception of the ear vein) irrespective of the particular α-adrenoceptor involved in mediating the contraction.

This work was supported by the Science and Engineering Research Council, through a Collaborative Project Grant in association with Roche Products, U.K. (C.J.D. & V.G.W.), and a CASE studentship in association with Dr A.F. Lever of the MRC Blood Pressure Unit (W.R.D.). We are grateful for support also to the Medical Research Funds of the University of Glasgow.

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(Received October 5, 1990
Revised January 14, 1991
Accepted January 28, 1991)

Br. J. Pharmacol. (1991), **103**, 1484–1492 © Macmillan Press Ltd, 1991

Postjunctional *a*-adrenoceptors in the rabbit isolated distal saphenous artery: indirect sensitivity to prazosin of responses to noradrenaline mediated via postjunctional α_2-adrenoceptors

[1]W.R. Dunn, J.C. McGrath & [2]V.G. Wilson

Autonomic Physiology Unit, Institute of Physiology, University of Glasgow, Glasgow, G12 8QQ

1 Under normal experimental conditions, the rabbit isolated distal saphenous artery appears to contain a homogeneous population of postjunctional α_1-adrenoceptors. Prazosin competitively antagonized responses to noradrenaline (NA) with a pA_2 value of 8.6, while a relatively high concentration of rauwolscine (1 μM), produced only a 2 fold rightward displacement of the NA cumulative concentration-response curve (CCRC).

2 Despite the fact that angiotensin II (AII) was without effect on responses to NA or phenylephrine in this preparation, this peptide made responses to NA less susceptible to the antagonistic action of prazosin. This was particularly evident on the lower portion of the CCRC for NA. These results suggest that in the presence of AII, NA produces contractile responses by an action mediated through a prazosin-resistant adrenoceptor.

3 An attempt was made to isolate a homogeneous population of postjunctional α_2-adrenoceptors by use of a receptor protection procedure involving the combination of rauwolscine and phenoxybenzamine. After the protection protocol no responses were observed to the α-adrenoceptor agonists NA, phenylephrine or UK-14304. In the presence of angiotensin II however, concentration-dependent contractions were observed to each of these agonists. Under these conditions the rank order of potency, UK-14304 > NA > phenylephrine, is consistent with that of an effect mediated through postjunctional α_2-adrenoceptors.

4 The responses to NA, after the protection protocol, in the presence of AII, were susceptible to the selective α_2-adrenoceptor antagonist, rauwolscine (1 μM), but resistant to the selective α_1-adrenoceptor antagonist prazosin (0.1 μM). Furthermore, the combination of rauwolscine (1 μM) and prazosin (0.1 μM) was no more effective in blocking responses to NA than was rauwolscine (1 μM) alone. These results are consistent with the presence of a homogeneous population of postjunctional α_2-adrenoceptors.

5 Inducing a small degree of tone with a low concentration of the selective α_1-adrenoceptor agonist, phenylephrine, markedly increased the threshold sensitivity to the selective α_2-adrenoceptor agonist UK-14304, in a manner analogous to that seen with AII.

6 The results in the present study indicate that responses mediated via postjunctional α_2-adrenoceptors in the rabbit isolated distal saphenous artery are dependent upon a degree of vascular smooth muscle stimulation by some other receptor system. It is hypothesized that under normal experimental conditions, this function is fulfilled by stimulation of α_1-adrenoceptors, while after α_1-adrenoceptor blockade the necessary positive influence can be provided by stimulation of AII receptors. The implications for such an interaction between postjunctional α-adrenoceptor subtypes in demonstrating prazosin-resistant, rauwolscine- or yohimbine-sensitive responses in isolated blood vessels is discussed.

Keywords: Noradrenaline; angiotensin II; postjunctional α_1-adrenoceptors; postjunctional α_2-adrenoceptors; rabbit isolated distal saphenous artery; interaction

Introduction

In contrast to the relative ease of demonstrating postjunctional α_2-adrenoceptor-mediated pressor responses in whole animals, it has been much more difficult to show prazosin-resistant, rauwolscine- or yohimbine-sensitive responses in isolated vascular preparations (McGrath *et al.*, 1989). Some success has been achieved in several isolated venous preparations such as the canine saphenous vein (Constantine *et al.*, 1982), rat saphenous vein (Cheung, 1985), rabbit saphenous vein (Daly *et al.*, 1988a) and most convincingly, the rabbit ear vein (Daly *et al.*, 1988b). It has been even more difficult however, to demonstrate unequivocally, responses mediated via postjunctional α_2-adrenoceptors in isolated arterial preparations, the likely source of α_2-adrenoceptor-mediated pressor responses *in vivo*.

Several explanations have been put forward to explain the elusive nature of this subtype *in vitro*. For example, it has been suggested that postjunctional α_2-adrenoceptors are located on small arterioles which, because of their size, are not normally accessible for examination *in vitro* (McGrath, 1982). This hypothesis is supported by the recent observations that functional postjunctional α_2-adrenoceptors are present on rat cremaster arterioles (Faber, 1988) and on human resistance vessels, where an inverse relationship between vessel diameter and magnitude of α_2-adrenoceptor-mediated responses was observed (Neilsen *et al.*, 1989). An additional explanation for the difficulty in demonstrating postjunctional α_2-adrenoceptors *in vitro* may be the lack of humoral agents

[1] Present address: Department of Pharmacology, University of Vermont, Given Medical Building, Burlington, Vermont, 05405, U.S.A.

[2] Author for correspondence at present address: Department of Physiology and Pharmacology, Medical School, Queens Medical Centre, Clifton Boulevard, Nottingham, NG7 2UH.

normally present in the whole animal (Schümann & Lues, 1983). For example it has been reported that the expression of postjunctional α_2-adrenoceptor-mediated responses to UK-14304 in the rabbit distal saphenous artery is dependent upon the presence of angiotensin II (AII) (Dunn *et al.*, 1989). Similarly, in the perfused isolated tail of the rat α_2-adrenoceptor-mediated responses are observed only after raising tone with, for example vasopressin or endothelin (Templeton *et al.*, 1989; MacLean & McGrath, 1990), while in the canine saphenous artery a non-contractile concentration of Bay K 8644 allowed the demonstration of responses mediated by the subtype (Sulpizio & Hieble, 1987).

The above mentioned studies with vasoactive agents have demonstrated the expression of α_2-adrenoceptor-mediated responses to synthetic agonists which are relatively selective for α_2-adrenoceptors. The present study has addressed the influence of AII on responses to the endogenous ligand NA in the rabbit isolated distal saphenous artery. The results demonstrate that functional postjunctional α_2-adrenoceptors, responding to NA, are present in this preparation, despite the fact that under 'standard' experimental conditions responses to NA are sensitive to low concentrations of the selective α_1-adrenoceptor antagonist, prazosin. Our observations suggest an interaction between postjunctional α_1- and α_2-adrenoceptors, which may be of more general applicability for other isolated vascular preparations.

Methods

White albino rabbits of either sex weighing 2.3–3.0 kg were killed by stunning followed by exsanguination. Segments of the distal saphenous artery were cleaned of fat and connective tissue *in situ* and placed in ice-cold physiological solution (PSS). When necessary, preparations were cleaned further with the aid of a dissecting microscope. Subsequently, 3–5 mm length segments were taken from each preparation and suspended between two 0.2 mm thick wire supports as described by Hooker *et al.* (1976). The upper support was connected by cotton to a Grass FT03 isometric transducer while the lower support was connected to a glass tissue holder. Each segment was then mounted in 30 ml isolated organ baths under an initial resting tension of 2 g wt. and allowed to relax. Each preparation was bathed in PSS maintained at 37°C and gassed with 95% O_2 plus 5% CO_2.

After a 60 min equilibration period, during which a steady resting tension was achieved, each preparation was exposed to 3 μM NA and allowed to contract for 10 min. Following complete washout, an additional equilibration period of 1 h was allowed before starting the experiment. This procedure was found to minimize changes in the sensitivity of the preparation to further addition of agonists and is similar to the method of Ruffolo *et al.* (1979). Basal tension following the sighting response remained stable for the rest of the experiment of 0.6–0.75 g wt. Isometric contractions were recorded by a Grass FT03 transducer connected to a Linseis 6025 pen recorder. In all experiments cumulative concentration-response curves (CCRCs) to agonists were constructed by increasing the concentration of agonist in the organ bath by approximately 3 fold increments. Following attainment of the maximal control contraction, preparations were washed until complete relaxation was effected and 30–60 min later one of the following experimental protocols adopted:

(1) Preparations were exposed to AII (0.05 μM) 15 min before the start of a second agonist CCRC.
(2) Preparations were exposed to various concentrations of prazosin for a minimum period of 45 min and the CCRC for NA repeated. In some tissues, AII (0.05 μM) was added to the organ bath 15 min before the start of the second CCRC to NA, in the presence of prazosin.
(3) An attempt was made to isolate a population of postjunctional α_2-adrenoceptors by use of a protocol similar to that used by Daly *et al.* (1988a). Briefly, tissues were exposed to rauwolscine (1 μM) 5 min prior to phenoxybenzamine (0.3 μM) which was left in contact with the tissues for a further 60 min. The tissues were then washed a minimum of seven times over a 45 min period. During the first two washes rauwolscine (1 μM) was present to maximize α_2-adrenoceptor protection. After a further 60 min period a CCRC was obtained to various agonists. In some experiments AII was added 15 min prior to the onset of a further CCRC. In others, antagonists were applied at least 45 min before the start of an agonist CCRC.
(4) A low concentration of the selective α_1-adrenoceptor agonist, phenylephrine, was applied to the distal saphenous artery to induce a small degree of tone. After a maintained contraction was achieved, a CCRC to the selective α_2-adrenoceptor agonist UK-14304 was obtained.

All responses are expressed as a percentage (mean ± s.e.mean) of the maximum response of the first concentration-response curve to NA (or UK-14304) obtained in each tissue. Differences between means were considered statistically different if $P < 0.05$ for either paired or unpaired observations (Student's t test). In examining the effects of antagonists, agonist concentration-ratio values were determined from the concentrations producing 50% of the maximum response in the absence and presence of each concentration of antagonist. In all experiments, one preparation was run in parallel with experimental tissues, but received no antagonist, and was used to determine time-dependent changes in agonist sensitivity (Furchgott, 1972). According to Arunlakshana & Schild (1959) if antagonism is competitive, a plot of the logarithm of dose-ratio − 1 against the negative logarithm of the molar concentration of the antagonists, yields a straight line with a slope of one and the intercept of which along the abscissa scale is the pA_2, which is equal to the antagonist dissociation constant (K_B) under equilibrium conditions. Antagonism was considered competitive if the 95% confidence limits for the slope of the Schild plot, drawn by linear regression, overlapped unity. In some experiments, the agonist concentration-ratio values in the presence of antagonist were determined at the 25%, 50% and 75% level of the maximum response to NA as described by Flavahan & Vanhoutte (1986).

The composition of the PSS was (mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4.7H_2O$ 1.2, $NaHCO_3$ 24.9, KH_2PO_4 1.2 and glucose 11.1. Na_2EDTA 23 μM was included in all experiments to prevent oxidative degradation of NA and 1 μM propranolol and 10 μM cocaine were also included to inhibit β-adrenoceptors and uptake$_1$, respectively. The following drugs were used: (−)-noradrenaline bitartrate (Sigma), phenoxybenzamine HCl (Smith, Kline & French), rauwolscine HCl (Roth), angiotensin II amide (Ciba), (±)-propranolol HCl (Sigma), cocaine HCl (Macarthys), (−)-phenylephrine HCl (Sigma), UK-14304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline bitartrate) (Pfizer) and prazosin HCl (Pfizer). With the exception of phenoxybenzamine, all drugs were dissolved in distilled water and added to the organ bath in volumes of 0.3 ml or less. Stock solutions of phenoxybenzamine (1 mM) were prepared in 20% absolute ethanol in distilled water and a drop of 1 N HCl was added to remove turbidity. Further dilutions were made in distilled water.

Results

Effects of antagonists on noradrenaline-induced contractions

As shown in Figure 1, prazosin produced a concentration-dependent, parallel rightward displacement of the CCRC to NA. The pA_2 value for prazosin was 8.57 and the 95% con-

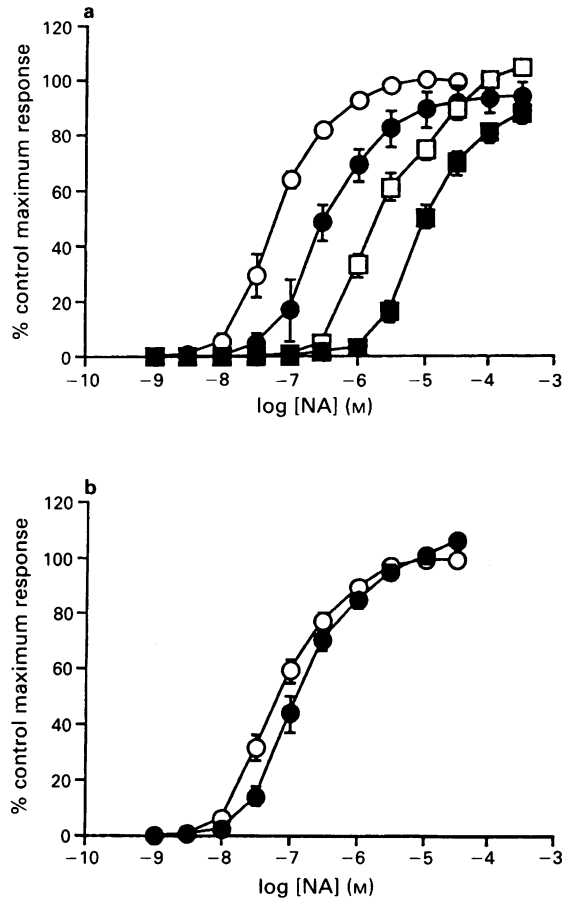

Figure 1 Effects of the α-adrenoceptor antagonists (a) prazosin 0.01 μM (●), 0.1 μM (□) and 1 μM (■) and (b) rauwolscine (1 μM) (●) on responses to noradrenaline (NA, ○) in the rabbit isolated distal saphenous artery. Results are expressed as a % of the initial maximum response to NA. Each point represents the mean of $n = 5$–6; s.e.mean shown by vertical bars.

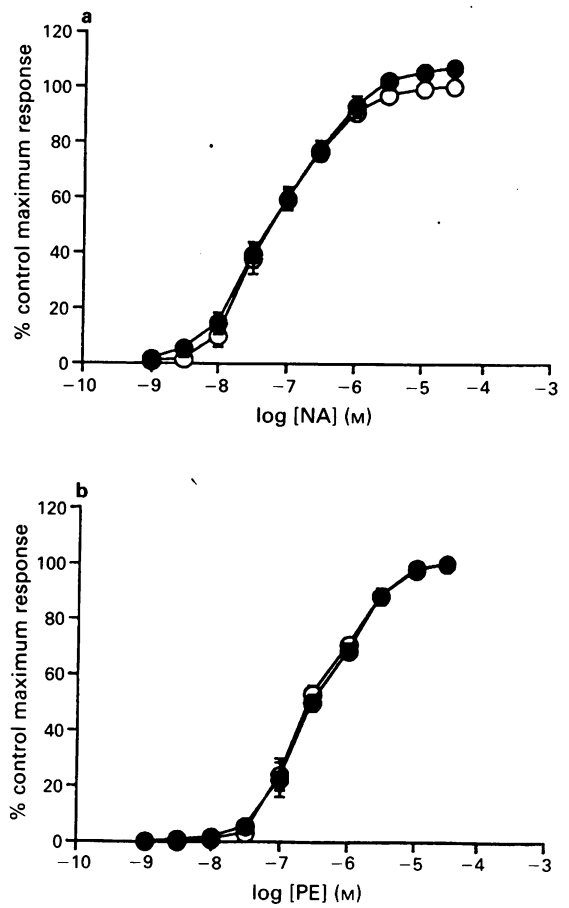

Figure 2 Effects of angiotensin II (0.05 μM) (●) on responses to the α-adrenoceptor agonists (a) noradrenaline (NA) and (b) phenylephrine (PE) in the rabbit isolated distal saphenous artery. Results are expressed as a % of the initial maximum response to each agonist (○). Each point represents the mean of $n = 5$–6; s.e.mean shown by vertical bars.

fidence interval for the slope of the Schild was not significantly different from unity, indicating competitive antagonism. Furthermore, prazosin (0.1 μM) produced an approximately 25 fold rightward displacement in the CCRCs to the α-adrenoceptor agonists, phenylephrine, Sgd 101/75 and UK-14304, an effect similar to that observed against NA. In contrast, a relatively high concentration of the selective α_2-adrenoceptor antagonist rauwolscine (1 μM), produced only a 2 fold rightward shift in the NA CCRC (Figure 1b).

Effects of angiotensin II on responses to noradrenaline and phenylephrine

AII (0.05 μM) produced a transient contraction, which returned to baseline after 12–15 min, in the rabbit isolated distal saphenous artery. The peak response to AII was equivalent to 37.8 ± 4.8% ($n = 13$) of the maximum response to NA in each preparation. This agent had no effect on responses to the agonists NA (non-selective α_1/α_2) (Figure 2a) phenylephrine (selective α_1) (Figure 2b) or Sgd 101/75.

AII (0.05 μM) did, however, have a marked effect on the ability of prazosin to antagonize responses to NA in the distal saphenous artery (Figure 3). This was particularly marked for the lower portion of the CCRC to NA (cf. Figure 1a), so that the log agonist concentration-ratio value at the level of 25% of the maximum response to NA, in the presence of prazosin (0.1 μM), was significantly reduced from control values in the absence of AII (Table 1).

Attempted isolation of postjunctional α_2-adrenoceptors

Phenoxybenzamine (0.3 μM) alone virtually abolished responses to NA in this preparation. The rationale employed therefore was to prevent phenoxybenzamine from binding to

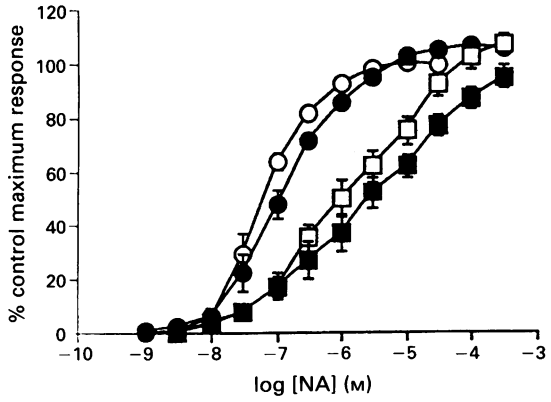

Figure 3 The effects of 0.01 μM (●), 0.1 μM (□), and 1 μM (■) prazosin on responses of the rabbit isolated saphenous artery to noradrenaline (NA, ○) in the presence of 0.05 μM angiotensin II. Results are expressed as a % of the initial maximum response to NA. Each point represents the mean of $n = 5$–6; s.e.mean shown by vertical bars. c.f. Figure 1a.

Table 1 Log agonist concentration-ratio values for noradrenaline (NA) in the presence of prazosin (0.1 μM) alone or in combination with angiotensin II (AII, 0.05 μM) calculated at the levels of 25%, 50% and 75% of the maximum response to NA

Antagonist	*25%*	*50%*	*75%*
Prazosin (0.1 μM) ($n = 6$)	1.55 ± 0.06	1.57 ± 0.08	1.63 ± 0.06
Prazosin (0.1 μM) with AII (0.05 μM) ($n = 6$)	1.08 ± 0.09*	1.32 ± 0.06	1.67 ± 0.07

* Represents a significant difference for the log agonist concentration-ratio value for NA calculated at the 25% level of the maximum response in the presence of AII.

any postjunctional α_2-adrenoceptors by masking the receptor with 1 μM rauwolscine. This concentration of rauwolscine was considered to be without effect at α_1-adrenoceptors, since it was without effect on responses to phenylephrine in this preparation.

The inclusion of rauwolscine (1 μM) prior to, and during, the incubation period with phenoxybenzamine (0.3 μM), did not result in part of the response to NA being 'spared' (Figure 4).

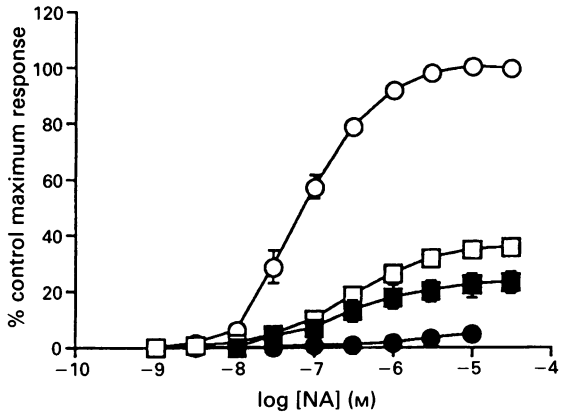

Figure 4 An examination of contractile responses of the rabbit isolated distal saphenous artery to noradrenaline (NA) following attempted isolation of post-junctional α_2-adrenoceptors with a combination of (●) 0.3 μM phenoxybenzamine and 1 μM rauwolscine, and the effect of 5 nM (■) and 0.05 μM (□) angiotensin II. Results are expressed as a % of the initial maximum response to (○) noradrenaline. Each point represents the mean of $n = 6$–11; s.e.mean shown by vertical bars. All responses to noradrenaline in the presence of angiotensin II (0.05 μM) after the protection protocol are significantly different from those after the protection protocol alone, $P < 0.05$, Student's t test.

Table 2 List of pD_2 values (with 95% confidence limits) for the α-adrenoceptor agonists, noradrenaline (NA), phenylephrine and UK-14304 under normal experimental conditions (control) and after attempted isolation of postjunctional α_2-adrenoceptors with the combination of rauwolscine (Rauw, 1 μM) and phenoxybenzamine (Pbz) (0.3 μM) in the presence of angiotensin II (AII, 0.05 μM), in the rabbit isolated distal saphenous artery

	NA	*Phenylephrine*	*UK-14304*
Control[a]	7.25 ± 0.09 ($n = 6$)	6.56 ± 0.08 ($n = 5$)	6.59 ± 0.12 ($n = 7$)
After Rauw/Pbz in the presence of AII (0.05 μM)	6.66 ± 0.29 ($n = 11$)	<5.15* ($n = 6$)	8.36 ± 0.31 ($n = 7$)

* A precise pD_2 value for phenylephrine has not been calculated since a true maximum response to this agonist was not achieved under these conditions.
[a] Values from Dunn *et al.* (1989).

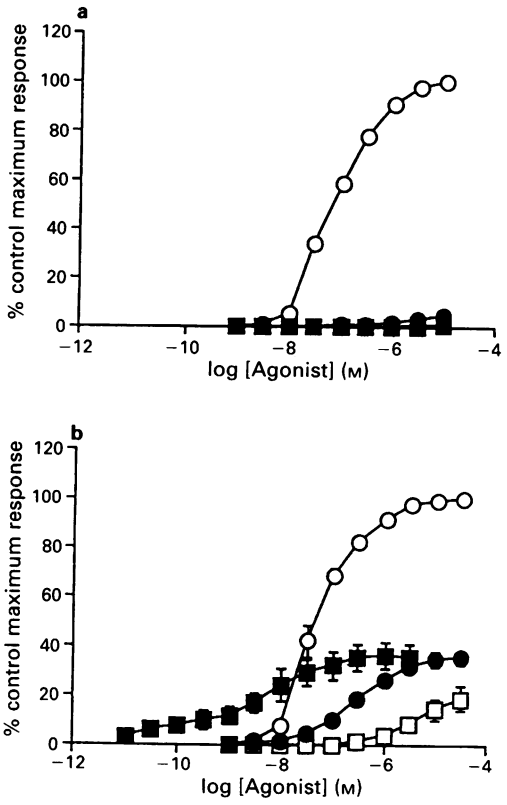

Figure 5 Responses to the α-adrenoceptor agonists noradrenaline (NA), (●), phenylephrine (□) and UK-14304 (■) in (a) the absence and (b) the presence of angiotensin II (0.05 μM) after attempted isolation of postjunctional α_2-adrenoceptors in the rabbit isolated distal saphenous artery. Results are expressed as a % of the maximum response to NA (○) prior to the protection protocol. Each point represents the mean of $n = 6$–11; vertical bars show s.e.mean.

The maximum response to NA was reduced to a similar degree by phenoxybenzamine alone and by the combination of rauwolscine and phenoxybenzamine (% reduction of NA

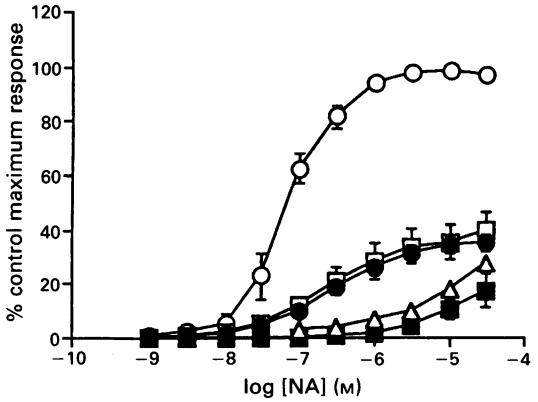

Figure 6 The effects of (□) 0.1 μM prazosin, (■) 1 μM rauwolscine and a combination of (△) 0.1 μM prazosin and 1 μM rauwolscine on responses to noradrenaline (NA), in the presence of 0.05 μM angiotensin II after attempted isolation of postjunctional α_2-adrenoceptors (●) in the rabbit isolated distal saphenous artery. Results are expressed as a % of the initial maximum response to NA (○) prior to the protection protocol. Each point represents the mean of $n = 5$–11; vertical bars show s.e.mean.

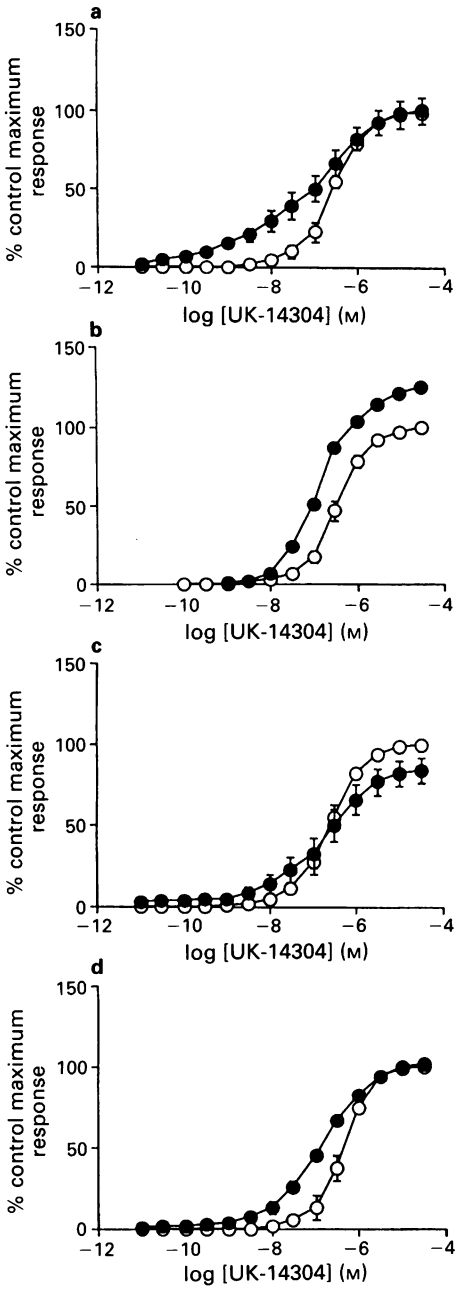

Figure 7 Contractile responses of the rabbit isolated saphenous artery to the selective α_2-adrenoceptor agonist UK-14304 in the (○) absence and the (●) presence of (a) the selective α_1-adrenoceptor agonist phenylephrine (10 nM), (b) 0.03 μM Bay K 8644, (c) 5-hydroxytryptamine (5-HT, 0.01–0.3 μM) and (d) KCl (17–20 mM). The 'active tone' induced by phenylephrine, 5-HT and KCl was approximately 15% of the maximum response to noradrenaline. Results are expressed as a % of the initial maximum responses to UK-14304 (○). Each point represents the mean of $n = 5$; vertical bars show s.e.mean.

maximum response: 97.4 ± 1.2 and 95.8 ± 2.3, respectively). If, however, the preparations were exposed to AII (5 nM–0.05 μM) after the combination of rauwolscine (1 μM) and phenoxybenzamine (0.3 μM), concentration-dependent responses to NA were observed (Figure 4). The maximum response after attempted isolation of postjunctional α_2-adrenoceptors was increased from 4.2 ± 2.3 to 35.1 ± 2.9% ($n = 11$) of the initial maximum response to NA in the absence and presence of 0.05 μM AII, respectively. Similarly, 5 nM AII allowed the expression of responses to NA equivalent to 23.3 ± 3.35% ($n = 9$) of the initial maximum response before treatment with the combination of rauwolscine and phenoxybenzamine (Figure 4).

AII was also found to be a necessary prerequisite for the expression of responses to the α-adrenoceptor agonists, phenylephrine and UK-14304, after the protection protocol (Figure 5). In the presence of AII all agonists, NA, phenylephrine and UK-14304, produced concentration-dependent contractions (Figure 5b). Under these conditions the relative potency of the agonists was UK-14304 > NA > phenylephrine, in contrast to the potency ratio of NA > phenylephrine = UK-14304 seen under normal experimental conditions (Table 2).

Effects of α-adrenoceptor antagonists

As shown in Figure 6, responses to NA in the presence of AII (0.05 μM), after attempted isolation of postjunctional α_2-adrenoceptors, were resistant to prazosin (0.1 μM) (pD_2 value for NA: 6.66 ± 0.29 and 6.42 ± 0.18, in the absence and presence of prazosin (0.1 μM) respectively, $n = 5$), but susceptible to rauwolscine (1 μM) which produced an approximately 100 fold rightward displacement of the CCRC to NA. Furthermore the combination of prazosin (0.1 μM) and rauwolscine (1 μM) was no more effective in antagonizing responses to NA than was rauwolscine (1 μM) alone (Figure 6).

Effect of other agents on responses to UK-14304

A concentration of phenylephrine selective for postjunctional α_1-adrenoceptors (10 nM), produced a sustained contraction equivalent to 13.8 ± 2.0% of the maximum response to UK-14304 in the rabbit isolated distal saphenous artery. Subsequently, exposure of the preparation to UK-14304 resulted in concentration-dependent contractions superimposed upon the induced tone. The sensitivity of the preparation to UK-14304 was markedly increased in a manner analogous to that seen with AII (see: Dunn *et al.*, 1989), with the threshold sensitivity of the preparation to UK-14304 being increased from approximately 0.01 μM to 0.03 nM in the presence of the α_1-adrenoceptor agonist-induced tone (Figure 7a).

Bay K 8644 (0.03 μM) did not alter resting tension in the saphenous artery, but significantly increased responses to UK-14304 in the preparation. This enhancement was associated with an approximately 25% increase in the maximum response and a 2 fold leftward displacement of CCRC to UK-14304 (Figure 7b). However, in spite of the potentiation produced by Bay K 8644, there was no increase in the threshold concentration of UK-14034 for contraction, which was approximately 0.01 μM in the absence and presence of Bay K 8644. Similar observations were made following induction of 'active-tone' with 5-hydroxytryptamine (5-HT, 0.01–0.3 μM), yielding a response approximately 15% of the NA maximum (Figure 7c). The presence of comparable 'active-tone' with KCl (17–20 mM) was associated with a significant increase in the sensitivity of the preparation to UK-14304 (Figure 7d). However, this was not as pronounced as that observed with either AII or phenylephrine.

Discussion

Under normal experimental conditions the rabbit isolated distal saphenous artery appears to possess a homogeneous population of postjunctional α_1-adrenoceptors. Prazosin produced a concentration-dependent rightward displacement of the CCRC to NA, with a pA_2 value of 8.57 and the slope of the Schild plot did not differ significantly from unity, indicating competitive antagonism. This pA_2 value for prazosin is consistent with reported pA_2 values against α_1-adrenoceptor-mediated responses in other vascular preparations from the rabbit such as the thoracic aorta (8.7)

(Docherty *et al.*, 1981), ear artery (8.6) (Hieble *et al.*, 1982) or pulmonary artery (8.8) (Kaposci *et al.*, 1987). Conversely, rauwolscine (1 μM), at a concentration approximately 100 times greater than that equivalent to its pA_2 value at postjunctional α_2-adrenoceptors in the rabbit ear vein (Daly *et al.*, 1988b) and its dissociation constant in the lateral saphenous vein after isolation of postjunctional α_2-adrenoceptors (Daly *et al.*, 1988a), produced only a small rightward displacement of the CCRC to NA in this preparation. The estimated $-\log K_B$ value for rauwolscine (6.32) in the rabbit distal saphenous artery is therefore slightly higher, but not dissimilar to its reported pA_2 value at postjunctional α_1-adrenoceptors in other rabbit vascular preparations, such as the thoracic aorta (6.1) and ear artery (5.5) (Daly *et al.*, 1988c).

The influence of angiotensin II

In a recent paper we demonstrated that AII uncovered responses to UK-14304 which were mediated via postjunctional α_2-adrenoceptors in the rabbit isolated distal saphenous artery (Dunn *et al.*, 1989). In the present study, AII did not influence responses to the selective α_1-adrenoceptor agonist phenylephrine and perhaps surprisingly, was also without effect on responses to the nonselective agonist NA. This peptide did, however, make responses to NA more resistant to the antagonistic action of prazosin, particularly on the lower portion of the CCRC, in effect introducing a prazosin-resistant component of responses to NA. The observations parallel results obtained in the rat tail artery, in which the lower third of the CCRC to NA is slightly 'resistant' to prazosin over a limited prazosin concentration range (Rajanayagam & Medgett, 1987) and in which postjunctional α_2-adrenoceptor-mediated contractile responses have subsequently been demonstrated (Templeton *et al.*, 1989). Furthermore, AII also reduces the potency of prazosin on responses to both B-HT 920 and NA in the rabbit lateral saphenous vein, another preparation in which postjunctional α_2-adrenoceptors have been clearly shown (Schümman & Lues, 1983; Daly *et al.*, 1988a; Dunn *et al.*, 1991). The introduction of a prazosin-resistant component of responses to NA, therefore, strongly indicates the presence of both postjunctional α_1- and α_2-adrenoceptors in the distal saphenous artery.

Attempted isolation of postjunctional α_2-adrenoceptors in rabbit isolated distal saphenous artery

Phenoxybenzamine (0.3 μM), an irreversible α-adrenoceptor antagonist (Furchgott, 1972), virtually abolished responses to NA in the rabbit isolated distal saphenous artery. The basis for receptor isolation, therefore, was to provide conditions whereby postjunctional α_2-adrenoceptors could be selectively protected by the inclusion of 1 μM rauwolscine in a manner analogous to that used in the rabbit isolated lateral saphenous vein (Daly *et al.*, 1988a). This concentration of α_2-adrenoceptor antagonist was considered to be selective since it was without effect on responses to phenylephrine in the distal saphenous artery.

In contrast to the rabbit lateral saphenous vein (see Daly *et al.*, 1988a), the inclusion of rauwolscine (1 μM) prior to and during exposure to phenoxybenzamine, did not result in part of the response to NA being spared from the action of phenoxybenzamine. In the presence of AII, however, concentration-dependent responses were observed to the α-adrenoceptor agonists UK-14304, NA and phenylephrine. The rank order of potencies for these agonists, UK-14304 > NA > phenylephrine differs from the potency order seen under normal circumstances, NA > phenylephrine > UK-14304 (Dunn *et al.*, 1989) and is similar to the potency order for agonists found in the rabbit ear vein, which contains predominantly postjunctional α_2-adrenoceptors (Daly *et al.*, 1988b).

Additionally, responses to NA after the receptor protection protocol in the presence of AII were resistant to 0.1 μM prazosin, a concentration approximately 30 times greater than for its pA_2 value on postjunctional α_1-adrenoceptors in this preparation. Furthermore, rauwolscine (1 μM) produced an approximately 100 fold rightward displacement of the CCRC to NA, yielding an estimated $-\log K_B$ value of 8.01. This value corresponds well with that obtained against responses to NA in the lateral saphenous vein after isolation of postjunctional α_2-adrenoceptors (Daly *et al.*, 1988a) and against responses to NA in the rabbit ear vein (Daly *et al.*, 1988b). Further evidence favouring the existence of a homogeneous population of postjunctional α_2-adrenoceptors, under these conditions, is provided from the observation that the combination of prazosin (0.1 μM) and rauwolscine (1 μM) was no more effective in inhibiting responses to NA than was rauwolscine (1 μM) alone. Essentially these results are similar to those obtained under normal experimental conditions, where the presence of AII was a prerequisite for the demonstration of prazosin-resistant responses to both UK-14304 (see: Dunn *et al.*, 1989) and NA (present study).

Interaction between postjunctional α_1- and α_2-adrenoceptors

A number of observations in the rabbit isolated distal saphenous artery suggest that postjunctional α_1- and α_2-adrenoceptors do not co-exist in a simple manner. For example, despite the fact that functional postjunctional α_2-adrenoceptors can be clearly demonstrated in this preparation, under appropriate conditions, no component of the response to NA is resistant to prazosin when both α-adrenoceptors are present. In addition, having clearly demonstrated a facilitatory role for AII on responses mediated via postjunctional α_2-adrenoceptors, we may then have expected this agent to enhance responses to NA, a non-selective agonist, when the full receptor complement was present. Such an action was not observed.

These apparently paradoxical observations could be reconciled if the two postjunctional α-adrenoceptors reside on the same cells and interact at the level of a common post-receptor site in the events leading to contraction. Thus, responses mediated by postjunctional α_2-adrenoceptors in the distal saphenous artery may be dependent upon simultaneous stimulation of the α_1-adrenoceptor population. With the latter predominating, this would explain the relatively high potency of prazosin, with no component of responses to NA (or other agonists) exhibiting resistance to the α_1-adrenoceptor antagonist, and the relatively low potency of rauwolscine. Since both α-adrenoceptors are present under normal experimental conditions, the positive influence required for the expression of α_2-adrenoceptor-mediated responses would be provided by stimulation of postjunctional α_1-adrenoceptors. Assuming a similar mechanism to be involved, the requirement for AII would therefore be superfluous, explaining the lack of a facilitatory action for this agent against responses to NA when the full α-adrenoceptor complement was active.

If, however, α_1-adrenoceptors are inhibited, by either prazosin or phenoxybenzamine, a positive influence for AII would be a prerequisite for the expression of α_2-adrenoceptor-mediated responses. This would explain the induction of prazosin-resistance produced by AII, and the requirement for the presence of this agent to produce responses to NA mediated via postjunctional α_2-adrenoceptors, after irreversible inactivation of postjunctional α_1-adrenoceptors with phenoxybenzamine. An interaction between the α-adrenoceptors in this preparation was directly demonstrated by inducing a small degree of tone in the distal saphenous artery with a selective concentration of phenylephrine (0.01 μM). Stimulation of α_1-adrenoceptors resulted in the introduction of a previously 'unseen' component of responses to UK-14304, at very low concentrations,

analogous to that introduced by AII (see Dunn et al., 1989). Attempting to examine the effects of α-adrenoceptor antagonists on these responses would have negated the experiment. However, it is reasonable to assume that the uncovered responses to UK-14304 were mediated via postjunctional α_2-adrenoceptors. Similarly, phenylephrine-induced tone has been shown to uncover responses to B-HT 920 in the canine portal vein (Furuta, 1988). The concept of interacting postjunctional α-adrenoceptors in the distal saphenous artery is represented schematically in Figure 8.

A similar interaction between postjunctional α-adrenoceptors has previously been implicated in the rabbit lateral saphenous vein (Daly et al., 1988d) although the dependency of α_2-adrenoceptor-mediated responses on α_1-adrenoceptor stimulation is somewhat less. This is apparent since NA can clearly sustain a response alone after isolation of postjunctional α_2-adrenoceptors (Daly et al., 1988a), contrasting with the distal saphenous artery, where responses are wholly dependent on the presence of a stimulatory agent. Again in this preparation, the facilitatory action of AII on postjunctional α_2-adrenoceptor-mediated responses is apparent only if α_1-adrenoceptors are blocked by prazosin or phenozybenzamine (Dunn et al., 1991). An even more subtle interaction between α_1- and α_2-adrenoceptors has been demonstrated in the cat nictitating membrane (Shepperson, 1984) and rat isolated tail artery (Xiao & Rand, 1989). In these preparations, contractile responses to selective α_1-adrenoceptor agonists were potentiated by prior exposure to a selective α_2-adrenoceptor agonist, even though these agonists failed to produce a functional response. This effect could be blocked by selective α_2-adrenoceptor antagonists.

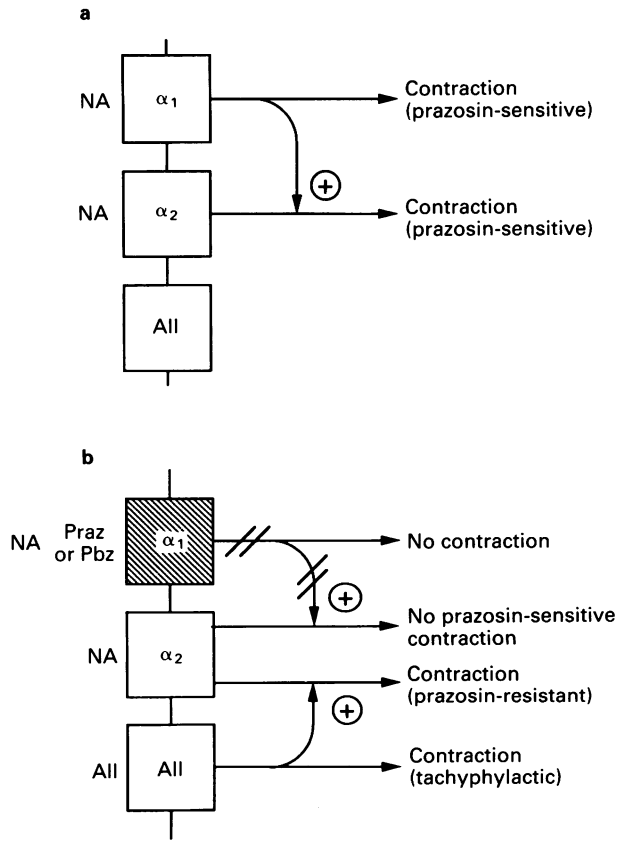

Figure 8 Diagrammatic representation of the proposed interaction between postjunctional α-adrenoceptor subtypes in the rabbit isolated distal saphenous artery. Note that under normal circumstances (a), stimulation of postjunctional α_1-adrenoceptors provides the positive input required for the expression of postjunctional α_2-adrenoceptors thereby rendering all responses sensitive to prazosin. Such a positive influence for angiotensin II (AII) is only necessary if α_1-adrenoceptors are blocked with either prazosin (Praz) or phenoxybenzamine (Pbz) (b).

Since the original demonstration of postjunctional α_2-adrenoceptors in the pithed rat (Drew & Whiting, 1979; Docherty et al., 1979), identification of this subtype in isolated vascular preparations, both responding to NA and resistant to prazosin or a suitable α_1-adrenoceptor antagonist, has proved very difficult. While a number of explanations have been put forward to explain the elusive nature of this receptor subtype (see McGrath et al., 1989), it is clear from the present study that one such factor that may conspire to render contractions mediated by postjunctional α_2-adrenoceptors 'prazosin-sensitive', is the dependency of α_2-adrenoceptor-mediated contractions on stimulation of α_1-adrenoceptors. Since other contractile agents can substitute for the positive influence of α_1-adrenoceptors, prazosin-resistant responses could be easily demonstrated in whole animals due to the presence of circulating vasoactive substances. In this respect, AII would assume great importance, particularly in models such as the pithed rat where the renin-angiotensin system is highly activated (Grant & McGrath, 1988).

Such an interaction between postjunctional α-adrenoceptors has widespread implications for the characterization of the α-adrenoceptor populations in blood vessels currently available for study *in vitro*. This is perhaps best illustrated by two further examples, which suggest that the clear demonstration of α_2-adrenoceptor-mediated responses has been obscured by their indirect sensitivity to prazosin.

Firstly, responses to NA in the isolated vascular bed of the rat tail are prazosin-sensitive, rauwolscine-resistant (α_1-mediated) under normal experimental conditions, but in contrast, a sizeable component of the response to NA becomes prazosin-resistant, rauwolscine-sensitive (α_2-mediated) in the presence of vasopressin-induced tone (Templeton et al., 1989). Similarly, inducing tone with phenylephrine or endothelin also introduced rauwolscine-sensitive responses to UK-14304 in this preparation (Templeton, 1989; MacLean & McGrath, 1990). Secondly, responses to NA in the rabbit femoral artery were shown to be prazosin-sensitive, rauwolscine-resistant, and therefore by definition α_1-adrenoceptor-mediated (Purdy & Weber, 1988). AII also introduces a component of responses to UK-14304 in the femoral artery in an analogous manner to that seen in the distal saphenous artery (authors unpublished observations). It would seem reasonable, therefore, to predict that further analysis would demonstrate unequivocally the presence of postjunctional α_2-adrenoceptors in the rabbit femoral artery.

Thus, the widespread use of a single concentration of an α_1-adrenoceptor antagonist (usually prazosin), as a basis for determining the characteristics of a population of α-adrenoceptors is no longer acceptable. Furthermore, any interaction between α_1- and 'silent' α_2-adrenoceptors has major implications for the use of phenoxybenzamine for inferential determination of agonst affinity (Furchgott, 1972). This irreversible antagonist possesses selectivity for vascular α_1-adrenoceptors (Constantine et al., 1982; Dunn et al., 1991), and a concentration that eliminates any 'receptor-reserve' (viewed as a reduction in the maximum response) will inevitably alter the relative contribution of α_1- and α_2-adrenoceptors to the observed response before and after inactivation. Hence, one of the underlying assumptions for the use of phenoxybenzamine in the quantitative analysis of the interaction between agonist and α-adrenoceptor (see: Besse & Furchgott, 1976) cannot be guaranteed in vascular smooth muscle.

Such an interaction between postjunctional α_1- and α_2-adrenoceptors may also partially explain the wide disparity in the relative potencies of α_1- and α_2-adrenoceptor antagonists found between vascular preparations (see Drew, 1985; McGrath et al., 1989). If, for example, the expression of postjunctional α_2-adrenoceptors is completely dependent upon α_1-adrenoceptor stimulation, then we may expect prazosin potently to antagonize responses (as is the case in the distal saphenous artery). The potency of α_2-adrenoceptor antagonists will, however, depend upon the relative predominance of this subtype. Therefore, with an increasing contribution of

postjunctional α_2-adrenoceptors to the total response, the pA_2 values for α_2-adrenoceptor antagonists will increase accordingly. It is interesting to note that in the human femoral artery and canine portal vein, in both of which there is some evidence suggesting the presence of postjunctional α_2-adrenoceptors (Glusa & Markwardt, 1983; Furuta, 1988), prazosin is only 11–35 fold more potent than yohimbine (De Mey & Vanhoutte, 1981; Shoji *et al.*, 1983). Similarly in the canine isolated splenic artery and vein the rauwolscine and prazosin potency ratio against noradrenaline (both agents produced competitive antagonism) was 12 and 3, respectively (Hieble & Woodward, 1984). This stands in contrast to the rabbit pulmonary artery (Starke, 1981), rat aorta or portal vein (Digges & Summers, 1983) where the differential is greater than 200 fold.

In summary, despite the fact that prazosin competitively antagonizes responses in the rabbit isolated distal saphenous artery and that no component of the CCRC to NA is resistant to the α_1-adrenoceptor antagonist, responses to NA mediated via postjunctional α_2-adrenoceptors can be demonstrated in this preparation under the appropriate experimental conditions. The susceptibility to prazosin of responses to NA can be explained by a functional interaction between the α-adrenoceptor subtypes, such that responses mediated via postjunctional α_2-adrenoceptors are dependent upon a degree of stimulation of α_1-adrenoceptors. In the presence of α_1-adrenoceptor blockade the necessary positive influence for α_2-adrenoceptor expression can be provided by stimulation of AII receptors.

This work was supported by the Science and Engineering Research Council, through a Collaborative Project Grant in association with Roche Products, U.K. (C.J.D. & V.G.W.), and a CASE studentship is association with Dr A.F. Lever of the MRC Blood Pressure Unit (W.R.D.). We are grateful for support also to the Medical Research Funds of the University of Glasgow.

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(Received October 5, 1990
Revised January 14, 1991
Accepted January 28, 1991)

Br. J. Pharmacol. (1991), **103**, 1493–1499 © Macmillan Press Ltd, 1991

The effects of nifedipine on α_2-adrenoceptor-mediated contractions in several isolated blood vessels from the rabbit

[1]W.R. Dunn, C.J. Daly, J.C. McGrath & [2]V.G. Wilson

Autonomic Physiology Unit, Institute of Physiology, University of Glasgow, Glasgow G12 8QQ

1 The effects of the dihydropyridine calcium channel blocker, nifedipine, on noradrenaline-induced contractile responses have been examined in several isolated blood vessels from the rabbit, with particular emphasis on responses mediated via postjunctional α_2-adrenoceptors.

2 In the isolated renal vein, ear vein, distal saphenous artery, saphenous vein and plantaris vein, 0.1 μM and 1 μM nifedipine reduced responses elicited by 54 mM KCl by more than 70%. The remaining responses were abolished by α-adrenoceptor blockade, suggesting the involvement of noradrenaline released from neurones activating a dihydropyridine-resistant mechanism.

3 In the renal vein (α_1-), ear vein (predominantly α_2-), distal saphenous artery (α_1- > α_2-), saphenous vein and plantaris vein (α_2- > α_1-), 0.01 μM and 0.1 μM nifedipine produced concentration-related reductions in the maximum response to noradrenaline. However, 1 μM nifedipine was no more effective than 0.1 μM nifedipine and the reduction in the maximum varied from 10–25% of the control response. Thus, a sizeable component of the α-adrenoceptor-medaited response in all blood vessels is resistant to dihydropyridine calcium channel blockers and this appears to be unrelated to the α-adrenoceptor subtype involved.

4 Following irreversible inactivation of α_1-adrenoceptors and isolation of functional α_2-adrenoceptors in the saphenous vein, plantaris vein and distal saphenous artery (the latter requiring the presence of angiotensin II), the effect of nifedipine on responses to noradrenaline was increased. However, a component of the α_2-adrenoceptor response in each preparation was present even after the concentration of nifedipine was increased to 1 μM.

5 In the saphenous vein, a preparation in which it has been demonstrated previously that α_2-adrenoceptor-mediated responses are highly dependent upon the presence of extracellular calcium ions, partial depolarization with 20 mM KCl failed to increase the inhibitory effect of 0.1 μM nifedipine. This suggests the involvement of dihydropyridine-resistant Ca^{2+} channels. The possible relationship between these dihydropyridine-resistant Ca^{2+} channels, α-adrenoceptor subtypes and 'receptor-operated' Ca^{2+} channels is discussed.

Keywords: Nifedipine; dihydropyridine-sensitive Ca^{2+} channels; dihydropyridine-resistant Ca^{2+} channels; α_1-adrenoceptors; α_2-adrenoceptors; vascular smooth muscle

Introduction

The introduction of dihydropyridine Ca^{2+} entry blockers, e.g. nifedipine, has provided a pharmacological basis for determining the involvement of L-type voltage-operated Ca^{2+} channels and receptor-operated, voltage-independent Ca^{2+} channels in contractile responses of vascular smooth muscle (Godfraind *et al.*, 1986). The α-adrenoceptor system in vascular smooth muscle and in particular responses mediated by the α_1- and α_2-adrenoceptor subtypes, has been a major focus of attention for the action of these agents because of their contribution to vascular tone in both normal and hypertensive states.

In the pithed rat, nifedipine and the dihydropyridine 'Ca^{2+} agonist', Bay K 8644, were found to inhibit and potentiate, respectively, pressor responses to the bolus injection of selective α_2-adrenoceptor agonists and 'low efficacy' selective α_1-adrenoceptor agonists (van Meel *et al.*, 1983; Wilffert *et al.*, 1984). Pressor responses to the bolus injection of 'high efficacy' selective α_1-adrenoceptor agonists were relatively unaffected (Timmermans & van Zwieten, 1982; Wilffert *et al.*, 1984). This was interpreted as evidence that while α_1-adrenoceptors mobilize intracellular Ca^{2+} and open dihydropyridine-sensitive Ca^{2+} channels, pressor responses to α_2-adrenoceptors are entirely dependent upon the entry of Ca^{2+} via dihydropyridine-sensitive Ca^{2+} channels (van Zwieten & Timmermans, 1987). However, in several other papers a different conclusion was reached. Calcium entry blockers failed to exhibit differential potency against responses arising from the infusion of various selective agonist in the spinal dog (Morita *et al.*, 1985), pithed rat (O'Brien & McGrath, 1987) and conscious unrestrained rat (Lefevre-Borg *et al.*, 1988). Thus, the manner in which α-adrenoceptor agonists are administered is a major factor in determining the sensitivity of the pressor responses to Ca^{2+} entry blockers and, therefore, the contribution of Ca^{2+} channels to responses via each subtype appears to be similar.

A systematic examination of the effect of Ca^{2+} entry blockers on α_1- and α_2-adrenoceptor-mediated responses in isolated blood vessels would both circumvent the problems of non-equilibrium responses and permit an examination of the contribution of cellular Ca^{2+} to responses. However, this has been hampered by the paucity of blood vessels that possess a functional population of postjunctional α_2-adrenoceptors. In the majority of isolated blood vessels with postjunctional α_1-adrenoceptors, a sizeable component of contractile responses is either resistant to dihydropyridine Ca^{2+} entry blockers or is still observed following removal of extracellular Ca^{2+} ions (see: Godfraind *et al.*, 1986). This lends support to the general view that α_1-adrenoceptor-mediated contractions are maintained by Ca^{2+} derived from two sources. In the few preparations with postjunctional α_2-adrenoceptors that have been examined, the results with Ca^{2+} entry blockers have varied greatly. Responses have been found to be either very sensitive to Ca^{2+} entry blockade, e.g. in the cat middle cerebral artery (Skärby, 1984) and the rat tail artery (Abe *et al.*,

[1] Present address: Department of Pharmacology, Given Medical Building, University of Vermont, College of Medicine, Burlington, Vermont 05405 U.S.A.

[2] Author for correspondence at present address: Department of Physiology and Pharmacology, The Medical School, Queen's Medical Centre, Clifton Boulevard, Nottingham NG7 2UH.

1987), or to exhibit marked resistance to Ca^{2+} entry blockers, e.g. in the canine isolated saphenous vein (DeMey & Vanhoutte, 1981; Matthews et al., 1984) or rabbit isolated saphenous vein (Schümann & Lues, 1983). However, direct comparisons versus different receptors under similar conditions in a single species have not been made.

We have recently characterized the α-adrenoceptors mediating contractions in several isolated blood vessels from the rabbit and identified preparations with either a mixture or a practically homogeneous population of α_1- or α_2-adrenoceptors (Daly et al., 1988a,b,c; Dunn et al., 1989). In addition, the contribution of cellular-bound calcium to contractions elicited by noradrenaline has been examined in each of these preparations (Daly et al., 1990). This has provided preliminary evidence that both α_1- and α_2-adrenoceptors can mediate the release of cellular Ca^{2+}. We have, therefore, examined the effects of nifedipine on noradrenaline-induced contractions in several blood vessels from the rabbit, both before and after pharmacological isolation of postjunctional α_2-adrenoceptors, to determine the relative contribution of dihydropyridine-sensitive and dihydropyridine-resistant Ca^{2+} channels to contractile responses.

Methods

White albino New Zealand rabbits of either sex weighing 2.3–3.0 kg were killed by stunning followed by exsanguination. Segments of the distal saphenous artery (DSA), the left renal vein (LV), the ear vein (EV), the lateral saphenous vein (SV) and the plantaris vein (PV) were cleaned of fat and connective tissue *in situ* and then placed in ice-cold modified Krebs-Henseleit saline (composition below). The ear vein was defined as the 10 mm region either side of the first bifurcation of the vein running parallel to the ear artery. The plantaris vein was taken as a 15 mm distal segment of the continuation of the lateral saphenous vein measured from the ankle. The distal saphenous artery was taken as a 20 mm segment of the saphenous artery (medial side) between the knee and ankle. Segments 3 mm in length were taken from each vein (5 mm for the ear vein) or artery and suspended between two 0.2 mm thick stainless steel supports as previously described (Daly et al., 1988b). The upper support was connected by surgical silk to a Grass FT03 isometric transducer while the lower support was connected to a rigid glass tissue holder. Preparations were then mounted in 30 ml isolated organ baths under an initial resting tension of 1.5 g wt. (distal saphenous artery), 0.5 g wt. (renal vein), 0.3 g wt. (ear vein), 1.5 g wt. (lateral plantaris vein), 2 g wt. (lateral saphenous vein) and allowed to relax. Each segment was bathed in modified Krebs-Henseleit saline maintained at 37°C and gassed with 5% CO_2 in O_2. No attempt was made to remove the endothelium in any preparation.

After a 90 min equilibration period, during which a steady resting tension was achieved, each preparation was exposed to 3 μM noradrenaline (NA) and allowed to contract for 10 min. After a complete washout, an additional 1 h equilibration period was allowed before starting the experiment. This procedure was found to minimize changes in the sensitivity of the preparation to subsequent additions of the agonist. Basal tension after the sighting response remained stable for the rest of the experiment; 0.5–0.6 g wt. (distal saphenous artery); 0.15–0.2 g wt. (renal vein); 0.1–0.15 g wt. (ear vein); 0.2–0.3 g wt. (lateral plantaris vein, lateral saphenous vein). Isometric contractions were recorded by a Grass FT03 transducer connected to a Linseis 6025 pen recorder.

A cumulative concentration-response curve (CRC) to NA was constructed in each preparation by increasing the concentration of NA by approximately a half log unit only after the preceding response had peaked or reached an equilibrium. Following attainment of the maximum response, preparations were washed repeatedly with Krebs-Henseleit saline to effect complete relaxation and then exposed to either 0.02% absolute ethanol or nifedipine (0.1 μM and 1 μM) for 40 min and the CRC repeated. Each preparation was exposed to only one concentration of nifedipine and all experiments were performed in subdued lighting. The effect of 0.1 μM nifedipine was also examined against responses to 54 mM KCl (no equimolar replacement of NaCl).

Isolation of postjunctional α_2-adrenoceptors

In one series of experiments, postjunctional α_2-adrenoceptors in the lateral saphenous vein, the plantaris vein and the distal saphenous artery were isolated by the receptor protection procedure described previously (Daly et al., 1988c). Briefly, preparations were exposed to 1 μM rauwolscine 10 min before the addition of 0.3 μM phenoxybenzamine. Both antagonists were applied to the tissue for either 30 min (veins) or 60 min (artery) and the irreversible antagonist was then washed out by replacing the bathing solution twice with modified Krebs-Henseleit saline containing 1 μM rauwolscine only. Finally, 1 μM rauwolscine was removed by replacing the bathing solution 5 times during a period of 60 min. A CRC to NA was constructed as described above, and repeated after 40 min exposure to either 0.02% absolute ethanol or nifedipine (0.01 μM to 1 μM). In the distal saphenous artery (only) α_2-adrenoceptor-mediated responses were observed only if the preparation was exposed to 0.05 μM angiotensin II (Dunn et al., 1991; see Figure 3). Thus, all responses to NA were elicited in the presence of angiotensin II. In a further series of experiments on the saphenous vein, preparations were exposed to 20 mM KCl (without replacement of equimolar NaCl) for 35 min of the equilibration period with 0.1 μM nifedipine. At the end of this period the CRC to NA was repeated.

The effects of 0.1 μM prazosin and 1 μM rauwolscine, concentrations selective for α_1- and α_2-adrenoceptors, respectively (Daly et al., 1988a,b), versus responses to NA in the distal saphenous vein and the plantaris vein were examined before and after pharmacological isolation of postjunctional α_2-adrenoceptors.

Calculation of results

Unless otherwise stated, responses to agonists are expressed as a percentage (mean ± s.e.mean) of the maximum response to noradrenaline in the first concentration-response curve. The negative logarithm of the concentration of the agonist required to produce 50% of the maximum contractile response ($-\log EC_{50}$ or pD_2) was also determined in the presence and absence of nifedipine. The maximum response to NA in the presence of nifedipine (E_{max}, relative to the 1st CRC) has also been determined after correction for time-related changes in parallel control preparations exposed to 0.02% absolute ethanol. The significance of differences between mean values was determined with either a paired or unpaired Student's t test as appropriate. Unless otherwise stated, $P < 0.05$ is considered significant.

Drugs and solutions

The composition of the modified Krebs-Henseleit saline was (mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4.7H_2O$ 1.2, $NaHCO_3$ 24.9, KH_2PO_4 1.2 and glucose 11.1. Na_2EDTA (23 μM) was included in all experiments to prevent oxidative degradation of NA. Propranolol 1 μM and cocaine, 10 μM, were also included to inhibit β-adrenoceptors and uptake$_1$, respectively. The following drugs were used: (−)-noradrenaline bitartrate (Sigma), prazosin HCl (Pfizer), rauwolscine HCl (Roth), phenoxybenzamine HCl (SK&F), nifedipine (Bayer), (±)-propranolol HCl (Sigma), angiotensin II amide (Hypertensin, Ciba) and cocaine HCl (Macarthys). Nifedipine and phenoxybenzamine were dissolved in 20% absolute ethanol and NA was dissolved in 23 μM Na_2EDTA. All other drugs were dissolved in distilled water and added to the organ baths in volumes of 0.1 ml.

Results

The effects of 0.1 μM nifedipine on responses to 54 mM KCl

The response to 54 mM KCl in control preparations of the renal vein, distal saphenous artery, saphenous vein, plantaris vein and ear vein varied between 20–80% of the maximum response to NA (Figure 1). Nifedipine, 0.1 μM reduced responses to KCl in all preparations by more than 70%. In the ear vein and plantaris vein a 10 fold increase in the concentration of nifedipine failed to produce a significantly greater reduction in responses. The nifedipine-resistant response appears to be due to neuronal release of noradrenaline, since these responses in the saphenous vein, distal saphenous artery and renal vein were abolished by 0.1 μM prazosin, while those in the ear vein and plantaris veins were blocked by 1 μM phentolamine. These observations indicate that 0.1 μM nifedipine effectively blocks all voltage-operated Ca^{2+} channels (activated by KCl) that participate in contractile events in rabbit isolated blood vessels.

The effects of nifedipine on noradrenaline-induced contractions

The vehicle used in these experiments (maximum concentration of 0.02% ethanol) exerted no effect on either the sensitivity or the maximum response to noradrenaline in any preparation, over that observed with time controls.

Figure 2 shows the effects of 0.1 μM and 1 μM nifedipine on noradrenaline-induced contractions. In the saphenous vein and ear vein (Figure 2c,e), both concentrations of nifedipine produced between 15–30% reduction in the maximum response to noradrenaline (relative to the time control, but there was no significant difference between the effects of the two concentrations, see also Table 1). This was not markedly different from the effect of 0.1 μM nifedipine on NA responses in the renal vein and plantaris vein (Figure 2a,d. Table 1). Furthermore, with the exception of the plantaris vein, the sensitivity of the venous preparations to NA was unaltered by 0.1 μM nifedipine (Table 1). Thus, the overall effect of nifedipine on noradrenaline-induced contraction in venous smooth muscle is essentially the same, irrespective of whether the receptors mediating the response belong to the α_1-subtype (renal vein, Daly *et al.*, 1988b), the α_2-subtype (ear vein, Daly *et al.*, 1988a), or a mixture of both (saphenous vein and plantaris vein, Daly *et al.*, 1988b).

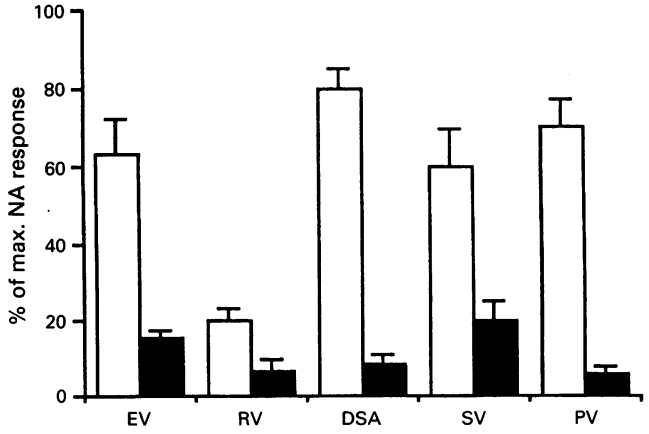

Figure 1 Contractile responses to 54 mM KCl in the ear vein (EV), renal vein (RV), distal saphenous artery (DSA), saphenous vein (SV) and plantaris vein (PV) isolated from the rabbit in the absence (open columns) and in the presence (closed columns) of 0.1 μM nifedipine. Responses have been expressed as a percentage of the maximum response to noradrenaline in each preparation and are shown as the mean of 4–6 observations; vertical bars show s.e.mean.

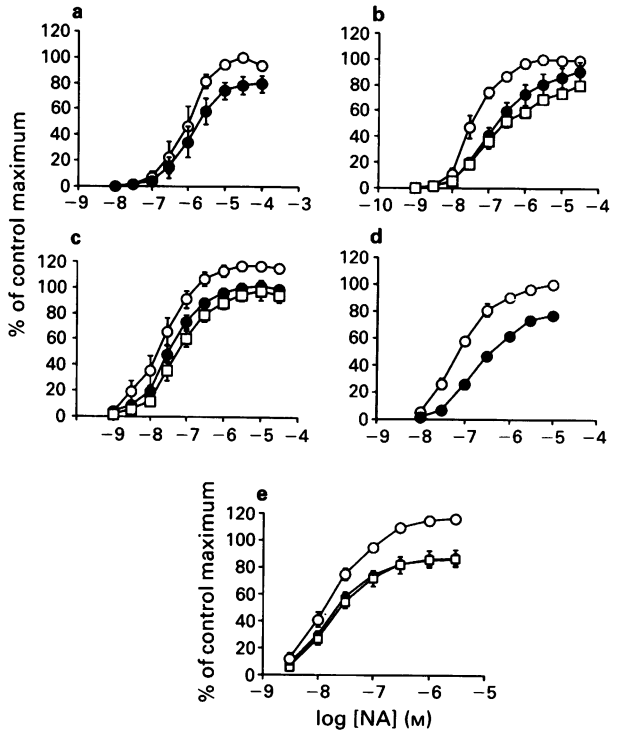

Figure 2 The effects of 0.1 μM (●) and 1 μM (□) nifedipine on contractile responses elicited by noradrenaline in (a) the renal vein, (b) the distal saphenous vein, (c) the saphenous vein, (d) the plantaris vein and (e) the ear vein isolated from the rabbit. All responses have been expressed as a percentage of the maximum response to noradrenaline in the first concentration-response curve, and are shown as the mean of a minimum of 5 observations: vertical bars show s.e.mean. Responses to noradrenaline in the presence of 0.02% absolute ethanol are shown by (○).

In the distal saphenous artery, 0.1 μM nifedipine significantly reduced the sensitivity of the preparation to NA (an effect also seen with 0.01 μM nifedipine) but failed to alter the maximum response (Table 1). However, 1 μM nifedipine significantly reduced both parameters (Figure 2b).

Table 1 Mean pD_2 values and E_{max} values (with s.e. of the mean) for noradrenaline (NA) in the presence of 0.1 μM nifedipine as compared to control values corrected for time-related changes in responses in the left renal vein (LRV), the distal saphenous artery (DSA), the saphenous vein (SV), the plantaris vein (PV) and ear vein (EV)

	pD_2 values		E_{max}
Preparation	*Control*	*Nifedipine*	*Nifedipine*
LRV (n = 5)	6.07 ± 0.20	5.90 ± 0.16	0.80 ± 0.07*
DSA (n = 5)	7.45 ± 0.15	6.88 ± 0.05*	0.91 ± 0.07
SV (n = 5)	7.47 ± 0.07	7.46 ± 0.09	0.86 ± 0.04**
PV (n = 6)	7.13 ± 0.09	6.72 ± 0.10*	0.75 ± 0.05**
EV (n = 6)	7.76 ± 0.07	7.77 ± 0.06	0.76 ± 0.06**
DSA–RP (n = 4)	6.66 ± 0.29	6.95 ± 0.16	0.30 ± 0.11**
PV–RP (n = 6)	6.93 ± 0.15	6.77 ± 0.13	0.42 ± 0.09**
SV–RP (n = 4)	6.66 ± 0.17	6.54 ± 0.24	0.63 ± 0.05**

Preparations exposed to 1 μM rauwolscine and 0.3 μM phenoxybenzamine to isolate the α_2-adrenoceptors are indicated by the abbreviation (–RP).

Differences between control and treated preparations were considered statistically significant if $P < 0.05$ for either paired or unpaired observations (Student's t test) and are denoted by: * $0.05 > P > 0.01$, ** $0.01 > P > 0.001$.

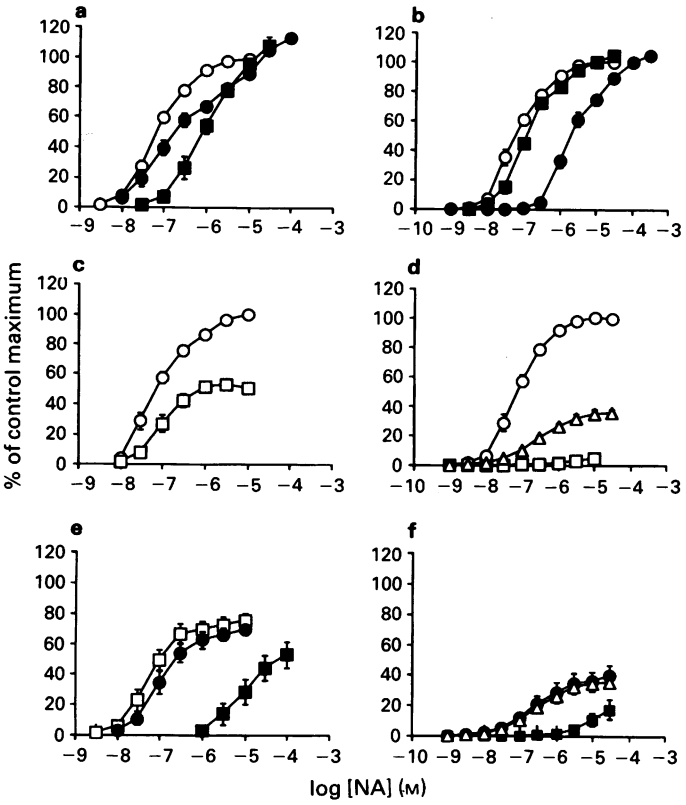

Figure 3 The effect of the receptor protection procedure with 1 μM rauwolscine and 0.3 μM phenoxybenzamine on the pharmacological characteristics of responses to noradrenaline in the isolated plantaris vein and isolated distal saphenous artery from the rabbit. (a and b) The effects of (●) 0.1 μM prazosin and (■) 1 μM rauwolscine on responses to noradrenaline in (a) the isolated plantaris vein and (b) the isolated distal saphenous artery. (c and d) Concentration-response curves to noradrenaline (○) before and (□) after the receptor protection procedure in (c) the isolated plantaris vein and (d) the isolated distal saphenous vein. The effect of 0.05 μM angiotensin II (△) on post-receptor protection responses to noradrenaline in the distal saphenous vein is also shown. (e and f) The effects of (●) 0.1 μM prazosin and (■) 1 μM rauwolscine on responses to noradrenaline following receptor protection in the (e) isolated plantaris vein and (f) isolated distal saphenous vein (in the presence of 0.05 μM angiotensin II) (cf. a and b). In (a–d), the control concentration-response curve to noradrenaline before receptor protection is represented (○) and after receptor protection by either (□ or △ if AII is present). Responses have been expressed as a percentage of the maximum response to noradrenaline in control preparations and are shown as the mean of a minimum of 5 observations; vertical bars show s.e.mean.

Isolation of α_2-adrenoceptor-mediated responses by irreversible inactivation of postjunctional α_1-adrenoceptors

Unlike in the renal and ear veins, the α-adrenoceptors in the distal saphenous artery, saphenous vein and plantaris vein consist of a mixture of α-adrenoceptor subtypes: $\alpha_1 > \alpha_2$–distal saphenous artery (Dunn *et al.*, 1989); $\alpha_2 > \alpha_1$–saphenous vein and plantaris vein (Daly *et al.*, 1988b). Thus, to investigate further the effect of nifedipine on α_2-adrenoceptor-mediated responses, we have isolated postjunctional α_2-adrenoceptors in these three preparations by use of the receptor protection experiment developed earlier (Daly *et al.*, 1988c).

Figure 3a and b shows the effects of 0.1 μM prazosin and 1 μM rauwolscine on NA-induced contractions in the plantaris vein and the distal saphenous artery. In both preparations, the inhibitory activity of 0.1 μM prazosin indicates the presence of α_1-adrenoceptors (distal saphenous artery > plantaris vein), while the limited effect of 1 μM rauwolscine is not consistent with a homogeneous population of postjunctional α_2-adrenoceptors. Following receptor protection with 1 μM rauwolscine and exposure to 0.3 μM phenoxybenzamine (RP) the maximum response to noradrenaline was reduced by approximately 45% in the plantaris vein (PV–RP) and 95% in distal saphenous artery (DSA–RP) (Figure 3c and d). However, concentration-dependent responses of DSA–RP were observed in the presence of 0.05 μM angiotensin II (Figure 3d) and the maximum responses to NA increased from approximately 5% to 30% of the maximum pre- RP control response. This peptide produced a transient contractile response (22.7 ± 4.9% of the pre-RP

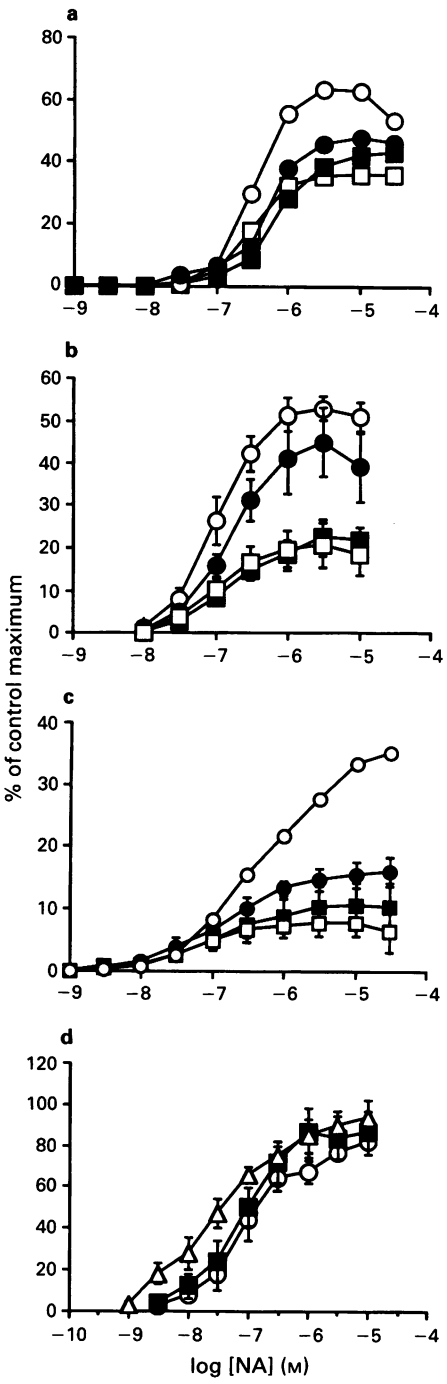

Figure 4 The effects of (●) 0.01 μM, (■) 0.1 μM and (□) 1 μM nifedipine on α_2-adrenoceptor-mediated responses in (a) the isolated saphenous vein, (b) the isolated plantaris vein and (c) the isolated distal saphenous artery. The effect of (■) 0.1 μM nifedipine on α_2-adrenoceptor responses of the rabbit isolated saphenous vein to noradrenaline (NA) in the presence of 20 mM KCl is shown in (d). The control concentration-response curve to noradrenaline following receptor protection is represented by (○) and that in the presence of 20 mM KCl by (△). All responses have been expressed as a percentage of the maximum response to noradrenaline prior to the receptor protection procedure, and are given as the mean of a minimum of 5 observations; s.e.mean shown by vertical bars. On graphs (a) and (c) the standard error bars (< 8%) have been omitted to increase clarity.

maximum responses to NA, $n = 5$) which returned to baseline after 15 min, and has previously been used to uncover α_2-adrenoceptor-mediated responses in this preparation (Dunn *et al.*, 1989).

After the receptor protection procedure (and addition of 0.05 μM angiotensin II to DSA–RP), the pharmacological profile of the response to NA in both the plantaris vein and the distal saphenous artery was consistent with that of an α_2-adrenoceptor, the concentration-response curves were resistant to 0.1 μM prazosin and displaced approximately 100 fold by 1 μM rauwolscine (Figure 3e,f). The effect of this protocol on α-adrenoceptors is essentially similar to that previously observed in the saphenous vein (Daly *et al.*, 1988c).

The effects of nifedipine on α_2-adrenoceptor-mediated responses after irreversible inactivation of postjunctional α_1-adrenoceptors

Nifedipine, 0.01 μM and 0.1 μM, produced a concentration-dependent reduction of responses to NA in the saphenous vein (Figure 4a), the plantaris vein (Figure 4b) and the distal saphenous artery (Figure 4c) following isolation of the postjunctional α_2-adrenoceptors. This effect was observed in the absence of a change in the potency of NA (Table 1). In the distal saphenous artery the transient contractile response to 0.05 μM angiotensin II in the presence of 0.1 μM nifedipine (18.8 ± 4.3% of the pre-RP maximum to NA) was not significantly different from control responses (see above). Hence the inhibitory effect of nifedipine in this preparation is not the consequence of an indirect effect on the angiotensin II contraction required to uncover α_2-adrenoceptor-mediated responses. Based upon the reduction in the maximum responses to NA produced by 0.1 μM nifedipine, the rank order of susceptibility to Ca^{2+} entry blockade was distal saphenous artery > plantaris vein > saphenous vein (Table 1). However, 1 μM nifedipine failed to produce a greater effect than 0.1 μM nifedipine (see Figure 4a,b,c). Thus a significant component of the α_2-adrenoceptor response in each of these preparations is resistant to nifedipine.

Figure 4d shows the effect of 35 min equilibration of the saphenous vein (RV–RP) with 0.1 μM nifedipine in partially depolarized Krebs-Henseleit solution (20 mM KCl excess) on responses to NA. In this particular series of experiments the reduction in the maximum responses following exposure to phenoxybenzamine and rauwolscine was less than that previously observed (approximately 20% in this series and approximately 40% for the results in Figure 4a). However, the residual response to NA was sensitive to rauwolscine and there was no evidence of a resistant component (indicative of the presence of postjunctional α_1-adrenoceptors). Under these conditions, the presence of 20 mM KCl failed to alter the effect of 0.1 μM nifedipine against α_2-adrenoceptor-mediated responses.

Discussion

The increase in intracellular calcium that supports α-adrenoceptor-mediated contractions of vascular smooth muscle can arise from three different sources: the release of cellular-bound calcium; entry via voltage-dependent Ca^{2+} channels (VOCs); entry via receptor-operated, voltage-independent Ca^{2+} channels (ROCs) (Bolton, 1979; Godfraind *et al.*, 1986). In a previous paper, we examined the relative contributions of cellular-bound Ca^{2+} ions and of the influx of extracellular Ca^{2+} ions to contractions mediated by α_1- and α_2-adrenoceptors in several isolated blood vessels from the rabbit (Daly *et al.*, 1989a; 1990). The basis of these experiments was that a buffered low concentration of extracellular Ca^{2+} (0.1 μM) abolished contractile responses to KCl, yet noradrenaline elicited initial transient contractions reflecting the release of cellular-bound Ca^{2+} ions. Pharmacological examination of the initial transient contraction with selective antagonists suggested that both α_1- and α_2-adrenoceptors can mediate the release of cellular-bound Ca^{2+} ions and, as such, represented a major departure with the prevailing view that α_2-adrenoceptors are entirely dependent upon the entry of extracellular Ca^{2+} ions (Timmermans & Zwieten, 1987; Nichols & Ruffolo, 1988). Our results are further supported by the observations of Michel and coworkers (1989), who demonstrated that activation of α_2-adrenoceptors in human erythroleukaemia cells results in the elevation of intracellular Ca^{2+} ion concentration in the absence of extracellular Ca^{2+} ions.

In the present study we have adopted a similar approach with the Ca^{2+} entry blocker, nifedipine. This has allowed us to determine the contribution of dihydropyridine-sensitive and dihydropyridine-resistant Ca^{2+} channels to the component of α_1- and α_2-adrenoceptor-mediated contractions dependent upon the entry of extracellular Ca^{2+} ions. In each of the blood vessels examined, KCl-induced contractions were markedly reduced by 0.1 μM nifedipine and a 10 fold increase in the concentration of the Ca^{2+} entry blocker failed to produce a greater effect. Furthermore, the residual response in each preparation was abolished by α-adrenoceptor blockade (and was practically absent in low Ca^{2+}) suggesting that NA, released from neurones by depolarization (hence Ca^{2+} influx and exocytosis), causes contraction through α-adrenoceptors linked to nifedipine-resistant Ca^{2+} entry.

Thus, responses observed in the presence of 0.1 μM nifedipine can be attributed either to the release of cellular-bound Ca^{2+} ions, to dihydropyridine-resistant ROCs and/or to dihydropyridine-resistant VOCs. The possibility of the latter is unlikely because T and N-type VOCs are inactivated too quickly to make a significant contribution to the elevation of intracellular Ca^{2+} ions required to support vascular contractions (Yatani *et al.*, 1987). Furthermore, we know of no example of an L-type VOC resistant to the dihydropyridine Ca^{2+} entry blockers. Nonetheless, while we readily concede that dihydropyridine-resistant responses to noradrenaline may reflect the activation of receptor operated, voltage-independent Ca^{2+} channels, this term has been avoided in relation to our own observations because: (i) the electrophysiological changes that accompany the contractile events in each tissue were not determined; (ii) there are examples of blood vessels in which Ca^{2+} entry blockers display potency against α-adrenoceptor-mediated contractions that occur in the absence of measurable changes in membrane potential (e.g. Häusler 1985; Abe *et al.*, 1987).

Contribution of dihydropyridine-sensitive Ca^{2+}-channels to α_1- and α_2-adrenoceptor-mediated contractions

The results in the present study, in agreement with several other reports with *in vivo* models (Morita *et al.*, 1985; O'Brien & McGrath, 1987; Lefevre-Borg *et al.*, 1989), indicate that α_1- and α_2-adrenoceptor-mediated contractions of vascular smooth muscle are similarly dependent upon the entry of Ca^{2+} ions through dihydropyridine-sensitive Ca^{2+} channels. For example, the reduction in the maximum response to noradrenaline in the left renal vein (α_1-) and the ear vein (almost exclusively α_2-) produced by 0.1 μM nifedipine were of similar magnitude (see Table 1). Furthermore, since 1 μM rauwolscine did not mimic the effect of nifedipine in the distal saphenous artery (compare Figures 3b and 2b), the reduction in potency of noradrenaline can be partly attributed to an effect on the α_1-adrenoceptor component. This is consistent with observations in two other preparations with predominantly α_1-adrenoceptors, the rabbit isolated femoral artery and isolated renal artery, where nifedipine also significantly reduced the potency of noradrenaline (Purdy & Weber, 1988; Hashimoto *et al.*, 1989).

Even after irreversible inactivation of postjunctional α_1-adrenoceptors and isolation of α_2-adrenoceptors, when the effect of nifedipine was more pronounced (see Table 1), a significant component of the contractile response to noradrenaline in the distal saphenous vein, the plantaris vein and the

saphenous vein was resistant to nifedipine. This is in agreement with other reports in which α_2-adrenoceptor-mediated responses, in the rabbit saphenous vein (Schümann & Lues, 1983) and canine saphenous vein (De Mey & Vanhoutte, 1981; Cooke et al., 1985), displayed considerable resistance to Ca^{2+} entry blockade. These observations contrast sharply with α_1-adrenoceptor-mediated contractions in the rat isolated longitudinal portal vein (Jetley & Weston, 1979), canine coronary artery (Rimele et al., 1983) and rabbit mesenteric resistance arteries (Cauvin et al., 1984), in which practically the whole contractile response to NA is sensitive to Ca^{2+} entry blockade.

Clearly, the prevailing view that α_2-adrenoceptor-mediated responses are invariably more sensitive to Ca^{2+} entry blockade than those elicited via α_1-adrenoceptors (Timmermans & van Zwieten, 1987; Nichols & Ruffolo, 1988), developed primarily from data derived from the bolus injection of synthetic α-adrenoceptor agonists in *in vivo* models, is no longer tenable. In the present study we identified suitable isolated blood vessels with functional α_1- and α_2-adrenoceptors and elicited responses with the endogenous agonist, noradrenaline. Furthermore, evidence from studies on human isolated digital arteries and hand veins with noradrenaline indicate that both α_1- and α_2-adrenoceptor-mediated responses exhibit similar sensitivity to Ca^{2+} entry blockade (Stevens & Moulds, 1986; Arner et al., 1988). Thus, there is no exclusive contract between subtypes of α-adrenoceptors and particular Ca^{2+} activation mechanisms.

Nifedipine-resistant contractions: evidence for the release of intracellular Ca^{2+} ions or dihydropyridine-resistant Ca^{2+} channels?

Triggle and coworkers (1988) have shown that the interaction of dihydropyridine Ca^{2+} entry blockers with their specific binding sites is a voltage-dependent phenomenon. Thus, the possibility exists that the resistance of α_1- and α_2-adrenoceptor-mediated contractions to nifedipine is attributable to the inability of noradrenaline to depolarize the tissue sufficiently to ensure nifedipine binding to essentially nifedipine-sensitive Ca^{2+} channels. However, the potency of nifedipine against α_2-adrenoceptor-mediated contractions in the saphenous vein (SV–RP) was not enhanced by equilibration in a partially depolarized Krebs-Henseleit solution (Figure 4d). Thus, the nifedipine-resistant contractions are due either to the release of intracellular Ca^{2+} ions or to the entry of Ca^{2+} ions via dihydropyridine-resistant channels.

Putney (1986) proposed a simple model for a link between the entry of extracellular Ca^{2+} ions via receptor-operated, voltage-independent channels and the release of cellular-bound Ca^{2+} ions, which has been refined further by Berridge & Irvine (1989). Basically, entry of Ca^{2+} ions is dependent upon prior emptying of the bound stores of Ca^{2+} ions by a receptor-linked intracellular messenger, e.g. inositol trisphosphate. Thus the superficial stores of bound Ca^{2+} ions act as a transit point for the entry of extracellular Ca^{2+} ions into the cell. Such a capacitive model for cellular Ca^{2+} movements is attractive because it obviates the need for an independent receptor-linked Ca^{2+} channel, unequivocal evidence for which has proved difficult to obtain (see: Benham & Tsien, 1987), and manages to accommodate several experimental findings.

First, contractile responses to noradrenaline in the rabbit isolated renal vein and rat isolated anococcygeus (α_1-adrenoceptors) and rabbit isolated ear vein (α_2-adrenoceptors) are not sustained in low ($<0.1\,\mu$M) concentrations of extracellular Ca^{2+} ions (McGrath, 1985; Daly et al., 1990), but are resistant to nifedipine in 2.5 mM Ca^{2+} (present study; Vila et al., 1985). Secondly, the effects of organic and inorganic (e.g. Ni^{2+} or La^{3+} ions) Ca^{2+} entry blockers on individual contractile responses show a characteristic difference: the latter inhibits all Ca^{2+} entry and, therefore, cellular stores are not refilled and the response is not sustained (Deth & van Breeman, 1974). Thirdly, α_1-adrenoceptor-mediated responses in the rat isolated longitudinal portal vein, canine coronary artery and the rabbit isolated mesenteric resistance vessels show marked sensitivity to Ca^{2+} entry blockers (Jetley & Weston, 1980; Rimele et al., 1983; Cauvin et al., 1984) and none of these preparations appear to have appreciable cellular-bound Ca^{2+} stores.

We have previously demonstrated that α_2-adreno- ceptor-mediated contractions of the rabbit isolated saphenous vein (SV–RP) are abolished following prolonged equilibration in nominally Ca^{2+}-free Krebs-Henseleit solution (Daly et al., 1989b) and are not associated with an initial transient contraction after a 10 min exposure to buffered low Ca^{2+} ($0.1\,\mu$M) Krebs-Henseleit solution (Daly et al., 1990). Thus, responses in this preparation appear to be entirely dependent upon the entry of extracellular Ca^{2+} ions and distinct from that proposed by Putney (1986), i.e. no passage via superficially located stores, but direct entry into the cytosol. Qualitatively similar observations have been reported in the arterial circulation of the rabbit isolated ear (Owen et al., 1987). The dependence of α-adrenoceptor-mediated contractions upon the presence of extracellular Ca^{2+} ions in this vascular bed was inversely related to the calibre of the arterial segment, yet this was not associated with a parallel increase in the sensitivity of responses to the dihydropyridine derivative, nimodipine. The possibility that these nifedipine-resistant Ca^{2+} channels are examples of receptor-operated, voltage-independent Ca^{2+} channels (Bolton, 1979; Godfraind et al., 1986) requires detailed electrophysiological examination, particularly in view of the recent report of a voltage-independent non-selective cation channel linked to α-adrenoceptor activation in the rabbit ear artery (Amedee et al., 1990).

In conclusion, we have provided evidence that α_1- and α_2-adrenoceptor-mediated contractions to noradrenaline in isolated blood vessels from the rabbit both utilize nifedipine-sensitive Ca^{2+} channels. Although no direct electrophysiological evidence has been provided, it seems likely that these channels are voltage-operated channels. These observations also complement our earlier findings that α_2-adrenoceptors, like those mediated by the α_1-subtype, can involve the mobilization of Ca^{2+} ions from cellular-bound stores. Furthermore, α_2-adrenoceptor-mediated contractions in the saphenous vein displayed considerable resistance to nifedipine, yet we found no evidence that this response was associated with the mobilization of intracellular Ca^{2+} ions. Could this be another example of receptor-operated, voltage-independent Ca^{2+} channels coupled to postjunctional α_2-adrenoceptors (see: Matthews et al., 1984)?

This work was supported by the Science and Engineering Research Council, through a Collaborative Project Grant in association with Roche Products, U.K. (C.J.D. & V.G.W.), and a CASE studentship in association with Dr A.F. Lever of the MRC Blood Pressure Unit (W.R.D.). We are grateful for support also to the Medical Research Funds of the University of Glasgow.

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(Received October 5, 1990
Revised January 14, 1991
Accepted January 28, 1991)

Br. J. Pharmacol. (1991), **103**, 1500–1508 © Macmillan Press Ltd, 1991

Differential effects of (±)-dobutamine and human α-CGRP on cardiac and on regional haemodynamics in conscious Long Evans rats

[1]S.M. Gardiner, A.M. Compton, P.A. Kemp, T. Bennett, *B. Hughes & *R. Foulkes

Department of Physiology and Pharmacology, Medical School, Queen's Medical Centre, Nottingham, NG7 2UH and *Pharmacology Department, Celltech Ltd, Slough, SL1 4EN

1 Comparisons were made of the full haemodynamic profiles of the known cardiostimulant, (±)-dobutamine, and the putative inotropic peptide, human α-calcitonin gene-related peptide (human α-CGRP), in conscious, chronically-instrumented Long Evans rats. Both substances were administered continuously i.v. for 60 min at two doses ((±)-dobutamine, 2 and 10 μmol kg^{-1} h^{-1}; human α-CGRP, 0.15 and 1.5 nmol kg^{-1} h^{-1}).

2 In spite of their similar (small) effects on mean arterial blood pressure, the low doses of (±)-dobutamine and human α-CGRP influenced cardiac haemodynamics differently. Thus, (±)-dobutamine caused an increase in cardiac index (due to a tachycardia), accompanied by rises in peak aortic flow, maximum rate of rise of aortic flow (dF/dt_{max}) and total peripheral conductance. However, the latter waned during the infusion, and after the infusion there was a significant systemic vasoconstriction and reductions in peak aortic flow, dF/dt_{max} and stroke index. Such 'off' effects following dobutamine infusion have not been described previously. The infusion of the lower dose of human α-CGRP caused only a transient fall in central venous pressure.

3 The rise in total peripheral conductance during infusion of the lower dose of (±)-dobutamine was associated with increases in hindquarters and common and internal carotid vascular conductances. The fall in total peripheral conductance after infusion was associated with renal vasoconstriction. Although there was no significant change in total peripheral conductance during the infusion of the lower dose of human α-CGRP there were hindquarters and carotid vasodilatations together with mesenteric vasoconstriction.

4 Infusion of the higher dose of (±)-dobutamine had greater effects than the lower dose on all cardiac haemodynamic variables and additionally, increased stroke index. However, the negative cardiac haemodynamic effects following the offset of infusion were also enhanced in association with marked renal and mesenteric vasoconstrictions. While infusion of the higher dose of human α-CGRP increased cardiac index, peak aortic flow, dF/dt_{max} and total peripheral conductance, stroke index fell together with central venous pressure.

5 (±)-Dobutamine caused greater cardiostimulation and increases in hindquarters blood flow than did human α-CGRP. However, the latter at the higher dose caused substantially greater common and internal carotid hyperaemia than did (±)-dobutamine, possibly indicating a selective and additional effect of human α-CGRP on cranial blood flow. Furthermore, there were no adverse cardiovascular effects following infusion of human α-CGRP.

Keywords: (±)-Dobutamine; human α-CGRP; regional haemodynamics; cardiac function

Introduction

In vivo studies in conscious dogs (Wang *et al.*, 1989) and man (Gennari *et al.*, 1989; 1990) indicate that human α-calcitonin gene-related peptide (human α-CGRP) can increase cardiac output. However, results of experiments in rats are less clear-cut, possibly because of limitations with the methodology used. Thus, there have been two studies (DiPette *et al.*, 1987; Ando *et al.*, 1990) using the radiolabelled microsphere technique in acutely prepared rats (3 h post surgery), in which single measurements of cardiac output were made at a fixed time point after bolus injections of CGRP. Using this approach DiPette *et al.* (1987) observed a small increase, while Ando *et al.* (1990) found no change, in cardiac output after administration of rat and human α-CGRP, respectively. In contrast, Sirén & Feuerstein (1988) measured cardiac output intermittently with the thermodilution technique in conscious rats and reported an increase of 95 ± 16 ml min^{-1} kg^{-1} in cardiac index in response to a bolus dose of 1.0 nmol kg^{-1} rat CGRP, but found doses of 0.1 and 10 nmol kg^{-1} were without significant effect. However, Lappe *et al.* (1987) recorded cardiac output continuously in conscious rats (chronically instrumented with electromagnetic flow probes on their ascending aortae), and observed that incremental bolus doses of rat CGRP (over the range 66–1320 pmol kg^{-1}) caused dose-related (maximum 28 ± 2%) increases in cardiac output.

Hence, to date, results are conflicting and there have been no studies on the profile of cardiac haemodynamic effects of sustained infusions of rat or human α-CGRP in chronically instrumented rats. Furthermore, none of the experiments performed so far have compared either rat or human α-CGRP to an established drug, such as (±)-dobutamine, which is used clinically for its ability to improve cardiac function (Ruffolo, 1987; Marcus *et al.*, 1987). The primary aim of the present work was, therefore, to measure the cardiac haemodynamic effects of intravenous infusions of two doses of human α-CGRP and to compare them with the effects of infusions of (±)-dobutamine in the same animals. Our initial intention was to compare doses of human α-CGRP and (±)-dobutamine with equal cardiotonic effects. However, from preliminary experiments it became clear that human α-CGRP only had slight effects on cardiac index over a wide dose range. Furthermore, the profiles of the haemodynamic effects of human α-CGRP and (±)-dobutamine were so different (see Results) that it was not possible to find doses that were

[1] Author for correspondence.

matched for their influences on all variables. Therefore, the lower doses of the substances were chosen to have similar, initial, but small, effects on mean arterial blood pressure, since this variable impinges on cardiac function (O'Rourke, 1982). The higher doses were chosen, likewise, to have similar initial hypotensive effects; in addition, the higher dose of human α-CGRP and the lower dose of (±)-dobutamine happened to be matched for their effects on total peripheral conductance.

Although (±)-dobutamine is used clinically and is administered by intravenous infusion (Ruffolo, 1987), when we came to devise the protocols for the present work we discovered that surprisingly little is known about the full haemodynamic profile of this drug during prolonged constant infusion either in man (Ruffolo, 1987; Leier *et al.*, 1977; 1978; Sonnenblick *et al.*, 1979) or in experimental animals (Liang & Hood, 1979). Thus, while a considerable literature on (±)-dobutamine exists, in large part it relates to bolus injections, brief infusions or incremental infusions of the drug (e.g. Vatner *et al.*, 1974; Schoemaker, 1989; Dorszewski *et al.*, 1990) often in anaesthetized or pithed animals (e.g. Robie & Goldberg, 1975; Ruffolo & Yaden, 1983; Ruffolo & Morgan, 1984; Maccarone *et al.*, 1984; Ruffolo & Messick, 1985a,b; Biro *et al.*, 1988). Furthermore, only incomplete data are available regarding the cardiovascular changes following the end of (±)-dobutamine infusion (Leier *et al.*, 1977; 1978). However, this period is of interest because dobutamine increases plasma renin activity (Jaski & Peters, 1988) and hence activation of the renin-angiotensin system could contribute to its post-infusion haemodynamic profile. Therefore, another objective of the present work was to delineate the extent to which changes in cardiac haemodynamics elicited by (±)-dobutamine were associated with regional vascular changes during and after infusion. To this end we monitored, continuously, renal, mesenteric, hindquarters, common carotid and internal carotid haemodynamics before, during and after infusion of (±)-dobutamine at the two dose levels.

In previous studies we have described some regional haemodynamic changes during and after 60 min infusions of human α-CGRP in Wistar rats (Gardiner *et al.*, 1989). However, in the present study in Long Evans rats it was important to monitor the full regional haemodynamic profile of human α-CGRP in the same animals that received (±)-dobutamine and to do this in a randomized, cross-over fashion, in order that the comparison of the cardiac haemodynamic effects of (±)-dobutamine and human α-CGRP was against a defined background of regional haemodynamic changes for the two substances.

Methods

Male, Long Evans rats (350–450 g) were used in all studies. Surgery was carried out under anaesthesia (sodium methohexitone, 60 mg kg^{-1} i.p., supplemented as required).

Animals with ascending thoracic aortic flow probes

Through a right intercostal (third and fourth rib space) incision an electromagnetic flow probe (Skalar MDL 1401, Delft, Netherlands) was placed around the ascending thoracic aorta (Smith & Hutchins, 1979; Smits *et al.*, 1982; Gardiner *et al.*, 1990c) of the rat.

Animals with renal, mesenteric and hindquarters flow probes

Through a midline, abdominal incision, pulsed Doppler probes (Haywood *et al.*, 1981) were sutured around the left renal and superior mesenteric arteries and the distal abdominal aorta, below the level of the ileocaecal artery, to monitor flow to the hindquarters of the rat (Gardiner *et al.*, 1989; 1990c).

Animals with common carotid and internal carotid flow probes

Through a midline, ventral, cervical incision both common carotid arteries were exposed and gently separated from the vagosympathetic nerve trunks. On the right side a probe was sutured around the common carotid artery, but on the left side the external carotid artery was ligated before placement of the probe on the common carotid artery. This arrangement permitted assessment of changes in total common carotid flow (i.e. external plus internal carotid flow) on the right side, together with changes in internal carotid flow alone on the left side (Gardiner *et al.*, 1990b).

In all animals the probe connections were exteriorised at the back of the neck. Following surgery, rats were given ampicillin (7 mg kg^{-1}, i.m.) and returned to their home cages for at least 7 days, with free access to food and water. Between 7 and 14 days after probe implantation animals were briefly re-anaesthetized (sodium methohexitone, 40 mg kg^{-1}, i.p.) and had intravascular catheters implanted (1 catheter in the abdominal aorta via the ventral caudal artery and 2 catheters in the right jugular vein). In animals with thoracic aortic probes, a third venous catheter was constructed of a short length (3 cm) of small bore polyethylene tubing (i.d. 0.28 mm; o.d. 0.61 mm) connected to a longer length (120 cm) of more rigid, wider bore, nylon tubing (i.d. 0.58 mm; o.d. 1.02 mm) and its tip was positioned close to the junction of the superior vena cava with the right atrium to permit recording of central venous pressure (Gardiner *et al.*, 1990c).

Catheters were tunnelled subcutaneously to emerge at the back of the neck. The catheters ran through a flexible spring connected to a harness worn by the rat and the linking cable to the flow probes was taped to the spring. The venous catheter that was to be used to monitor central venous pressure was connected to a slow infusion pump (SRI) through a fluid-filled swivel (Brown *et al.*, 1976). Isotonic saline (NaCl 157 mmol l^{-1}) was infused (0.3 ml h^{-1}) through this catheter to ensure it remained patent until recordings were started (Gardiner *et al.*, 1990c). Animals were allowed to recover in their home cages overnight before experiments were begun.

All protocols involved a 30 min baseline recording period followed by a 60 min infusion period and a 60 min post-infusion period. Animals receiving no infusion or vehicle infusion over this time showed no consistent cardiovascular changes. From preliminary experiments we found that human α-CGRP and (±)-dobutamine infused at 0.15 nmol kg^{-1} h^{-1} and 2 μmol kg^{-1} h^{-1}, respectively caused similar, slight, initial falls in mean systemic arterial blood pressure, whereas human α-CGRP and (±)-dobutamine at 1.5 nmol kg^{-1} h^{-1} and 10 μmol kg^{-1} h^{-1}, respectively caused similar, sustained reductions in mean systemic arterial blood pressure. Therefore, in the full experimental protocols animals were randomized to receive human α-CGRP or (±)-dobutamine on the first day and the other substance on the second day. The lower dose infusion was always given before the higher dose infusion, with the infusions being separated by at least 2.5 h.

In those experiments involving assessment of cardiac haemodynamics, the intra-arterial blood pressure, thoracic aortic flow and central venous pressure signals were fed into a Tandon 386 microcomputer interfaced with a custom-built haemodynamics microprocessor (University of Limburg, Department of Instrument Services). The latter provided mean cardiac output, peak aortic flow, maxium rate of rise of aortic flow (dF/dt_{max}), total peripheral conductance, stroke volume and mean central venous pressure (Gardiner *et al.*, 1990c). Subsequent analysis provided group mean values for the changes in these variables (relative to baseline). Mean cardiac output and stroke volume were factored by body weight to give cardiac index and stroke index.

In those experiments in which regional haemodynamic changes were assessed, continuous recordings of mean and phasic arterial blood pressure and Doppler shift signals (VFI system, Crystal Biotech, Holliston, U.S.A.) were made,

together with instantaneous heart rate (all on a Gould ES 1000 system). At selected time points, changes in regional blood flows and vascular conductances were calculated (Gardiner et al., 1990c).

Drugs

Infusions were given in isotonic saline (containing 1% bovine serum albumin for human α-CGRP) at a rate of 0.3 ml h^{-1}. Human α-CGRP was obtained from Celltech Ltd (Lot No ZD 949) while (±)-dobutamine hydrochloride (Eli Lilly Ltd; Dobutrex) was obtained from University Hospital Pharmacy (Nottingham). Solutions of human α-CGRP and (±)-dobutamine were prepared immediately before use.

Data analysis

Within group changes were analysed by Friedman's test (Theodorsson-Norheim, 1987); between group differences (i.e human α-CGRP versus (±)-dobutamine) were assessed by Wilcoxon's ranks sums test applied to areas under or over curves (Gardiner et al., 1990c). A P value <0.05 was taken as significant.

Results

Low dose infusions

Cardiac haemodynamics (±)-Dobutamine (2 μmol kg^{-1} h^{-1}) caused a transient, small fall in mean arterial blood pressure, but there were sustained increases in heart rate, cardiac index, peak aortic flow and dF/dt_{max}. Although total peripheral conductance was elevated throughout the infusion period, by 60 min the magnitude of the vasodilatation was only about half that at 5 min (Figure 1). Stroke index and central venous pressure showed no significant changes (Figure 1). Following infusion there were transient undershoots in peak aortic flow, dF/dt_{max}, total peripheral conductance and stroke index (Figure 1).

Human α-CGRP (0.15 nmol kg^{-1} h^{-1}) caused slight hypotension but produced no significant tachycardia (Figure 1). Furthermore, there were no significant changes in cardiac index, peak aortic flow, dF/dt_{max}, total peripheral conductance or stroke index (Figure 1), although central venous pressure showed an early, transient fall (Figure 1).

During infusion of (±)-dobutamine the overall changes in heart rate, cardiac index, peak aortic flow, dF/dt_{max} and total peripheral conductance (over the first 30 min of infusion) were greater ($P < 0.05$ for areas under curves) than seen during infusion of human α-CGRP. There was no significant difference between the overall changes in mean arterial blood pressure. Table 1 summarizes the maximum changes in cardiovascular variables during infusion of human α-CGRP and (±)-dobutamine.

Table 1 Mean maximum changes (% of baseline) in cardiovascular variables during infusions of human α-calcitonin gene-related peptide (α-CGRP) or (±)-dobutamine in the same conscious, Long Evans rats

	(±)-Dobutamine (μmol kg^{-1} h^{-1})		Human α-CGRP (nmol kg^{-1} h^{-1})	
	2	10	0.15	1.5
Heart rate	19	22	3	18
Mean arterial blood pressure	−6	−18	−9	−20
Cardiac index	23	43	3	9
Peak aortic flow	20	31	3	9
dF/dt_{max}	45	53	5	16
Total peripheral conductance	31	58	11	35
Stroke index	5	19	−3	−10
Central venous pressure	11	18	−23	−27

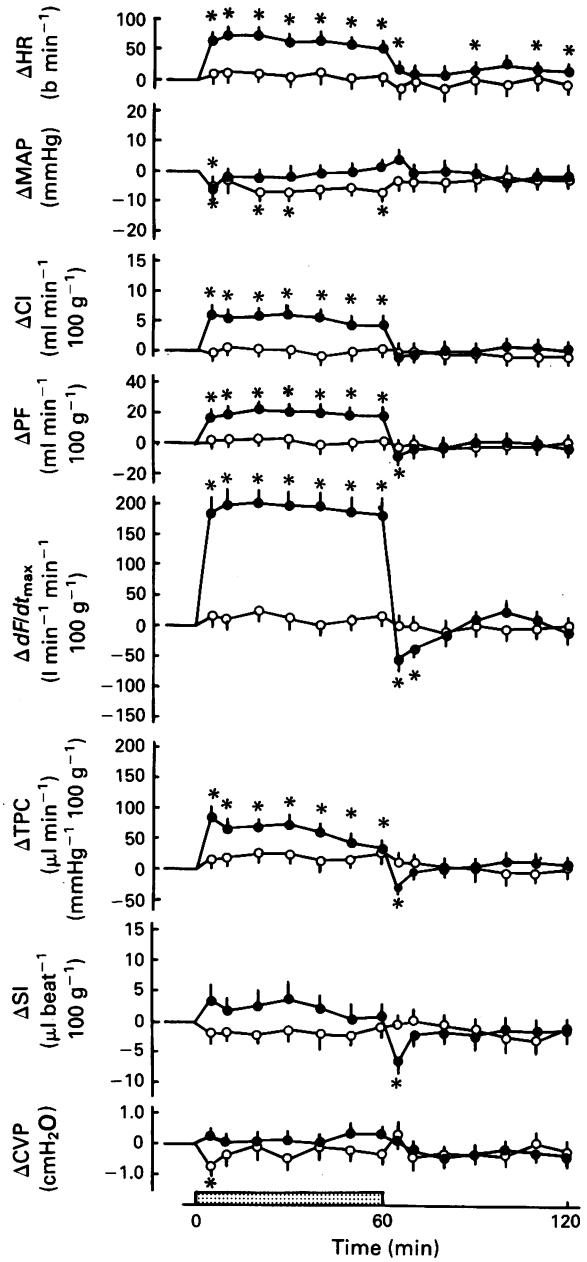

Figure 1 Cardiovascular changes (Δ) during and after a 60 min infusion of human α-calcitonin gene-related peptide (α-CGRP, ○) (0.15 nmol kg^{-1} h^{-1}) or (±)-dobutamine (●) (2 μmol kg^{-1} h^{-1}) in conscious, Long Evans rats ($n = 9$), chronically instrumented with intravascular catheters and ascending thoracic aortic electromagnetic flow probes. Values are mean and the bars show s.e.mean; * $P < 0.05$ versus baseline (Friedman's test). Statistics for comparisons between human α-CGRP and (±)-dobutamine are given in the text. HR = heart rate; MAP = mean arterial blood pressure; CI = cardiac index; PF = peak thoracic aortic flow; dF/dt_{max} = maximum rate of rise of thoracic aortic flow; TPC = total peripheral conductance; SI = stroke index; CVP = central venous pressure.

Regional haemodynamics

Renal, mesenteric and hindquarters haemodynamics. In the animals with renal, mesenteric and hindquarters probes the changes in mean arterial blood pressure and heart rate were not different from those above, during or after (±)-dobutamine infusion (2 μmol kg^{-1} h^{-1}). However, in this group the small overshoot in mean arterial blood pressure after the infusion was stopped did reach significance (Figure 2). There were no significant changes in renal or mesenteric blood flows

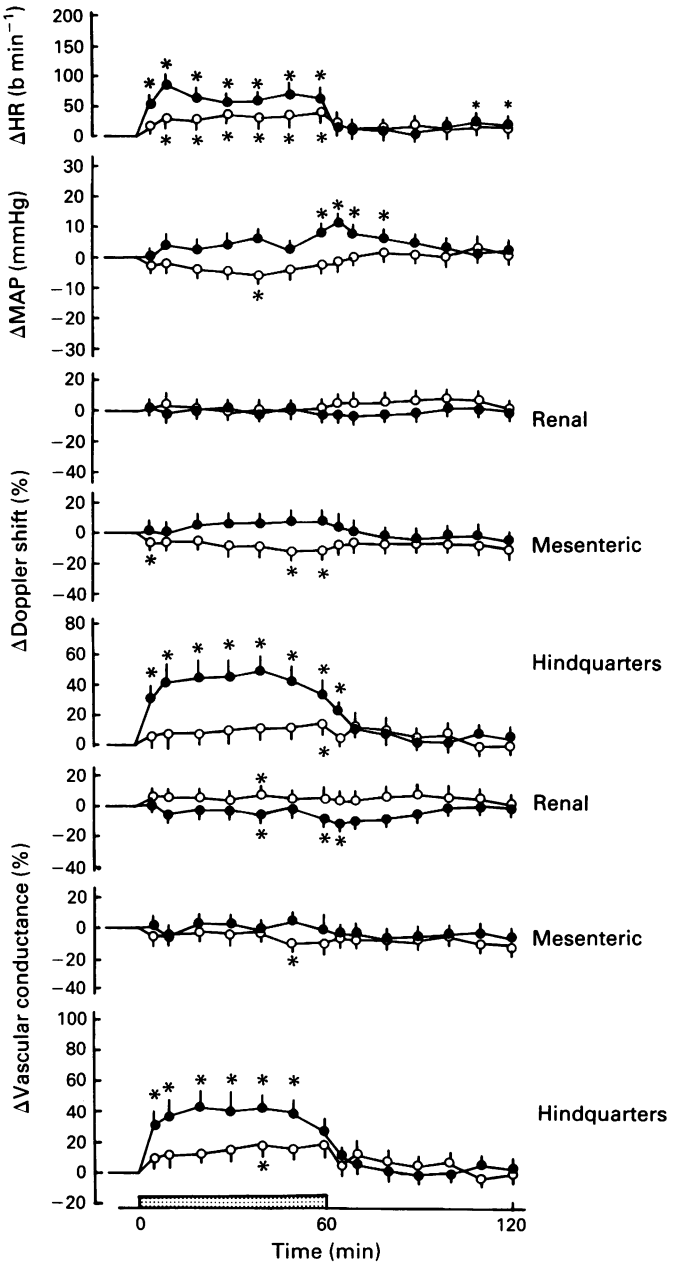

Figure 2 Cardiovascular changes during and after a 60 min infusion of human α-calcitonin gene-related peptide (α-CGRP, ○) (0.15 nmol kg^{-1} h^{-1}) or (±)-dobutamine (●) (2 μmol kg^{-1} h^{-1}) in conscious, Long Evans rats ($n = 8$), chronically instrumented with intravascular catheters and renal, mesenteric and hindquarters pulsed Doppler flow probes. Values are mean and bars are s.e.mean; * $P < 0.05$ versus baseline (Friedman's test). Statistics for comparisons between human α-CGRP and (±)-dobutamine are given in the text. Abbreviations as in Figure 1.

during infusion, but there was a marked hindquarters hyperaemia (Figure 2). There was a slight renal vasoconstriction during, and a transient renal vasoconstriction after, (±)-dobutamine infusion. The marked hindquarters vasodilator effect of (±)-dobutamine faded towards the end of the infusion period (Figure 2).

Infusion of human α-CGRP (0.15 nmol kg^{-1} h^{-1}) caused slight tachycardia and hypotension (Figure 2). There was no change in renal flow, but a modest decrease in mesenteric flow and an increase in hindquarters flow (Figure 2). These changes were associated with slight renal and hindquarters vasodilatations and mesenteric vasoconstriction (Figure 2). Following infusion all variables were not different from baseline.

The overall changes in heart rate, mesenteric and hindquarters flows, and hindquarters vascular conductance during infusion of (±)-dobutamine were significantly different from those during infusion of human α-CGRP ($P < 0.05$ for areas under or over curves). There was no significant difference between the overall changes in mean arterial blood pressure or renal haemodynamics or mesenteric vascular conductance. Table 2 summarizes the maximum changes in cardiovascular variables during infusion of human α-CGRP and (±)-dobutamine.

Table 2 Mean maximum changes (% of baseline) in cardiovascular variables during infusions of human α-calcitonin gene-related peptide (α-CGRP) or (±)-dobutamine in the same conscious, Long Evans rats

	(±)-Dobutamine (μmol kg^{-1} h^{-1})		*Human α-CGRP* (nmol kg^{-1} h^{-1})	
	2	10	0.15	1.5
Heart rate	19	41	12	31
Mean arterial blood pressure	5	−12	−6	−22
Renal flow	−2	−18	4	−12
Mesenteric flow	7	−8	−14	−26
Hindquarters flow	49	66	15	35
Renal conductance	−9	−15	7	13
Mesenteric conductance	−5	10	−11	−7
Hindquarters conductance	42	81	18	68

Common and internal carotid haemodynamics. In animals with probes monitoring common and internal carotid blood flows, infusion of (±)-dobutamine (2 μmol kg^{-1} h^{-1}) caused tachycardia and transient hypotension (Figure 3) not different from those in the groups described above. There was a slight overshoot in mean blood pressure when the infusion was stopped (Figure 3). In addition, there were marked increases in common and internal carotid blood flows that persisted for 30 min after cessation of (±)-dobutamine infusion (Figure 3).

Infusion of human α-CGRP (0.15 nmol kg^{-1} h^{-1}) caused modest tachycardia and slight hypotension (Figure 3). There was no significant increase in internal carotid blood flow, but common carotid flow increased slightly. Internal and common carotid vascular conductances increased during infusion, but returned to baseline when the infusion was stopped (Figure 3).

The overall changes in heart rate and internal and common carotid blood flows during infusion of (±)-dobutamine were greater than those seen during infusion of human α-CGRP ($P < 0.05$ for areas under curves), but the overall changes in mean arterial blood pressure and in the internal and common carotid vascular conductances were not different during the two infusions. Table 3 summarizes the maximum changes in cardiovascular variables during infusion of human α-CGRP and (±)-dobutamine.

Table 3 Mean maximum changes (% of baseline) in cardiovascular variables during infusions of human α-calcitonin gene-related peptide (α-CGRP) or (±)-dobutamine in the same conscious, Long Evans rats

	(±)-Dobutamine (μmol kg^{-1} h^{-1})		*Human α-CGRP* (nmol kg^{-1} h^{-1})	
	2	10	0.15	1.5
Heart rate	35	45	7	24
Mean arterial blood pressure	5	−23	−7	−22
Internal carotid flow	40	33	15	47
Common carotid flow	51	35	18	86
Internal carotid conductance	37	46	20	89
Common carotid conductance	48	48	28	139

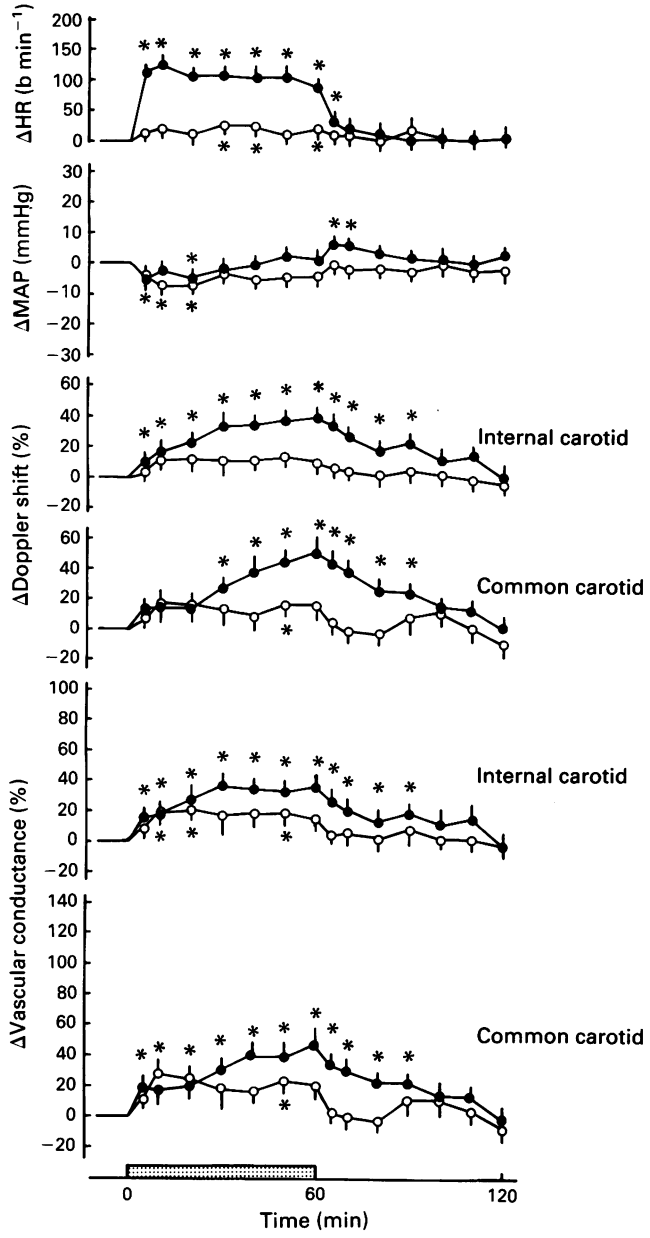

Figure 3 Cardiovascular changes during and after a 60 min infusion of human α-calcitonin gene-related peptide (α-CGRP, ○) (0.15 nmol kg^{-1} h^{-1}) or (±)-dobutamine (●) (2 μmol kg^{-1} h^{-1}) in conscious, Long Evans rats ($n = 8$), chronically instrumented with intravascular catheters and internal and common carotid pulsed Doppler flow probes. Values are mean and bars are s.e.mean; * $P < 0.05$ versus baseline (Friedman's test). Statistics for comparisons between human α-CGRP and (±)-dobutamine are given in the text. Abbreviations as in Figure 1.

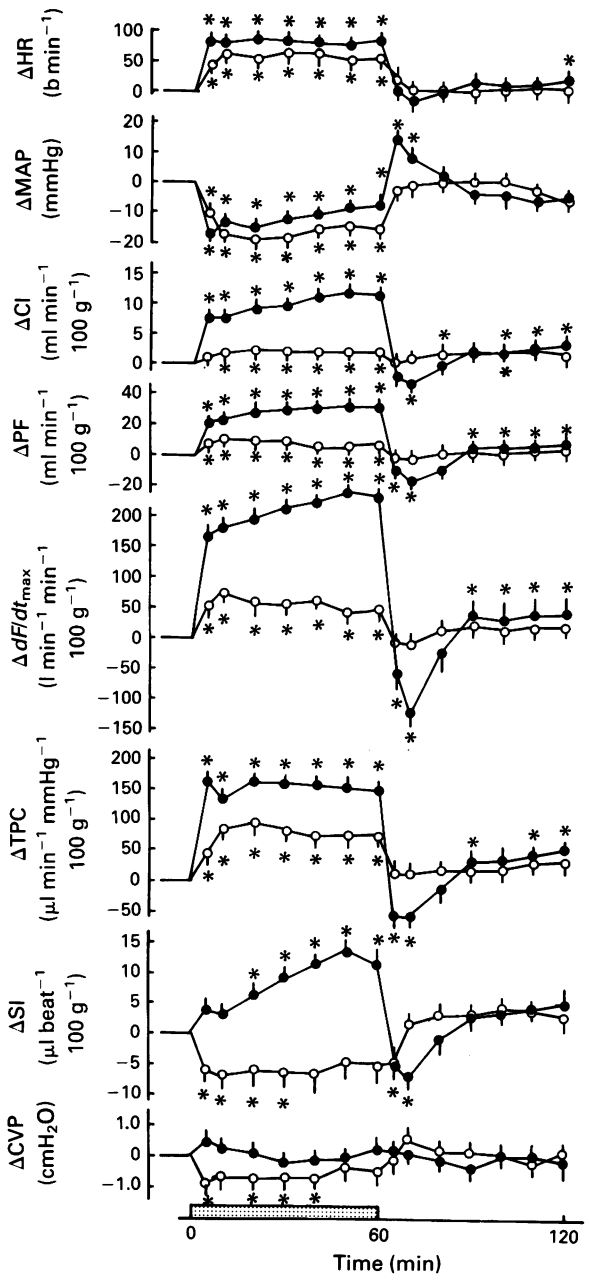

Figure 4 Cardiovascular changes during and after a 60 min infusion of human α-calcitonin gene-related peptide (α-CGRP, ○) (1.5 nmol kg^{-1} h^{-1}) or (±)-dobutamine (●) (10 μmol kg^{-1} h^{-1}) in conscious, Long Evans rats ($n = 9$), chronically instrumented with intravascular catheters and ascending thoracic aortic electromagnetic flow probes. Values are mean and bars are s.e.mean; * $P < 0.05$ versus baseline (Friedman's test). Statistics for comparisons between human α-CGRP and (±)-dobutamine are given in the text. Abbreviations as in Figure 1.

High dose infusions

Cardiac haemodynamics During infusion of (±)-dobutamine (10 μmol kg^{-1} h^{-1}) hypotension was accompanied by sustained tachycardia and a maintained increase in total peripheral conductance. However, there were progressive increases in cardiac index, peak aortic flow, dF/dt_{max}, and stroke index; there was no significant change in central venous pressure (Figure 4). After the end of the infusion there was a transient overshoot in mean arterial blood pressure accompanied by reductions in cardiac index, peak aortic flow, dF/dt_{max} and stroke index; these events coincided with a fall in total peripheral conductance (Figure 4). Thereafter, total peripheral conductance rose above baseline and blood pressure fell; at this time there were increases in cardiac index, peak aortic flow and dF/dt_{max} (Figure 4).

Infusion of human α-CGRP (1.5 nmol kg^{-1} h^{-1}) caused hypotension accompanied by sustained increases in heart rate, cardiac index, peak aortic flow, dF/dt_{max} and total peripheral conductance (Figure 4). However, there were reductions in stroke index and central venous pressure (Figure 6). Within 5 min of the end of human α-CGRP infusion all variables were back to baseline levels (Figure 4).

During and after infusion of (±)-dobutamine the overall changes in cardiac index, peak aortic flow, dF/dt_{max}, total peripheral conductance and stroke index were greater ($P < 0.05$ for areas under curves) than the changes seen during infusion of human α-CGRP (Figure 4). Although the overall changes in mean arterial blood pressure or in heart rate during infusion of human (±)-dobutamine and α-CGRP were not significantly

different, there was a significant difference in the overall changes in mean arterial blood pressure following the infusions (Figure 4). Table 1 summarizes the maxium changes in cardiovascular variables during infusion of human α-CGRP and (±)-dobutamine.

Regional haemodynamics

Renal, mesenteric and hindquarters haemodynamics In animals with renal, mesenteric and hindquarters probes the changes in heart rate and mean arterial blood pressure during and after (±)-dobutamine (10 μmol kg^{-1} h^{-1}) infusion (Figure 5) were as described above in animals with thoracic aortic flow pobes. During the infusion period, renal blood flow was reduced while there was a hindquarters hyperaemia, but no significant change in mesenteric blood flow (Figure 5). There

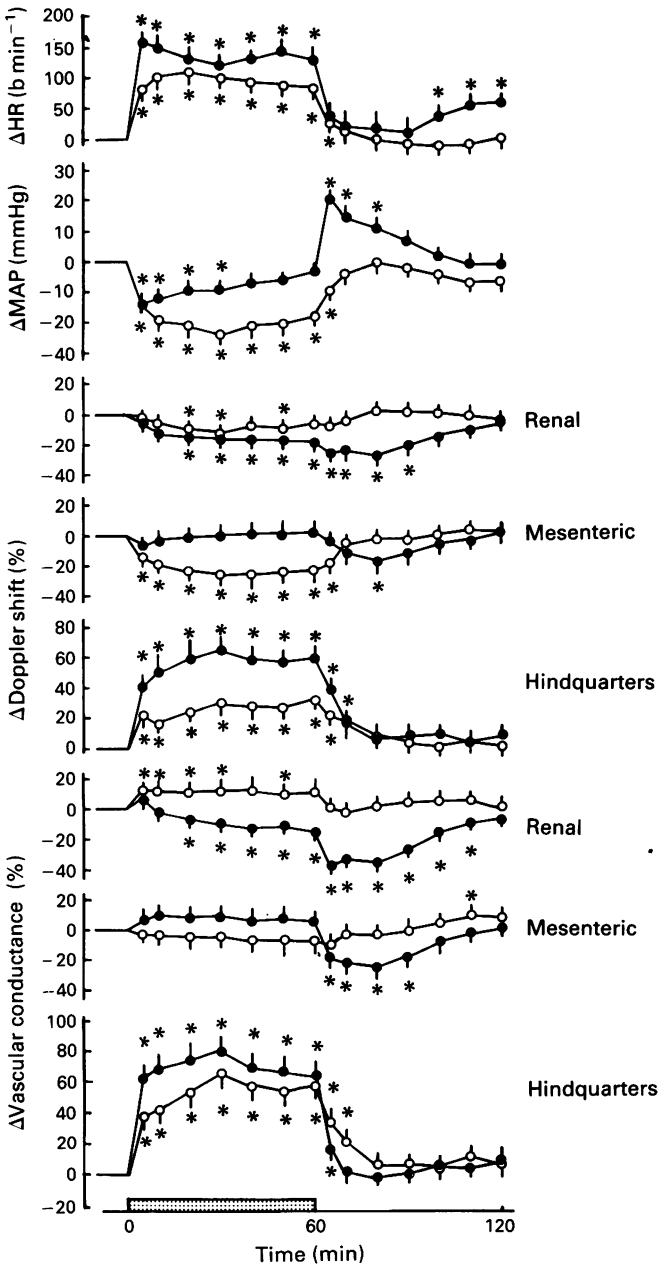

Figure 5 Cardiovascular changes during and after a 60 min infusion of human α-calcitonin gene-related peptide (α-CGRP, ○) (1.5 nmol kg^{-1} h^{-1}) or (±)-dobutamine (●) (10 μmol kg^{-1} h^{-1}) in conscious, Long Evans rats ($n = 8$), chronically instrumented with intravascular catheters and renal, mesenteric and hindquarters pulsed Doppler flow probes. Values are mean and bars are s.e.mean; * $P < 0.05$ versus baseline (Friedman's test). Statistics for comparisons between human α-CGRP and (±)-dobutamine are given in the text. Abbreviations as in Figure 1.

was renal vasoconstriction and hindquarters vasodilatation; when the infusion was stopped further renal vasoconstriction occurred, together with mesenteric vasoconstriction (Figure 5).

The changes in heart rate and mean arterial blood pressure during and after infusion of human α-CGRP (1.5 nmol kg^{-1} h^{-1}) were not different from those described above in animals with thoracic aortic flow probes. During infusion of human α-CGRP there were reductions in renal and mesenteric flows, together with an increase in hindquarters blood flow (Figure 5). There were renal and hindquarters vasodilatations but no change in mesenteric vascular conductance, although there was an upwards drift in the latter following infusion (Figure 5).

During infusion of (±)-dobutamine, the overall changes in heart rate, mean blood pressure, mesenteric and hindquarters flows and renal vascular conductance were different ($P < 0.05$ for areas under or over curves) from those seen during human α-CGRP infusion. Furthermore, the overall changes in mean blood pressure and renal and mesenteric vascular conductances were different ($P < 0.05$ for areas under or over curves) following infusion of human α-CGRP and (±)-dobutamine (Figure 5). Table 2 summarizes the maximum changes in cardiovascular variables during infusion of human α-CGRP and (±)-dobutamine.

Common and internal carotid haemodynamics In animals with common and internal carotid flow probes, the changes in heart rate and mean arterial blood pressure during infusion of (±)-dobutamine (10 μmol kg^{-1} h^{-1}) were similar to those in the corresponding groups above. There were increases in internal and common carotid blood flows and vascular conductances (Figure 6). Following infusion there was an overshoot in mean arterial blood pressure, but carotid haemodynamics remained above baseline levels at that time (Figure 6).

Infusion of human α-CGRP (1.5 nmol kg^{-1} h^{-1}) caused tachycardia, hypotension and increases in internal and common carotid blood flows and vascular conductances (Figure 6). The changes in carotid haemodynamics were still significant for about 10 min after the infusion was stopped (Figure 6).

During infusion of (±)-dobutamine the overall changes in carotid flows and vascular conductances were less, but the tachycardia was greater ($P < 0.05$ for areas under curves) than during infusion of human α-CGRP. Table 3 summarizes the maximum changes in cardiovascular variables during infusion of human α-CGRP and (±)-dobutamine.

Discussion

The major objective of the present work was to compare the full haemodynamic profiles of infusions of human α-CGRP and (±)-dobutamine at two dose levels. As explained in the Introduction, our initial intention of matching the doses of human α-CGRP and (±)-dobutamine for their cardiotonic influences was confounded by the relative lack of effect of human α-CGRP on cardiac index. Therefore, since cardiac performance is affected by systemic arterial blood pressure (O'Rourke, 1982), we matched the doses of human α-CGRP and (±)-dobutamine for their initial effects on this variable. As it turned out, the higher dose of human α-CGRP and the lower dose of (±)-dobutamine had similar effects on total peripheral conductance but otherwise generally had dissimilar actions.

The lower dose infusion of human α-CGRP did not cause any increase in cardiac function. Hence the slight increases in flows through hindquarters and carotid vascular beds under these conditions must have been met by the reduced flow through the mesenteric vascular bed (and, possibly, through other, unmeasured, regions). With the higher dose of human α-CGRP there were increases in cardiac index and indices of contractility (peak flow and dF/dt_{max}) and, thus, a positive ino-

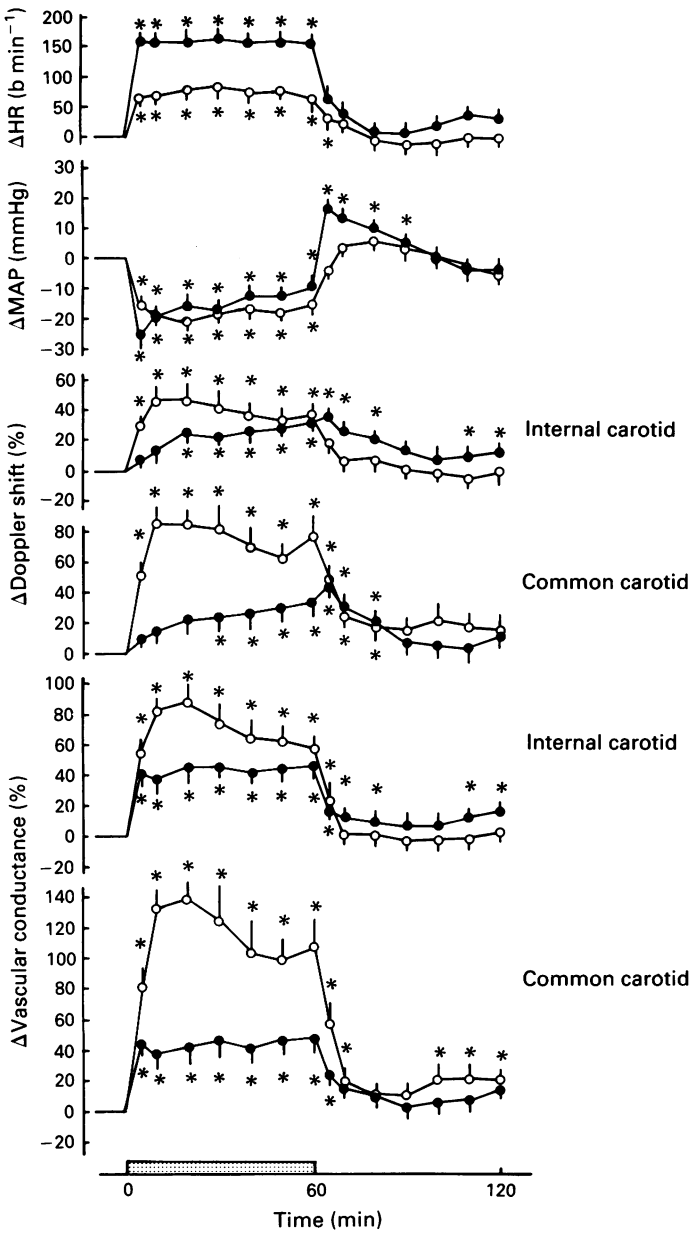

Figure 6 Cardiovascular changes during and after a 60 min infusion of human α-calcitonin gene-related peptide (α-CGRP, ○) (1.5 nmol kg^{-1} h^{-1}) or (±)-dobutamine (●) (10 μmol kg^{-1} h^{-1}) in conscious, Long Evans rats ($n = 8$), chronically instrumented with intravascular catheters and internal and common carotid pulsed Doppler flow probes. Values are mean and bars are s.e.mean; * $P < 0.05$ versus baseline (Friedman's test). Statistics for comparisons between human α-CGRP and (±)-dobutamine are given in the text. Abbreviations as in Figure 1.

tropic effect of human α-CGRP, together with redirection of blood flow from the mesenteric vascular bed, could have contributed to the increase in carotid and hindquarters blood flows. Since there was a significant reduction in afterload it is feasible this contributed to the increase in cardiac index. However, there was a clear-cut reduction in stroke index, possibly resulting from reduced cardiac filling, since central venous pressure fell. Thus, it is feasible that any putative positive inotropic effects of human α-CGRP did not result in more marked increases in cardiac index because of a reduction in venous return. It is likely the latter was due to a venodilator action of human α-CGRP (Törnebrandt *et al.*, 1987; Crossman *et al.*, 1987; but see Al-Kazwini *et al.*, 1987; Marshall *et al.*, 1988). The present results, therefore, provide some explanation for the conflicting data in the literature regarding the cardiac haemodynamic effects of CGRP (DiPette *et al.*, 1987; Lappe *et al.*, 1987; Sirén & Feuerstein, 1988; Ando *et al.*, 1990) (see Introduction), since it is apparent that the effects of the peptide depend on the dose used. Moreover, it may be that the cardiac haemodynamic responses to bolus injections of CGRP would give a less clear picture of the interrelated changes that were seen in the present study, particularly with the infusion of the higher dose of human α-CGRP.

The lower dose of (±)-dobutamine had potent, positive chronotropic and inotropic effects, together with marked hindquarters and carotid vasodilator actions. Although the vasodilatations were similar to those seen with the lower dose of human α-CGRP, the hyperaemias in these vascular beds were greater than those observed following the lower dose of human α-CGRP, probably because they were supported by the increase in cardiac index. This proposal is consistent with the finding that superior mesenteric blood flow was not reduced under these conditions. The results indicate that, in the conscious rat, (±)-dobutamine may have substantial β_1- (i.e. cardiac) and β_2- (i.e. hindquarters, Gardiner & Bennett, 1988) adrenoceptor-stimulating properties, although reflex responses to the increased cardiac output may have contributed to changes in regional vascular conductance (Ruffolo & Messick, 1985b; Ruffolo, 1987).

The present results, showing that the infusion of the lower dose of (±)-dobutamine (i.e. 2 μmol kg^{-1} h^{-1}) caused an increase in cardiac index that was primarily due to a tachycardia rather than an increase in stroke index are the opposite of the findings of Ruffolo & Messick (1985b), but they studied the responses to bolus doses of (±)-dobutamine (0.01 μmol kg^{-1}) in anaesthetized rats. In general, our results are compatible with those of Schoemaker (1989), although she found that (±)-dobutamine (4–40 μmol kg^{-1} h^{-1}) caused no significant increase in stroke volume at any dose level, whereas our higher dose infusion of (±)-dobutamine (i.e. 10 μmol kg^{-1} h^{-1}) caused a substantial rise in stroke index. However, under these conditions there was a large increase in total peripheral conductance and hence mean arterial blood pressure fell. This finding contrasts with those of the majority of previous studies in which administration of (±)-dobutamine caused no change (Vatner *et al.*, 1974, boluses 1.6–8.0 μmol kg^{-1}, infusions 1.6 μmol kg^{-1} h^{-1}; Robie & Goldberg, 1975, 0.4–6.4 μmol kg^{-1} h^{-1}; Leier *et al.*, 1977, 1978, 0.5–3.0 μmol kg^{-1} h^{-1}; Liang & Hood, 1979, 2–8 μmol kg^{-1} h^{-1}; Dorszewski *et al.*, 1990, 0.2–0.8 μmol kg^{-1} h^{-1}) or an increase (Ruffolo & Messick, 1985b) in mean arterial blood pressure. However, these differences are likely to be due to differences in administered dose, since Biro *et al.* (1988), Schoemaker (1989) and Dorszewski *et al.* (1990) using doses of (±)-dobutamine (6, 12 and 4 μmol kg^{-1} h^{-1}, respectively) similar to those employed here, also observed hypotension.

The increase in total peripheral conductance seen in response to infusion of (±)-dobutamine was found to be associated with marked hindquarters and common and internal carotid vasodilatations. Under the same conditions, mesenteric and renal vascular conductances were either unchanged or, in the case of the latter during the higher dose infusion, reduced. While a relatively selective hindquarters vasodilator effect of (±)-dobutamine has been described previously (Vatner *et al.*, 1974; Robie & Goldberg, 1975; Liang & Hood, 1979) this has not been observed invariably (Drexler *et al.*, 1987; Biro *et al.*, 1988), even in studies that did demonstrate a cerebral vasodilator response to (±)-dobutamine (Drexler *et al.*, 1987).

Although there was a greater hindquarters hyperaemia and a better maintenance of mesenteric blood flow during infusion of (±)-dobutamine than of human α-CGRP at either dose level, carotid hyperaemia was greater during infusion of the latter at the higher dose. This could indicate an additional, selective, carotid vasodilator effect of human α-CGRP compared to (±)-dobutamine. It was also apparent that renal blood flow during infusion, and renal and mesenteric flows

after infusion were more compromised when (±)-dobutamine was administered than when human α-CGRP was given. While there is evidence that administration of CGRP activates the renin-angiotensin system (Kurtz *et al.*, 1988; Gardiner *et al.*, 1990a) the present results are most likely explained by (±)-dobutamine having a much more potent direct action in this regard, since it is relatively devoid of pre-junctional actions on noradrenaline release (Marcus *et al.*, 1987; Fischer *et al.*, 1989). It seems likely that activation of neurohumoral vasoconstrictor mechanisms, such as the renin-angiotensin system (Jaski & Peters, 1988), was responsible for the apparent desensitization to the vasodilator effects of (±)-dobutamine during infusions of the lower dose (see Figures 1 and 2) since there was no evidence for down-regulation of cardiac effects, even during infusion of the higher dose of (±)-dobutamine. The initial reduction in cardiac function following the end of (±)-dobutamine infusion was probably secondary to the increased afterload (i.e. decreases in renal and mesenteric vascular conductances and overshoot in mean blood pressure), since, subsequently, mean blood pressure fell as total peripheral conductance increased and cardiac function rose again above baseline. It is likely this series of events was due to a relatively more rapid offset of the direct vasodilator effects of (±)-dobutamine than of the vasoconstrictor effects of an activated renin-angiotensin system. Such deleterious cardiovascular effects have not been described previously following prolonged infusion of (±)-dobutamine in clinical studies (Leier *et al.*, 1977; 1978), but in those investigations the first measurements were made 30 min after the end of infusion in patients with congestive heart failure who were being treated with other drugs at the time.

In conclusion, the present results in conscious rats indicate that, in doses matched for their initial effects on mean arterial blood pressure, (±)-dobutamine has a more marked influence on cardiac function than does human α-CGRP. The increase in cardiac index caused by the latter is attenuated by a reduction in stroke index, resulting from a fall in central venous pressure, and probably due to venodilatation. Nonetheless, human α-CGRP can produce more potent carotid hyperaemias than (±)-dobutamine and, furthermore, does not trigger the substantial overshoot in blood pressure, and associated renal or mesenteric vasoconstrictions, or the reduction in cardiac function seen following (±)-dobutamine administration, at least at the highest doses used here.

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(Received June 1, 1990
Revised January 22, 1991
Accepted January 28, 1991)

Br. J. Pharmacol. (1991), **103**, 1509–1514 © Macmillan Press Ltd, 1991

Regional haemodynamic effects of prolonged infusions of human α-calcitonin gene-related peptide in conscious, Long Evans rats

[1]S.M. Gardiner, A.M. Compton, P.A. Kemp, T. Bennett, *R. Foulkes & *B. Hughes

Department of Physiology and Pharmacology, Queen's Medical Centre, Nottingham, NG7 2UH and *Department of Pharmacology, Celltech Ltd, Slough, SL1 4EN

1 Haemodynamic measurements were made in conscious, Long Evans rats chronically instrumented for the assessment of changes in regional blood flows (renal, mesenteric and hindquarters, or internal and common carotid) and systemic arterial blood pressure and heart rate, before, during and after 3 day infusions of vehicle or human α-calcitonin gene-related peptide (CGRP) (1.5 or 15 nmol $kg^{-1} h^{-1}$).

2 In animals with renal, mesenteric and hindquarters flow probes ($n = 8$), during the first day of infusion of human α-CGRP (1.5 nmol $kg^{-1} h^{-1}$) there was sustained tachycardia and hypotension, a sustained reduction in renal flow, a transient reduction in mesenteric flow and a relatively well-maintained increase in hindquarters flow. All these effects were significantly different from the changes seen in vehicle-infused rats ($n = 8$), but calculation of vascular conductances showed only the late mesenteric vasodilatation and the sustained hindquarters vasodilatation were different from the changes in vehicle-infused rats. However, by the second day of infusion and thereafter cardiovascular variables in the animals receiving vehicle and those receiving human α-CGRP were not different.

3 Nine animals instrumented with probes to monitor changes in internal and common carotid haemodynamics initially received human α-CGRP infused at a rate of 1.5 nmol $kg^{-1} h^{-1}$. Three of these animals still showed some response to the human α-CGRP (tachycardia, hypotension, hyperaemic vasodilatation) throughout the second day of infusion and hence were taken through the 3 day infusion protocol. When the infusion was stopped on the fourth day all these animals showed reversal of the effects of human α-CGRP. The other 6 animals in the original group showed complete desensitization to the effects of human α-CGRP by the end of the second day of infusion (as seen in the group instrumented with renal, mesenteric and hindquarter probes). Therefore, in order to assess the extent of desensitization, at the beginning of the third day the dose of human α-CGRP was increased to 15 nmol $kg^{-1} h^{-1}$. The resulting tachycardia, hypotension and hyperaemia in the carotid vascular beds were significant, (but no greater than the initial responses to human α-CGRP at 1.5 nmol $kg^{-1} h^{-1}$). These effects were maintained throughout the third infusion day, but there was some desensitization to the effects of this higher dose of human α-CGRP by the beginning of the fourth infusion day. However, when the infusion was stopped there was clear reversal of the effects of human α-CGRP.

4 The results indicate substantial inter-individual variation in the haemodynamic effects of prolonged infusions of human α-CGRP in conscious, Long Evans rats. However, since increasing the dose of human α-CGRP overcame the desensitization, it is feasible that, in the clinical setting, maintained increases in internal carotid blood flow could be achieved by individually-adjusted infusions of human α-CGRP.

Keywords: Human α-CGRP; regional haemodynamics; vasodilatation

Introduction

There is some evidence that prolonged infusions of human α-calcitonin gene-related peptide (CGRP) can cause sustained hypotension in conscious rats (Marshall *et al.*, 1987), although the associated tachycardia wanes. Several aspects of those studies are worth noting: (1) a very high dose of human α-CGRP was used, causing a fall in systemic arterial blood pressure that would be clinically unacceptable; (2) only female rats were studied; (3) although normotensive and hypertensive rats were used in the protocols involving infusions up to 24 h duration, only hypertensive animals were investigated when the effects of 3 day infusions were studied; (4) peptide delivery was achieved with an implanted osmotic minipump system in the experiments involving 3 day infusions; and (5) systemic arterial blood pressure and heart rate were the only cardiovascular variables measured. Subsequently we found that, in normotensive male rats, a 1 h infusion of rat α-CGRP at the same dose as used by Marshall *et al.* (1987) caused sustained hypotension, but the associated regional haemodynamic profile was not stable (Gardiner *et al.*, 1989a). Similar observations were made with 1 h infusions of lower doses of human α- and β-CGRP (Gardiner *et al.*, 1989b).

Putative clinical uses of human α-CGRP include the treatment of cerebral vasospasm (Gardiner *et al.*, 1990a; Johnston *et al.*, 1990) following subarachnoid haemorrhage, i.e. a situation which would require sustained administration of human α-CGRP with maintained effect. Therefore, the aims of the present work were: (1) to extend our previous observations and to determine if there were any progressive changes in the effects of human α-CGRP on renal, mesenteric and hindquarters haemodynamics and systemic arterial blood pressure when infusions were continued over 3 days, in normotensive rats; and (2) to assess the effects of prolonged infusions of human α-CGRP on carotid haemodynamics in normotensive rats.

Methods

All experiments were carried out on male, Long Evans rats (aged 3–4 months, 350–450 g). Under sodium methohexitone anaesthesia (60 mg kg^{-1}, i.p., supplemented as required), miniaturized, pulsed Doppler flow probes (Haywood *et al.*, 1981) were implanted around the left renal and superior mesenteric

[1] Author for correspondence.

artery and the distal abdominal aorta (Gardiner et al., 1989a,b). In other animals probes were implanted around both common carotid arteries after the external carotid artery had been ligated on the left side to allow assessment of changes in ipsilateral internal carotid flow (Gardiner et al., 1990a). Following surgery all animals received ampicillin (7 mg kg^{-1}, i.m.; Penbritin, Beecham), and were returned to their home cages for 7–14 days. Thereafter, under brief anaesthesia (sodium methohexitone, 40 mg kg^{-1}, i.p.) intra-arterial (distal abdominal aorta via ventral caudal artery) and intravenous (right jugular vein) catheters were implanted and tunnelled subcutaneously to emerge at the back of the neck, together with the pulsed Doppler wires. The catheters were protected by a flexible, counter-balanced spring attached to a harness fitted to the rat and a connector to the pulsed Doppler lead was taped to this spring (Gardiner et al., 1988a,b; 1990a). Experiments began at least 24 h after catheter implantation.

Continuous intravenous infusions (0.3 ml h^{-1}) were given through a swivel system (Brown et al., 1976) that allowed animals free movement. One group of animals ($n = 8$) instrumented with renal, mesenteric and hindquarters probes received a 3 day infusion of isotonic saline containing 1% bovine serum albumin (BSA) (Sigma), this being the vehicle in which human α-CGRP was dissolved. Another group of rats ($n = 8$) with renal, mesenteric and hindquarters probes, and a further group of rats ($n = 9$) with internal carotid and common carotid probes were given infusions of human α-CGRP at a rate of 1.5 nmol kg^{-1} h^{-1}.

Infusions started at 07 h 00 min on day 1 after 30 min baseline recording. The infusion pumps were switched off at 07 h 00 min on day 4 and recordings continued for a further 5 h. In all experiments involving infusion of human α-CGRP the infusate was prepared fresh twice daily at 07 h 00 min and 16 h 00 min from aliquots of the same stock solution that was kept at −80°C (solution of 200 nmol ml^{-1}). Between 07 h 00 min and 16 h 00 min each day recordings were made of heart rate, mean arterial blood pressure and mean Doppler shift signals (Crystal Biotech VFI system). Changes (%) in mean Doppler shift signals were taken as changes in blood flow (Haywood et al., 1981; Gardiner et al., 1989a,b; 1990a) and % changes in vascular conductances were calculated from mean Doppler shift and mean arterial blood pressure signals (Gardiner et al., 1990b).

Experiments were organized so that animals with renal, mesenteric and hindquarters probes receiving vehicle or human α-CGRP infusions were run in tandem. Analysis of the results of this experiment showed there was marked tachyphylaxis to the effects of human α-CGRP (see Results). On the basis of this experiment the protocol involving assessment of the carotid haemodynamic effects of human α-CGRP was devised with the results being assessed during the experiment to detect the occurrence of tachyphylaxis. Of the 9 animals studied, 3 showed relatively sustained responses to human α-CGRP and went through the protocol described above. However, 6 animals showed marked tachyphylaxis during the second day of infusion (see Results) and so in these animals at 07 h 00 min on the third day of infusion the rate of administration of human α-CGRP was increased 10 fold (i.e. to 15 nmol kg^{-1} h^{-1}) in order to determine if there was complete desensitization to the peptide.

Data analysis

Within-group analysis was carried out by Friedman's test (Theodorsson-Norheim, 1987) and areas under or over curves (Gardiner et al., 1990b), were compared by the Mann-Whitney U test.

Peptides

Human α-CGRP (Lot Number ZD 949) (Celltech, U.K.) was used throughout the study. The peptide was dissolved in isotonic saline containing 1% BSA (Sigma).

Results

Cardiovascular effects of 3 day infusions of vehicle or human α-CGRP in rats with renal, mesenteric and hindquarters probes

During infusion of vehicle, heart rate and mean blood pressure showed no consistent changes (Figure 1). Renal blood flow showed some variation during the day, and hindquarters flow showed a downwards drift over the first day, but was relatively steady thereafter (Figure 1). However, mesenteric blood flow showed a clear rhythm with flow being highest in the morning at 07 h 00 min and falling subsequently to reach its lowest level at about 15 h 00 min. This variation we attribute to feeding-induced gastrointestinal hyperaemia. Rats are nocturnal and eat most of their daily intake when the lights are out (i.e. between 18 h 00 min and 06 h 00 min), and thus, when the recordings started at 06 h 30 min, the animals were post-prandial.

In spite of the variations during vehicle infusions there were substantial and significant differences between those and the changes seen during infusion of human α-CGRP on the first day (Figures 1 and 2). In the latter circumstance, initially, there was marked tachycardia, hypotension, reductions in renal and mesenteric flows and an increase in hindquarters flow (Figure 1). However, over the course of the day the reduction in mesenteric blood flow waned, so that, 9 h after the onset of infusion, mesenteric vascular conductance had changed from being below to being above baseline (Figure 2). There was a hindquarters hyperaemia and vasodilatation at this time, but the reduction in renal flow was not associated with a significant change in renal vascular conductance (Figures 1 and 2). During the second and third days of infusion and following the cessation of infusion, there were no significant differences between any variables in the vehicle and human α-CGRP-treated groups (Figures 1 and 2).

Examination of individual responses showed some quantitative variation, but similar patterns of change occurred in all animals studied with the exception of one animal that had relatively well-maintained responses during the 3 day infusion of human α-CGRP.

Cardiovascular effects of 3 day infusions of human α-CGRP in rats with internal and common carotid probes

In 3 animals there was a relatively sustained hypotensive response to human α-CGRP over the infusion period, although the tachycardia waned (Figure 3). However, while there were still substantial hypotensive effects and internal and common carotid vasodilatations on the second and third days of infusion, there was a clear reduction in response between the first and second days, during the time recordings were not made (i.e. between 16 h 00 min and 07 h 00 min the next day). Furthermore, at the beginning of the fourth day (i.e. just before the infusion pumps were switched off) there had been further, marked reductions in the effects of human α-CGRP, relative to the responses seen at the end of the third day (Figure 3).

In the other sub-group (6 animals), during the first day of infusion and at the beginning of the second day, the haemodynamic effects of human α-CGRP were the same as in the sub-group that showed a relatively maintained response to the peptide (see Figures 3 and 4). However, during the second infusion day (i.e. during the time that recordings were being made) there was desensitization to all the effects of human α-CGRP in these 6 animals. At the beginning of the third day the concentration of human α-CGRP in the infusate was increased 10 fold (i.e. to 15 nmol kg^{-1} h^{-1}) and all variables showed marked responses, but these effects were no greater than those seen at the start of infusion of the lower dose (Figure 4). The responses to human α-CGRP at 15 nmol kg^{-1} h^{-1} were maintained throughout the day, but by the beginning of the next day there was a clear, but incomplete

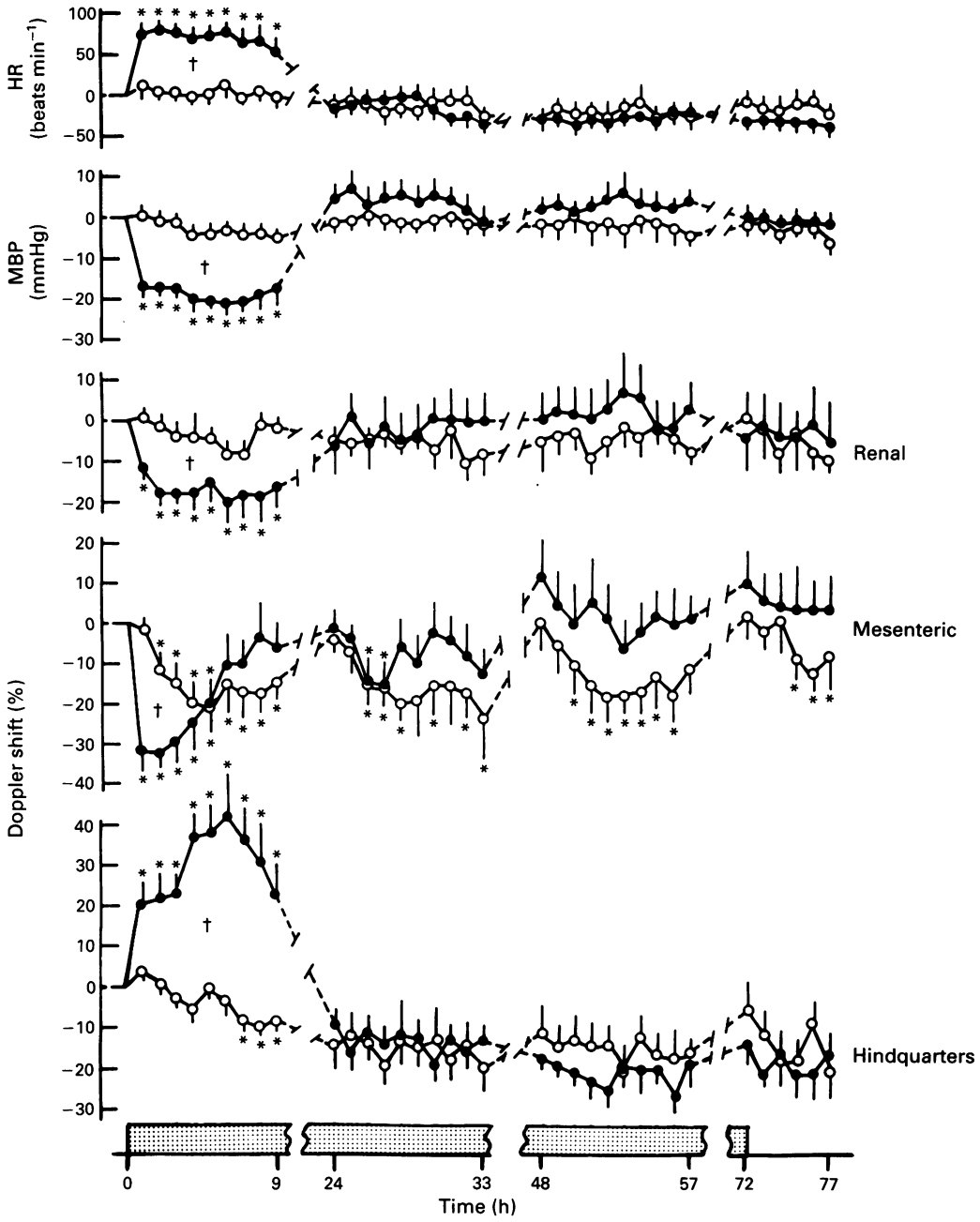

Figure 1 Cardiovascular changes during and after a 3 day infusion of human α-calcitonin gene-related peptide (α-CGRP, ●, 1.5 nmol kg^{-1} h^{-1}, $n = 8$) or vehicle (○, 0.3 ml h^{-1}, $n = 8$). Variables were recorded at 1 h intervals (averaged over 10 min) for 9 h each day (i.e. start 07 h 00 min, end 16 h 00 min). Values are mean and bars are s.e.mean. * $P < 0.05$ versus original baseline: † $P < 0.05$ for human α-CGRP versus vehicle (assessed from corresponding areas under or over curves). Infusion period indicated by shaded area on time axis. HR = heart rate; MBP = mean blood pressure.

desensitization to the effects of the peptide (Figure 4). Thus, human α-CGRP was still exerting measurable influences at the end of the infusion period and these were reversed when the infusion was stopped (Figure 4).

Discussion

In the present work marked desensitization has been shown to the haemodynamic effects of human α-CGRP when the peptide is infused over a period of 3 days in conscious, normotensive rats. There was some inter-individual variability but even the animals that showed the greatest sensitivity and most sustained responses to human α-CGRP administration demonstrated substantial tachyphylaxis by the end of the 3-day infusion period. These diminished responses were not due to the infusion system used delivering diminishing amounts of human α-CGRP over time since radioimmunoassay of the human α-CGRP concentration of the infusate showed no consistent change (unpublished observations).

The present results contrast with those of Marshall *et al.* (1987) who found that human α-CGRP administered at a nominal rate of 15 nmol kg^{-1} h^{-1} for 3 days caused sustained hypotension in female, spontaneously hypertensive rats. The differences between the observations and ours do not seem to be due to the dose of peptide administered, since, in the present study, when the infusion rate for human α-CGRP was increased to 15 nmol kg^{-1} h^{-1} (i.e. the rate used by Marshall *et al.*, 1987), tachyphylaxis was still observed. Furthermore, Marshall *et al.* (1987) administered human α-CGRP over 3 days from an implanted osmotic minipump and it is likely that the amount of human α-CGRP delivered by such a system is substantially less than calculated and not constant because of peptide degradation (unpublished observations).

It is feasible that the results obtained by Marshall *et al.*

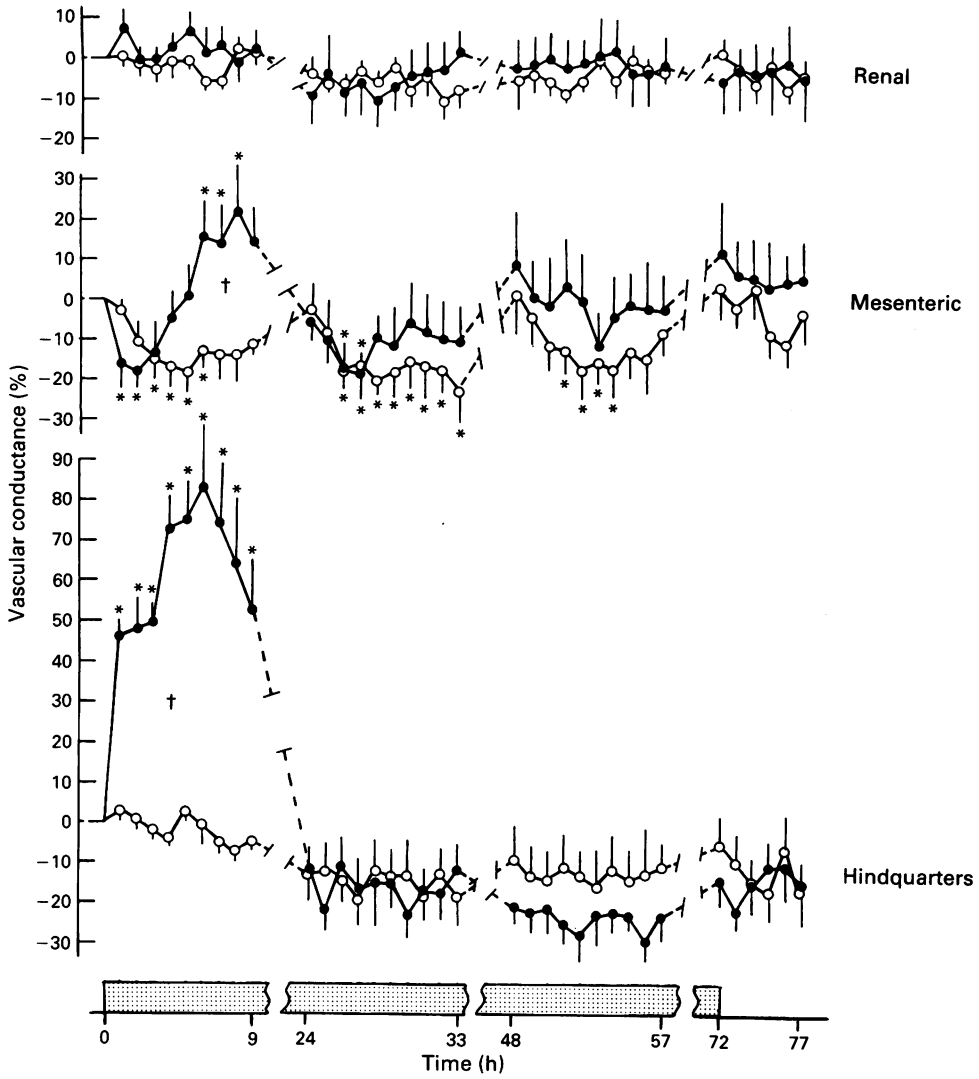

Figure 2 Changes in vascular conductance during infusion of human α-calcitonin gene-related peptide (α-CGRP, (●) or vehicle (○) calculated from the data in Figure 1. Values are mean and bars are s.e.mean. * $P < 0.05$ versus original baseline; † $P < 0.05$ for human α-CGRP versus vehicle (assessed from corresponding areas under or over curves). Infusion period indicated by shaded area on the time axis.

(1987) were due to the use of hypertensive animals, since it is well known that such animals are more sensitive to acute hypotensive stimuli (Takata & Hutchinson, 1983), possibly due to impaired baroreflexes (Thoren *et al.*, 1979; Verberne *et al.*, 1988; but see also Unger *et al.*, 1987). In addition, impaired baroreflexes might have contributed to a chronic failure of hypertensive rats to counteract the hypotensive effects of human α-CGRP. Thus, the apparent desensitization to human α-CGRP observed in normal rats in the present study could have been due to baroreflex-mediated recruitment of cardiovascular compensatory mechanisms in response to the hypotension caused by the peptide. However, this is not likely to be the full explanation, since tachyphylaxis was apparent also in the heart rate response to human α-CGRP, a component of which is reflex in origin (Sirén & Feuerstein, 1988). Furthermore, following cessation of human α-CGRP infusion there was no evidence of rebound effects that would have been expected if there was on-going stimulation of compensatory mechanisms such as the renin-angiotensin system (Bennett *et al.*, 1989). Hence, it appears more likely that the tachyphylaxis to the effects of prolonged infusions of human α-CGRP was due to receptor desensitization (i.e. loss of receptors or inhibition of the transducer mechanism, or both).

Han *et al.* (1990) have shown that exposure of the isolated mesenteric vasculature of the rat to rat CGRP at a concentration of 10^{-6} M for 2.5 h causes significant inhibition of vasodilator responses to rat CGRP at 10^{-8} M. However, they did not determine if higher doses of rat CGRP could overcome the desensitization. In the present work we found that increasing the infusion rate of human α-CGRP could re-establish the effects of the peptide. However, the response achieved was no greater than that seen with the initial, 10 fold lower dose of human α-CGRP and was considerably less than that achieved when naive rats were exposed to such a high dose of CGRP (Gardiner *et al.*, 1989a,b). Thus, the desensitization to the effects of human α-CGRP seen in the present work could have represented a shift in the dose-effect curve of the sort seen with competitive antagonism. In this context it is notable that human α-CGRP [8–37] can act as an antagonist of the *in vivo* haemodynamic actions of exogenous human α-CGRP (Gardiner *et al.*, 1990c). However, human α-CGRP [8–37] is not a likely candidate as an antagonist generated *in vivo* (Grèves *et al.*, 1989). Furthermore, since the N-terminal fragments of human α-CGRP show little affinity for CGRP binding sites (Dennis *et al.*, 1989), then human α-CGRP [28–37] might be considered as a candidate (Maton *et al.*, 1990; Chakder & Rattan, 1990), but we have found this peptide does not act as an antagonist of the *in vivo* haemodynamic effects of human α-CGRP (unpublished observations). Another possibility is that prolonged infusions of human α-CGRP induce peptidase activity that inactivates the peptide. Additional experiments are required to determine

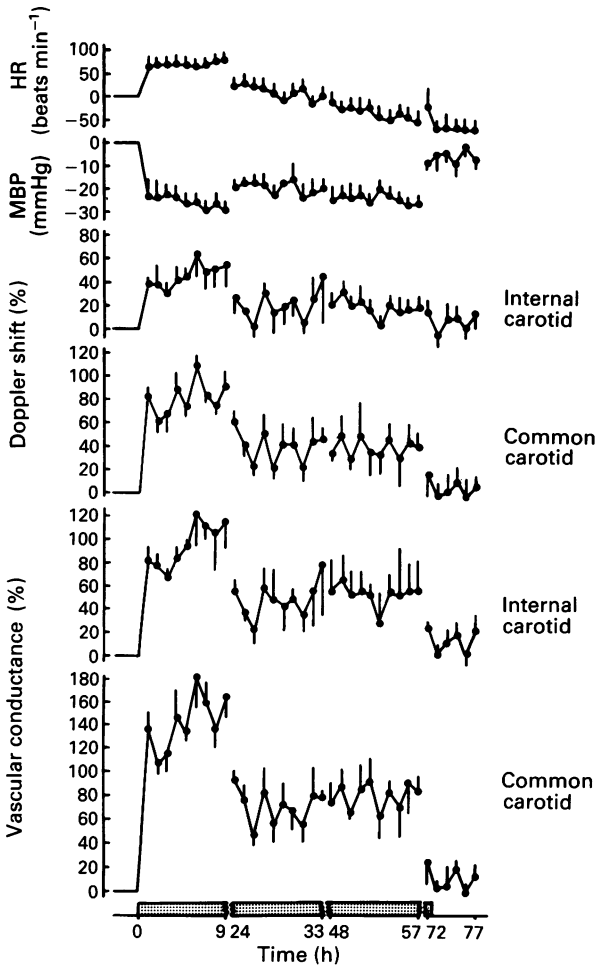

Figure 3 Cardiovascular changes during and after a 3-day infusion of human α-calcitonin gene-related peptide (α-CGRP, 1.5 nmol kg^{-1} h^{-1}) in conscious, Long Evans rats ($n = 3$). These animals showed a relatively well-maintained response to the peptide (compare Figure 4) but due to the small group size data were not analysed statistically. Infusion period indicated by shaded area on time axis. Values are mean and bars are s.e.mean. HR = heart rate; MBP = mean blood pressure.

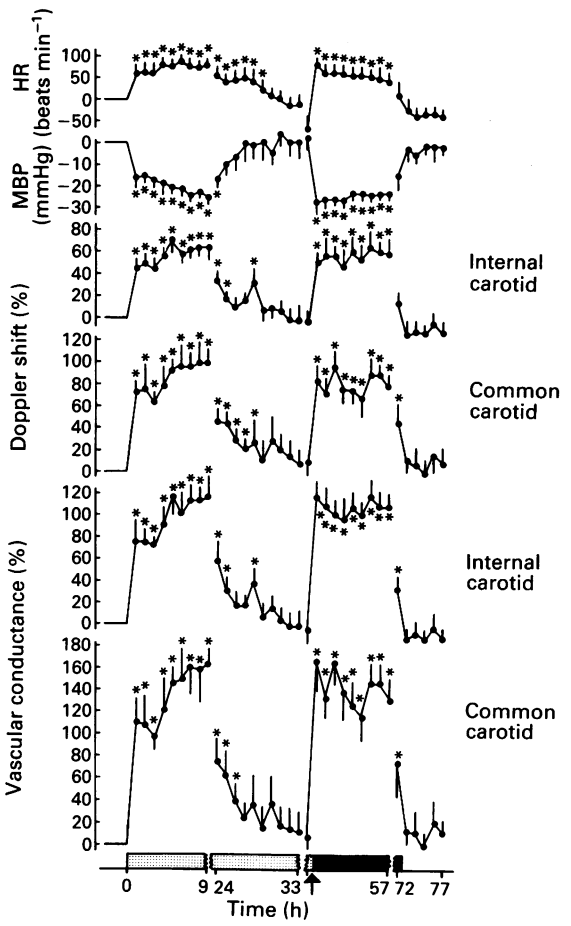

Figure 4 Cardiovascular changes during and after infusion of human α-calcitonin gene-related peptide (α-CGRP) in conscious, Long Evans rats ($n = 6$). Over the first 2 days human α-CGRP was infused at 1.5 nmol kg^{-1} h^{-1} and for the third day it was infused at 15 nmol kg^{-1} h^{-1} (onset at arrow). Values are mean and bars are s.e.mean; * $P < 0.05$ versus original baseline; infusion period indicated by shaded area on time axis. HR = heart rate; MBP = mean blood pressure.

if this phenomenon and/or receptor changes (see above) occur with prolonged administration of human α-CGRP.

At the outset of this study we thought it was possible that desensitization to the tachycardic, hypotensive and renal, mesenteric and hindquarters haemodynamic effects of human α-CGRP might not reflect what was happening in the carotid vascular bed, since the latter is the most sensitive to the vasodilator effects of exogenous human α-CGRP (Gardiner *et al.*, 1989a,b; 1990a). However, the present results have demonstrated that there can also be marked loss of the carotid vasodilator effects during prolonged infusions of human α-CGRP. If these findings can be extrapolated to the clinical setting it is likely that in order to achieve sustained effects of human α-CGRP on the carotid vasculature in patients, it would be necessary to infuse individually-adjusted and increasing amounts of the peptide.

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(Received December 3, 1990
Revised January 21, 1991
Accepted January 28, 1991)

Br. J. Pharmacol. (1991), **103**, 1515–1519 © Macmillan Press Ltd, 1991

Time-dependent synergistic interactions between the vasodilator neuropeptide, calcitonin gene-related peptide (CGRP) and mediators of inflammation

T.L. Buckley, [1]S.D. Brain, [2]M. Rampart & T.J. Williams

Department of Applied Pharmacology, National Heart and Lung Institute, Dovehouse Street, London

1 The action of the long lasting neuropeptide vasodilator, calcitonin gene-related peptide (CGRP), in potentiating oedema formation and neutrophil accumulation was investigated in the dorsal skin of the rabbit, *in vivo*. Combinations of agents under test were administered by intradermal (i.d.) injection. Oedema formation and neutrophil accumulation were then measured by quantitative radiolabel techniques.

2 CGRP (1×10^{-11} mol per site) potentiated neutrophil accumulation induced by zymosan activated plasma, (used as a source of C5a des Arg), N-formyl-methionyl-leucyl-phenylalanine (FMLP) and leukotriene B_4 (LTB_4). In contrast CGRP did not induce neutrophil accumulation when injected alone.

3 Oedema formation induced by a series of chemically distinct mediators of increased microvascular permeability; bradykinin, platelet activating factor (PAF), FMLP and zymosan-activated plasma; measured 0–30 min after i.d. injection was potentiated by CGRP (1×10^{-11} mol per site).

4 Oedema formation induced by zymosan activated plasma and FMLP but not bradykinin and PAF, was also significantly potentiated by CGRP when oedema was measured 30–60 min after i.d. injection. The potentiation of oedema induced by zymosan activated plasma measured 30–60 min after i.d. injection was not observed in the presence of the shorter acting prostanoid vasodilator prostacyclin (PGI_2, 3×10^{-10} mol per site).

5 These results suggest that CGRP, as a consequence of its sustained vasodilator activity could have prolonged potentiating effects on neutrophil accumulation and oedema formation in inflammatory conditions.

Keywords: Calcitonin gene-related peptide (CGRP); vasodilatation; neutrophils; oedema; inflammation

Introduction

The neuropeptide, calcitonin gene-related peptide (CGRP), a product of alternative processing of mRNA from the calcitonin gene, has been localised in the peripheral nervous system (Rosenfeld *et al.*, 1983). It is found in small unmyelinated sensory C-fibres that are associated with the smooth muscle of blood vessels and as free nerve endings in the epidermis and dermis of human skin (Gibbins *et al.*, 1987; Wallengren *et al.*, 1987). Activation, or antidromic stimulation, of sensory C-fibres leads to the release of immunoreactive CGRP (Martling *et al.*, 1988; Franco-Cereceda *et al.*, 1989).

Our studies have shown that CGRP is a very potent vasodilator. When injected intradermally (i.d.) it significantly increases microvascular blood flow at fmol doses (Brain *et al.*, 1985a). CGRP has a long duration of action in rabbit skin as well as human skin, when compared to the prostaglandin vasodilators PGE_2 and PGI_2 (Brain *et al.*, 1985; 1986b). The localisation and potency of CGRP strongly suggest that it could have an important role in regulating blood flow.

Mediators that increase microvascular permeability induce a small amount of oedema when injected i.d. at low doses in the rabbit skin. However, when combined with a vasodilator such as PGE_2, vasoactive intestinal polypeptide or CGRP, a substantial potentiation in plasma extravasation is observed in rabbit skin (Williams & Peck, 1977; Williams, 1982; Brain & Williams, 1985; Rampart & Williams, 1986). Mediators of increased microvascular permeability either act via a direct interaction with endothelial cells e.g. bradykinin, platelet activating factor (PAF), the tachykinins and histamine) or act via a mechanism that is dependent on the presence of neutrophils (e.g. the lipoxygenase product leukotriene B_4 (LTB_4), the bacterial peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP), tumour necrosis factor (TNF), or the complement fragment C5a des Arg (Wedmore & Williams, 1981; Rampart *et al.*, 1989). In previous studies we have measured oedema formation 0–30 min after i.d. injection of mediators under test (Brain & Williams, 1985; Rampart & Williams, 1986). In this study we have investigated the effect of CGRP on oedema formation and neutrophil accumulation and examined the possibility that CGRP can influence these responses for longer periods.

C5a, or the more physiologically stable product C5a des Arg, are potent mediators of neutrophil accumulation and oedema formation in rabbit skin (Jose *et al.*, 1981). C5a des Arg, has been detected in high concentrations in inflammatory exudates (Ward & Zvaifler, 1971; Jose *et al.*, 1983; 1990; Forrest *et al.*, 1986) and has been implicated in many inflammatory disease processes such as rheumatoid arthritis and lung injury (Ward & Zvaifler, 1971; Henson *et al.*, 1982). In a previous study (Brain & Williams, 1985) we demonstrated that CGRP can potentiate oedema induced by C5a des Arg. In this study we have used zymosan activated plasma as the source of C5a des Arg and we have investigated time-dependent interactions between zymosan activated plasma and CGRP.

Methods

Animals

Male New Zealand White (NZW) rabbits (2–2.5 kg) were purchased from Froxfield Farm, Hampshire and Hacking & Churchill, Huntingdon, Cambridgeshire.

Preparation of ^{111}In-neutrophils

Rabbits were anaesthetized with intravenous (i.v.), sodium pentobarbitone (Sagatal) 30 mg kg^{-1} and bled via the carotid artery into acid citrate dextrose. Neutrophils were isolated, as

[1] Author for correspondence at present address: Pharmacology Group, Biosciences Division, Kings College, Manresa Road, London SW3 6LX.

[2] Present address: Division of Pharmacology, Universitaire Instelling, Antwerpen – B2610, Wilrijk, Belgium.

described by Rampart & Williams (1988), by centrifugation over a two-layer discontinuous (50:70%) Percoll-plasma gradient after an initial red blood cell sedimentation with 3% hydroxyethyl starch. Neutrophils were incubated with ^{111}In (2–8 MBq) chelated with 2-mercaptopyridine-N-oxide (40 μg in 0.1 ml of 50 mM phosphate buffered saline) for 15 min at room temperature. The cells were then washed by suspending in 11 ml autologous plasma, followed by centrifugation. This process was repeated once and then the cells ($2.4–4.1 \times 10^7$) were resuspended in 3 ml autologous citrated plasma for i.v. injection into a recipient rabbit.

Preparation of C5a des Arg

Zymosan activated plasma (a source of C5a des Arg) was prepared by incubating heparinised (10 u ml^{-1}) rabbit plasma with zymosan (1 mg ml^{-1}) for 30 min at 37°C. Zymosan was removed by centrifugation (2×10 min: 2500 g). Activated plasma was stored at −25°C. The C5a des Arg content of activated plasma used in this study was 3×10^{-10} mol ml^{-1} as measured by radioimmunoassay (Jose *et al.*, 1983).

Measurement of neutrophil and plasma extravasation in the skin in vivo

In some experiments oedema formation alone was measured at injected sites in the rabbit dorsal skin (Williams, 1976) whereas in other experiments neutrophil accumulation and oedema formation were measured simultaneously (Rampart & Williams, 1988).

Rabbits were anaesthetized with sodium pentobarbitone and the dorsal fur was clipped. Neutrophil accumulation was measured as the local accumulation of ^{111}In-labelled neutrophils injected i.v. and oedema formation was measured as the accumulation of [^{125}I]-human serum albumin (HSA) (0.2 MBq; mixed with 1 ml of a 2.5% Evans Blue dye solution) injected i.v. The agents under investigation were injected i.d. in 0.1 ml volumes, each treatment having six replicates with injections given according to a balanced site plan. At the end of the accumulation period a 10 ml cardiac blood sample was collected into heparin (10 u ml^{-1}). The animal was then killed by an overdose of sodium pentobarbitone, the dorsal skin was removed and the injection sites punched out with a 17 mm diameter punch. The injected sites, together with plasma samples, were counted in a gamma counter (LKB, Wallac 1260 Multigamma II). Exudate volumes were expressed in terms of microlitres (μl) of plasma by dividing ^{125}I counts in each skin sample by ^{125}I counts in 1 μl of plasma. The ^{111}In counts per neutrophil were determined and the results were expressed as ^{111}In-neutrophils accumulating per skin site (^{111}In-neutrophil per site).

Experiments were designed as follows to study oedema formation and neutrophil accumulation over specific periods after intradermal injections. The potentiation of oedema formation and neutrophil accumulation by CGRP over 0–30 min (see Figure 1) was investigated by injecting the mediators i.d. alone or in combination, 5 min after the i.v. injection of [^{125}I]-HSA and ^{111}In-labelled neutrophils. The animal was then killed 30 min later. When investigating the long lasting potentiating action of CGRP (see Figure 2), a second identical set of i.d. test agents was injected at different sites, 30 min after the first set and then the animal was killed 60 min after the initial injections. This allowed oedema formation and neutrophil accumulation to be measured for 30 and 60 min after i.d. injection in the same animal. Accumulation for the 30–60 min period in the sites injected at time zero was then determined by subtraction. Oedema formation was measured directly over the 30–60 min period, as well as over the 0–30 min periods, in experiments where the ability of CGRP to potentiate oedema induced by a range of mediators of increased microvascular permeability was determined (see Figure 3). This was achieved by injecting i.d. the first set of test agents 30 min before i.v. [^{125}I]-HSA. A second identical set of test agents was then injected i.d. at different sites after the i.v. [^{125}I]-HSA. The animal was killed at 30 min after the i.v. [^{125}]-HSA (i.e. 60 min after the first set of i.d. test agents).

Materials

FMLP, 2-mercaptopyridine-*N*-oxide, histamine acid phosphate and bradykinin were purchased from Sigma Chemical Co., Poole, Dorset. 1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphorycholine (PAF) was purchased from Bachem, Saffron Walden, Essex. Evans blue was from BDH, Poole, Dorset; Sagatal (pentobarbitone sodium) was from May & Baker, Dagenham, Essex. Steriflex (sterile, pyrogen-free isotonic saline solution) was from The Boots Co. Nottingham. Hespan (6% hyroxy-ethyl starch in 0.9% NaCl) was from American Hospital Supply, Didcot, Oxfordshire. Percoll was from Pharmacia Fine Chemicals, Uppsala, Sweden. $^{111}InCl_3$ (0.74 kBq in 0.2 ml sterile, pyrogen-free 0.04 M hydrochloric acid) and [^{125}I]-human serum albumin (18.5 MBq, 20 mg albumin in 1 ml sterile isotonic saline) were from Amersham International, Amersham, Buckinghamshire. The following were generous gifts: LTB_4 was from Dr S.J. Foster, ICI, Macclesfield, recombinant human alpha CGRP from Dr U. Ney, Celltech, Slough; prostacyclin (PGI_2) was from Dr B.J.R. Whittle, Wellcome, Beckenham.

Working solutions were freshly made up from stocks each day in either sterile, pyrogen-free saline or when PGI_2 was used, 1.4% sodium bicarbonate solution pH 8.5.

Statistical analysis

The data were analysed by two way analysis of variance. Significant differences ($^* P < 0.05$, $^{**} P < 0.01$) between groups were determined by the Newman-Keuls procedure.

Results

Increasing doses of CGRP potentiate oedema formation induced by zymosan activated plasma and bradykinin over a 30 min accumulation period after i.d. injection as shown in Figure 1a. These results support our previous studies which demonstrate that CGRP, as a consequence of its vasodilator activity, potentiates inflammatory oedema formation induced by mediators of increased microvascular permeability (Brain & Williams, 1985; Brain *et al.*, 1986a). The effect of CGRP on neutrophil accumulation is shown in Figure 1b. Zymosan activated plasma, used as a source of C5a des Arg, caused significant neutrophil accumulation ($P < 0.05$, $n = 4$) when injected alone, in contrast to bradykinin which had no chemoattractant activity. Figure 1b shows that CGRP potentiated, in a dose-dependent manner, the neutrophil accumulation induced by zymosan activated plasma. The injection of CGRP (1×10^{-11} mol per site) alone did not induce oedema formation. CGRP (1×10^{-11} mol per site) also potentiated oedema induced by the chemoattractants LTB_4 (5×10^{-10} mol per site) and FMLP (5×10^{-11} mol per site). Results, expressed as ^{111}In-neutrophil per site, were as follows: FMLP 2889 ± 417; FMLP + CGRP 7352 ± 549; LTB_4 1469 ± 310; LTB_4 + CGRP 5878 ± 435; CGRP 433 ± 72; saline (control) 439 ± 88; mean ± s.e.mean, $n = 6$ replicate sites.

Figure 2 shows results from an experiment which was designed to investigate the potentiation of oedema and neutrophil accumulation induced by either the long acting vasodilator CGRP or the shorter acting vasodilator PGI_2. The responses were measured for either a 0–30 min or 0–60 min period after i.d. injection of test agents and results for the 30–60 min accumulation calculated by subtraction as described in the legend to Figure 2. The results show that co-injection of the vasodilators with zymosan activated plasma caused a potentiation of oedema formation and neutrophil accumulation when compared with responses to zymosan activated

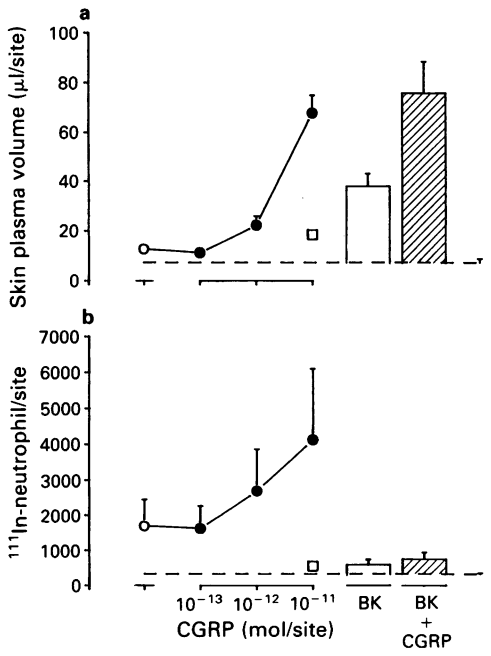

Figure 1 Potentiation by calcitonin gene-related peptide (CGRP, 1×10^{-11} mol per site) of (a) oedema formation (μl per site) (b) neutrophil accumulation (^{111}In-neutrophil per site) induced by zymosan activated plasma containing C5a des Arg (3×10^{-11} mol per site) and bradykinin (1×10^{-10} mol per site) over 30 min. The symbols represent: zymosan activated plasma (○), zymosan activated plasma + CGRP (●) and CGRP (□). Responses to bradykinin (BK) are shown by open columns, and BK + CGRP by hatched columns. The dashed line indicates responses at sites injected with saline. Results are expressed as mean (with s.e.mean shown by vertical bars) of $n = 4$ rabbits.

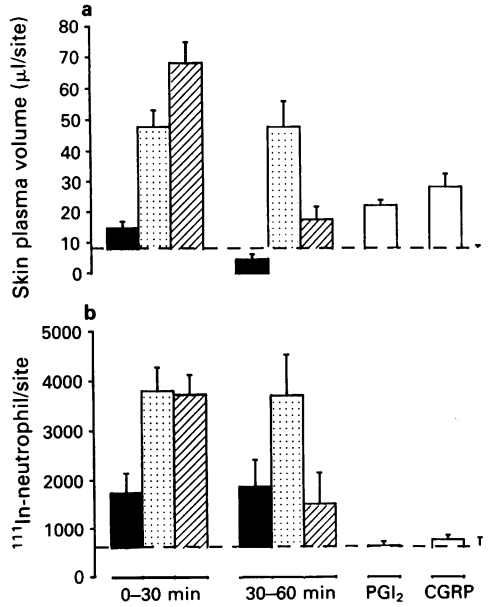

Figure 2 The effect of calcitonin gene-related peptide (CGRP, 1×10^{-11} mol per site) and prostacyclin (PGI_2, 3×10^{-10} mol per site) on (a) oedema formation (μl per site) and (b) neutrophil accumulation (^{111}In-neutrophil per site) induced by zymosan activated plasma containing C5a des Arg (3×10^{-11} mol per site). Both neutrophil accumulation and oedema formation were measured over 0–30 and 0–60 min response. Results for 30–60 min were determined by subtraction (see Methods). Responses to zymosan activated plasma are shown by solid columns, zymosan activated plasma + CGRP stippled columns, and zymosan activated plasma + PGI_2 hatched columns. Responses to CGRP and PGI_2 alone were measured for 0–60 min. The dashed line indicates responses at saline-injected sites. Results are from 1 experiment expressed as mean of $n = 6$ replicate sites (with s.e.mean shown by vertical bars). Three other experiments showed a similar trend in results.

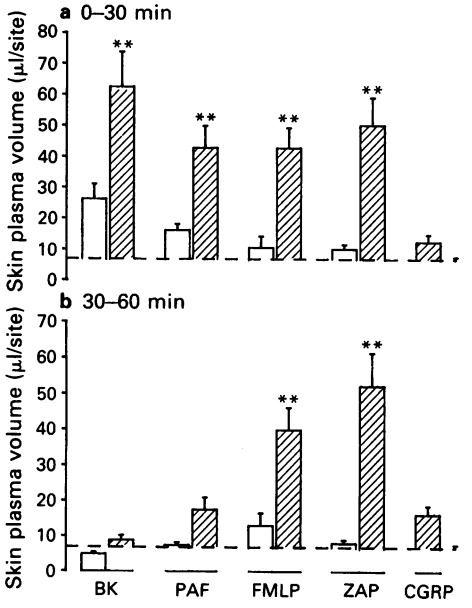

Figure 3 Potentiation by calcitonin gene-related peptide (CGRP) of oedema induced by mediators of increased microvascular permeability. Oedema induced by mediators (bradykinin, BK, 1×10^{-10} mol per site; PAF 1×10^{-9} mol per site; zymosan activated plasma (ZAP), containing C5a des Arg, 3×10^{-11} mol per site; and FMLP, 5×10^{-11} mol per site) alone (open columns) was compared to oedema in the presence of CGRP (10^{-11} mol per site; hatched columns) over (a) 0–30 min and (b) 30–60 min. In these experiments oedema formation was measured directly over the 0–30 min and 30–60 min periods after i.d. test agents (see Methods). The dashed line indicates responses at saline-injected sites. Results are expressed as mean of $n = 6$ rabbits (with s.e.mean shown by vertical bars). Significant differences between oedema induced by the mediator of increased microvascular permeability alone and in the presence of CGRP are indicated: ** $P < 0.01$.

plasma alone, measured over the 0–30 min period. However, PGI_2 failed to potentiate the responses to zymosan activated plasma during the 30–60 min period. In contrast, CGRP potentiated zymosan activated plasma-induced responses to a similar extent over both the 0–30 and 30–60 min periods. This representative experiment is supported by three other experiments which showed similar results as defined by statistical evaluation of the grouped data. CGRP (1×10^{-11} mol per site) significantly potentiated neutrophil accumulation ($P < 0.05$, $n = 4$) and oedema formation ($P < 0.05$, $n = 4$) induced by zymosan activated plasma over both the 0–30 and 30–60 min periods. In contrast PGI_2 only significantly potentiated oedema responses and neutrophil accumulation induced by zymosan activated plasma measured over 0–30 min (0–30 min $P < 0.05$; 30–60 min not significant, $n = 4$ rabbits).

The ability of CGRP to potentiate oedema induced by a range of mediators of increased microvascular permeability is shown in Figure 3; experiments were designed to measure directly the ability of CGRP to potentiate oedema formation over the 0–30 and 30–60 min periods. CGRP potentiated oedema induced by all the mediators over the 0–30 min period. However, in the 30–60 min period only oedema formation induced by the neutrophil-dependent mediators FMLP and zymosan activated plasma was significantly enhanced by CGRP.

Discussion

Our results suggest that there is a long lasting synergistic interaction between the neutrophil-dependent mediators of increased microvascular permeability (zymosan activated

plasma and FMLP) and CGRP in rabbit skin. The results show that the shorter acting vasodilator PGI_2 did not cause a long lasting potentiation. This suggests that the protracted potentiating effect of CGRP is due to its sustained vasodilator activity in skin. Our group has previously shown, by measuring the time taken for each mediator to lose half its activity in rabbit skin ($t_{1/2}$) that C5a des Arg ($t_{1/2}$ 100 min) and FMLP ($t_{1/2}$ 50 min) are both long acting mediators (Jose *et al.*, 1981; Wedmore & Williams, 1981). Histamine and bradykinin are known to be substantially shorter acting, $t_{1/2}$ 3–6 min (Wedmore & Williams, 1981) and their effects were not potentiated by CGRP from the 30–60 min after i.d. injections of mediators. Therefore, we suggest that a sustained potentiation of oedema formation is only observed when vasodilators and mediators of increased microvascular permeability both possess long duration of action.

Vasodilatation is an important component of inflammation. The protracted vasodilator action of neurally-derived CGRP could facilitate not only the continued supply of neutrophils to an inflammatory site but could also allow potentiation of oedema formation induced by various chemoattractants. The results suggest that the increased neutrophil accumulation induced by CGRP is due to its potent vasodilator activity as we show here that CGRP is not directly chemotactic for neutrophils in rabbit skin.

The human synovium is richly innervated by sensory nerve fibres and CGRP has been detected in small diameter nerves supplying the knee joint (Gronblad *et al.*, 1988). Recently it has been demonstrated that depletion of neuropeptides from the sensory C-fibres by pretreatment of the knee joint with the neurotoxin capsaicin, appears to moderate the severity of inflammation in a model of antigen-induced arthritis (Inman *et al.*, 1989). Furthermore, the release of CGRP by capsaicin from dorsal horn slices, is enhanced in adjuvant arthritic rats suggesting that CGRP-containing fibres are activated in rheumatoid arthritis (Nanayama *et al.*, 1989). CGRP-like immunoreactivity has also been observed in the upper and lower airways in many mammalian species including man (Cadieux *et al.*, 1986; Martling *et al.*, 1988) and it has been suggested that inflammatory neutrophil accumulation and oedema formation play a role in the pathogenesis of asthma. Therefore, CGRP could have an important potentiating role in inflammatory conditions in the joint and lung as well as in skin.

We have previously investigated mechanisms by which CGRP is metabolized *in vivo*. It is stable to incubation in plasma and blood (Brain & Williams, 1985). However, it is often co-localized in nerve endings with substance P, a potent mast cell activator in rat and human skin (Gibbins *et al.*, 1985; Lundberg *et al.*, 1985). We have shown that the protracted vasodilator response of CGRP is attenuated when substance P and CGRP are injected together into skin. Our studies have shown that this is due to mast cell proteases such as tryptase and chymase which break down CGRP (Brain & Williams, 1988). We have suggested that this could be an important mechanism for regulating the vasodilator activity of CGRP (Brain & Williams, 1988). This metabolizing mechanism could occur in types of inflammatory responses where mast cell activation is evident e.g. acute allergic responses. However, in other non-mast cell-dependent pathophysiological conditions, CGRP could remain active for long periods.

In conclusion, our results suggest that CGRP, as a consequence of its long duration of vasodilator action, could cause protracted potentiation of neutrophil accumulation and oedema formation induced by inflammatory mediators in skin and possibly in other organs such as the joint and lung.

We thank the Wellcome Trust for support. T.L.B. is an M.R.C. scholar. M.R. is a research associate of the Belgium National Science Foundation. T.J.W. is supported by the National Asthma Campaign.

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(Received January 8, 1991
Accepted January 30, 1991)

Br. J. Pharmacol. (1991), **103**, 1520–1524 © Macmillan Press Ltd, 1991

The effect of cetirizine on antigen-dependent leucopenia in the guinea-pig

[1]Anthony J. Coyle, Jean Lefort & [2]B. Boris Vargaftig

Unité de Pharmacologie Cellulaire, Unité Associée Institut Pasteur, INSERM No. 285, 25 Rue du Dr. Roux, Paris, 75015 France

1 Intravenous administration of ovalbumin (1 mg kg^{-1}) to guinea-pigs that had previously been injected with 3.5×10^9 platelets from actively sensitized animals induced an approximately 40% decrease in the number of circulating leucocytes 30–60 min later, whereas the number of platelets was not affected.

2 In contrast, there was no change in the leucocyte number following antigen challenge of guinea-pigs that had received platelets from non-immunised animals.

3 This platelet-dependent leucopenia was inhibited by prior treatment of the recipient animal with cetirizine (10–30 mg kg^{-1}, i.v.). Terfenadine (50 mg kg^{-1}, p.o.) and mepyramine (2 mg kg^{-1}, i.v.) were completely inactive in this respect. All doses of anti-histamines were used at concentrations which completely inhibited the bronchoconstriction to an i.v. injection of 5 μg kg^{-1} of histamine.

4 The site of action of cetirizine is most likely to be the platelet as leucopenia induced by the neutrophil agonists leukotriene B_4 (LTB_4) (30 ng kg^{-1}) and platelet activating factor (PAF) (30 ng kg^{-1}) were not modified by cetirizine treatment.

5 In these experiments, we failed to support a role for lipoxygenase products as mediators of the platelet-dependent leucopenia, as the selective lipoxygenase inhibitor BWA4C (50 mg kg^{-1}, p.o.) was ineffective.

6 Our present results confirm and extend previous data demonstrating that antigen stimulated platelets can induce leucopenia in non-immunised animals and this can be inhibited by the anti-allergic agent, cetirizine, by an action which is probably unrelated to its anti-histamine properties. The precise nature of the platelet derived factor(s) and their target of action remains to be determined.

Keywords: Platelet; leucocyte; cetirizine; anti-histamine; lipoxygenase

Introduction

Following the identification of low affinity receptors for IgE on the surface of the platelet (Capron *et al.*, 1986), there has been increasing interest in the possibility that immunological activation of the platelet may contribute to allergic reactions (Joseph, 1988). We have previously shown that platelets from actively sensitized guinea-pigs when transferred to non-immunized animals will induce a marked leukopenia following antigen challenge (Pretolani *et al.*, 1990). A central role for lipoxygenase products was proposed as the platelet-dependent leucopenia was attenuated by the dual lipoxygenase-cyclooxygenase inhibitor BW755C, but not by indomethacin. Thus it was suggested that antigen-stimulated platelets release a product of the lipoxygenase pathway which alters leucocyte and/or endothelial cell function resulting in leucocyte margination and the observed leucopenia.

Cetirizine is a metabolite of hydroxyzine which has recently been demonstrated as effective in the management of allergic disorders, including chronic idiopathic urticaria (Alomar *et al.*, 1990) and allergic rhinitis (Backhouse *et al.*, 1990). However, the precise mechanisms underlying its beneficial effect remain unknown.

In the present series of experiments, we have investigated the effect of cetirizine on antigen-induced platelet-dependent leucopenia and compared its effect to related agents with anti-histaminergic properties. In addition, we have attempted to verify the role of lipoxygenase products in mediating this response with a more selective lipoxygenase inhibitor, BWA4C (Tateson *et al.*, 1988).

[1] Author for correspondence at present address: Dept. of Pulmonary Medicine, National Jewish Hospital, 1400 Jackson Street, Denver, CO 80206, U.S.A.

[2] Reprint requests to Prof. B.B. Vargaftig.

Methods

Sensitization procedure

Hartley guinea-pigs of either sex (400–600 g) were sensitized by a s.c. injection of 0.5 ml 0.9% NaCl containing 10 μg ovalbumin dispersed in 1 mg $Al(OH)_3$ (modified from Andersson & Brattsand, 1981). The injection was repeated after 2 weeks and the animal used 7–10 days after the second injection. Untreated guinea-pigs were used as controls.

Preparation of washed platelets

Control or actively sensitized guinea-pigs were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.p.) and the carotid artery cannulated to allow blood collection. Blood was taken into 1/10th final volume sodium citrate (3.8% w/v) in the presence of 1 μM prostacyclin (PGI_2). Washed platelets were prepared as described previously (Pretolani *et al.*, 1990). Briefly, platelet rich plasma (PRP) was prepared by centrifugation at 200*g* for 15 min at 30°C. The platelets washed twice with Tyrode-gelatin buffer and resuspended in 1 ml of saline containing 1 μM PGI_2. The number of platelets were counted with a Coulter counter (ZBI) and adjusted to give a final concentration of 3.5×10^9 ml^{-1} and 1 ml of this platelet suspension injected i.v. to non-immunized animals.

In vivo *experiments*

The day after the transfer of the sensitized platelets, guinea-pigs were anaesthetized as described above, the carotid artery and the jugular vein cannulated for collection of blood and i.v. administration of drugs respectively. Aliquots of blood (200 μl) were taken before and at 15 min intervals for up to 90 min

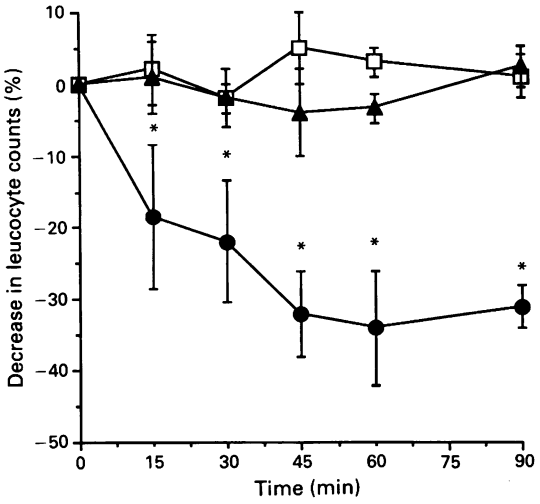

Figure 1 Time course of the decrease in leucocyte number following ovalbumin injection in non-immunized animals that received platelets from immunized (●) or non-immunized (▲) guinea-pigs. The change in the number of platelets following antigen challenge of guinea-pigs that received platelets from sensitized animals is also shown (□). Results are expressed as the percentage change in the number of circulating leucocytes and platelets, representing the mean (s.e.mean shown by vertical bars) of $n = 6$ experiments for sensitized cells and $n = 3$ for control transfer experiments. Significance was determined by Student's t test and a value of $P < 0.05$ (*) considered significant.

after i.v. injection of $1\,mg\,kg^{-1}$ ovalbumin and the number of circulating leucocytes and platelets determined with a Coulter counter. Where indicated, guinea-pigs were treated with cetirizine (10–30 $mg\,kg^{-1}$, i.v.), terfenadine ($50\,mg\,kg^{-1}$, p.o.), mepyramine ($2\,mg\,kg^{-1}$, i.v.) or BWA4C ($50\,mg\,kg^{-1}$, p.o.) 1 h before ovalbumin administration. In some experiments, lung function was assessed as described previously (Lefort & Vargaftig, 1978), and a bolus i.v. injection of $5\,\mu g\,kg^{-1}$ of histamine given before, and 1 h after administration of the antihistamines.

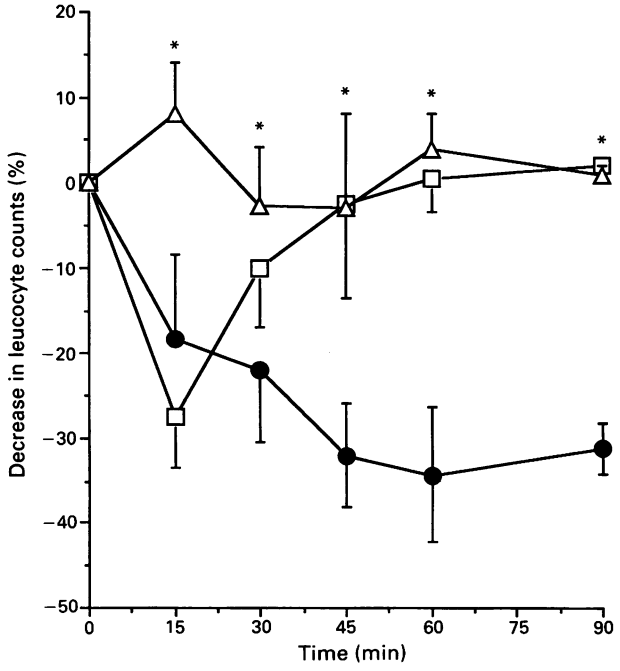

Figure 2 The effect of cetirizine, $10\,mg\,kg^{-1}$ (□), $30\,mg\,kg^{-1}$ (△) on antigen-induced leucopenia in non-immunized guinea-pigs that received platelets from sensitized animals. Control animals were injected with saline (●). Results are expressed as the percentage change in the number of circulating leucocytes and represent the mean (s.e.mean shown by vertical bars) of $n = 5$–6 experiments and a P value of < 0.05 (*) considered significant.

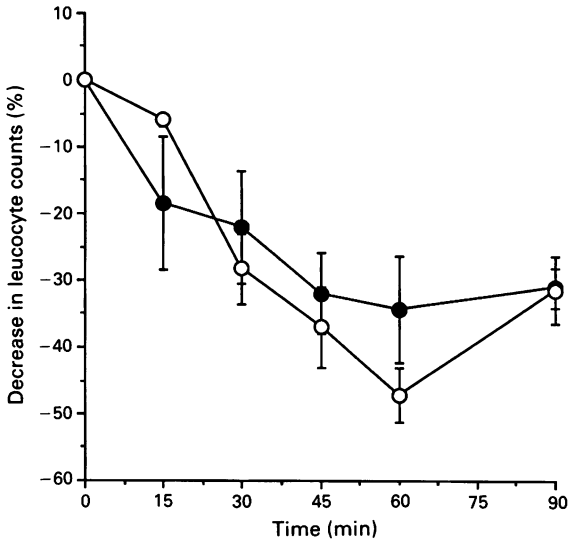

Figure 3 The effect of mepyramine, $2\,mg\,kg^{-1}$ (○), on antigen-induced leucopenia in non-immunized guinea-pigs that received platelets from sensitized animals. Control animals were injected with saline (●). Results are expressed as the percentage change in the number of circulating leucocytes and represents the mean (s.e.mean shown by vertical bars) of $n = 5$ experiments.

Reagents

Reagents used were as follows: sodium pentobarbitone (Sanofi, Montpellier, France), ovalbumin (Miles, Naperville, IL, U.S.A.), gelatin (Merck, Darmstadt), PGI_2, EGTA, KCl, $MgCl_2$, NaCl, $NaHCO_3$, glucose, mepyramine, (Sigma, St. Louis, MO, U.S.A.), carboxymethylcellulose (CMC) (Prolabo, France), cetirizine (2-(4-((4-chlorophenyl) phenylmethyl)-1-piperazinylethoxy) acetic acid, UCB, France), terfenadine (Merrell Dow, France). BWA4C (N-(3-benzylcinnamyl)acetohydroxamic acid) was kindly supplied by Dr S. Moncada from the Wellcome Research Labs, Beckenham, Kent. PAF

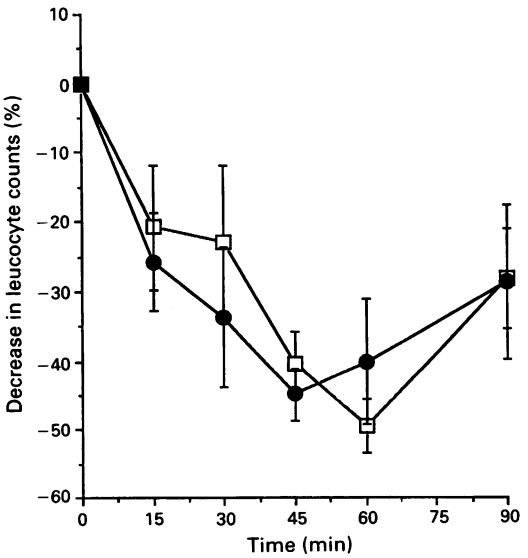

Figure 4 The effect of terfenadine $50\,mg\,kg^{-1}$, p.o. (□) on antigen-induced leucopenia in non-immunized guinea-pigs that received platelets from sensitized animals. Control animals were treated orally with carboxymethylcellulose (●). Results are expressed as the percentage change in the number of circulating leucocytes and represent the mean (s.e.mean shown by vertical bars) of $n = 3$–5 experiments.

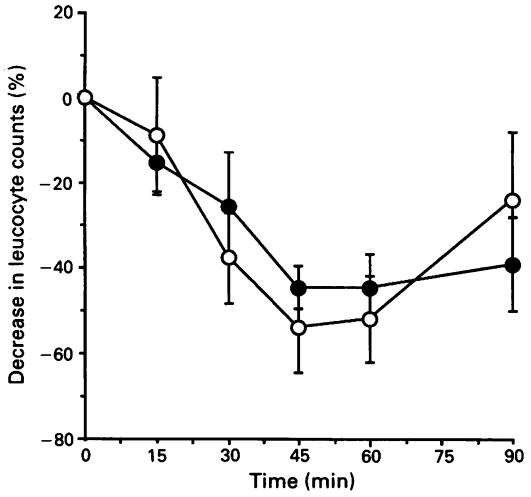

Figure 5 The effect of BWA4C 50 mg kg^{-1}, p.o. (○) on antigen-induced leucopenia in non-immunized guinea-pigs that received platelets from sensitized animals. Control animals were treated orally with carboxymethylcellulose (●).

was supplied by Bachem, Switzerland and LTB_4 from Cascade Biochemicals, U.K.

Statistical analysis

Results are expressed as the mean ± s.e.mean of the indicated number of experiments and statistical significance determined by Student's *t* test for unpaired data. A *P* value of less than 0.05 was considered as significant.

Results

Effect of antigen administration on the number of circulating leucocytes in guinea-pigs injected with platelets from immunized or non-immunized animals

The i.v. injection of 1 mg kg^{-1} of ovalbumin induced a 40% decrease in the number of circulating leucocytes which was maximal 60 min after challenge and evident for at least 90 min (Figure 1). Antigen challenge of animals that received platelets from non-immunized animals failed to induce any significant leucopenia. In accordance with results previously reported, there was no change in the number of platelets following antigen challenge (Figure 1).

Pharmacological modulation of antigen-induced leucopenia

Pretreatment with cetirizine 1 h before antigen administration induced a dose-related inhibition of the platelet-dependent leucopenia, with the maximal dose used achieving a complete inhibition (Figure 2). Terfenadine and mepyramine failed to inhibit the leucopenia (Figures 3 and 4). All doses of antihistamine used achieved a complete inhibition of bronchoconstriction induced by an intravenous injection of 5 μg kg^{-1} of histamine (data not shown). Pretreatment with the selective lipoxygenase inhibitor of BWA4C, also failed to inhibit the antigen-induced platelet dependent leucopenia (Figure 5).

Effect of cetirizine on leukotriene B_4- and PAF-induced leucopenia

Pretreatment with cetirizine (30 mg kg^{-1}) failed to modify leucopenia induced by LTB_4 (−44.3 ± 8.1% fall in leucocytes in control animals; −38.6 ± 9.5% in cetirizine-treated animals, $n = 3$, NS) or PAF (−43.5 ± 4.1% fall in leucocytes in control animals; −45.9 ± 5.2% in cetirizine treated animals, $n = 5$, NS).

Discussion

There is an increasing body of evidence to implicate the platelet as an important effector cell in inflammatory and allergic diseases (Joseph, 1988; Page, 1988). In 1986, Capron and coworkers identified receptors for IgE on the surface of the platelet, opening the possibility that platelets can directly participate in immunological reactions. Experimental animal studies have demonstrated a pulmonary recruitment of platelets following antigen challenge (Pinckard *et al.*, 1977; Pretolani *et al.*, 1986; Lellouch-Tubiana *et al.*, 1987). Furthermore, active participation of platelets is suggested by the observation that selective platelet depletion inhibits the late asthmatic response and heightened airway responsiveness in IgE immunized rabbits (Coyle *et al.*, 1990). Platelet activation is also associated with antigen provocation in man. Thus elevated levels of β-thromboglobulin and Platelet Factor 4 have been reported in the circulation following antigen provocation (Knauer *et al.*, 1981; Gresele *et al.*, 1985). Moreover, activated platelets have been identified in the bronchoalveolar lavage fluid after antigen challenge (Metzger *et al.*, 1987). In addition, several groups have demonstrated platelet abnormalities in asthmatic individuals such as prolonged bleeding time and reduced platelet survival (Szczeklik *et al.*, 1986; Taytard *et al.*, 1986). However, this is still a controversial area and other workers have failed to provide evidence of platelet activation in asthma (Ind *et al.*, 1985; Hemmendinger *et al.*, 1989).

In this paper we confirm previous observations demonstrating that antigen challenge of non-immunized guinea-pigs which 24 h previously received platelets from actively sensitized animals, induces a marked leucopenia in the absence of any change in the number of circulating platelets. Such observations may help to explain some of the discrepancies in the literature as most of these studies investigating a role of the platelet in asthma have been directed towards detecting markers of classical platelet aggregation and secretion. Our results demonstrate that platelets can undergo activation that is distinct from platelet aggregation as revealed by the absence of any overt thombocytopenia following antigen challenge.

We have extended our initial observations to demonstrate that cetirizine will inhibit the platelet-dependent leucopenia. The precise mechanism of action of cetirizine is at present unclear. However, it is unlikely to be related to its H_1 histamine receptor antagonism as terfenadine and mepyramine at a dose that inhibits both the bronchoconstrictor response to i.v. histamine (data not shown) and histamine-induced wheal response in guinea-pig skin (De Vos *et al.*, 1987) were completely ineffective. However, it is important to note that cetirizine is 1.5 times more potent as a histamine antagonist than mepyramine following i.v. administration and 10 times more potent than terfenadine (De Vos *et al.*, 1987) and although the response to exogenous histamine was inhibited, the possibility that high local concentrations of histamine were able to overcome H_1 receptor blockade cannot be ruled out.

Cetirizine has been demonstrated to inhibit the synthesis of PGD_2 (Charlesworth *et al.*, 1988), PAF and histamine (Michel *et al.*, 1988) after antigen challenge in the skin of allergic subjects. However, it is unlikely that inhibition of the cyclooxygenase pathway of arachidonic acid metabolism or inhibition of PAF generation is responsible for the efficacy of cetirizine as it has previously been demonstrated that indomethacin and the selective PAF antagonist, WEB 2086, were ineffective in inhibiting the antigen-dependent leucopenia (Pretolani *et al.*, 1990). Cetirizine has also been reported to inhibit the accumulation of leucocytes in the skin of allergic individuals following antigen challenge *in vivo* (Fadel *et al.*, 1987; Charlesworth *et al.*, 1988; Michel *et al.*, 1988; Henocq

& Rihoux, 1990) and will inhibit both PAF and FMLP-induced chemotaxis of human eosinophils *in vitro* (De Vos *et al.*, 1989). However, we were unable to demonstrate any inhibitory effect of cetirizine on either PAF- or LTB_4-induced leucopenia suggesting that the ability of cetirizine to inhibit the platelet-dependent leucopenia is not an effect on the leucocyte, but is most likely an action mediated on the platelet itself.

The platelet can liberate a variety of substances which can affect leucocyte function (reviewed by Page, 1988). Indeed, it has been suggested that platelet-derived 5-hydroxytryptamine is important in enhancing leucocyte adhesion to vascular endothelium (Boogarts *et al.*, 1982). Conversely, the antigen-activated platelets may express activities which alter endothelial cell function. Such a substance is IL-1 which has recently been demonstrated to be expressed by activated platelets (Hawrylowicz *et al.*, 1989) and is known to facilitate the adhesion of leucocytes to vascular endothelium (Bevilacqua *et al.*, 1985).

Previous work has suggested an important role for lipoxygenase products as the platelet-dependent leucopenia was inhibited by the mixed lipoxygenase inhibitor BW755C but not indomethacin (Pretolani *et al.*, 1990). In the present series of experiments however, we failed to support a role for lipoxygenase products in this response, as the selective lioxygenase inhibitor BWA4C (Tateson *et al.*, 1988) was ineffective at a concentration previously demonstrated to inhibit the 'leukotriene-dependent' component of bronchial anaphylaxis in the guinea-pig (Payne *et al.*, 1988). This observation suggests that the inhibitory effect of BW775C is by an action unrelated to its ability to inhibit the lipoxygenase pathway. In this regard, BW755C, is a powerful antioxidant, inhibiting lipid peroxidation in rat basophilic leukaemic cell homogenates (Thody *et al.*, 1987), human neutrophils and macrophages (Muller-Peddinghaus & Wurl, 1987). In contrast, BWA4C has considerably less efficacy as a scavenger of oxygen free radicals, although it will inactivate peroxyl radicals (Darley-Usmar *et al.*, 1989) opening up the possibility that generation of oxygen free radicals plays an important role in this response. It is therefore of considerable interest that antigen stimulation of platelets from allergic individuals results in the generation of oxygen free radicals and the expression of platelet cytotoxic functions (Joseph *et al.*, 1986). Furthermore, cetirizine (De Vos *et al.*, 1989) and nedocromil sodium (Joseph *et al.*, 1986) have been reported to inhibit oxygen free radical generation following IgE dependent activation and the subsequent platelet cytotoxicity. However, whether an analogous mechanism is responsible for the platelet-dependent leukopenia remains to be determined. In addition, the site of action of the factor(s) liberated by the platelet is unknown and maybe an action on the leucocyte and/or the vascular endothelium. Further investigation into the interaction between antigen-activated platelets and other components of the inflammatory response may be a rewarding area for future research.

We would like to acknowledge the generous support of Dr J.P. Rihoux (U.C.B., Belgium).

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(Received October 25, 1990
Revised January 29, 1991
Accepted February 4, 1991)

Br. J. Pharmacol. (1991), **103**, 1525–1529 © Macmillan Press Ltd, 1991

Early and late contraction induced by ouabain in human umbilical arteries

[1]Koichi Sato & K. Aoki

Second Department of Internal Medicine, Nagoya City University Medical School, Mizuho-ku, Nagoya 467, Japan

1 Ouabain (3×10^{-7}–10^{-4} M) evoked a biphasic contraction in human umbilical artery that consisted of an early and a late contraction. The Ca^{2+}-antagonists, verapamil (10^{-7}–10^{-5} M), diltiazem (10^{-7}–10^{-5} M) and nifedipine (10^{-9}–10^{-7} M) inhibited the early but not the late contraction. Caffeine (5 mM) changed neither the magnitude of the peak of the biphasic contraction nor the time needed to reach it.

2 Sodium concentration reduction (140 to 0 mM, replaced by N-methyl-D-glucamine, NMG) produced dose-dependent contraction of the arterial strip in 2.5 mM Ca^{2+} solution after the first treatment with verapamil (10^{-5} M) and caffeine (5 mM), but not in Ca^{2+}-free solution.

3 After prior treatment with verapamil and caffeine, the amplitude of the ouabain (10^{-4} M)-induced late contraction varied, depending on the concentration of Ca^{2+} (0–2.5 mM) in the medium.

4 Amiloride (5×10^{-5} M–5×10^{-4} M), an inhibitor of the Na^+–Ca^{2+} exchange system, produced dose-dependent inhibition of the late contraction induced by ouabain (10^{-5} M) after prior treatment with verapamil and caffeine.

5 The time needed to reach the peak tension induced by monensin (5×10^{-7} M) together with ouabain (10^{-6} M) was less than that with ouabain alone, but the magnitude of the peak tension was not changed.

6 These results suggest that the early and late contractions caused by ouabain respectively produce a Ca^{2+} influx through voltage-sensitive Ca^{2+} channels and Ca^{2+} entry through sodium-calcium (Na^+–Ca^{2+}) exchange.

Keywords: Ouabain; human umbilical artery; early contraction; late contraction; amiloride; monensin; Ca^{2+}-antagonist; Na^+ reduction; Na^+-Ca^{2+} exchange

Introduction

Human umbilical arteries are devoid of sympathetic innervation (von Euler, 1938; Davignon *et al.*, 1965; Somylo *et al.*, 1965; Burton & Godfraind, 1974) and are therefore very useful for the study of the myogenic contraction of arterial smooth muscle (Gopalakrishnan *et al.*, 1985).

We have shown in human umbilical arteries, that a biphasic contraction is evoked by ouabain or a K^+-free solution (Sato & Aoki, 1988; 1989). This contraction consists of a transient rapid early contraction and a slow continuous late one. The Ca^{2+}-antagonist, nifedipine, inhibits the early contraction and a Ca^{2+}-free solution inhibits both. Therefore, we suggested that the ouabain-induced early contraction is caused by a Ca^{2+} influx through voltage-sensitive Ca^{2+} channels. No inhibition of the early contraction by any other Ca^{2+}-antagonist has been demonstrated, and the mechanism of the late contraction has yet to be demonstrated.

We have now investigated the myogenic mechanisms of the ouabain-induced biphasic contraction after prior treatment with verapamil and caffeine in order to inhibit Ca^{2+} influx and Ca^{2+} release from the sarcoplasmic reticulum. We here discuss the contribution of Na^+–Ca^{2+} exchange to the late contraction produced by the reduction of the Na^+ concentration in the medium.

Methods

Umbilical arteries

Human umbilical arteries were taken from 50 umbilical cords of mothers aged 22 to 33 years at normal full-term uncomplicated deliveries. Mothers who had hypertension, diabetes, Rh factor problems, or other overt diseases or who were on medication such as antihistamines, morphine, and adrenergic drugs, were not included. At delivery 10 cm segments were cut from the umbilical cords midway between the placenta and the infant and immediately placed in modified Krebs-bicarbonate solution (Krebs solution) at 4°C. After removal of the surrounding tissue, the arteries were cut into 2 by 8 mm helical strips within 12 h of delivery.

Tension development

Strips were mounted vertically between hooks in a water-jacketed muscle bath containing 20 ml of Krebs solution at pH 7.4, then bubbled with a mixture of 95% O_2 and 5% CO_2. The upper end of each strip was connected to the lever of a force-displacement transducer (TB-612T, Nihon Koden Kogyo Co., Tokyo, Japan) in order to record the tension. Each strip was stretched passively to 200% of its original length. The respective initial and final tensions were 4.4 ± 0.7 (mean ± s.d.) and 2.1 ± 0.3 g. The strips were allowed to equilibrate in the Krebs solution for 5 h the solution being changed at 15 min intervals. Before the start of the experiments, the solution was replaced with 60 mM K^+ solution in order to evoke the maximum contraction of 2.2 ± 0.2 g which was considered as the 100% response. The high K^+ solution was then replaced with the Krebs solution, and the tension relaxed to the basal value. Phenoxybenzamine (Pbz, 2×10^{-6} M) was added to the bath fluid 30 min before inducing contractions in order to inhibit endogenous α-adrenoceptor-mediated effects.

Contractile response to ouabain

In Krebs solution, ouabain (10^{-6} M) induced early and late contractions. The late contraction was evoked only after a 30 min prior treatment with verapamil (10^{-5} M) which inhibited Ca^{2+} influx through the Ca^{2+} channels. The dose-response curves for the ouabain-induced contractions were determined from a single-dose stimulation.

[1] Author for correspondence.

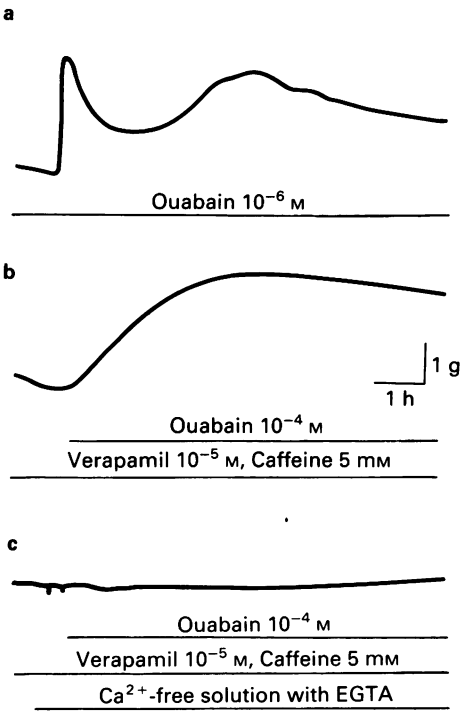

Figure 1 Recordings of contractions: ouabain (10^{-6} M)-induced contraction (a), the late phase of ouabain (10^{-4} M)-induced contraction in Krebs solution (b) and Ca^{2+}-free solution containing ouabain with 0.2 mM EGTA (c).

Ca^{2+}-antagonists

The umbilical strips were treated with a Ca^{2+}-antagonist for 30 min then contracted with ouabain (10^{-6} M). Concentrations of the Ca^{2+}-antagonists were 10^{-7} to 10^{-5} M for verapamil, 10^{-7} to 10^{-5} M for diltiazem and 10^{-9} to 10^{-7} M for nifedipine.

Contractile response to low Na^+

The strips were incubated in a verapamil-caffeine solution for 30 min then contracted by reducing the Na^+ concentration.

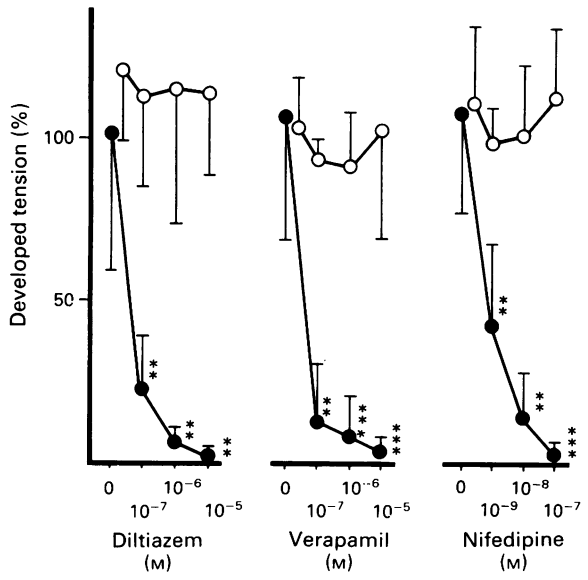

Figure 2 Effects of the Ca^{2+}-antagonists verapamil (10^{-7}–10^{5} M) (a), diltiazem (10^{-7}–10^{-5} M) (b) and nifedipine (10^{-9}–10^{-7} M) (c) on the early (●) or late (○) phase induced by ouabain (10^{-6} M). Results are expressed as percentages of contractions obtained with a high-K^+ solution. Points show mean and vertical bars show s.d. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Na^+ was replaced iso-osmotically with N-methyl-D-glucamine (NMG) and pH was adjusted to 7.4 with HCl. After the strips had been incubated in the verapamil-caffeine solution for 30 min and then immersed in the Ca^{2+}-free EGTA solution for 10 min, they were treated with 14 mM Na^+ in verapamil Ca^{2+}-free solution.

Ca^{2+}

The effect of extracellular Ca^{2+} on a Ca^{2+}-free solution containing 0.2 mM ethylenglycol-*bis*-(β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) was analysed for 10 min before ouabain-induced contraction. The strips were incubated in a verapamil-caffeine solution for 30 min after which they were immersed in Ca^{2+}-free 0.2 mM EGTA solution for 10 min. The Ca^{2+}-free EGTA solution was replaced with verapamil solution containing $CaCl_2$ (0–2.5 mM) after which the strips were contracted with ouabain (10^{-4} M).

Amiloride

After a 30 min treatment with verapamil and caffeine in the absence or presence of amiloride (5×10^{-5}–5×10^{-4} M), the strips were contracted with ouabain (10^{-6} M).

Monensin

The strips were immersed in verapamil-caffeine solution for 30 min after which they were contracted with ouabain (10^{-6} M) in the absence or presence of monensin (5×10^{-7} M). The time needed to reach the peak tension and the magnitude of the maximum tension were measured.

Bath fluid

The composition of the Krebs solution at pH 7.4 (in mM) was: 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 60 mM K^+-depolarizing solution was made by equimolar substitution of KCl for NaCl. The Ca^{2+}-free solution was produced by replacing $CaCl_2$ with equimolar $MgCl_2$. Ouabain was dissolved in distilled water on the day of the experiment. A stock solution of Pbz was prepared in saline (0.9% NaCl)-ascorbic acid (0.01%) solution, distilled water and ethanol (99.5%) and was kept at −20°C. Solutions of 10^{-1} M verapamil or diltiazem were made by dissolving each in distilled water. A solution of nifedipine (10^{-3} M) was made in ethanol and kept at −20°C and protected from light. Monensin was dissolved in ethanol and diluted with Krebs solution to give a final ethanol concentration of less than 0.2% (Yang *et al.*, 1986). For use, these solutions were diluted with Krebs solution to the desired concentration.

Drugs

The drugs used were verapamil HCl (Eisei Co., Tokyo, Japan), diltiazem (Tanabe Co., Osaka, Japan), nifedipine (Bayer AG, Tokyo, Japan), caffeine (Sigma Chemical Co., Tokyo, Japan), ouabain octahydrate (Sigma Chemical Co., Tokyo, Japan), phenoxybenzamine (Nakarai Chemical Co., Tokyo, Japan), monensin sodium (Sigma Chemical Co., Tokyo, Japan) and amiloride (Merck & Sharp & Dohme S.p.A., Milano, Italy).

Statistical analysis

Data are expressed as means ± s.d. Statistical significance was assumed when $P < 0.05$. Student's paired t test was used.

Results

Ouabain-induced early and late contraction

Biphasic contraction Ouabain-induced biphasic, dose-dependent contractions at concentration from 3×10^{-7} to

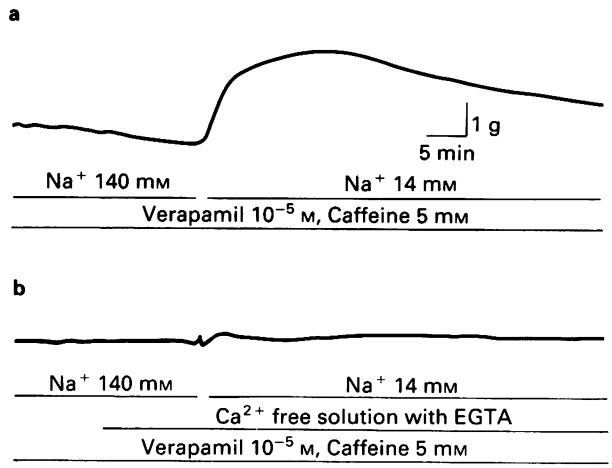

Figure 3 Recordings of 14 mM Na^+-induced contractions in Krebs solution (a) and in Ca^{2+}-free solution containing verapamil and caffeine (b).

10^{-4} M (Sato & Aoki, 1988; 1989). The contraction consisted of a transient rapid early contraction and a slow continuous late one (Figure 1a).

Early contraction in Ca^{2+}-antagonist and Ca^{2+}-free EGTA solution The early contraction was inhibited by the Ca^{2+}-antagonists verapamil, diltiazem and nifedipine (Figures 1b and 2), with complete inhibition of the contraction by Ca^{2+}-free 0.2 mM EGTA solution (Figure 1c). The Ca^{2+}-antagonists did not inhibit the late contraction (Figures 1b and 2); whereas, the Ca^{2+}-free EGTA solution completely inhibited both the early and late contractions (Figure 1c).

Reduction of Na^+ concentration-induced contractions

The reduction of Na^+ from 140 to 0 mM in the verapamil solution produced dose-dependent contraction of the strips (Figures 3a and 4a). The 14 mM Na^+-induced contraction was inhibited by Ca^{2+}-free solution after prior treatment with verapamil and caffeine and subsequent treatment with Ca^{2+}-free EGTA solution for 10 min (Figure 3b). The relationship between the rate of tension development and Na^+ is plotted in Figure 4b. The rate when Na^+ was reduced cannot be

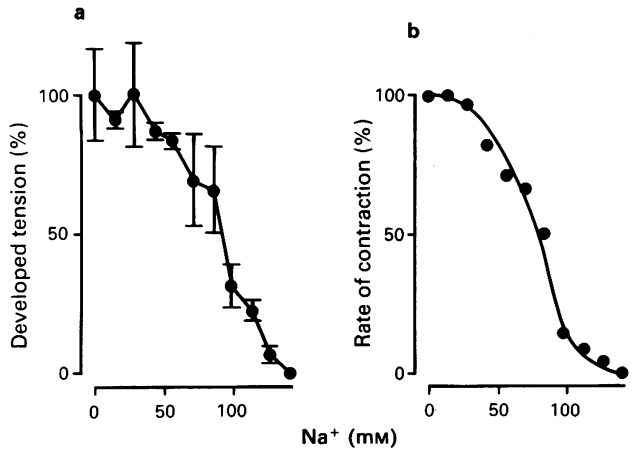

Figure 4 Dose-respone curve for Na^+ reduction after treatment with verapamil together with caffeine (a). The tension development in 0 mM Na^+ was taken as 100%. Points show mean and vertical bars show s.d. ($n = 5$). Relation between Na^+ and the relative rate of tension development after treatment with verapamil and caffeine (b). The rate of tension development was calculated from the rising slope, 100% being taken as the development in 0 mM Na^+. Each symbol represents the mean of data for two to four strips. The absolute rates of tension development in 0 mM Na^+ were 740.0 ± 42.4 mg min^{-1} ($n = 3$).

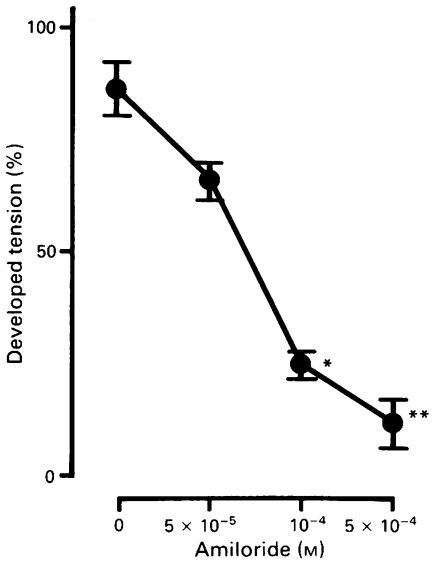

Figure 5 The effect of amiloride on the late phase of ouabain (10^{-6} M)-induced contraction. The results are expressed as a percentage of the contraction obtained with high-K^+ solution. Five strips were used for each amiloride concentration. Points show mean and vertical lines slow s.d. * $P < 0.05$; ** $P < 0.01$.

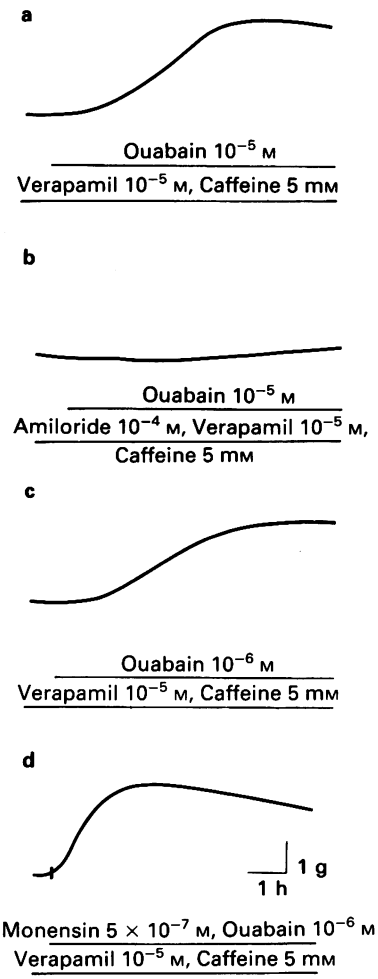

Figure 6 Recording showing the late phase of ouabain (10^{-5} M)-induced contraction in the presence of verapamil and caffeine (a). The effects of amiloride (10^{-4} M) on the late phase of ouabain (10^{-5} M)-induced contraction (b). Recording (c) shows the late phase of ouabain (10^{-6} M)-induced contraction. The effects of monensin (5×10^{-7} M) on the late phase of ouabain (10^{-6} M)-induced contraction (d).

attributed to Ca^{2+} entry through voltage-sensitive Ca^{2+} channels or to Ca^{2+} release from the sarcoplasmic reticulum because the media contained verapamil and caffeine. Nevertheless, the low Na^+ contractions were entirely dependent upon the external Ca^{2+}.

Effects of Ca^{2+} concentration on the late contraction

After a 30 min treatment with verapamil and caffeine followed by a 10 min treatment with the Ca^{2+}-free EGTA solution, the strips were contracted with ouabain (10^{-4} M) in a solution containing Ca^{2+} up to 2.5 mM. The late contraction was dose-dependent (Figure 1b,c).

Effects of amiloride and monensin on the late contraction

After a 30 min treatment with amiloride (5×10^{-5} M to 5×10^{-4} M), in the presence of both verapamil and caffeine, amiloride inhibited the ouabain (10^{-5} M)-induced contraction dose-dependently (Figures 5 and 6a,b).

Effects of monensin on the late contraction

The time needed to reach the peak tension induced by monensin (5×10^{-7} M) together with ouabain (10^{-6} M) was less than with ouabain alone, but the magnitude of the peak tension was not changed (Figure 6c,d).

Discussion

Ouabain-induced contractions are myogenic or neurogenic depending on the type of artery. A myogenic mechanism for the contraction of arterial smooth muscle has been shown in rat aorta (Karaki *et al.*, 1978) and cat cerebral artery (Marin *et al.*, 1988); whereas a neurogenic mechanism has been demonstrated in dog mesenteric artery and rat tail artery (Bonaccorsi *et al.*, 1977) and in cat femoral artery (Marin *et al.*, 1988). Human umbilical arteries are devoid of sympathetic innervation (Davignon *et al.*, 1965) which suggests that ouabain-induced contractions are myogenic.

Ouabain-induced contractions have been reported to be monophasic (Ozaki *et al.*, 1982; Marin *et al.*, 1988) or biphasic (Hayashi & Park, 1984; Sato & Aoki, 1988; 1989). Ouabain-induced contraction of dog mesenteric arteries was shown to produce a biphasic contraction that consisted of an early and late contraction. The early contraction was neurogenic because of its inhibition by phentolamine, and the late one myogenic because it was not inhibited by phentolamine, atropine, chlorpheniramine, cimetidine, saralasin, aspirin or methysergide (Hayashi & Park, 1984). We earlier reported that in contrast to the contraction of dog mesenteric arteries, the ouabain-induced biphasic contraction of human umbilical arteries is myogenic for both the early and late contractions (Sato & Aoki, 1988; 1989). There is evidence that ouabain-induced contraction of arterial smooth muscle is evoked by the release of catecholamine from adrenergic nerves (Katsuragi *et al.*, 1977), release of Ca^{2+} from sarcoplasmic reticulum (Lamb *et al.*, 1988); that there is either an increase in Ca^{2+} entry or a decrease in Ca^{2+} extrusion via the Na^+–Ca^{2+} exchange system (Bohr *et al.*, 1969; Ito *et al.*, 1981; Ozaki *et al.*, 1978; 1982); and that membrane depolarization leads to activation of voltage-sensitive Ca^{2+} channels (Hermsmeyer & Harder, 1986). Our study has demonstrated that in human umbilical arteries, the Ca^{2+} antagonists verapamil, diltiazem and nifedipine inhibit ouabain-induced early contraction but not late contraction and that a Ca^{2+}-free EGTA solution completely inhibits both the early and late contractions. These findings suggest that the ouabain-induced early contraction may depend on a Ca^{2+} influx through voltage-sensitive Ca^{2+} channels. Prior treatment with verapamil and caffeine inhibited the early ouabain contraction but not the late one. As the effect of this treatment is to inhibit the influx of Ca^{2+} through voltage-sensitive Ca^{2+} channels and to release Ca^{2+} from the sarcoplasmic reticulum, the mechanism of late contraction may not be dependent on Ca^{2+} influx through voltage-sensitive Ca^{2+} channels.

Ca^{2+} entry through Na^+–Ca^{2+} exchange has been reported to evoke the contraction of arterial smooth muscle (Blaustein, 1974; Aicken *et al.*, 1984; Bolton, 1985; Ashida & Blaustein, 1987; Bova *et al.*, 1988). We have demonstrated here that ouabain-induced contraction is dependent on the Ca^{2+} concentration in the bath fluid and that this contraction is inhibited dose-dependently by amiloride.

The time needed to reach the maximum contraction induced by monensin together with ouabain was less than with ouabain alone. The Na^+ reduction-induced contraction was inhibited by a Ca^{2+}-free solution. Taken together, these results suggest that the mechanism for the late contraction probably depends on Ca^{2+} entry through Na^+–Ca^{2+} exchange in human umbilical arteries.

It has been reported that Na^+ had to be markedly reduced to obtain a significant increase in tension development in guinea-pig aorta (Ozaki *et al.*, 1978); but, in our study on human umbilical arteries half maximal contraction took place with about 90 mM Na^+. This shows that the artery is very sensitive to changes in Na^+. The relation between the rate of tension development and Na^+ in human umbilical arteries showed a shift to the right as compared to the relation in rat aorta or bovine tail artery (Ashida & Blaustein, 1987).

In conclusion, in human umbilical arteries ouabain-induced contraction consists of an early and late contraction, the former probably being dependent on a Ca^{2+} influx through voltage-sensitive Ca^{2+} channels and the latter on Ca^{2+} entry through Na^+–Ca^{2+} exchange.

We thank Prof. Y. Yagami of the Department of Obstetrics of this hospital and Dr Seigo Fujimoto of Nagoya City University Medical School for the supply of umbilical cords and for their helpful comments.

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(Received September 20, 1990
Revised January 24, 1991
Accepted February 4, 1991)

Br. J. Pharmacol. (1991), **103**, 1530–1534 © Macmillan Press Ltd, 1991

Mechanical responses to catecholamines in the longitudinal muscle of guinea-pig gastric fundus

[1]A.B. Parekh, *M.Md. Syed & *T. Tomita

Department of Pharmacology, Oxford University, Oxford OX1 3QT, and *Department of Physiology, School of Medicine, Nagoya University, Nagoya 466, Japan

1 In the longitudinal muscle of guinea-pig gastric fundus, adrenaline and phenylephrine (1–30 μM) both produced a slow contraction preceded by a relaxation. The slow contraction was strongly inhibited by prazosin (0.1 μM), but only weakly by yohimbine (1 μM), suggesting main contribution of α_1-adrenoceptors.

2 Most of the slow contraction was blocked by meclofenamate or indomethacin (0.1–0.3 μM). Both these drugs also inhibited spontaneously generated muscle tone. In some preparations, obtained from the apical fundus, a small contraction remained in the presence of meclofenamate.

3 During contraction induced by prostaglandin E_2, adrenaline produced sustained relaxation and phenylephrine often transient relaxation, in the presence of meclofenamate. The transient relaxation, but not the sustained relaxation, was suppressed by prazosin.

4 In the presence of prostaglandin E_2 (5 nM), after treating with phenoxybenzamine (30 μM) for 30 min, isoprenaline and adrenaline produced concentration-dependent relaxation, with IC_{50}s of 3.9 nM and 64 nM, respectively. Propranolol shifted these concentration-response curves to the right, with apparent pA_2s of 8.15 and 7.34, respectively.

5 It is suggested that in the fundic longitudinal muscle, adrenaline-induced contraction is mediated mainly by an increase in endogenous prostaglandin production through activation of α_1-adrenoceptors and that adrenaline produces transient relaxation through α_1-adrenoceptors and sustained relaxation through β-adrenoceptors. The β-adrenoceptors in the longitudinal muscle are more sensitive to adrenaline and isoprenaline than those in the circular muscle.

Keywords: Stomach; smooth muscle; catecholamine; α-adrenoceptor; β-adrenoceptor; meclofenamate

Introduction

In a previous paper, we have shown that adrenaline and phenylephrine, acting on α_1-adrenoceptors, produce a complex mechanical response in the circular muscle of guinea-pig gastric fundus. In the presence of the prostaglandin biosynthesis inhibitor, meclofenamate, this response is converted to a simple slow contraction (Syed *et al.*, 1990). During the experiments, it was noted that slow contractions of the longitudinal muscle induced by catecholamines were almost completely abolished by meclofenamate. Some agonist-receptor interactions are found to increase prostaglandin biosynthesis in several tissues, by releasing arachidonic acid, through activation of either phospholipase A_2 or diglyceride lipase (human platelets: Bell *et al.*, 1979; rabbit vas deferens: Trachte, 1987; cultured pineal gland: Ho & Klein, 1987). Therefore, in the guinea-pig stomach muscle, it is possible that activation of α_1-adrenoceptors liberates prostaglandins. The difference between circular and longitudinal muscles would then be that in the former prostaglandins modify the catecholamine-induced contraction, whereas in the latter the prostaglandins produced seem almost entirely responsible for catecholamine-induced contraction. The main aim of this paper was to examine this possibility.

Another aim of the present experiments was to study some pharmacological properties of β-adrenoceptors in the longitudinal muscle of gastric fundus. In the circular muscle, when muscle tone is raised by prostaglandin E_2 in the presence of meclofenamate and 5 μM propranolol, adrenaline can still produce relaxation. This relaxation has been considered to result from stimulation of β-adrenoceptors, as isoprenaline produced relaxation (Syed *et al.*, 1990). In the present experiments, relaxant effects of isoprenaline and adrenaline were also studied under the same conditions used for the experiments on the circular muscle.

[1] Author for correspondence.

Methods

The methods employed were essentially the same as those previously described by Syed *et al.* (1990). Hartley guinea-pigs (250–350 g) of either sex were killed by stunning and bleeding. The stomach was excised, cut in the longitudinal direction along the greater curvature, and the mucosa was removed. Four muscle strips (two each from apical and distal fundus, about 1 mm width, 7 mm length) were dissected in the direction of the longitudinal muscle fibres from the ventral wall of the fundus at a distance of 1–4 mm from the greater curvature. The fundus was arbitrarily defined as a region of the rostral one third of the stomach. In most of the experiments, muscle strips with a circular muscle layer attached were used, but in some experiments, the circular muscle layer was removed by gently pulling with forceps. No essential differences were found between these preparations.

Preparations, suspended in small glass tubes (1 ml capacity), were superfused with a physiological solution at a constant rate (2.5 ml min^{-1}) at 35°C and isometric contractions were recorded simultaneously from 4 preparations. The physiological solution contained (mM): NaCl 130, $KHCO_3$ 6, $CaCl_2$ 2.4, $MgCl_2$ 1.2, glucose 12, Tris-HCl 7, the pH being adjusted to 7.4 at 35°C. Most of the experiments were carried out in the presence of propranolol (3–5 μM) in order to observe mainly responses to α-adrenoceptor activation, except for the experiments in which effects of isoprenaline were studied. When muscle tone was inhibited with meclofenamate or indomethacin, application of other drugs started after 20 min in the continuous presence of these cyclo-oxygenase inhibitors. Numerical values are expressed as mean ± s.e.mean with the number of preparations in parentheses (n).

Drugs used were (±)-adrenaline, (±)-isoprenaline, (±)-noradrenaline, (−)-phenylephrine, (±)-propranolol, prazosin, yohimbine (all HCl salts), atropine sulphate, tetrodotoxin, indomethacin (all from Sigma), phenoxybenzamine HCl (Tokyo Kasei), BHT-920 (2-amino-6-allyl-5,6,7,8-tetrahydro-

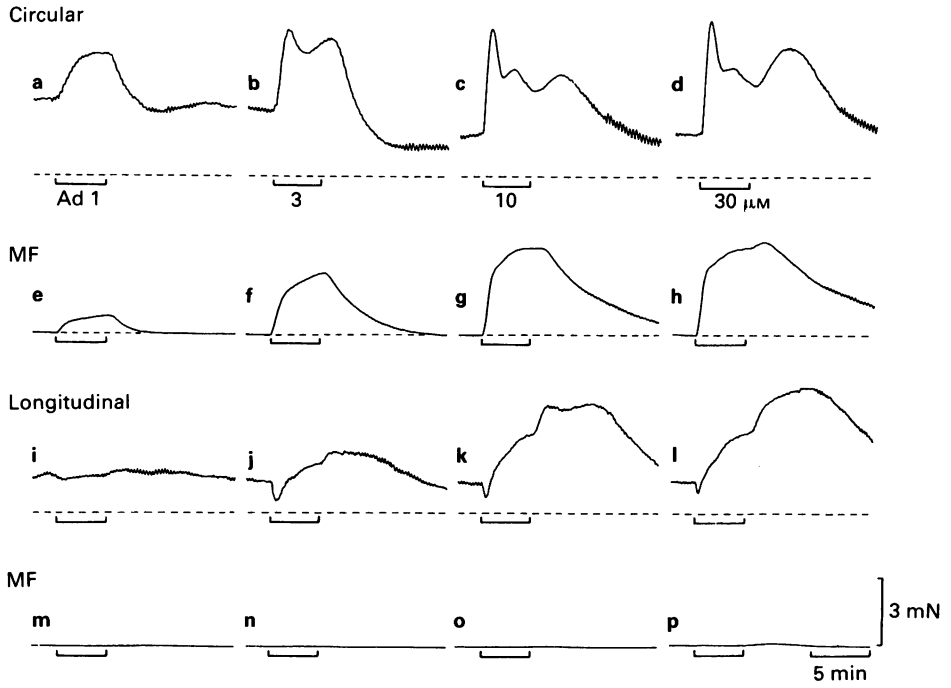

Figure 1 Mechanical responses to adrenaline (Ad, 1–30 μM) in circular (a–h) and longitudinal (i–p) muscle strips, obtained from the same stomach, in the absence (a–d and i–l) and in the presence (e–h, and m–p) of meclofenamate (MF, 0.1 μM). Adrenaline was applied for 4 min at intervals of 20–25 min. Propranolol (3 μM) was present throughout the experiments. Dotted lines indicate the lowest level of muscle tone. Note that meclofenamate suppressed muscle tone and that in the presence of meclofenamate, adrenaline produced a slow contraction in the circular, but no response in the longitudinal muscle.

4H-thiazolo-(4,5-d)-azepine) (a gift from Boehringer), sodium meclofenamate monohydrate (a gift from Parke-Davis), and prostaglandin E_2 (a gift from Ono Pharmaceutical Co.). Catecholamines were dissolved in diluted HCl solution (pH about 3) as stock solutions and renewed every week.

Results

Figure 1 shows an example of mechanical responses to four concentrations of adrenaline in a circular (a-h) and a longitudinal muscle strip (i-p) obtained from the same gastric fundus in the presence of propranolol (3 μM). Responses to adrenaline varied between circular and longitudinal muscle strips. In the circular muscle, adrenaline (3–100 μM) produced a complex response and meclofenamate inhibited spontaneously generated muscle tone, leaving a slow adrenaline-induced contraction, as previously described (Syed *et al.*, 1990).

The general pattern of response to adrenaline (10 μM) in the longitudinal muscle was an early relaxation followed by slow contraction which was increased after removal of adrenaline. In some preparations (13 out of 156), the relaxation was prolonged until the end of adrenaline application (4 min), but the relaxation usually tended to decrease, often reversing to contraction, and contraction followed on adrenaline wash out, as shown in Figure 1 (j–l). Responses to adrenaline were affected neither by tetrodotoxin (1 μM) nor by atropine (1 μM) both in the circular and longitudinal muscles (each examined in four preparations).

In the majority of longitudinal muscles (134 out of 156), adrenaline failed to produce any clear response after blockade of muscle tone by meclofenamate ($n = 114$), as shown in Figure 1 (m–p) or indomethacin ($n = 20$) both at 0.1–0.3 μM. In the remaining preparations, all from the apical fundus (22 out of 78), adrenaline could produce a small slow contraction in the presence of meclofenamate (Figure 2, f–h). The contraction was not due to the presence of circular muscle, because this was observed in the preparations ($n = 3$) from which the circular muscle layer was stripped off.

The contraction induced by adrenaline was strongly inhibited by 0.1 μM prazosin, a blocking agent selective for α_1-adrenoceptors at this concentration (Figure 3, e–h). In the presence of prazosin and also 3 μM propranolol, adrenaline caused relaxation. When adrenaline concentrations were higher than 10 μM, there was a small slow contraction following the wash out of adrenaline, but this was further reduced by increasing the prazosin concentration to 1 μM (not shown). Compared with prazosin, yohimbine, a relatively selective antagonist of α_2-adrenoceptors, had much weaker inhibitory effects. The contraction induced by 30 μM adrenaline was reduced to $86 \pm 2\%$ with 0.1 μM and $61 \pm 3\%$ with 1 μM yohimbine ($n = 8$).

In preparations in which muscle tone was relatively high, the early transient relaxation induced by adrenaline was prominent even at 1–3 μM and the rate of relaxation was fast, reaching the minimum in less than 10 s. Since the rate of relaxation produced by lower concentrations of adrenaline was reduced by prazosin, probably activation of α_1-adrenoceptors is involved in the early phase of the relaxation. In the following experiments, receptors responsible for relaxation were further studied during maintained contraction with prostaglandin E_2 to facilitate analysis.

In Figure 4, relaxations caused by adrenaline and phenylephrine applied for 4 min were compared in the presence of prostaglandin E_2. In normal solution, adrenaline (10 μM) produced relaxation which slowly subsided during its application and slow contraction on washout (Figure 4a), whereas phenylephrine (10 μM) produced transient relaxation followed by slow contraction during its application (Figure 4f). On average, the duration at 50% relaxation was 2.6 ± 0.2 min for adrenaline and 0.6 ± 0.1 min for phenylephrine in the absence of propranolol ($n = 12$). In the presence of meclofenamate, no clear contraction was observed in response to adrenaline (Figure 4b), but a small contraction to phenylephrine (Figure 4g) in this preparation. When the muscle tone was increased by prostaglandin E_2 in the presence of meclofenamate, adrenaline produced sustained (Figure 4c), but phenylephrine transient (Figure 4h), relaxation both at 1 and 10 μM. Propranolol (3 μM) altered the sustained relaxation caused by 1 μM adrenaline to a transient relaxation but had no significant

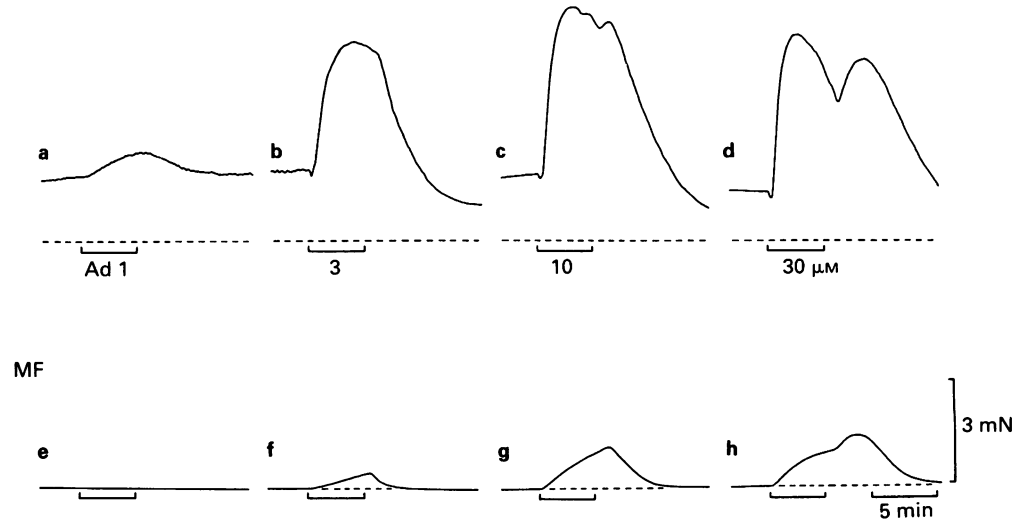

Figure 2 Contractions produced by adrenaline (Ad, 1–30 μM) in a longitudinal muscle strip from which the circular muscle layer was removed. (a–d) before and (e–h) after application of meclofenamate (MF, 0.1 μM). Propranolol (3 μM) was present throughout. The muscle strip was obtained from the apical fundus near the greater curvature.

effect on relaxations induced by adrenaline at 10 μM (Figure 4d) and phenylephrine at 1 and 10 μM (Figure 4i). An agonist selective for α_2-adrenoceptors, BHT-920 (10 μM), failed to produce any clear response ($n = 8$, not shown). Addition of prazosin (0.1 μM) blocked the transient relaxation caused by 1 μM adrenaline (Figure 4e) and phenylephrine (Figure 4j). In the presence of propranolol and prazosin, 10 μM phenylephrine produced a small contraction followed by relaxation on wash out in two preparations, but in six preparations no clear response was observed with phenylephrine under these conditions. In the presence of prazosin or phenoxybenzamine (1–5 μM), phenylephrine at concentrations higher than 30 μM produced sustained relaxation in many preparations (10 out of 12) in which phenylephrine failed to produce contraction in the presence of meclofenamate.

Figure 5 shows adrenaline responses in longitudinal muscle strips obtained from the distal (a–e) and apical fundus (f–j). At the distal fundus, where adrenaline produced no contraction in the presence of meclofenamate (Figure 5b), adrenaline produced sustained relaxation during prostaglandin-induced contraction (Figure 5c) and this was not affected by propranolol (Figure 5d) or by phenoxybenzamine (Figure 5e) both at 1 μM. On the other hand, at the apical fundus, where adrenaline produced contraction after blockade of muscle tone by meclofenamate (Figure 5g), adrenaline produced contraction after transient relaxation in the presence of prostaglandin (Figure 5h). The adrenaline-induced contraction was potentiated by propranolol (Figure 5i), but converted to sustained relaxation by 1 μM phenoxybenzamine (Figure 5j).

To examine the possibility that β-adrenoceptors are responsible for relaxation with high concentrations of adrenaline, effects of isoprenaline were studied. When preparations were treated with phenoxybenzamine (50 μM for 30 min followed by a 20 min period of washing) to block α-adrenoceptors, neuronal and extraneuronal uptake (O'Donnell & Wanstall, 1976) and muscle tone was raised with prostaglandin E_2 (5 nM), both isoprenaline and adrenaline caused only relaxation. The experiments were carried out in the presence of 0.1 μM meclofenamate. Both isoprenaline and adrenaline, applied cumulatively for 5 min, were very effective in inhibiting the prostaglandin-induced contraction. Concentration-response curves were constructed by averaging

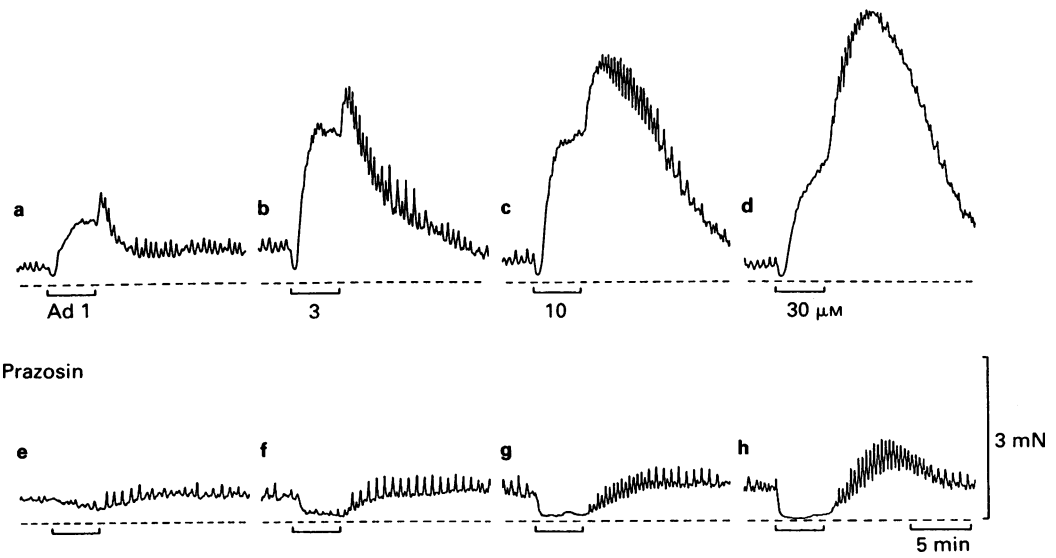

Figure 3 Effects of prazosin (0.1 μM) on responses of longitudinal muscle to adrenaline (Ad, 1–30 μM), in the presence of propranolol (3 μM). Note that prazosin strongly inhibited the adrenaline-induced contraction and that adrenaline produced sustained relaxation in the presence of prazosin and propranolol.

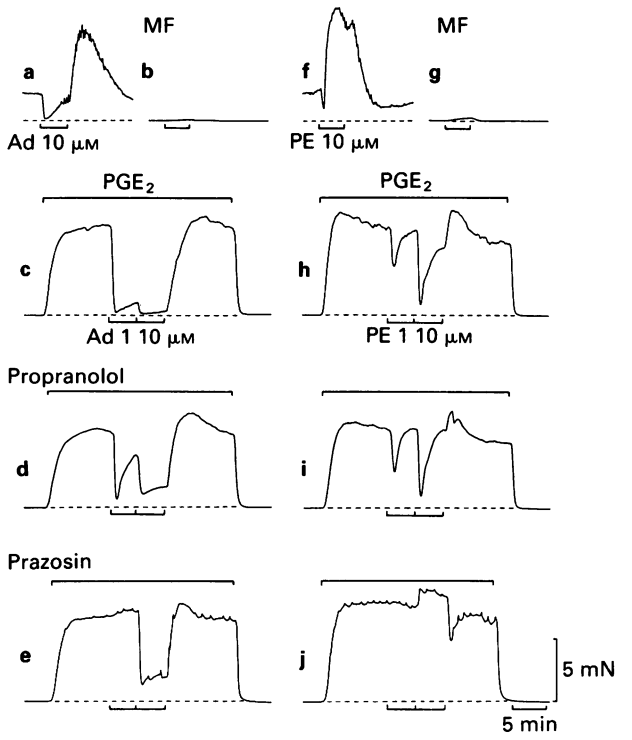

Figure 4 Comparison between adrenaline (Ad) (a–e) and phenylephrine (PE)-induced (f–j) contractions in the same preparation (from apical fundus). After observing control responses (a, f), meclofenamate (MF 0.1 μM) was applied throughout the experiment. During contraction produced by prostaglandin E_2 (PGE_2, 10 nM), two different concentrations (1 and 10 μM) of adrenaline (c–e) or phenylephrine (h–j) were applied successively as shown by horizontal bars below tracings (c, h); (d, i) similar to c, h, but in the presence of propranolol (3 μM), and (e, j) after further addition of prazosin (0.1 μM).

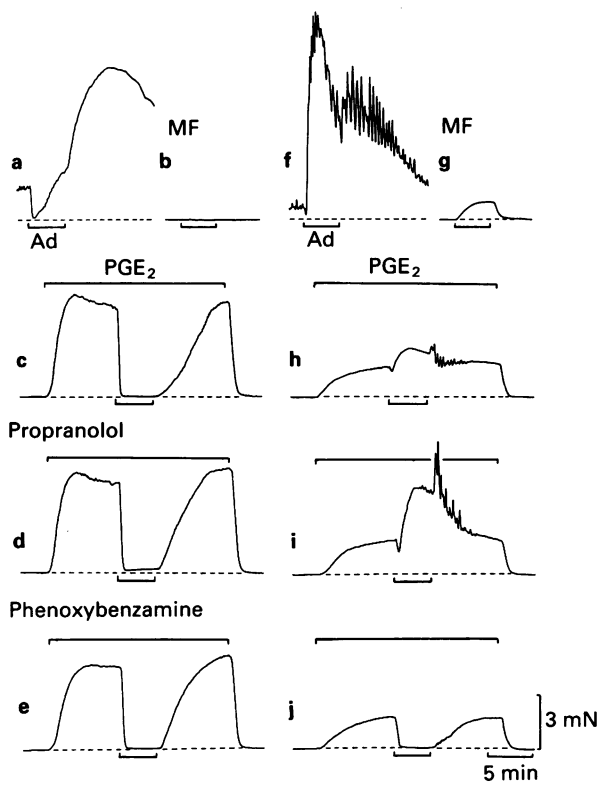

Figure 5 Adrenaline (Ad, 10 μM) responses recorded simultaneously from longitudinal strips obtained from distal (a–e) and apical (f–j) regions of the same fundus. (a, f) Control responses and (b, g) after application of meclofenamate (MF, 0.3 μM). (c, h) Adrenaline application in the presence of prostaglandin E_2 (PGE_2, 10 nM) and meclofenamate; (d, i) similar to (c, h) but in the presence of propranolol (1 μM) and (e, j) in the presence of propranolol and phenoxybenzamine (1 μM).

the maximum relaxation for each concentration ($n = 12$), as shown in Figure 6. In the absence of propranolol, the IC_{50} (the concentration to produce 50% relaxation) of isoprenaline and adrenaline estimated from the curves was 3.9 and 64 nM, respectively. When higher muscle tone was produced by increasing the prostaglandin concentration to 10 nM, the concentration-relaxation curve to isoprenaline was shifted to the right, IC_{50} being increased to 7.3 nM for isoprenaline and 84 nM for adrenaline. The potency of noradrenaline was nearly the same as that of adrenaline, the ICD_{50} of noradrenaline being 69 nM (n = 8) in the presence of 5 nM prostaglandin E_2.

Propranolol, after 20 min pretreatment, shifted the concentration-response curves to the right in parallel, and the degree of shift was almost the same, independent of prostaglandin concentrations between 2–10 nM. Since the slope of Schild plot was significantly less than unity (0.57–0.63) between 0.1 and 1 μM of propranolol, pA_2 values could not be calculated. When apparent pA_2 values were estimated as the intercept on the abscissa scale of the Schild plots (Tallarida & Jacob, 1979); they were 8.15 for isoprenaline and 7.34 for adrenaline.

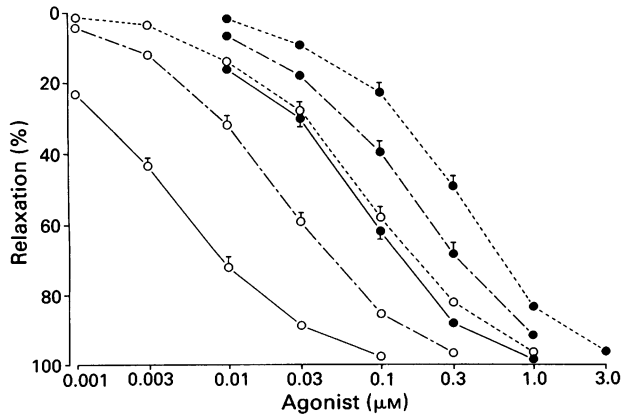

Figure 6 Effects of propranolol on the concentration-relaxation curves of isoprenaline (○) and adrenaline (●) in the presence of 5 nM prostaglandin E_2 and meclofenamate (0.1 μM). Preparations were pretreated with 50 μM phenoxybenzamine for 30 min. The catecholamines were applied cumulatively for 5 min. Continuous lines: in the absence, broken lines: in the presence of 0.1 μM, and dotted lines: 1 μM propranolol. Each point represents the mean and vertical bars indicate s.e.mean ($n = 12$).

Discussion

The main difference in responses to catecholamine between circular and longitudinal muscles is the very strong blocking action of meclofenamate on the adrenaline-induced contraction in the longitudinal muscle. In the circular muscle, a slow contraction can always be observed by activating α_1-adrenoceptors with adrenaline, after suppressing muscle tone with meclofenamate (Syed *et al.*, 1990). On the other hand, in most of the longitudinal muscles, adrenaline-induced contractions disappear almost completely in the presence of meclofenamate. As in the circular muscle, the response to adrenaline of the longitudinal muscle seems mainly mediated through α_1-adrenoceptors, since prazosin is very effective in blocking the response. A weak inhibitory effect of yohimbine

could be partly due to a blocking action on α_1-adrenoceptors (Timmermans *et al.*, 1979). Thus, it is likely in the longitudinal muscle that adrenaline activates phospholipase A_2 through α_1-adrenoceptors and releases arachidonic acid, which is then metabolized to prostaglandins, as found in rabbit vas deferens (Trachte, 1987) and that endogenous production of prostaglandin is almost entirely responsible for the contraction.

It appears that there are two independent processes after activation of α_1-adrenoceptors (or two different subtypes of α_1-adrenoceptors) in the stomach fundic muscles. One is to stimulate biosynthesis of prostaglandins and the other is to increase Ca^{2+} influx directly. The former is mainly responsible for the contraction in the longitudinal muscle, whereas both processes are involved in the circular muscle. Some longitudinal strips obtained from the apical fundus of stomach show a weak contraction in response to adrenaline, even after blockade of muscle tone with meclofenamate. This could be due to the presence of the circular muscle type of adrenoceptors in this region of longitudinal muscle.

In the longitudinal muscle, as in the circular muscle (Syed *et al.*, 1990), both α_1- and β-adrenoceptors seem to be involved in causing relaxation with adrenaline. Transient relaxation produced by phenylephrine (1–10 μM) and adrenaline (1 μM), which is resistant to propranolol, but susceptible to prazosin, is likely to be due to activation of α_1-adrenoceptors. On the other hand, sustained relaxation produced by 10 μM adrenaline is only slightly reduced by prazosin and is considered to be due to activation of β-adrenoceptors not blocked by 3 μM propranolol.

In the longitudinal muscle, contracted by 10 nM prostaglandin E_2, the IC_{50} of isoprenaline and adrenaline is 7.4 and 84 nM, respectively. In the circular muscle, contracted by the same concentration of prostaglandin E_2, the IC_{50} of isoprenaline and adrenaline for relaxation is 40 nM and 1.6 μM, respectively (Syed *et al.*, 1990), so that the longitudinal muscle is about 5 to 20 times more sensitive to these catecholamines than the circular muscle in causing relaxation. In other smooth muscles, the IC_{50} of isoprenaline for relaxation is found to be in a range of 3.8 to 60 nM (guinea-pig taenia caeci, contracted by histamine: 3.8 nM, Koike & Takayanagi, 1988; guinea-pig trachea: 4.5 nM, O'Donnell & Wanstall, 1979; arterial smooth muscles, contracted by KCl: 6–48 nM, O'Donnell & Wanstall, 1985; guinea-pig ileum, contracted by histamine: 60 nM, Bond & Clarke, 1988). The β-adrenoceptors in the stomach longitudinal muscle belong to one of the more sensitive groups.

In the present experiments, the antagonistic action of propranolol was not carefully studied. The slope of the Schild plot was much lower than unity (about 0.6), suggesting existence of a mixture of different subtypes of receptor. In the longitudinal muscle of the guinea-pig ileum, the presence of inhibitory adrenoceptors with pharmacological properties distinct from α- and β-adrenoceptors has been reported (Bond & Clarke, 1988). It is possible that the atypical adrenoceptor, which is rather resistant to propranolol blockade, is responsible for relaxation caused by high concentrations of catecholamines in both muscles.

This work was supported in part by the Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, and by ICI. A.B.P. was a recipient of Rushton and Sasakawa awards during the course of this study.

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(Received October 19, 1990
Revised January 19, 1991
Accepted February 7, 1991)

Br. J. Pharmacol. (1991), **103**, 1535–1541 © Macmillan Press Ltd, 1991

Tachykinin antagonists and capsaicin-induced contraction of the rat isolated urinary bladder: evidence for tachykinin-mediated cotransmission

Carlo Alberto Maggi, Riccardo Patacchini, Paolo Santicioli & Sandro Giuliani

Pharmacology Department, A. Menarini Pharmaceuticals, Via Sette Santi 3, 50131 Florence, Italy

1 The possible involvement of tachykinins (TKs) in the contraction produced by capsaicin in the rat isolated urinary bladder was addressed on the hypothesis that co-release of substance P (SP) and neurokinin A (NKA) occurs from sensory nerve terminals.

2 A low concentration of SP (30 nM) produced a rapid contraction which faded to baseline within 10 min. A low concentration of NKA (10 nM) produced a slowly developing contraction which was still evident at 10 min. Capsaicin (1 μM) produced a rapid phasic response and a tonic response (late response to capsaicin). Co-administration of SP and NKA mimicked the response to capsaicin more than each TK alone.

3 Fading of the response to SP was not caused by receptor desensitization and was partially prevented by peptidase inhibitors.

4 Spantide (3 μM) selectively antagonized the SP-induced contraction while L-659,877 (3–10 μM) or MEN 10,376 (10–30 μM) which are NK_2 receptor selective antagonists selectively blocked the response to NKA. Co-administration of spantide and L-659,877 inhibited the response to both SP and NKA by an amount not greater than that produced by each antagonist alone.

5 Spantide selectively reduced the peak response to capsaicin, while leaving the late response unaffected. L-659,877 (3 μM) and MEN 10,376 (10 μM) selectively inhibited the late response to capsaicin while, at higher concentrations, also reduced the peak response to capsaicin. Co-administration of spantide and L-659,877 reduced the peak response to capsaicin more than that produced by each antagonist alone.

6 Bombesin (10 nM) produced a tonic contraction similar to that induced by NKA. The response to bombesin was not affected by spantide, L-659,877 or MEN 10,376.

7 P_{2x} purinoceptor desensitization by repeated administration of α,β-methylene ATP depressed the twitch response to electrical stimulation of postganglionic nerves but did not affect the peak or the late response to capsaicin.

8 We conclude that multiple TKs are coreleased by capsaicin in the rat bladder and mediate the capsaicin-induced contraction by activating both NK_1 and NK_2 receptors. Endogenous TK with preferential affinity for the NK_1 receptor (putatively SP) are selectively involved in the peak response to capsaicin while endogenous TK with preferential affinity for the NK_2 receptor (putatively NKA) are selectively involved in the late response to capsaicin and partly contribute to the peak response. These findings provide pharmacological evidence for tachykinin-mediated cotransmission in the rat urinary bladder. ATP is unlikely to be involved in the efferent function of capsaicin-sensitive sensory nerves in the rat bladder.

Keywords: Tachykinins; tachykinin receptors; tachykinin antagonists; substance P; neurokinin A; ATP; bombesin

Introduction

Several tachykinins (TKs), such as substance P (SP) and neurokinin A (NKA), are present in sensory nerves of the urinary bladder in rats and other mammalian species (Maggi & Meli, 1986; 1988; Maggi 1991, for reviews). The mRNAs which encode SP, NKA and other elongated forms of NKA (e.g. neuropeptide K) are derived from a single gene (Nawa *et al.*, 1984; Krause *et al.*, 1987). Alternative RNA splicing of the SP gene primary transcript results in the generation of 3 mRNAs termed alpha-, beta- and gamma-preprotachykinin (PPT) mRNA. The latter two encode both SP and NKA while alpha PPT encodes SP but not NKA. In both central and peripheral tissues of adult rats the expression of beta- and gamma-PPT mRNA dominates over that of the alpha PPT and this accounts for the observation of a generalized coexistence of these two peptides (Carter & Krause, 1990). In rat dorsal root ganglia SP and NKA are synthesized in a ratio of 2–3:1 and a similar ratio in content of these peptides has been found in several peripheral tissues, including the urinary bladder (Harmar & Keen, 1984; Hua *et al.*, 1985; Dalsgaard *et al.*, 1985; Ogawa *et al.*, 1985; Takano *et al.*, 1986; Too *et al.*, 1989; Takeda *et al.*, 1990).

Capsaicin produces a contraction of the rat isolated bladder muscle which exhibits desensitization, a specific feature of the action of this drug on sensory nerves, and is abolished by extrinsic bladder denervation (Santicioli *et al.*, 1986). There is compelling evidence suggesting the involvement of TKs in this response: the height of the capsaicin-evoked contraction, but not that of the twitch response produced by electrical stimulation of postganglionic nerves, is directly proportional to the bladder content of TK-like immunoreactivity (Maggi *et al.*, 1987a; 1988a). Furthermore this contraction is not observed in the isolated bladder of newborn rats but it becomes more and more evident during the postnatal development, in parallel to an increase of bladder SP levels (Santicioli *et al.*, 1986; Maggi *et al.*, 1988b). Exogenously administered TKs are powerful spasmogens of the rat isolated urinary bladder: two types of TK receptors are involved (Maggi *et al.*, 1988c) i.e. NK_1 receptors for which SP has greater affinity than NKA and NK_2 receptors for which the reverse is true (Lee *et al.*, 1986; Regoli *et al.*, 1986).

We reported that [D-Pro^4, D-Trp^{7,9}, Leu^{11}]-SP(4–11), a substance P antagonist, selectively reduces, by about 35%, the capsaicin-induced contraction of the rat isolated bladder (Maggi *et al.*, 1985). Although quantitatively small, this inhibitory effect further suggested an involvement of TKs. However, the 'first generation' of TK antagonists, developed by insertion of multiple D-Trp on the backbone of SP, suffers from several drawbacks, including (a) a poor ability to discriminate between NK_1 and NK_2 receptors (Buck & Shatzer, 1988) and (b) ability to antagonize peptides not related to TKs, such as bombesin (Jensen *et al.*, 1984). The latter finding is particularly important for the topic for capsaicin-induced contraction of the rat bladder, inasmuch as bombesin-like peptides have been described as present in capsaicin-sensitive primary afferents (see Holzer 1988 for review) and bombesin itself is a powerful spasmogen in the rat isolated urinary bladder (Abdel-Hakim *et al.*, 1981). Some SP antagonists of the first generation have been shown to act as bombesin antagonists in the rat isolated urinary bladder (Mizrahi *et al.*, 1985).

Another candidate which may be considered as a possible mediator of the capsaicin-induced contraction of the rat bladder is adenosine triphosphate (ATP). ATP produces a rapidly developing phasic bladder contraction and is considered a likely noncholinergic excitatory transmitter at this level (Brown *et al.*, 1979; Bo & Burnstock, 1990; Brading & Williams, 1990; Luheshi & Zar, 1990). After the early suggestion of Holton & Holton (1954) and Holton (1959), a role for ATP as a sensory transmitter has been recently reconsidered (Jahr & Jessell, 1983; Fyffe & Perl, 1984). In particular, it appears that certain primary sensory neurones which are capsaicin-sensitive may utilize ATP as a transmitter (Nagy & Daddona, 1985) and ATP release by capsaicin in the dorsal half of spinal cord has been reported upon application of capsaicin (Sweeney *et al.*, 1989). However, excluding the early findings of Holton & Holton (1954) and Holton (1959), there is no available indication that ATP may be involved in the efferent function of capsaicin-sensitive afferents in the periphery.

The aim of this study was to readdress the topic of a possible involvement of TKs in the capsaicin-induced contraction of the rat isolated bladder. The working hypothesis was that both SP and NKA contribute to the overall response to capsaicin and that both NK_1 and NK_2 receptors may be involved. With this aim, we first evaluated the effect of low concentrations of SP and NKA alone and in combination in comparison with the response to capsaicin and then assessed the effect of spantide, a SP antagonist of the first generation endowed with some selectivity for the NK_1 receptor (Leander *et al.*, 1981; Buck & Shatzer, 1988) and of some newly developed selective antagonists which discriminate between NK_1 and NK_2 receptors (McKnight *et al.*, 1988; Williams *et al.*, 1988; Maggi *et al.*, 1990a). The effects of bombesin and P_{2x} purinoceptor desensitization by the stable ATP analogue α,β-methylene ATP were also investigated.

Methods

Male albino rats of Wistar strain (300–350 g) were stunned and bled. The urinary bladder was excised and placed in oxygenated (96% O_2 and 4% CO_2, pH 7.4 at 37°C) Krebs solution as described previously (Maggi *et al.*, 1985; Santicioli *et al.*, 1986). A small strip (about 1 cm long and 2 mm wide) of detrusor muscle was excised along the longitudinal axis and prepared for isometric tension recording (load 5 mN) in a 5 ml isolated organ bath. Transmural electrical field stimulation was performed by means of two wire platinum electrodes placed at the top and the bottom of the organ bath, connected with a GRASS S88 stimulator. Square wave pulses were automatically delivered at a frequency of 0.1 Hz (0.5 ms pulse width, 60 V maximal voltage).

Experiments started after a 45 min equilibration period. Contractile responses were expressed as a percentage of the response to 40 mM KCl, added to the bath.

Contact time of the antagonists was 15 min. The amino acid sequences of TK antagonists studied are shown in Table 1: spantide is a well known SP antagonist with some selectivity (about 10 times) for NK_1 over NK_2 receptors (Buck & Shatzer, 1988); L-659,877 is a cyclic antagonist endowed with high selectivity for NK_2 receptors (McKnight *et al.*, 1988; Williams *et al.*, 1988); MEN 10,376 or [Tyr^5, D-Trp^{6,8,9}, Lys^{11}]-NKA(4–10), is a close analogue of MEN 10,207 (indicated as Peptide I in Maggi *et al.*, 1990a) in which Arg in position 11 has been replaced by Lys. Both MEN 10,207 and MEN 10,376 are highly selective NK_2 receptor antagonists which discriminate between putative NK_2 receptor subtypes (Maggi *et al.*, 1990a; Van Giesbergen *et al.*, 1991; Maggi *et al.*, unpublished data). In preliminary experiments we found that MEN 10,207 antagonized the response to capsaicin of the rat isolated bladder but only at concentrations (10–30 μM) at which the peptide produced some agonist effect of its own. This drawback was not present with MEN 10,376 which was therefore used in this study. MEN 10,376 competitively antagonizes TK-mediated responses in various bioassays with affinity for the various TK receptor types and NK_2 receptor subtypes similar to those reported previously for MEN 10,207 (Maggi *et al.* unpublished data).

Some experiments were performed in the presence of peptidase inhibitors (bestatin, captopril and thiorphan 3 μM each, 15 min beforehand). For desensitization of P_{2x} purinoceptors, the stable ATP analogue, α,β-methylene ATP was administered in a total dose of 50 μM (10 + 10 + 30 μM) at 10 min intervals. All contractions were expressed relative to that elicited by 40 mM KCl.

Evaluation of data

Each value in the text, tables and Figures is mean ± standard error of the mean (s.e.mean). Statistical analysis was performed by means of Student's *t* test for paired or unpaired data or by means of analysis of variance, when applicable.

Peptides

Spantide, substance P, neurokinin A and bombesin were from Peninsula (St Helens, England). L-659,877 (Table 1) from Cambridge Research Biochemicals (Cambridge, England). Capsaicin and α,β-methylene ATP were from Sigma. MEN 10,376 (Table 1) was synthesized by Dr P. Rovero, Chemistry Department, Menarini Pharmaceuticals by conventional solid phase methods. Concentrated (10 mM) solutions of the antagonists were prepared in dimethyl sulphoxide (DMSO) and diluted in water. Concentrations of L-659,877 higher than 10 μM were not tested because of precipitation in the bath. Administration of the vehicle had no effect on the responses under study.

Results

Comparison of the response to substance P and neurokinin A alone or in combination with the response to capsaicin

The concentrations of SP (30 nM) and NKA (10 nM) were

Table 1 Amino acid sequences of the antagonists used in the study

Spantide	D-Arg-Pro-Lys-Pro-Gln-Gln-D-Trp-Phe-D-Trp-Leu-Leu-NH_2
L-659,877	cyclo(Leu-Met-Gln-Trp-Phe-Gly)
MEN 10,376	H-Asp-Tyr-D-Trp-Val-D-Trp-D-Trp-Lys-NH_2

chosen with the following criteria in mind: (a) to produce a distinct contraction of the rat bladder submaximal to that produced by capsaicin; and (b) to give a SP:NKA ratio of 3:1. SP (30 nM) produced a rapidly developing phasic contraction which peaked in 1–2 min from administration and was followed by a slow decay toward baseline (Figure 1). The peak contraction to 30 nM SP averaged about 50% of the response to KCl (Table 2). By 10 min, tension had virtually declined to baseline (Figure 1 and Table 2). The peak response to 30 nM SP was about 30% of the maximal response to the peptide (30 μM).

NKA (10 nM) produced a slowly developing tonic contraction (about 40% of the response to KCl) which peaked in 3–4 min from administration (Figure 1 and Table 2). Some fading of the response was evident also for NKA but the decline was much slower than for SP. At 10 min, a tonic response with superimposed phasic contraction was still evident and tension averaged about 25% of the response to KCl. The peak response to 10 nM NKA was about 20% of the maximal response to the peptide (10 μM).

Capsaicin (1 μM) produced a rapid contraction of the isolated bladder which consisted of a phasic and tonic component, the latter with superimposed phasic contraction. This concentration of capsaicin was selected on the basis of previous experiments (Santicioli *et al.*, 1986) as producing a near-to-maximal response. Peak response to capsaicin (80–90% of that to KCl) was achieved in 1–2 min from administration and usually coincided with the phasic response (Figure 1 and Table 2). Some decline of developed tension was evident afterward but a distinct response was still clearly evident at 10 min for capsaicin administration (hereafter referred to as the late response to capsaicin) (Figure 1, Table 2).

Over all, the response to capsaicin had features which resemble the response to both SP and NKA and for this reason the effect of coadministration of the peptides was studied. Data in Table 2 and Figure 1 show that the co-administration of SP (30 nM) and NKA (10 nM) closely matched the response to capsaicin from both a qualitative and quantitative point of view. Thus the response to SP plus NKA was more similar in terms of peak tension developed, time to peak and decay, to that produced by capsaicin than those produced by SP or NKA alone.

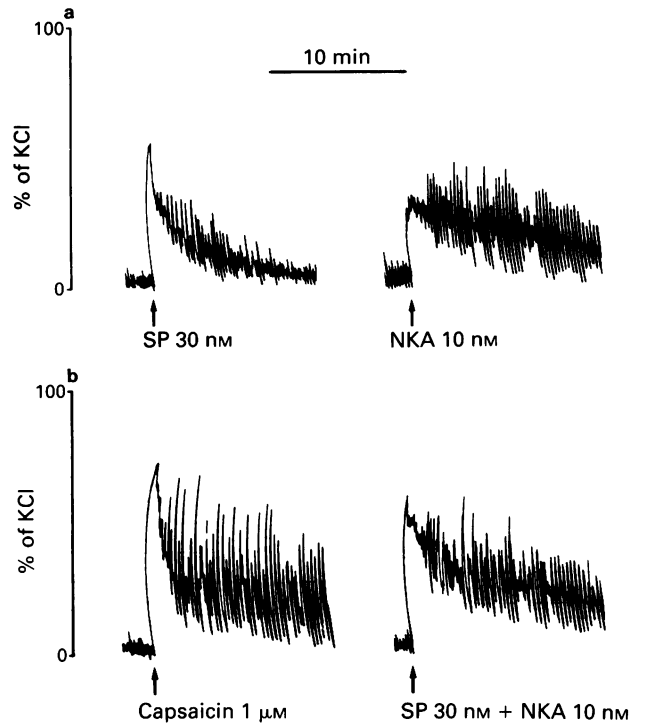

Figure 1 Original tracings showing the contractile response to substance P (SP), neurokinin A (NKA) capsaicin or coadministration of SP and NKA. See the text for details.

Effect of repeated substance P administration and of peptidase inhibitors

The question was raised as to whether the fading of the response to SP might involve receptor desensitization: to assess this point second and third doses of SP (30 nM) were administered 10 and 20 min after the first one. In these experiments ($n = 4$) the first dose produced a peak response averaging 48 ± 5% of that to KCl which faded to baseline; the second and third doses produced responses averaging 44 ± 5 and 40 ± 6% of that to KCl, respectively (Figure 2). Thus no desensitization to SP occurred in the present experimental conditions.

In other experiments the effect of SP (30 nM), NKA (10 nM) or capsaicin (1 μM) was studied in the presence of peptidase inhibitors (bestatin, captopril and thiorphan 3 μM each). Peptidase inhibitors enhanced the peak response to SP and delayed the fading of contraction (Figure 2 and 3). The peak response to capsaicin was slightly enhanced compared with controls, although not significantly, while the late response to capsaicin was larger in the presence of peptidase inhibitors (Figure 3). The response to NKA was totally unaffected by the peptidase inhibitors (Figure 3).

Effect of antagonists on the response to substance P or neurokinin A

Neither spantide (3 μM) nor MEN 10,376 (30 μM) produced any significant effect on the twitch response to electrical field stimulation or contracted the unstimulated bladder. L-659,877 produced a slight and transient enhancement (about 15% increase) of twitches and slightly contracted the unstimulated bladder (< 10% of KCl) at 10 μM while it had no effect at 3 μM. Co-administration of spantide (3 μM) and L-659,877 (10 μM)

Table 2 Comparison of the contractile response to substance P (SP, 30 nM) neurokinin A (NKA, 10 nM) capsaicin (1 μM) or co-administration of SP and NKA in the rat isolated urinary bladder

	Peak response (% of 40 mM KCl)	*Time to peak* (min)	*Response at 10 min after administration* (% of 40 mM KCl)
SP	52 ± 8	1.5 ± 0.2	7 ± 2
NKA	37 ± 5	3.5 ± 0.3	25 ± 3
Capsaicin	82 ± 12	1.6 ± 0.3	45 ± 8
SP + NKA	79 ± 9*	1.7 ± 0.3**	29 ± 4***

Each value is mean ± s.e. of 8 experiments.
* Significantly greater than the response to SP or NKA alone.
** Significantly different from the response to NKA alone.
*** Significantly greater than the response to SP alone.

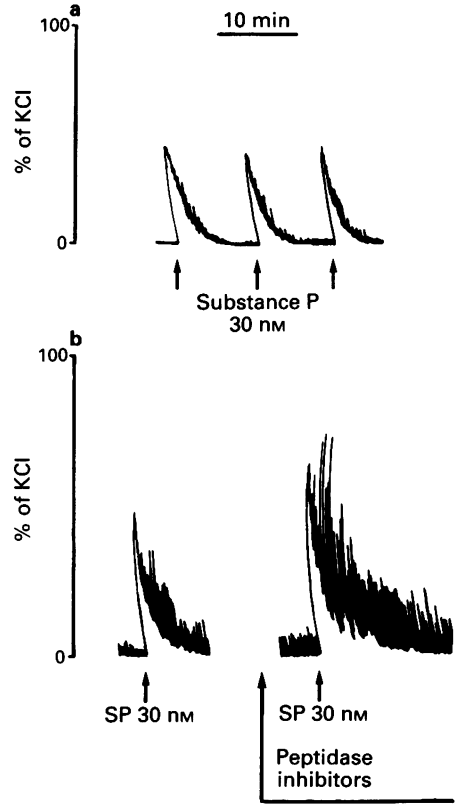

Figure 2 Original tracings showing the response to repeated administration of substance P (SP) at 10 min intervals (a) and the effect of peptidase inhibitors (bestatin, thiorphan and captopril 3 μM each, 15 min beforehand) on the response to SP (b).

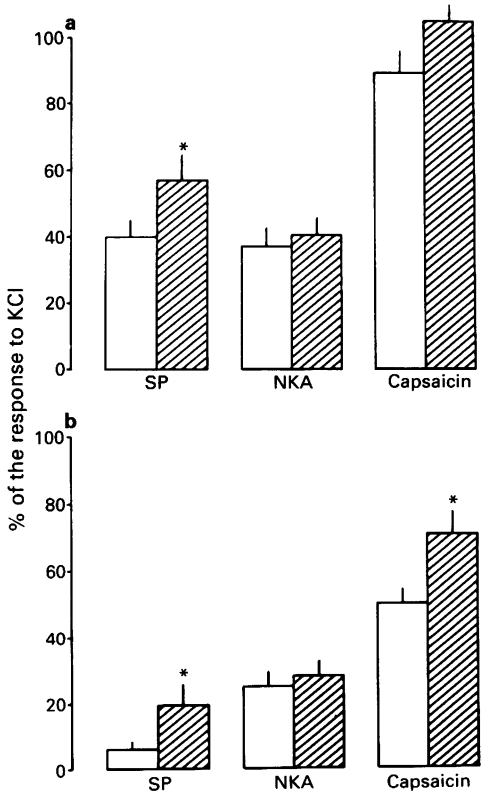

Figure 3 Effect of peptidase inhibitors (bestatin, thiorphan and captopril, 3 μM each, 15 min beforehand) on the peak (a) and 'late' (residual tension at 10 min from administration) (b) response to substance P (SP, 30 nM), neurokinin A (NKA, 10 nM) or capsaicin (1 μM). Each value is mean of 6 experiments: vertical bars show s.e. Open columns, control; hatched columns, peptidase inhibitors present. * Significantly different from the response obtained in the absence of peptidase inhibitors, $P < 0.05$.

slightly and transiently increased twitches as observed with L-659,877 alone.

Spantide (3 μM, 15 min beforehand) significantly reduced the response to SP (47% inhibition) while leaving the response to NKA unaffected (Figure 4). MEN 10,376 (10–30 μM, 15 min beforehand) and L-659,877 (3–10 μM, 15 min beforehand) significantly reduced the response to NKA while leaving the response to SP unaffected (Figure 4). The inhibition induced by MEN 10,376 at 10 and 30 μM was 36 and 76%, respectively, and the inhibition for L-659,877 at 3 and 10 μM was 49 and 94% respectively.

Co-administration of spantide and L-659,877 (3 and 10 μM respectively, 15 min beforehand) reduced the response to SP (43% inhibition) and NKA (84% inhibition) to an extent not significantly different from that produced by each antagonist alone (47% inhibition for spantide vs. SP and 94% inhibition for L-659,877 vs. NKA, Figure 4).

Effect of antagonists on the response to capsaicin

Spantide (3 μM) significantly reduced (43% inhibition) the peak response to 1 μM capsaicin while leaving the late response unaffected (Table 3). The lower concentration of MEN 10,376 (10 μM) or L-659,877 (3 μM) selectively inhibited the late response to capsaicin while leaving the peak tension development unaffected (Table 3). At a higher concentration, the two antagonists significantly reduced the peak response to capsaicin (about 50% for MEN 10,376 30 μM and 49% for L-659,877 10 μM) and abolished the late response (Table 3). Co-administration of spantide and L-659,877 (3 and 10 μM respectively) depressed the peak response to capsaicin (78% inhibition) to an extent larger than that produced by each antagonist alone (Table 3).

Effect of bombesin

Bombesin (10 nM) produced a tonic and sustained contraction (time to peak 2–4 min) similar to that produced by the same concentration of NKA (33 $\pm$ 5% of KCl, $n = 11$). Spantide (3 μM), MEN 10,376 (30 μM) or L-659,877 (10 μM) did not affect the response to bombesin (29 $\pm$ 4, 32 $\pm$ 4 and 30 $\pm$ 6 of KCl for the various antagonists, respectively $n = 4$–6).

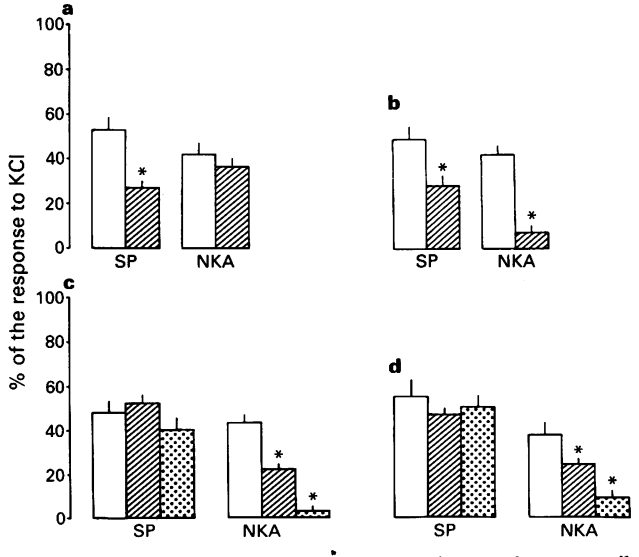

Figure 4 Effect of several tachykinin antagonists on the contractile response produced by substance P (SP, 30 nM) or neurokinin A (NKA, 10 nM). Open columns, control; in (a) hatched column, spantide 3 μM; in (b) hatched columns, spantide 3 μM plus L-659,877 10 μM; in (c) hatched columns, L-659,877 3 μM, stippled columns L-659,877 10 μM; in (d) hatched columns MEN 10,376 10 μM, stippled columns, MEN 10,376 30 μM. Each value is mean of at least 5 determinations; vertical lines show s.e.mean. * Significantly different from control $P < 0.05$.

Table 3 Effect of tachykinin antagonists on the peak and late response of the rat isolated urinary bladder to capsaicin

			% of response to KCl (40 mM)	
		n	Peak response to capsaicin	Response at 10 min after administration
Control		22	90 ± 6	52 ± 4
Spantide	3 μM	6	52 ± 8*	42 ± 8
L-659,877	3 μM	6	104 ± 16	16 ± 5*
	10 μM	6	46 ± 10*	6 ± 4*
MEN 10,376	10 μM	6	88 ± 6	29 ± 4*
	30 μM	6	45 ± 6*	13 ± 6*
Spantide plus L-659,877	3 μM 10 μM	5	20 ± 4*,**	1 ± 1*

Each value is mean ± s.e.mean.
* Significantly different from control, $P < 0.05$.
** Significantly different from spantide or L-659,877 alone, $P < 0.05$.

Effect of P_{2x} purinoceptor desensitization with α,β-methylene ATP on the response to capsaicin

α,β-methylene ATP (10 μM) produced a rapid phasic contraction of the bladder which approached 100% of that to KCl. The response declined to baseline in 10 min and a second dose of 10 μM produced a response ranging between 5–15% of that to KCl. Application of a further dose of α,β-methylene ATP (30 μM), 10 min later, produced a further small response (about 10% of that to KCl). This protocol of P_{2x} purinoceptor desensitization reduced the twitch response of the rat bladder to transmural electrical stimulation by about 60%. Addition of 1 μM capsaicin 10 min after the last dose of α,β-methylene ATP produced a contraction (peak response = 101 ± 12% of KCl; late response = 50 ± 6% of KCl, $n = 4$) not significantly different from that observed in controls.

Discussion

The rat urinary bladder contains not only SP, but also NKA and both shorter and elongated forms of NKA (see Introduction for references). All these peptides are stored in capsaicin-sensitive afferents from which they are putatively co-released upon application of capsaicin. In spite of previous negative results with capsaicin (Maggi *et al.*, 1987b) we recently observed a small but significant release of SP-LI from the rat isolated bladder in response to resiniferatoxin, an ultrapotent capsaicin analogue (Maggi *et al.*, 1990b). In addition, the calcitonin gene-related peptide (CGRP) is also present in the rat bladder where it is co-stored with TKs in capsaicin-sensitive afferents (Sundler *et al.*, 1985; Su *et al.*, 1986). CGRP levels in the rat bladder are very high and a huge release of CGRP-LI is observed upon application of capsaicin (Maggi *et al.*, 1990b). However CGRP has no motor effect on the rat isolated bladder muscle because of the absence of CGRP receptors on smooth muscle cells (Maggi *et al.*, 1987c; Nimmo *et al.*, 1988).

In the rat bladder muscle both NK_1 and NK_2 receptors are present, as evidenced by radioligand binding, autoradiographic and functional experiments (Burcher & Buck, 1986; Maggi *et al.*, 1988c; Dion *et al.*, 1987; Morrison *et al.*, 1990). Present findings confirm our earlier observation (Maggi *et al.*, 1985) involving TK release in the capsaicin-induced contraction of the rat isolated urinary bladder and provide pharmacological evidence that multiple TKs having differential affinity for NK_1 and NK_2 receptors are involved.

The contraction produced by capsaicin is about 60–70% of that produced by maximally effective concentrations of added SP or NKA. Assuming that the response to capsaicin is entirely mediated by endogenous TKs, it could be concluded that submaximal concentrations of endogenous TKs are released by capsaicin. At high concentrations, SP and NKA stimulate equally well NK_1 and NK_2 receptors but, at the low concentrations which putatively mimic those released by capsaicin, SP might preferentially interact with NK_1 receptors and NKA might preferentially interact with NK_2 receptors. Such an hypothesis, suggested by the results of earlier studies in which phenoxybenzamine was used to alkylate NK_2 receptors preferentially (Maggi *et al.*, 1988a,c), has been fully supported by the present results with receptor selective antagonists. Spantide selectively reduced the response to SP while L-659,877 or MEN 10,376 selectively antagonized the response to NKA. Coadministration of spantide and L-659,877 antagonized both SP and NKA, but the extent of this inhibition was not larger than that produced by each antagonist alone, further confirming their selectivity. In sharp contrast, co-administration of spantide and L-659,877 depressed the peak response to capsaicin to a larger extent than that produced by each antagonist alone, indicating that multiple transmitters acting selectively on NK_1 and NK_2 receptors were released following sensory nerve stimulation.

A few points about the results with TK antagonists should also be made: although rather high concentrations of these peptides were used, we found a remarkable selectivity in antagonizing SP (spantide) or NKA (L-659,877 and MEN 10,376), i.e. a selectivity somewhat greater than that one could expect on the basis of the available information about their affinities at NK_1 and NK_2 receptors. While no definitive statement about this can be made on the basis of our results, we suspect that diffusion of the peptides within the bladder muscle may be lower than in other preparations (e.g. guinea-pig ileum). In addition, the existence of pharmacologically distinct NK_2 receptor subtypes could have had a bearing on our results. The available information does not yet allow a subtyping of NK_2 receptors present in the rat urinary bladder.

The response produced by exogenous SP developed rapidly and then faded to baseline within 10 min from application. This is not due to receptor desensitization but may have involved metabolic breakdown by tissue peptidases. In contrast, the response to NKA developed more slowly and persisted for a longer time. These differences in the response to exogenous TKs are not observed when SP or NKA are administered at high concentrations (when both peptides produce a rapidly developing response, data not shown). Co-administration of low doses of SP and NKA mimicks more closely the response to capsaicin than administration of each peptide alone. This observation, along with the result of co-administration of spantide (weakly NK_1-selective) and L-659,877 (NK_2-selective) strongly supports the idea that both SP (which is NK_1-selective in low concentrations) and NKA (NK_2-selective at low concentrations) are simultaneously released by capsaicin and act through distinct receptor types.

In the presence of peptidase inhibitors, the late response to capsaicin was enhanced: this might be due to a reduced degradation of endogenous SP and summation of its effect with that of NKA. On the other hand, we cannot exclude that some other TK-like peptide (e.g. shorter or elongated forms of

NKA, see Introduction for References) might be released by capsaicin along with SP and NKA and that these additional TK-like peptides are more susceptible to degradation by tissue peptidases than NKA itself.

Our results allow us to exclude an involvement of ATP through P_{2x} purinoceptors in the effect of capsaicin in the rat bladder, while confirming previous studies implicating ATP as an excitatory transmitter released from postganglionic nerves (Bo & Burnstock, 1990; Luheshi & Zar, 1990; Brading & Williams, 1990). The P_{2x} receptor is the dominant subtype of purinoceptor in rat urinary bladder (Bo & Burnstock, 1990). These findings do not exclude the possibility that in other species or in other tissues ATP may contribute to the efferent function of sensory nerves (Holton & Holton, 1954; Sweeney et al., 1989) and further studies on this topic are warranted.

Bombesin-LI is present in the rat urinary bladder (Ghatei et al., 1985) and bombesin-LI has been shown to be expressed in some capsaicin-sensitive primary afferents (see Holzer, 1988). To date there is no evidence for a release of bombesin-like peptide from sensory nerves. Our data indicate that, contrary to what has been observed for some other TK antagonists (Jensen et al., 1981; Mizrahi et al., 1985), the TK antagonists used in this study do not inhibit the effect of bombesin.

In conclusion, the results of the present experiments provide strong pharmacological evidence for the involvement of SP and NKA (or other NKA-related peptides preferentially acting at NK_2 receptors) as cotransmitters in the rat urinary bladder muscle. In this species the local contraction produced by stimulation of sensory nerves can be largely, if not totally, accounted for by release of TKs. Therefore, in addition to acetylcholine and ATP, TKs must also be considered as excitatory transmitters in the rat urinary bladder. However, owing to the modalities of activation of the efferent function of these sensory nerves (Maggi & Meli, 1988; Maggi, 1991) the local motor response produced by TK release from sensory nerves is likely to be more important during inflammatory conditions (for example in contributing to the overall symptoms of cystitis) rather than in normal micturition. The results of this and preceding studies (Maggi et al., 1987a; 1988a) indicate that both prejunctional and postjunctional factors are important in determining the local motor response produced by sensory nerve activation in the rat urinary bladder muscle (see Maggi et al., 1988d for a comparison with the guinea-pig urinary bladder). In the rat bladder, an important postjunctional factor is the absence of CGRP receptors on smooth muscle cells (Nimmo et al., 1988) which explains the lack of motor response to CGRP (Maggi et al., 1987c). The contemporary expression of NK_1 and NK_2 receptors and the simultaneous release of endogenous ligands with differential affinities for these receptors enables the sensory nerves to transmit a complex message to muscle cells the full expression of which requires multiple components at both the pre- and postjunctional level.

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(Received December 2, 1990
Revised February 5, 1991
Accepted February 7, 1991)

Br. J. Pharmacol. (1991), **103**, 1542–1546 © Macmillan Press Ltd, 1991

Renal handling and effects of S(+)-ibuprofen and R(−)-ibuprofen in the rat isolated perfused kidney

[1]P.G.F. Cox, W.M. Moons, F.G.M. Russel & C.A.M. van Ginneken

Department of Pharmacology, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

1 The renal handling and effects of S(+)- and R(−)-ibuprofen have been studied in the isolated perfused kidney (IPK) of the rat.

2 Both ibuprofen enantiomers were extensively reabsorbed and accumulated in the kidney in a concentration-dependent manner. No pharmacokinetic differences were observed between the two enantiomers.

3 S(+)-ibuprofen concentrations ranging from 0.25 to 25 $\mu g\,ml^{-1}$ (1.2 to 120 μM) caused a decrease in urinary flow, glomerular filtration rate (GFR) and electrolyte excretion. Urinary pH and excretion of glucose were not influenced. R(−)-ibuprofen concentrations ranging from 2.5 to 25 $\mu g\,ml^{-1}$ (12 to 120 μM) also decreased urinary flow and electrolyte excretion. This decrease, however, was less than observed with S(+)-ibuprofen. GFR, urinary pH and glucose excretion were not affected by R(−)-ibuprofen. Prostaglandin E_2 (PGE_2) concentrations of 133 $ng\,ml^{-1}$ reversed the effects on renal function of both enantiomers.

4 Very high S(+)- and R(−)-ibuprofen concentrations (>400 $\mu g\,ml^{-1}$) resulted in an increase in urinary flow and fractional excretion of sodium, chloride, potassium, glucose and calcium.

5 It is concluded that the pharmacokinetic behaviour of ibuprofen in the kidney is not stereoselective. Relatively high concentrations of both enantiomers increased the urinary flow and electrolyte excretion in a nonstereoselective manner. Lower concentrations of S(+)-ibuprofen decreased urinary flow and electrolyte excretion. The pharmacologically inactive R(−)-ibuprofen was also able to affect renal function in a similar way, but at different concentrations. These effects on renal function are probably caused by inhibition of PGE_2 synthesis.

Keywords: Rat isolated perfused kidney; renal handling; S(+)-ibuprofen; R(−)-ibuprofen

Introduction

An important group of nonsteroidal anti-inflammatory drugs (NSAIDs) are the 2-arylpropionic acids. These drugs possess an asymmetrical carbon atom and can therefore occur either as the S(+)- or R(−)-isomer. They are marketed as racemates, except for naproxen, which is marketed as the pure S(+)-enantiomer. The S(+)-enantiomers have been shown to possess essentially all the pharmacological activity (Adams *et al.*, 1976; Hutt & Caldwell, 1983). *In vivo*, chiral inversion occurs from the pharmacologically inactive R(−)-enantiomer to the active S(+)-enantiomer, predominantly in the liver (Hutt & Caldwell, 1983; Jamali, 1988; Knadler & Hall, 1990; Knihinicki *et al.*, 1989).

NSAIDS are well known to influence renal function and are usually associated with a decrease in urinary flow and electrolyte excretion, which is related to the inhibition of the prostaglandin synthesis (Clive & Stoff, 1984; Dunn & Zambraski, 1980). (S,R)-ibuprofen is widely used as an analgesic, but its effects on renal function are not clearly documented. Allon *et al.* (1990) reported a decrease in urinary flow and sodium excretion caused by (S,R)-ibuprofen. Brater *et al.* (1985) observed that (S,R)-ibuprofen affected only the chloride excretion, while Passmore *et al.* (1989, 1990) found no influence on kidney function. The varying results might be caused by the use of different (S,R)-ibuprofen doses and by different study protocols. The physical condition of the human beings or animals under investigation is also important (Clive & Stoff, 1984). The influence of the separate stereoisomers S(+)- and R(−)-ibuprofen on renal function is unknown.

Renal handling might also play a role in the nephrotoxic action of NSAIDS. It probably involves glomerular filtration, active secretion and passive reabsorption. As a result of active secretion, drugs can accumulate in the proximal tubular cells and exert a damaging effect.

[1] Author for correspondence.

Previously we have shown that the IPK is a good model for investigating renal handling and influence of NSAIDs on renal function (Cox *et al.*, 1990a). Using this model, we have studied both enantiomers of naproxen (Cox *et al.*, 1990b). The aim of the present investigation was to provide detailed data on the renal handling of the active S(+)- and the inactive R(−)-enantiomer of ibuprofen. We also looked at the influence of both enantiomers on renal function with and without concomitant administration of prostaglandin E_2 (PGE_2).

Methods

Isolated perfused kidney experiments

The IPK experiments were performed as described in detail elsewhere (Cox *et al.*, 1990a). In brief, male Wistar rats (225–275 g) were used in all experiments. The right kidney was isolated without interruption of the blood flow and placed in a fluid bath with a constant temperature of 37.5°C. The perfusion fluid was recirculated and continuously oxygenated. It was based on a Ringer solution supplemented with amino acids and did not contain any protein. Instead of albumin, the perfusate contained pluronic F108 (25 $g\,l^{-1}$) as colloid osmotic agent. Glomerular filtration rate (GFR) was determined instantaneously and continuously, with cyanocobalamin used as a marker, as described in detail elsewhere (Brink & Slegers, 1979).

After isolation, the kidney was allowed to stabilize for 30 min. During this period no urine or perfusate samples were taken. Kidneys with a fractional water reabsorption of less than 93% were excluded. The subsequent experimental time was 150 min, during which urine was collected over 10 min periods. The first 30 min were used as control values for renal function. In this control period total perfusate volume was 500 ml. After this period a new perfusate reservoir was connected with a total perfusate volume of 150 ml, in which S(+)-

ibuprofen or its enantiomer were already dissolved. Both enantiomers were added as the sodium salt. Perfusate samples (50–150 μl) were taken from the perfusate reservoir at the midpoint of these urine collection periods. Two additional perfusate samples were taken. One, before perfusion with drug and one at the end of the experiment.

At the end of the experiment the kidney was removed from the system, blotted, weighed and frozen. S(+)- or R(−)-ibuprofen concentrations in the kidney, in the perfusate samples, and in the urine samples were determined.

Average values are expressed as mean ± s.d. Statistical differences between means were established by Student's t test. A difference was considered significant if $P < 0.05$.

Analytical methods

Urine samples and perfusate were analyzed for sodium, potassium, chloride, glucose, magnesium, calcium, and (S,R)-ibuprofen. Sodium and potassium were determined by flame photometry. Chloride was determined by electrochemical titration, magnesium by atomic absorption flame photometry. Calcium was determined by a colorimetrical method, based on the formation of a deep blue calcium complex of methylthymol blue (RAPID STAT diagnostic kit of Lancer, Athy, Ireland). The GLUCO-QUANT glucose kit from Boehringer (Mannheim, FRG) was used for glucose analysis. This analysis is based on the enzymatic transformation of glucose and the simultaneous formation of NADPH which is measured by u.v. spectroscopy.

Perfusate and urine (S,R)-ibuprofen concentrations are determined by a high-performance liquid chromatographic method which is a modification of the method described by Askholt & Nielsen-Kudsk (1986). Chromatography was performed on a stainless steel column (10 cm × 4.6 mm i.d.) packed with LiChrosorb RP-18 (particle size 5 μM) with a mixture of 60% methanol and 40% H_3PO_4 (50 mM, pH 4.6) used as eluent. The elution rate was 1 ml min^{-1} and ibufenac was used as the internal standard. The compounds were detected by u.v. absorption at 220 nm. Under these conditions the retention times of ibufenac and (S,R)-ibuprofen were 8 and 12 min, respectively.

Depending on the drug concentration, 0.4 to 4 μg ibufenac was used in each sample, and 20 to 100 μl urine or perfusate was added. After addition of 50 μl 1.5 M HCl, total volume was adjusted to 150 μl with distilled water. Di-ethyl ether (1.35 ml) was added, the mixture was vortexed for 10 s and centrifuged for 10 min at 6000g. The di-ethyl ether layer was removed, evaporated under a stream of dry filtered air on ice, 0.2 to 1 ml of the elution fluid was added and the mixture was vortexed. An aliquot (20 μl) of the solution obtained was injected onto the column. Calibration was performed using peak area ratios of (S,R)-ibuprofen and ibufenac after spiking samples of blank perfusate with various amounts of (S,R)-ibuprofen. Linear calibration curves were obtained in all cases ($R^2 > 0.980$).

(S,R)-ibuprofen concentrations in the kidneys were determined in a similar way. The kidney was homogenized in 5 to 10 ml distilled water with a Polytron homogenizer on setting 10 for 2 times 60 s. To 250 μl of the homogenate, 1 to 5 μg ibufenac and 50 μl 0.004 M HCl were added. The mixture was vortexed, 1.2 ml di-ethyl ether was added and it was vortexed again for 15 s. After centrifugation for 30 min at 6000g, the di-ethyl ether layer was removed and the extraction was repeated. The di-ethyl ether fractions were collected and evaporated under a stream of dry filtered air on ice, 0.5 to 1 ml elution fluid was added and the solution was vortexed. An aliquot (20 μl) of the clear solution obtained was injected onto the column. Calibration was performed using peak area ratios of (S,R)-ibuprofen and ibufenac after spiking samples of blank kidney homogenates with various amounts of (S,R)-ibuprofen (recovery >90%). Linear calibration curves were obtained in all cases ($R^2 > 0.987$).

The limit of detection for the determinations of (S,R)-ibuprofen was 0.5 μg per ml fluid injected onto the column.

Materials

Sodium pentobarbitone was obtained from Apharma (Apharma, The Netherlands). Heparin, insulin and aldosterone were from Organon (Oss, The Netherlands). Pluronic F-108 was purchased from BASF (Arnhem, The Netherlands), lysine-vasopressin from Sandoz (Basel, Switzerland) and angiotensin II from Beckmann (Palo Alto, Ca., USA). Synthamin 14 was from Travenol (Thetford, Norfolk, England). LiChrosorb RP-18 was from Merck (Darmstadt, F.R.G.). All other chemicals were of analytical grade and were purchased either from Sigma (St. Louis, Mo., U.S.A.) or Merck (Darmstadt, F.R.G.).

Results

Renal handling

The kinetic data of several S(+)- and R(−)-ibuprofen doses are shown in Table 1. The excretion of both enantiomers into the urine was very low. As a result, the perfusate concentration showed practically no decrease during the experiments. The excretion rate was nearly constant after its maximal value was reached (usually within the first 30 min after addition).

The amount of S(+)- and R(−)-ibuprofen in the kidney was determined at the end of the experiment. In order to determine whether the drugs accumulated, the kidney to perfusate

Table 1 Renal handling of S(+)- and R(−)-ibuprofen[a]

Dose (μg)	S(+)/R(−)	*Perfusate concentration*[b] (μg ml^{-1})	*Filtration rate*[cd] (μg min^{-1})	*Excretion rate*[c] (μg min^{-1})	*FE*[e] (%)	*Amount in kidney* (μg g^{-1})	*K/P ratio*[f]
37500	S(+)	199 ± 16	57 ± 9	2.11 ± 0.19	3.7	276 ± 19	1.44 ± 0.18
3750	S(+)	20.5 ± 1.2	5.4 ± 1.2	0.22 ± 0.03	4.0	31 ± 3	1.6 ± 0.2
375	S(+)	1.9 ± 0.2	0.50 ± 0.14	0.019 ± 0.003	3.8	4.0 ± 0.5	2.4 ± 0.3
375[g]	S(+)	1.8 ± 0.2	0.62 ± 0.13	0.031 ± 0.003**	5.0	3.7 ± 0.2	2.46 ± 0.18
37500	R(−)	183 ± 12	55 ± 11	1.8 ± 0.3	3.3	303 ± 13	1.17 ± 0.13
3750	R(−)	18 ± 2	4.9 ± 1.0	0.19 ± 0.02	3.8	31 ± 4	1.86 ± 0.16
375	R(−)	1.7 ± 0.3	0.44 ± 0.12	0.023 ± 0.005	5.1	3.1 ± 0.4	2.3 ± 0.5
375[g]	R(−)	2.0 ± 0.4	0.58 ± 0.17	0.026 ± 0.004	4.5	4.0 ± 0.9	2.7 ± 0.2

[a] $n = 4$ for all doses. ** $P < 0.01$ vs. experiments in which no PGE_2 was added.
[b] Mean values of the period 0–120 min are presented.
[c] Mean values of the period 30–120 min are presented.
[d] Filtration rate = GFR * perfusate concentration.
[e] FE = fractional excretion = 100 * excertion rate/filtration rate.
[f] K/P ratio = amount drug (μg) in kidney per g kidney divided by the perfusate concentration.
[g] Perfusate contained PGE_2 133 ng ml^{-1}.

Table 2 Renal function of control experiments and experiments with S(+)-ibuprofen[a]

Parameter	Exp	Time (min) c0–c30	0[b]–30	30–60	60–90	90–120
Urinary flow ($\mu l\,min^{-1}$)	—	25 ± 5	24 ± 5	22 ± 5	21 ± 5	21 ± 7
	S(+)	26 ± 5	22 ± 4	15 ± 3**	13 ± 2**	13 ± 2**
GFR ($\mu l\,min^{-1}$)	—	430 ± 80	400 ± 70	340 ± 50	290 ± 50	240 ± 50
	S(+)	480 ± 60	400 ± 50	300 ± 40	240 ± 20*	210 ± 40
Urinary pH	—	5.8 ± 0.2	5.7 ± 0.2	5.8 ± 0.2	5.8 ± 0.3	5.9 ± 0.3
	S(+)	5.9 ± 0.2	5.9 ± 0.2	5.9 ± 0.2	5.9 ± 0.2	5.9 ± 0.2
FE of glucose (%)	—	2.5 ± 0.4	2.9 ± 0.8	3.7 ± 1.5	6 ± 3	10 ± 6
	S(+)	3.1 ± 0.9	3.8 ± 1.4	5.0 ± 1.7	7.2 ± 2.6	12 ± 3
FE of sodium (%)	—	2.6 ± 0.6	2.5 ± 0.6	2.6 ± 0.7	2.8 ± 0.8	3.9 ± 1.4
	S(+)	2.8 ± 0.8	2.4 ± 0.7	1.7 ± 0.4*	1.6 ± 0.3**	1.8 ± 0.4***
FE of chloride (%)	—	3.2 ± 0.8	3.4 ± 0.8	3.6 ± 0.9	3.8 ± 1.0	5.0 ± 1.6
	S(+)	2.8 ± 1.1	3.2 ± 1.0	2.3 ± 0.8*	1.7 ± 0.6***	1.8 ± 0.6***
FE of potassium (%)	—	38 ± 13	45 ± 17	46 ± 18	39 ± 15	41 ± 15
	S(+)	29 ± 8	37 ± 6	23 ± 7**	15 ± 5***	16 ± 5***
FE of calcium (%)	—	4.5 ± 1.4	4.6 ± 1.4	4.5 ± 1.5	4.4 ± 1.8	6 ± 3
	S(+)	4.0 ± 1.2	4.4 ± 0.8	3.3 ± 0.5*	2.9 ± 0.5*	3.0 ± 0.9*
FE of magnesium (%)	—	47 ± 9	48 ± 10	41 ± 9	34 ± 8	30 ± 7
	S(+)	39 ± 10	39 ± 11	27 ± 9*	19 ± 7**	16 ± 5**

[a] Mean values of three urine 10 min periods are given. — = control experiments ($n = 12$) and S(+) = experiments with $1.9 \pm 0.2\,\mu g\,ml^{-1}$ S(+)-ibuprofen ($n = 4$). FE = fractional excretion. c = control period. * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$ vs. control experiments.

[b] Time at which the reservoir is changed to perfusate with or without S(+)-ibuprofen.

(K/P) ratio was calculated. This ratio is the drug amount (μg) per g kidney divided by the perfusate concentration. A ratio higher than one indicates accumulation of drug. As shown in Table 1, S(+)- and R(−)-ibuprofen accumulated in the kidney in a concentration-dependent manner. Increasing the perfusate concentration decreased the K/P ratio.

The presence of $133\,ng\,ml^{-1}$ PGE_2 in the perfusate resulted in a significantly higher excretion rate of S(+)-ibuprofen. The excretion rate of R(−)-ibuprofen was also increased, although not significantly.

No significant difference between the kinetic data of either stereoisomer was observed.

Effects on kidney function

Renal function was monitored by determination of the GFR, urinary flow, urinary pH and fractional excretion of sodium, chloride, potassium, glucose, magnesium and calcium. S(+)-ibuprofen decreased the GFR, urinary flow and fractional excretion of all electrolytes (Table 2). Urinary pH and fractional excretion of glucose were not influenced. The change in renal function occurred mainly in the first 30 min after drug addition, thereafter it was practically stable.

The effects of a broad concentration range of S(+)- and R(−)-ibuprofen on renal function are shown in Figure 1. The functional parameters are expressed as normalized values. S(+)-ibuprofen concentrations ranging from 0.0025 to $0.025\,\mu g\,ml^{-1}$ did not affect renal function. At concentrations between 0.25 and $25\,\mu g\,ml^{-1}$ GFR, urinary flow (Figure 1) and fractional excretion of electrolytes (Figure 2) decreased as compared to control experiments. The presence of $133\,ng\,ml^{-1}$ PGE_2 in the perfusate completely abolished the effects of S(+)-ibuprofen on kidney function.

An R(−)-ibuprofen concentration of $0.25\,\mu g\,ml^{-1}$ did not affect renal function. Concentrations ranging from 2.5 to $25\,\mu g\,ml^{-1}$ caused a decrease in urinary flow and fractional excretion of electrolytes, which was less than observed with S(+)-ibuprofen. GFR, urinary pH and fractional excretion of glucose were not affected. The effects of R(−)-ibuprofen on urinary flow and fractional excretion of sodium, chloride, magnesium and calcium were reversed by PGE_2 ($133\,ng\,ml^{-1}$). Fractional excretion of potassium, however, was decreased in the presence of PGE_2, although not statistically significant as compared to R(−)-ibuprofen experiments without PGE_2. In the absence of (S,R)-ibuprofen, PGE_2 did not influence kidney function.

High concentrations of S(+)- and R(−)-ibuprofen ($250\,\mu g\,ml^{-1}$) resulted in the same parameter values as in control experiments (Figures 1 and 2). S(+)- and R(−)-ibuprofen concentrations above $400\,\mu g\,ml^{-1}$ increased significantly the urinary flow and fractional excretion of sodium, potassium, chloride, glucose and calcium. GFR and fractional excretion of magnesium were not affected. The experiments with these very high concentrations were performed only once, because of the limited amounts of the enantiomers available.

Neither the perfusion flow, nor pressure were affected by S(+)- or R(−)-ibuprofen. The mean perfusion flow of all ibuprofen experiments ($n = 48$) was $14.3 \pm 1.5\,ml\,min^{-1}$ and the mean perfusion pressure was 86 ± 5 mmHg.

Discussion

A very broad concentration range (0.0025 to $500\,\mu g\,ml^{-1}$) of both ibuprofen enantiomers was studied in the IPK. The normal therapeutic range is $1–50\,\mu g\,ml^{-1}$ (Aarons *et al.*, 1983). *In vivo*, ibuprofen is highly bound to plasma proteins (99%). The concentration-range we examined included the therapeutically used total as well as unbound concentrations.

Like other 2-arylpropionic acids, R(−)-ibuprofen can be inverted to its active S(+)-enantiomer, whereas stereoinversion from S(+)- to R(−)-ibuprofen does not occur (Hutt & Caldwell, 1983; Jamali, 1988; Knihinicki *et al.*, 1989). The inversion from R(−)- to S(+)-enantiomer predominantly takes place in the liver and there are no indications of the occurrence of inversion in the kidney (Knihinicki *et al.*, 1989; Knadler & Hall, 1990). In the present study, we added S(+)- and R(−)-ibuprofen separately to the IPK and analysed urine samples, perfusate samples and kidneys for (S,R)-ibuprofen using a nonstereoselective high-performance liquid chromatographic assay.

S(+)- and R(−)-ibuprofen must be reabsorbed extensively in the IPK, since only a small fraction of the filtered amount was excreted into the urine (Table 1). Reabsorption is probably a passive process, dependent on the urinary pH and flow. Active reabsorption, however, cannot be excluded although this seems unlikely because no saturation of the reabsorption could be detected over the broad concentration range studied.

PGE_2 ($133\,ng\,ml^{-1}$) reversed the decrease in urinary flow caused by both ibuprofen enantiomers. The higher urinary flow resulted in a decreased passive reabsorption and an increased excretion rate.

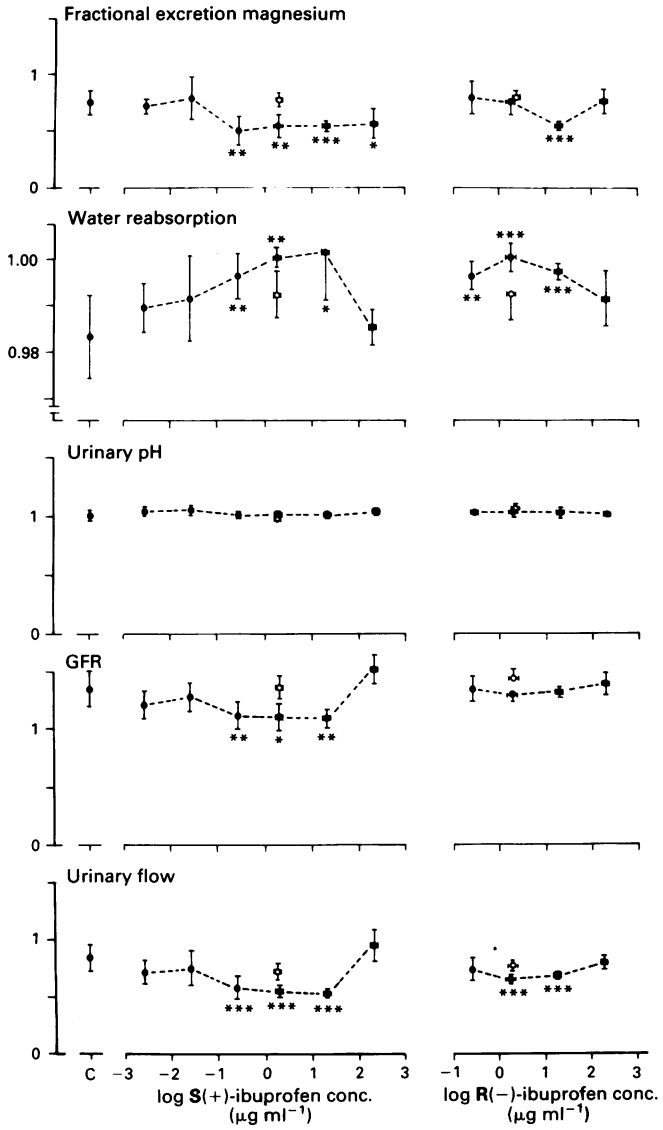

Figure 1 Effects of S(+)- and R(−)-ibuprofen on urinary flow, glomerular filtration rate (GFR), urinary pH, water reabsorption and fractional excretions of magnesium. Normalized parameter values are shown. Normalization is performed by dividing the mean parameter value of the period 30 to 120 min by the mean parameter value of the control period c0–c30. C = normalized parameter value of control experiments ($n = 12$). For all concentrations: $n = 4$. Only perfusate concentrations of the three highest doses could be determined, the other concentrations were below the detection limit of the analytical method and are determined by dividing the dose by total perfusate volume. Open circles represent experiments with perfusate containing 133 ng ml^{-1} PGE_2. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. control experiments.

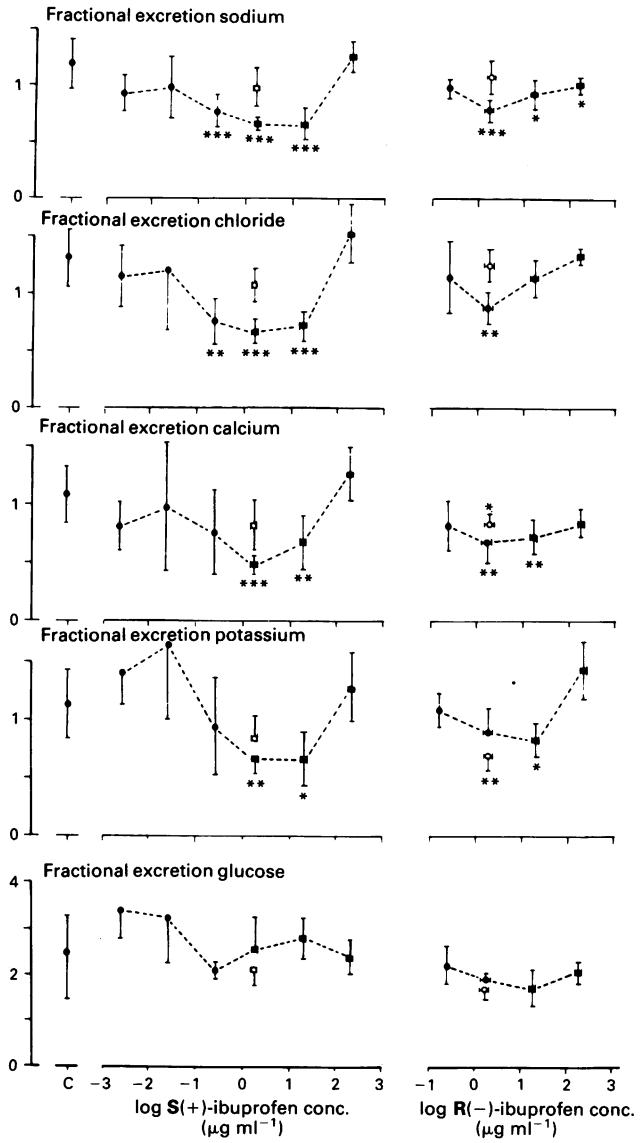

Figure 2 Effects of S(+)- and R(−)-ibuprofen on fractional excretions of glucose, potassium, calcium, chloride and sodium. Normalized parameter values are shown. Conditions as in Figure 1.

Both stereoisomers accumulated to the same extent in the IPK as can be deduced from the K/P ratios. Accumulation is probably the result of active secretion. Saturation of the active secretion at higher perfusate concentrations would lead to a decrease in K/P ratio, as observed in our experiments.

S(+)- and R(−)-ibuprofen did not possess different kinetic parameters (Table 1). This indicates nonstereoselective pharmacokinetics in the kidney. *In vivo*, stereoselective pharmacokinetics are observed, which at least partly is caused by stereoinversion of R(−)- to S(+)-ibuprofen (Lee *et al.*, 1985; Knihinicki *et al.*, 1989; Geisslinger *et al.*, 1989; 1990; Knadler & Hall, 1990). Stereoselective metabolism, predominantly occurring in the liver and other nonrenal factors probably also play a role in the *in vivo* observed stereoselective pharmacokinetics.

NSAIDs are usually associated with a decrease in urinary flow and electrolyte excretion (Clive & Stoff, 1984). In the IPK, S(+)-ibuprofen concentrations ranging from 0.25 to 25 μg ml^{-1} decreased the urinary flow, GFR and fractional excretion of electrolytes. Urinary pH and fractional excretion of glucose were not influenced. Besides the decrease of GFR, S(+)-ibuprofen probably exerts a distal tubular effect causing the decrease in electrolyte excretion and urinary flow. This is in agreement with the proposed most probable site of effect of NSAIDs: the thick ascending limb of Henle (Kaojarern *et al.*, 1983).

R(−)-ibuprofen concentrations ranging from 2.5 to 25 μg ml^{-1} affected renal function in a similar way. As compared to S(+)-ibuprofen, the lowest concentration which was able to affect kidney function was at least ten times higher.

Because the effects of NSAIDs on renal function might be related to the inhibition of prostaglandin synthesis, we investigated the influence of PGE_2. The presence of 133 ng ml^{-1} PGE_2 in the perfusate reversed the renal effects of both ibuprofen enantiomers. The effects, therefore, are probably caused by the reduction of PGE_2 synthesis.

Flower (1974) reported that 0.3 μg ml^{-1} (S,R)-ibuprofen was able to inhibit 50% of the prostaglandin synthesis. Humes *et al.* (1983) observed a similar value of 0.2 μg ml^{-1}. Although racemic mixtures were used in these studies, these values are comparable to the lowest S(+)-ibuprofen concentration at which an effect on renal function was observed (0.25 μg ml^{-1}).

Previously, we investigated the effects of naproxen and its enantiomer on renal function (Cox *et al.*, 1990b). Naproxen

caused similar effects on kidney function as S(+)-ibuprofen. Also the concentrations needed to bring about the effects were similar (0.25 to 25 $\mu g\,ml^{-1}$). The inactive enantiomer of naproxen did not affect renal function. In contrast, at the same concentration (2.5 $\mu g\,ml^{-1}$) R(−)-ibuprofen did affect renal function in a similar way to its S(+)-enantiomer and naproxen. R(−)-ibuprofen is 160 times less potent in inhibiting PGE_2 synthesis than S(+)-ibuprofen (Adams *et al.*, 1976). The inactive enantiomer of naproxen, however, is only 75 times less potent as compared to naproxen (Tomlinson *et al.*, 1972). The difference in potency in inhibiting the prostaglandin synthesis, therefore, cannot explain the different effects of R(−)-ibuprofen and the enantiomer of naproxen. Stereoinversion of R(−)- to S(+)-ibuprofen could explain the difference. However, there are several arguments against such an inversion. Firstly, it has been shown that inversion does not occur or occurs only to a very small extent in the kidney (Knadler & Hall, 1990; Knihinicki *et al.*, 1989). Secondly, the lowest R(−)-ibuprofen concentration needed to affect kidney function is at least 10 times higher than the lowest S(+)-ibuprofen concentration. If inversion would be the cause of the R(−)-ibuprofen effects on renal function, then at least 10% of the R(−)-ibuprofen amounts must have been inverted. Such a large scale inversion seems unlikely in the IPK, especially considering the relatively short experimental time. Thus, although unlikely, inversion of R(−)- to S(+)-ibuprofen cannot be excluded and further studies in this direction are needed.

Besides the decrease in urinary flow and electrolyte excretion, high concentrations of S(+)- and R(−)-ibuprofen increased the urinary flow and electrolyte excretion in a non-stereoselective manner. At concentrations of 250 $\mu g\,ml^{-1}$ these opposite influences are practically balanced, leading to an unchanged renal function as compared to control experiments (Figures 1 and 2). At very high concentrations of both enantiomers (>400 $\mu g\,ml^{-1}$) the nonstereoselective influence prevails, resulting in an increase in urinary flow and fractional excretion of sodium, chloride, potassium, glucose and calcium. GFR and fractional excretion of magnesium were not affected. Previously, we have shown that very high concentrations of naproxen and its enantiomer (>500 $\mu g\,ml^{-1}$) caused a similar nonstereoselective influence on renal function (Cox *et al.*, 1990b).

In summary, renal handling of S(+)- and R(−)-ibuprofen does not appear to be stereospecific. Very high concentrations of S(+)- and R(−)-ibuprofen (>400 $\mu g\,ml^{-1}$) increased the urinary flow and fractional excretion of electrolytes (except magnesium) in a nonstereoselective manner. Lower S(+)-ibuprofen concentrations decreased GFR, urinary flow and fractional excretion of electrolytes. However, R(−)-ibuprofen affected kidney function in a similar way at higher concentrations. These effects on renal function are probably caused by the inhibition of the PGE_2 synthesis. Because R(−)-ibuprofen is pharmacologically inactive and because it affects renal function, it might be beneficial to use the pure S(+)-enantiomer clinically, instead of the racemate.

This work was supported by the Dutch League against Rheumatism ("Het Nationaal Rheumafonds"). S(+)-ibuprofen (S(+)-2-(4-isobutylphenyl)propionic acid), R(−)-ibuprofen (R(−)-2-(4-isobutylphenyl)propionic acid) and ibufenac (4-isobutylphenyl acetic acid) were generous gifts from The Boots Company (Nottingham, U.K.).

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(Received October 25, 1990
Revised January 12, 1991
Accepted February 7, 1991)

Br. J. Pharmacol. (1991), **103**, 1547–1555 © Macmillan Press Ltd, 1991

Characterization of the 5-HT receptor subtypes involved in the motor behaviours produced by intrathecal administration of 5-HT agonists in rats

[1]K.C.F. Fone, A.J. Robinson & C.A. Marsden

Department of Physiology and Pharmacology, Queens Medical Centre, Clifton Boulevard, Nottingham, NG7 2UH

1 The motor behavioural effects of intrathecal injections of 5-hydroxytryptamine (5-HT) and a variety of 5-HT receptor agonists were examined in adult Wistar rats to establish; (a) which 5-HT receptor subtype/s elicit each behaviour and (b) whether these receptors are located within the spinal cord.

2 Intrathecal injection of 5-methoxy-*N*,*N*'-dimethyltryptamine (5-MeODMT), (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI) or 2,5-dimethoxy-α,4-dimethylbenzene ethamine hydrochloride (DOM) produced dose-related back muscle contractions (BMC) and wet dog shakes (WDS) which were both markedly attenuated by intraperitoneal pretreatment with either ritanserin ($1\,mg\,kg^{-1}$), ketanserin ($0.16\,mg\,kg^{-1}$) or mianserin ($0.6\,mg\,kg^{-1}$) indicating the involvement of $5\text{-}HT_2$ receptors in both these motor behaviours. Both fluoxetine ($1–20\,mg\,kg^{-1}$, i.p.) and high doses of 5-HT ($50\,\mu g$) following fluoxetine ($5\,mg\,kg^{-1}$, i.p.) also elicited BMC, further confirming the involvement of 5-HT in this behaviour.

3 Intrathecal 5-carboxamidotryptamine (5-CT) evoked a marked wet-dog shake response without producing any BMC. Intrathecal pretreatment with 8-hydroxy-2-(di-n-propylamino)tetraline (8-OH-DPAT) enhanced, while in contrast 2-methyl-5-HT pretreatment attenuated, 5-HT agonist-induced BMC without affecting WDS. These data suggest that the spinal $5\text{-}HT_2$ receptors mediating BMC are positively modulated by $5\text{-}HT_{1A}$ but negatively influenced by $5\text{-}HT_3$ receptor activation and may be of a different subtype to the supra-spinal $5\text{-}HT_2$ receptors which elicit WDS.

4 A contrast, reciprocal forepaw treading, lateral head weaving, flat body posture and Straub-tail were evoked by 5-MeODMT, 8-OH-DPAT or 5-CT but not by DOI or DOM indicating that these behaviours were not produced by $5\text{-}HT_2$ receptor activation alone. Ritanserin ($1\,mg\,kg^{-1}$, i.p.) or ketanserin ($0.16\,mg\,kg^{-1}$, i.p.) pretreatment reduced the reciprocal forepaw treading induced by high intrathecal doses of either 5-MeODMT ($25\,\mu g$) or 5-CT ($50\,\mu g$) suggesting that this behaviour may be facilitated by $5\text{-}HT_2$ receptor activation.

5 Intrathecal injection of 5-HT (0.05–$50\,\mu g$, after systemic fluoxetine, $5\,mg\,kg^{-1}$, i.p.), or 1-(3-chlorophenyl)piperazine (mCPP) produced dose-related forepaw-licking and grooming, neither of which were attenuated by ketanserin ($0.16\,mg\,kg^{-1}$, i.p.) pretreatment suggesting these behaviours may be mediated by $5\text{-}HT_{1C}$ receptors. In contrast, 2-methyl-5-HT (50 and $100\,\mu g$) produced sideward tail-flicks, not evoked by any other 5-HT agonist and could therefore be mediated by spinal $5\text{-}HT_3$ receptor activation.

6 These data provide behavioural evidence for the existence of spinal $5\text{-}HT_2$ receptors which produce a novel motor behaviour, BMC. Ligand binding studies and dose-response studies with a range of selective 5-HT antagonists are required to establish whether BMC and WDS are mediated by different subtypes of $5\text{-}HT_2$ receptors.

Keywords: Bulbospinal 5-hydroxytryptaminergic neurones; $5\text{-}HT_2$ receptors; 5-HT behavioural syndrome

Introduction

Within the central nervous system ligand binding studies have identified $5\text{-}HT_1$, $5\text{-}HT_2$, $5\text{-}HT_3$ and recently $5\text{-}HT_4$ binding sites, furthermore subtypes of $5\text{-}HT_{1(A\text{ to }E)}$ and possibly $5\text{-}HT_{2(A\text{ and }B)}$ have also been proposed (for recent reviews see Peroutka, 1988; Hartig, 1989; Frazer *et al.*, 1990; Bobker & Williams, 1990). Three of the putative 5-HT receptors appear to be linked to different second messenger systems; all the $5\text{-}HT_1$ subtypes (apart from $5\text{-}HT_{1C}$) are coupled to adenylate cyclase (Barbaccia *et al.*, 1983), the $5\text{-}HT_2$ (and $5\text{-}HT_{1C}$) to phosphoinositide hydrolysis (Conn & Sanders-Bush, 1984; 1986) and the $5\text{-}HT_3$ forms an ion gating channel (Bobker & Williams, 1990), while the $5\text{-}HT_4$ receptor is positively coupled to adenylate cyclase (Dumuis *et al.*, 1989). Of all these different 5-HT receptors, however, only four ($5\text{-}HT_{1A}$, $5\text{-}HT_{1C}$, $5\text{-}HT_{1D}$ and $5\text{-}HT_2$) have so far been cloned (Hartig, 1989; Branchek *et al.*, 1990) so much effort is currently being made to identify functional correlates for each 5-HT receptor subtype, a problem addressed in the current behavioural study.

It is well established that bulbospinal neurones from the nuclei raphe obscurus and pallidus, which contain 5-hydroxytryptamine (5-HT), project to the ventral horn of the spinal cord (Fuxe, 1965) where many of these neurones make synaptic contact with α-motoneurones (Ulfhake *et al.*, 1987). There is considerable evidence to suggest that 5-HT released from these descending raphe neurones facilitates spinal motoneurone excitability. For example the iontophoretic application of 5-HT onto spinal motoneurones augments the effect of excitatory amino acids (White & Neuman, 1980) and stimulation of bulbospinal raphe neurones produces excitatory postsynaptic potentials in motoneurones (Fung & Barnes, 1989). In addition, Barbeau & Bédard (1981) showed the precursor to 5-HT, 5-hydroxytryptophan, augments hindlimb EMG in spinalised rats, an effect which was enhanced by destruction of bulbospinal raphe neurones with the 5-hydroxytryptaminergic neurotoxin, 5,7-dihydroxytryptamine (5,7-DHT). However, the physiological role of these bulbospinal raphe neurones in the control of normal spinal motor function is poorly understood but could be more readily investigated if a motor behavioural model was available to examine the effect of 5-HT on spinal motor function in conscious animals.

[1] Author for correspondence.

The various components of the 5-HT behavioural syndrome appear to involve activation of the caudal raphe neurones which innervate cranial and spinal motor nuclei (Jacobs, 1976; McCall & Aghajanian, 1979). However, of all the components of the 5-HT syndrome, only Straub-tail and tremor are unaffected by transverse lesions of the CNS down to the spinal cord while hindlimb abduction is abolished by spinal transection (Jacobs & Klemfuss, 1975), implying that these may be the only behaviours resulting from an action of 5-HT at the spinal level. More recent studies have shown that intrathecal administration of relatively high doses of the non-selective 5-HT agonist 5-methoxy-N,N'-dimethyltryptamine (5-MeODMT) induce dose-related contractions of the muscles of the back in rats (Fone *et al.*, 1989). These back muscle contractions (BMC) are potentiated by intrathecal pretreatment with 5,7-DHT, as would be expected if this behaviour results from the activation of 5-HT receptors located postsynaptic in relation to bulbospinal raphe neurones (Fone *et al.*, 1989). The present study attempts to characterize more fully the 5-HT receptor subtype/s involved in the production of BMC by examining the effect of, and interaction between, a variety of 5-HT agonists given intrathecally and 5-HT antagonists administered systemically to conscious rats.

Methods

Intrathecal cannulation and behavioural monitoring

Male Wistar rats (weighing between 260 and 320 g) were maintained on a 12 h light-dark cycle and given food and water *ad libitum*. Indwelling intrathecal cannulae (7.5 cm long polyethylene tubing, 0.63 mm o.d. Portex) were implanted along the spinal subarachnoid space of rats under sodium methohexitone anaesthesia (Brietal 60 mg kg^{-1}, i.p.) as described previously (Yaksh & Rudy, 1972; Fone *et al.*, 1987). Animals were habituated to their behavioural recording chamber during the post-operative week and again for 30 min before each of a maximum of ten intrathecal injections of the selected 5HT agonist/s, which were given at three or four day intervals.

Following each intrathecal injection (10 μl washed in with 15 μl saline) the number of back muscle contractions (BMC, brief caudo-rostral contractions of the superficial muscles of the back), wet dog shakes (WDS, including head shakes) and yawns were counted separately and the time spent forepaw-licking and face washing (collectively referred to as forepaw-licking) and the total time spent grooming (including forepaw-licking) were recorded for 30 min post-injection. In addition lateral head weaving, reciprocal forepaw treading, flat body posture and Straub-tail were each scored separately on a scale of 0–3 (0 absent, 1 present < 50%, 2 present > 50% and 3 present all of the time) for 20 s once every min for 20 min (maximum score being 60 for each behaviour).

Drug administration

Dose-response studies to examine the effect of each selected 5-HT agonist were performed in eight separate groups of rats which received either, 8-hydroxy-2-(di-n-propylamino)tetraline (8-OH-DPAT, 0.1 to 100 μg, $n = 9$), 1-(3-chlorophenyl)piperazine (mCPP, 10 to 100 μg, $n = 5$), 5-MeODMT (2 to 100 μg, $n = 8$), 5-carboxamidotryptamine (5-CT, 0.1 to 100 μg, $n = 6$), (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI, 1 to 50 μg, $n = 6$), 2,5-dimethoxy-α,4-dimethylbenzene ethamine (DOM, 2 to 25 μg, $n = 7$), 2-methyl-5-HT (2-Me-5-HT, 1 to 100 μg, $n = 7$) or 5-HT (0.5 to 200 μg, $n = 6$ given 30 min after clorgyline, 1 mg kg^{-1} i.p. or 0.05 to 50 μg, $n = 5$ given 30 min after fluoxetine, 5 mg kg^{-1}, i.p.). Subsequent to these dose-response studies the behavioural effect of a selected dose of some of the 5-HT agonists (groups above) was re-examined following pretreatment (−30 min) with either a 5-HT$_2$ antagonist or another 5-HT agonist. The behavioural effects of mCPP (50 μg) and 5-HT (50 μg) were re-examined after ketanserin (0.16 mg kg^{-1}, i.p.), 5-MeODMT (25 μg) and 2-Me-5-HT (50 μg) after ritanserin (1 mg kg^{-1} and 0.16 mg kg^{-1}, i.p., respectively) and DOI (10 μg) after ketanserin (0.16 mg kg^{-1}, i.p.) and mianserin (0.6 mg kg^{-1}, i.p.) while the behavioural response to 8-OH-DPAT (0.1 to 100 μg) was re-examined after pretreatment with DOI (2 μg). In two additional groups of rats the behavioural response to DOI (2, 10 and 50 μg) was recorded following intrathecal pretreatment (−30 min) with saline and either 8-OH-DPAT (2 μg, $n = 8$) or 2-Me-5-HT (2 μg, $n = 7$). In a further group of rats, BMC were counted for 30 min following repeated intrathecal injection of DOI (10 μg, $n = 4$), the second injection being 24 h after the first, followed by further twice daily injections for four days. Five rats were decapitated 9 min after intrathecal injection of a mixture of cold and [^{125}I]-DOI (10 μg containing 6.85 kBq in 10 μl saline washed in with 15 μl saline) and the distribution of radioactivity was examined in the intrathecal cannula, brain and spinal cord which were removed, sonicated (10–15 s MSE sonicator) in 90% methanol and counted (200 s) on a gamma counter (Mini-Instruments Ltd, type 6-20). In another group of six rats, which did not have indwelling intrathecal cannula, the behavioural response to fluoxetine (1 to 20 mg kg^{-1}, i.p.) was examined for 30 min post-injection and compared with the effect of saline. A final group of rats was decapitated 35 min after a single intrathecal injection of either saline ($n = 5$) or 8-OH-DPAT (2 μg, $n = 5$), and the thoraco-lumbar spinal cord removed and stored at −60°C prior to determining levels of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) by high performance liquid chromatography with electrochemical detection as described in detail by Fone *et al.* (1989).

Drugs

The following drugs were used: 8-hydroxy-2-(di-n-propylamino)tetraline hydrobromide (8-OH-DPAT), 1-(3-chlorophenyl)piperazine dihydrochloride (mCPP), (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI) and 3-(2-aminoethyl)-2-methyl-1-1H-indol-5-ol maleate (2-Me-5-HT) (Research Biochemicals Inc.); [^{125}I]-(±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride ([^{125}I]-DOI) (New England Nuclear), 5-methoxy-N,N'-dimethyltryptamine hydrochloride (5-MeODMT) 5-hydroxytryptamine hydrochloride (5-HT) and N-methyl-N-propargyl-3-(2,4-dichlorophenoxy) propylamine hydrochloride (clorgyline) (Sigma); 2,5-dimethoxy-α,4-dimethylbenzene ethamine hydrochloride (DOM provide by Janssen), 5-carboxamidotryptamine maleate (5-CT, Glaxo), fluoxetine hydrochloride (Eli Lily & Co.) and mianserin hydrochloride (Research Biochemicals Inc.): these were all dissolved in saline (0.154 M sodium chloride). Ketanserin and ritanserin (Janssen) were dissolved in a minimum volume of 0.1 M tartaric acid or 0.04 M lactic acid in dextrose, respectively before being diluted to the required concentration in saline (0.154 M sodium chloride).

Analysis of results

Behavioural experiments were performed using a blind protocol as described previously (Fone *et al.*, 1987) and values given are mean (± s.e.mean for parametric data). Analysis of variance (ANOVA) was used for the statistical analysis of the behavioural studies followed by Wilcoxan rank test (behaviours which were scored on a rating scale) or Dunnett's test (behaviours counted or timed).

Results

Effect of systemic fluoxetine and clorgyline on intrathecal 5-hydroxytryptamine-induced behaviours

Systemic administration of the 5-HT uptake inhibitor fluoxetine (1, 5 and 20 mg kg^{-1}, i.p.) evoked readily quantifiable,

dose-related, brief contractions of the superficial muscles of the back (BMC) resulting in caudal-rostral movements of the skin without any movements of the vertebrae. These BMC began within 2–3 min of injection, lasted for 20–30 min (Figure 1), and were not accompanied by any WDS. Even the highest dose of fluoxetine failed to elicit any of the previously well characterized components of the 5-HT behavioural syndrome (scores for reciprocal forepaw treading 2, lateral head weaving 3, flat body posture 8 and Straub-tail 0 were not significantly different from those obtained with saline, being 1, 2, 4 and 5 respectively, from a possible maximum of 60 each). In addition, 30 min after a submaximally effective dose of fluoxetine (5 mg kg^{-1}, i.p.), intrathecal injection of 5-HT re-initiated BMC in a dose-related manner (Figure 1), once again without eliciting WDS (even 50 μg 5-HT evoked only 2 ± 2 in 30 min) or any of the other components of the 5-HT behavioural syndrome. Following fluoxetine (5 mg kg^{-1}, i.p.), intrathecal 5-HT produced significantly more forepaw-licking ($P < 0.02$, ANOVA) and grooming ($P < 0.001$, ANOVA) than saline (which caused 63 ± 14 s and 114 ± 32 s in 30 min, respectively) both these behaviours being maximal (117 ± 25 s, $P < 0.001$ and 283 ± 55 s, $P < 0.05$, respectively) with the highest dose of 5-HT.

In contrast, following pretreatment with the monoamine oxidase A inhibitor, clorgyline, intrathecal administration of 5-HT (0.05–200 μg) elicited WDS, which exhibited a bell-shaped response to increasing dose (being maximal with 100 μg 5-HT), but did not produce any of the other behaviours monitored (Table 1).

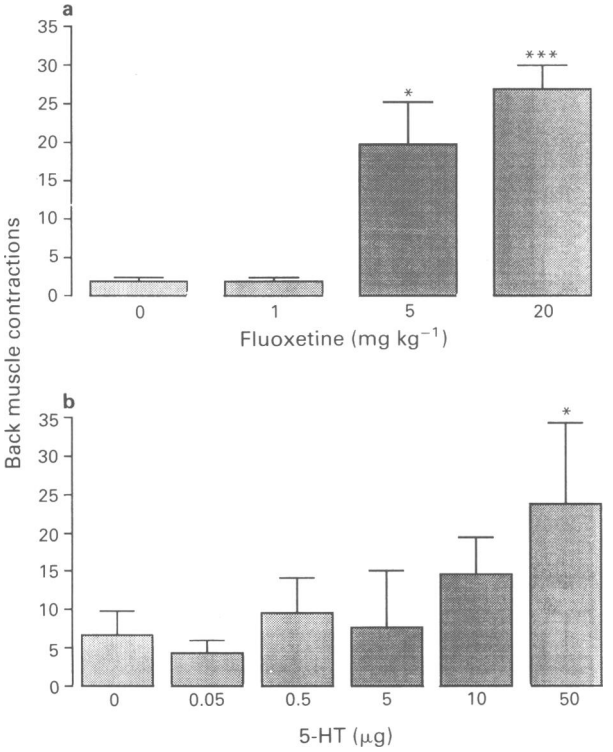

Figure 1 Total number (mean with s.e.mean shown by vertical bars) of back muscle contractions (BMC) observed in 30 min following: (a) intraperitoneal injection of saline (0.1 ml 100 g^{-1}) or fluoxetine (1, 5 and 20 mg kg^{-1} as indicated, $n = 6$) or (b) intrathecal injection of saline (10 μl washed in with 15 μl saline) or 5-hydroxytryptamine (0.05 to 50 μg, $n = 5$) given 30 min after fluoxetine (5 mg kg^{-1}, i.p.). In (b) column 0 is the number of BMC elicited by intrathecal saline from 30–60 min after fluoxetine (5 mg kg^{-1}, i.p.). Note the re-initiation of BMC by 5-HT. * $P < 0.05$ and *** $P < 0.001$ Dunnett's test following a significant ANOVA, from the effect of the appropriate saline control.

Behavioural effects of intrathecal 5-hydroxytryptamine agonists

The most marked behavioural effect, which began within the first 2 min of the intrathecal injection of the non-selective 5-HT agonist 5-MeODMT, or DOI or DOM which are selective agonists for both 5-HT$_{1C}$ and 5-HT$_2$ receptors (Glennon *et al.*, 1988; Hoyer, 1988) was BMC (Figure 2). The number of BMC elicited by 5-MeODMT ($r = 0.959$), DOI ($r = 0.958$) and DOM ($r = 0.997$) were linearly correlated to $\log_{10}$ dose according to the method of least squares ($P < 0.001$), the ED$_{50}$ values being 10.6, 8.5 and 11.0 μg, respectively. With all three agonists BMC were accompanied by WDS, which typically began after, but within 2–3 min of, the first BMC and were approximately ten fold fewer in number (Figure 2). Irrespective of whether BMC or WDS were initiated by 5-MeODMT, DOI or DOM they were attenuated by pretreatment with low doses of one of the 5-HT$_2$ receptor antagonists, ketanserin, ritanserin or mianserin (Table 2). Although the higher doses of 5-MeODMT also evoked flat body posture, reciprocal forepaw treading and Straub-tail, as previously documented (Fone *et al.*, 1989), none of these behaviours were quantitatively different from values obtained with saline treatment with any dose of DOI or DOM (data not shown). Following twice daily intrathecal injection the number of BMC produced by DOI (10 μg, 93 ± 27 in 30 min) rapidly declined with the second (70 ± 23 at +24 h), fourth (26 ± 12 at +48 h, $P < 0.05$ Dunnett's test following a significant ANOVA $F = 3.55$, $P = 0.038$, compared with the response to the first injection) and sixth (13 ± 11 at +72 h, $P < 0.05$) intrathecal injections. Intrathecal injection of 8-OH-DPAT (0.1 to 100 μg) a selective 5-HT$_{1A}$ receptor agonist (Tricklebank *et al.*, 1985) failed to evoke any marked behavioural effects until the highest dose (100 μg), which produced significantly more flat body posture, reciprocal forepaw treading and lateral head weaving than saline (Table 3), but these

Table 1 Behavioural effects of intrathecal 5-hydroxytryptamine (5-HT) following clorgyline

5-HT dose (μg)	0	0.05	5	50	100	200
Wet dog shakes	1 ± 1	1 ± 1	1 ± 1	8 ± 2	40 ± 6***	24 ± 7***
Back muscle contract.	9 ± 2	7 ± 3	11 ± 4	8 ± 3	6 ± 2	6 ± 1
Flat body posture	1.5	0.2	0.0	0.0	11.0	0.3
	(0–6)	(0–1)	(0)	(0)	(0–36)	(0–2)
Reciprocal forepaw	0.0	0.0	0.8	0.7	0.3	0.5
treading	(0)	(0)	(0–3)	(0–3)	(0–1)	(0–1)
Lateral head weaving	2.3	3.0	2.4	1.3	1.3	1.3
	(0–5)	(0–6)	(1–5)	(0–4)	(1–2)	(1–4)
Straub tail	0	0.4	0.6	0.0	0.7	0.7
	(0)	(0–1)	(0–2)	(0)	(0–3)	(0–3)

Comparison of the effect of intrathecal saline with that of 5-HT (0.05–200 μg, $n = 6$) given 30 min after clorgyline (1 mg kg^{-1}, i.p.) on the total number of wet dog shakes and back muscle contractions (mean ± s.e.mean in 30 min) counted separately. All other behaviours were scored separately on a scale of 0–3, (0 = absent to 3 = present 100% of the time, for 20 s once every minute for the first 20 min post-injection, maximum score = 60), values given are mean (range) from six rats. *** $P < 0.001$ Dunnett's test from the effect of saline after a significant ANOVA ($F = 16.099$, $P < 0.001$).

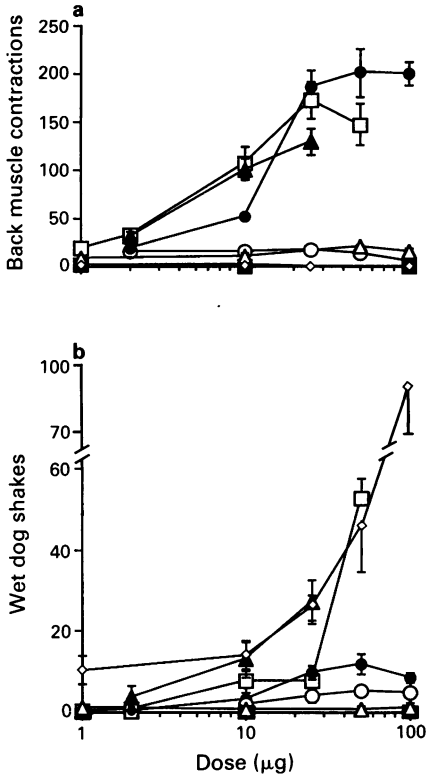

Figure 2 Dose-response studies showing the total number (mean with s.e.mean shown by vertical bars) of back muscle contractions (BMS, a) and wet dog shakes (WDS, b) observed in 30 min following the intrathecal administration (10 μl washed in with 15 μl saline) of; 8-OH-DPAT (■, 0.1 to 100 μg, $n = 9$), mCPP (○, 10 to 100 μg, $n = 5$), 5-MeODMT (●, 2 to 100 μg, $n = 8$), 5-CT (◇, 0.1 to 100 μg, $n = 6$), DOI (□, 1 to 50 μg, $n = 6$), DOM (▲, 2 to 25 μg, $n = 7$) or 2-Me-5-HT (△, 1 to 100 μg, $n = 7$). mCPP (10 to 100 μg, $P < 0.01$), 5-MeODMT (25 to 100 μg, $P < 0.001$), 2-Me-5-HT (50 μg, $P < 0.01$), DOI (10 to 50 μg, $P < 0.001$) and DOM (2 to 25 μg, $P < 0.001$) elicited significantly more BMC and mCPP (50 and 100 μg, $P < 0.05$), 5-MeODMT (25 to 100 μg, $P < 0.001$), 5-CT (50 μg, $P < 0.05$; 100 μg, $P < 0.01$), DOI (50 μg, $P < 0.001$) and DOM (10 and 25 μg, $P < 0.001$) produced significantly more WDS than saline, Dunnett's test following a significant ANOVA. The $\log_{10}$ dose-response curves for BMC and WDS were linearly correlated for 5-MeODMT, DOM and DOI according to the method of least squares. See text for abbreviations.

behaviours were typically only observed from 3 to 4 min after the injection. No WDS or BMC were evoked by any dose of 8-OH-DPAT used in the present study (Figure 2). Intrathecal pretreatment with DOI (2 μg, −30 min) did not significantly affect any of the behaviours produced by any dose of 8-OH-DPAT, as illustrated for the highest dose of DOI in Table 3. In contrast, intrathecal pretreatment of rats with 8-OH-DPAT (2 μg, −30 min) approximately doubled the total number of BMC produced by the lowest and highest doses of DOI ($P < 0.05$ at both 2 and 50 μg), although it did not significantly enhance the effect of intermediate doses (Figure 3). In contrast, 8-OH-DPAT pretreatment did not affect the WDS concomitantly evoked by intrathecal injection of DOI (Figure 3). In a separate acute biochemical study, a single intrathecal injection of 8-OH-DPAT (2 μg given 35 min prior to decapitation) significantly increased ($P < 0.05$ Student's t test) the levels of both 5-HT (from 40.7 ± 3.4 to 57.1 ± 6.1 pmol mg^{-1} protein) and 5-HIAA (from 24.0 ± 2.0 to 32.3 ± 3.0 pmol mg^{-1} protein) in the ventral portion of the

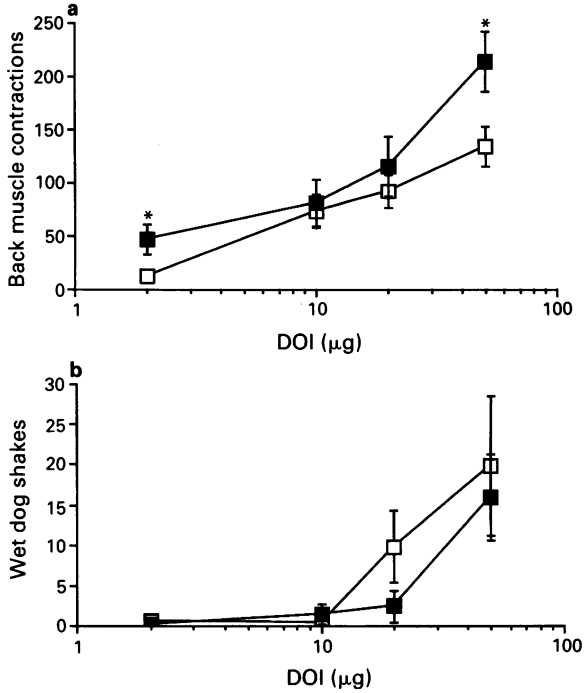

Figure 3 Comparison of the effect of pretreatment (−30 min intrathecally) with either saline 25 μl (□) or 8-hydroxy-2-(di-n-propylamine)tetraline (8-OH-DPAT, 2 μg, ■) on the total number (mean with s.e.mean shown by vertical bars) of back muscle contraction (a) and wet dog shakes (b) recorded in 30 min following the intrathecal injection of (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI, 2 to 50 μg, $n = 8$). * $P < 0.05$ from saline pretreated controls, Dunnett's test following a significant ANOVA.

Table 2 Effect of selected 5-hydroxytryptamine (5-HT) antagonists on 5-HT agonist-induced back muscle contractions and wet dog shakes

	5-HT agonist					
Pretreatment	*DOI* (10 μg)	*DOM* (10 μg)	*5-MeODMT* (25 μg)	*mCPP* (50 μg)	*2-Me-5-HT* (50 μg)	*5-CT* (50 μg)
Control	108 ± 16	101 ± 11	186 ± 18	18 ± 3	20 ± 2	0 ± 0
	(8 ± 3)	(3 ± 5)	(10 ± 2)	(5 ± 1)	(1 ± 1)	(46 ± 11)
Ketanserin	38 ± 14*	—	—	5 ± 2**	—	0 ± 0
	(0 ± 0)	—	—	(1 ± 1)	—	(7 ± 2)*
Ritanserin	—	1 ± 1**	17 ± 10**	—	†1 ± 1*	—
	—	(1 ± 1)	(0 ± 0)	—	(0 ± 0)	—
Mianserin	4 ± 1**	—	—	—	—	—
	(0 ± 0)	—	—	—	—	—

Effect of intraperitoneal pretreatment with either ketanserin (0.16 mg kg^{-1}), ritanserin (1 mg kg^{-1} and †0.16 mg kg^{-1}) or mianserin (0.6 mg kg^{-1}) on the total number (mean ± s.e.mean) of back muscle contractions (upper value) and wet dog shakes (lower value in parentheses) elicited in 30 min following the intrathecal administration of the selected 5-HT agonist ($n = 5$ to 8) at the dose shown in parentheses. * $P < 0.05$ and ** $P < 0.01$ from agonist alone.

DOI, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane; DOM, 2,5-dimethoxy-α,4-dimethylbenzene ethamine hydrochloride; 5-MeODMT, 5-methoxy-N,N'-dimethyltryptamine; mCPP, 1-(3-chlorophenyl)piperazine; 2-Me-5-HT, 2-methyl-5-HT; 5-CT, 5-carboxamidotryptamine.

Table 3 Behavioural effects of intrathecal 8-hydroxy-2-(di-n-propylamino)tetraline (8-OH-DPAT)

8-OH-DPAT dose (μg)	0	0.1	1	10	100	100 plus DOI
Flat body posture	1.7	2.8	3.4*	2.9	35.0**	35.8**
	(0–4)	(0–7)	(1–6)	(1–5)	(32–37)	(31–40)
Reciprocal forepaw treading	1.4	1.2	1.9	2.6	10.1**	9.1*
	(0–3)	(0–3)	(0–5)	(0–7)	(7–14)	(4 – 13)
Lateral head weaving	2.0	3.0	3.4	2.9	14.0**	13.5**
	(0–4)	(0–5)	(0–8)	(0–7)	(11–17)	(10–17)

Behavioural effects of 8-OH-DPAT (0.1–100 μg intrathecally, in 10 μl washed in with 15 μl saline) compared with the effect of saline (25 μl). Each behaviour was scored on a scale of 0–3 (0 = absent to 3 = present 100% of the time) for 20 s once every minute for the first 20 min post-injection (maximum score = 60), values given are mean (range) from nine rats. * $P < 0.05$ and ** $P < 0.01$ Wilcoxon rank test from the effect of saline after a significant Kruskal-Wallis test ($P < 0.001$ for each behaviour). 100 plus DOI = 8-OH-DPAT (100 μg) given 30 min after intrathecal injection of (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI, 2 μg), all other 8-OH-DPAT injections were given 30 min after saline (25 μl, intrathecally).

thoraco-lumbar spinal cord, without affecting either indoleamine level in the dorsal spinal cord or brainstem (data not shown).

Intrathecal administration of mCPP which shows some selectivity for 5-HT_{1C} over the other 5-HT_1 ligand binding sites (Hoyer, 1988) produced a limited number of WDS and BMC, which were significantly greater ($P < 0.05$, 50 and 100 μg and $P < 0.01$, 10 to 100 μg, respectively) than observed with saline but neither of these behaviours was linearly correlated to $\log_{10}$ dose according to the method of least squares (Figure 2). The few WDS and BMC evoked by mCPP (50 μg) were, however, significantly reduced ($P < 0.05$, Table 2) by pretreatment with the selective 5-HT_2 receptor antagonist ketanserin (0.16 mg kg^{-1}, i.p.) at a dose which shows some selectivity for 5-HT_2 rather than 5-HT_{1C} receptors (Hoyer, 1988; Kennett & Curzon, 1988). Figure 4 shows that mCPP also induced grooming of the body and, in particular, licking of the forepaws and face-washing behaviour (collectively referred to as forepaw-licking) which both occurred predominantly between 2 and 10 min from injection and increased with dose. Neither of these grooming behaviours was significantly altered by ketanserin pretreatment (Figure 4). Intrathecal mCPP injection also elicited significantly more yawns than saline (Figure 4). However, yawning usually commenced after the grooming behaviours, typically being maximal 10 to 20 min after the injection and was significantly reduced ($P < 0.05$) by ketanserin pretreatment (Figure 4).

5-CT which has high affinity for 5-HT_1-like receptor subtypes (Connor *et al.*, 1986; Hoyer, 1988) was the most potent of all the 5-HT agonists in producing WDS, which were significantly greater in incidence than observed with saline, increased with dose and were significantly reduced by ketanserin (Figure 2 and Table 2). 5-CT also evoked marked flat body posture (without accompanying hindlimb abduction) and some lateral head weaving, neither of which was altered by ketanserin (Figure 5). Reciprocal forepaw treading demonstrated a bell-shaped relationship to the intrathecal dose of 5-CT, the maximum effect (with 50 μg 5-CT) being attenuated by ketanserin pretreatment (Figure 5). No BMC were observed with any dose of 5-CT (Figure 2).

Intrathecal injection of even the highest dose of the 5-HT_3 receptor agonist, 2-Me-5-HT (100 μg), did not elicit significantly more reciprocal forepaw treading (mean score of 2 from a maximum of 60), lateral head weaving (3), flat body posture (0), Straub-tail (0) or yawns (11 ± 2 mean ± s.e.mean in 30 min) than saline (being 2, 3, 2, 0 and 17 ± 3, respectively). The limited number of BMC evoked by 2-Me-5-HT were significantly greater than observed with saline at all doses used but failed to increase with dose, being maximal with 50 μg (Figure 2), and were significantly attenuated by ritanserin (0.16 mg kg^{-1}, i.p.) pretreatment (Table 2). The highest two doses of 2-Me-5-HT (50 and 100 μg) produced sideward tail-flicks, (each flick lasting for less than a second) which appeared with almost clockwork regularity with the highest dose (reaching 442 ± 90 jerks in 30 min following 100 μg) and yet were not evident with any of the other 5-HT agonists used. Intrathecal pretreatment with 2-Me-5-HT (2 μg, which elicited little behavioural effect alone) produced a small but significant attenuation of the number of the BMC evoked by the highest dose (50 μg) of the selective 5-HT_2 receptor agonist DOI (from 179 ± 14 to 127 ± 14 in 30 min, $P < 0.05$ Dunnett's test following ANOVA), without affecting the response to 2 or 10 μg DOI (data not shown).

Distribution of [^{125}I]-DOI given intrathecally

Following intrathecal injection, only 20 ± 7% of the total radioactive DOI administered was recovered from the brain and spinal cord while 19 ± 4% remained in the cannula, with

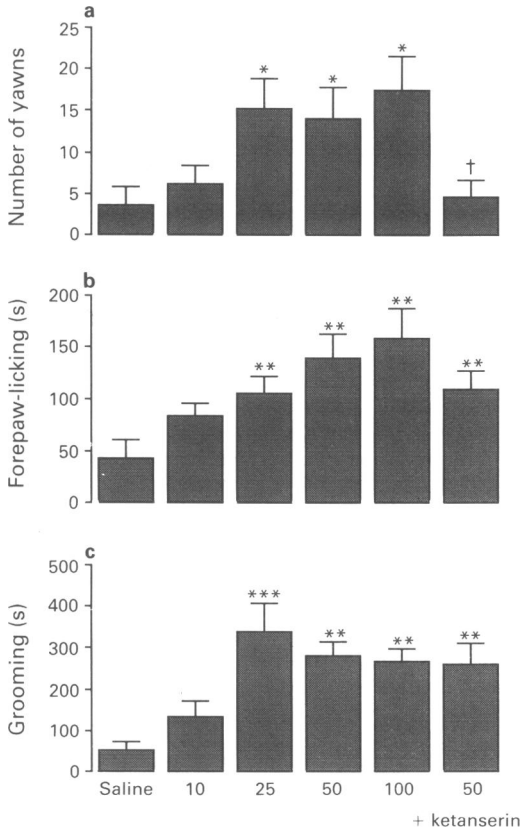

Figure 4 Effect of intrathecal injection of 1-(3-chlorophenyl)piperazine (mCPP, 10 to 100 μg, $n = 5$) alone or following pretreatment with ketanserin (0.16 mg kg^{-1}, i.p., −30 min) on the total number of yawns (a) and time (s) spent forepaw-licking and face washing (b) and grooming (c) i 30 min post-injection, values given represent mean (s.e.mean shown by vertical bars) in nine rats; 50 + ketanserin = mCPP (50 μg) given 30 min after ketanserin (0.16 mg kg^{-1}, i.p.). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ from saline-treated controls and † $P < 0.05$ from mCPP (50 μg) alone, Dunnett's test following ANOVA.

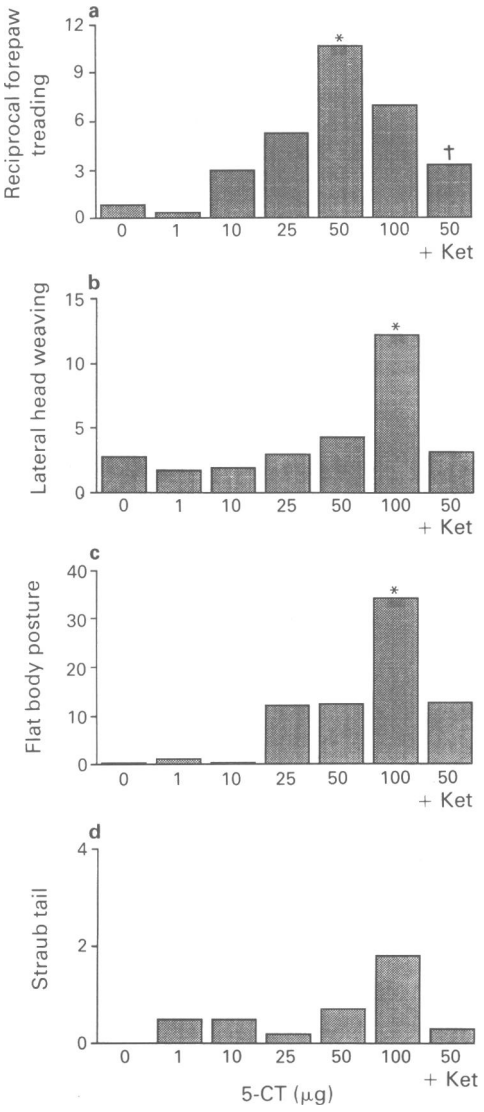

Figure 5 Effect of ketanserin (Ket) pretreatment (0.16 mg kg^{-1}, i.p., −30 min) on (a) the reciprocal forepaw treading, (b) lateral head weaving, (c) flat body posture and (d) Straub-tail (given as a mean score with a maximum of 60, rated on a scale of 0–3 once every min for 20 min post-injection, see methods for further details) evoked by the intrathecal injection of 5-carboxamidotryptamine (5-CT, 1 to 100 μg, $n = 6$). * $P < 0.05$ Wilcoxan rank test following a significant Kruskall-Wallis from the effect of saline and † $P < 0.05$ from 50 μg 5-carboxamidotryptamine without ketanserin.

the rest presumably being in the cerebrospinal fluid which was lost during the dissection procedure. Nine minutes after intrathecal injection of [^{125}I]-DOI, 97 ± 2% of the total radioactivity recovered from the entire CNS tissue was within the spinal cord, highest levels (68 ± 8%) being in the thoracic segments.

Discussion

The present study examined the behavioural effects of intrathecal injection of a variety of 5-HT agonists which show some selectivity for different 5-HT receptor subtypes (Bradley *et al.*, 1986; Hoyer, 1988; Bobker & Williams, 1990). Although relatively high doses (1 to 10 μg) of the various 5-HT agonists were required to elicit many of the behaviours reported, the strikingly different effects suggest that some pharmacological selectivity was maintained. Furthermore the present study shows that [^{125}I]-DOI is principally restricted to the spinal cord for at least 9 min post-intrathecal injection, in agreement with a previous study (Johnson *et al.*, 1989) using intrathecal [^{14}C]-RX 77368 (a thyrotrophin-releasing hormone analogue), and also suggested that a large proportion of the total dose of DOI administered remains either in the cerebrospinal fluid or the cannula at this time.

Considerable evidence suggests that head twitches and WDS in rats are mediated by 5-HT$_2$ receptors (Bédard & Pycock, 1977; Yap & Taylor, 1983; Lucki *et al.*, 1984; Yocca *et al.*, 1990) and in the present study both WDS and BMC were attenuated by a low dose of one of the 5-HT$_2$ antagonists, ketanserin, ritanserin or mianserin, irrespective of the initiating agonist. Furthermore, the rank order of potency of the 5-HT agonists at producing BMC (DOI ⩾ DOM = 5-MeODMT ⩾ 5-HT = mCPP = 2-Me-5-HT > 8-OH-DPAT = 5-CT) was similar to that for WDS (5-CT > DOI = DOM > 5-MeODMT > mCPP > 5-HT = 2-Me-5-HT = 8-OH-DPAT), consistent with both behaviours being 5-HT$_2$ receptor-mediated. However, 5-CT potently evoked WDS without eliciting any BMC and intrathecal 5-HT only evoked WDS when given following clorgyline and BMC after fluoxetine treatment, suggesting these two behaviours are produced by different mechanisms. Although McKenna & Peroutka (1989) proposed the existence of two 5-HT$_2$ receptor subtypes (DOI binding 5-HT$_{2A}$ while ketanserin labels both 5-HT$_{2A}$ and 5-HT$_{2B}$ sites) such differences in ligand binding are probably due to separate G-protein-dependent agonist affinity states of one common receptor (Lyon *et al.*, 1987; Leonhardt & Titeler, 1989). Without determining dose-response curves for WDS and BMC using a range of antagonists it is impossible to determine whether these behaviours are mediated by different 5-HT$_2$ receptor subtypes or one receptor at two anatomically distinct locations.

The present results, together with the previously observed potentiation of BMC following intrathecal 5,7-DHT (Fone *et al.*, 1989) suggests that this motor behaviour is elicited by the activation of 5-HT$_2$ receptors located postsynaptic to bulbospinal raphe nerve terminals, either on spinal motoneurons or interneurones. Iontophoretic studies show that 5-HT depolarizes spinal motoneurones (Connell & Wallis, 1988; Roberts *et al.*, 1988), an effect not mimicked by 2-Me-5-HT, 8-OH-DPAT or mCPP, and 5-HT$_2$ agonists excite both facial (Rasmussen & Aghajanian, 1990) and spinal motoneurones (Dixon & Fone, 1991) all of which correlate with the current pharmacological profile reported for 5-HT agonist-induced BMC. However, 5-CT is a full excitatory agonist on motoneurones, an effect poorly antagonized by ketanserin (Roberts *et al.*, 1988; Connell & Wallis, 1989); which fails to agree with the current findings on BMC. Following intrathecal administration, 5-HT agonists presumably affect dorsal horn neurones that are responsive to 5-HT (El-Yassir *et al.*, 1988) which in turn may indirectly influence the activity of motoneurones. It would therefore be surprising if all 5-HT agonists and antagonists had an identical pharmacological profile of activity on a single cell (α-motoneurones) as on a motor behaviour (BMC), even if motoneurone discharge produces this behaviour.

One of the criteria empirical to classifying a functional response as 5-HT$_1$-like, is that it is mimicked by 5-CT (Bradley *et al.*, 1986; Frazer *et al.*, 1990). Although 5-CT has greater binding affinity for 5-HT$_1$ than 5-HT$_2$ receptors (Hoyer, 1988), the present study suggests that this selectivity may be lost *in vivo*, since 5-CT was the most potent agonist in eliciting WDS. A similar conclusion was made by Toda & Okamura (1990) who showed that 5-CT-induced contractions of primate arteries are ketanserin-sensitive. In anaesthetized rats, intrathecal injection of 5-CT (25–200 μg) and low doses of 5-HT (20–100 μg) enhance while α-methyl-5-HT and high doses of 5-HT (100–600 μg) attenuate sympathetic neuronal discharge, but ketanserin pretreatment only prevents the inhibitory effects (Yusof & Coote, 1988). Thus at comparable doses to those used in the present study, 5-CT mimicked the effect of 5-HT on sympathetic discharge yet it failed to evoke BMC. It is therefore unlikely that BMC directly result from

these cardiovascular effects of 5-HT agonists. Alteration in local spinal cord blood flow could, however, contribute to some of the observed behaviours, but intrathecal pretreatment with 5,7-DHT, which enhanced 5-HT agonist-induced BMC (Fone *et al.*, 1989), would not be expected to potentiate the vascular effects of 5-HT agonists. Flat body posture and reciprocal forepaw treading are the only behaviours produced by the selective 5-HT_{1A} agonist 8-OH-DPAT (Middlemiss & Fozard, 1983), thought to be mediated by 5-HT_{1A} receptor activation (Tricklebank *et al.*, 1985). In the present intrathecal study, only the highest dose of 8-OH-DPAT (100 μg) produced flat body posture and reciprocal forepaw treading which with our previous finding that these behaviours are unaffected by intrathecal 5,7-DHT (Fone *et al.*, 1989) implies that they are mediated by supraspinal receptors. Intrathecal administration of DOI or DOM, which have high affinity for 5-HT_2 receptors, evoked only WDS and BMC in agreement with previous studies showing that the complete 5-HT behavioural syndrome is not elicited by 5-HT_2 receptor activation alone (Lucki *et al.*, 1984; Goodwin & Green, 1985). Although simultaneous administration of DOI has been shown to potentiate the reciprocal forepaw treading induced by systemic 8-OH-DPAT (Arnt & Hyttel, 1989; Berendsen *et al.*, 1989), this was not observed in the present intrathecal study. In agreement with previous studies (Goodwin & Green, 1985; Jackson & Kitchen, 1989) however, systemic pretreatment with 5-HT_2 antagonists reduced the reciprocal forepaw treading evoked by intrathecal 5-MeODMT or 5-CT as expected if the positive interaction between 5-HT_2 and the 5-HT_{1A} receptors which cause reciprocal forepaw treading occurs at the supraspinal level. Conversely the 5-HT_{1A} agonists 8-OH-DPAT and buspirone inhibit the ketanserin-sensitive head twitches evoked by DOI (Arnt & Hyttel, 1989), quipazine (Yocca *et al.*, 1989) or 5-HTP following carbidopa (Goodwin & Green, 1985) suggesting that 5-HT_{1A} receptor activation attenuates 5-HT_2 receptor function. In contrast, in the present study intrathecal pretreatment with 8-OH-DPAT (which alone did not elicit BMC) significantly enhanced DOI-induced BMC. One explanation for this discrepancy is that positive coupling exists between the 5-HT_{1A} and 5-HT_2 receptors in the ventral spinal cord which elicit BMC, but not at supraspinal sites involved in mediating other 5-HT-induced behaviours. The present biochemical study showed ventral horn 5-HT levels were elevated 35 min after intrathecal 8-OH-DPAT (an equivalent time to the potentiation of DOI-induced BMC caused by 8-OH-DPAT), as expected if 5-HT release from bulbospinal raphe neurones had decreased as previously documented following 8-OH-DPAT (Hjorth & Magnusson, 1988). The potentiation of BMC produced by 8-OH-DPAT pretreatment could thus result from postsynaptic supersensitivity to the 5-HT_2 agonist precipitated by an acute reduction in 5-HT release from bulbospinal raphe neurones. In support of this view, tolerance rapidly developed to repeated 5-HT_2 agonist injection in the present study, as previously noted for DOM-induced WDS (Leysen *et al.*, 1989) and supersensitivity occurs following depletion of ventral horn 5-HT by intrathecal 5,7-DHT (Fone *et al.*, 1989).

Although forepaw-licking and whole body grooming were apparent with 5-HT following fluoxetine, of all the other 5-HT agonists used only mCPP, which has slightly higher affinity for 5-HT_{1C} than other 5-HT receptor subtypes (Hoyer, 1988), elicited dose-related grooming as formerly noted in 5 day old rats (Jackson & Kitchen, 1989). Pretreatment with ketanserin (0.16 mg kg^{-1}, i.p.), a dose that selectively antagonizes 5-HT_2 and not 5-HT_{1C} receptors (Kennett & Curzon, 1988), abolished the few BMC produced by mCPP without affecting grooming, implicating 5-HT_{1C} receptors in grooming. Hylden & Wilcox (1983) demonstrated similar biting and scratching in mice given intrathecal 5-HT, which was potentiated by fluoxetine (Larson & Wilcox, 1984). Although Berendsen *et al.*, (1990) recently suggested hindlimb scratching may involve 5-HT_{1D} receptors, the existence of this receptor subtype in the rat is controversial (Hoyer & Middlemiss, 1989).

Intrathecal injection of the selective 5-HT_3 receptor agonist 2-Me-5-HT (Kilpatrick *et al.*, 1987; Barnes *et al.*, 1989) produced sideward tail-flicks, not observed with any other 5-HT agonist used. Systemic administration of 8-OH-DPAT to rats with tails hanging freely from restraining cylinders also produces tail-flicks (Millan *et al.*, 1989) but none was observed with the current intrathecal injection in unrestrained rats, suggesting that spinal 5-HT_{1A} receptors are not involved in this behaviour. Even large intrathecal doses of 2-Me-5-HT failed to evoke the classical 5-HT behavioural syndrome and the few BMC were attenuated by ritanserin, however, 2-Me-5-HT reduced DOI-induced BMC in a non-competitive manner. Although rodent spinal motoneurones are unresponsive to iontophoresis of 2-Me-5-HT (Connell & Wallis, 1989), ligand binding studies show 5-HT_3 receptors concentrated throughout the grey matter of the spinal cord (Waeber *et al.*, 1989), providing a mechanism for the observed behavioural interaction.

Figure 6 summarizes the mechanisms involved in the induction of BMC. BMC are evoked by activation of 5-HT_2 receptors located postsynaptic to bulbospinal raphe neurones, possibly on thoracic α-motoneurones. Activation of 5-HT_{1A} receptors located on raphe soma in the brainstem facilitates 5-HT_2 receptor-induced BMC, while activation of 5-HT_3 receptors possibly located on spinal interneurones or dorsal horn neurones attenuate BMC.

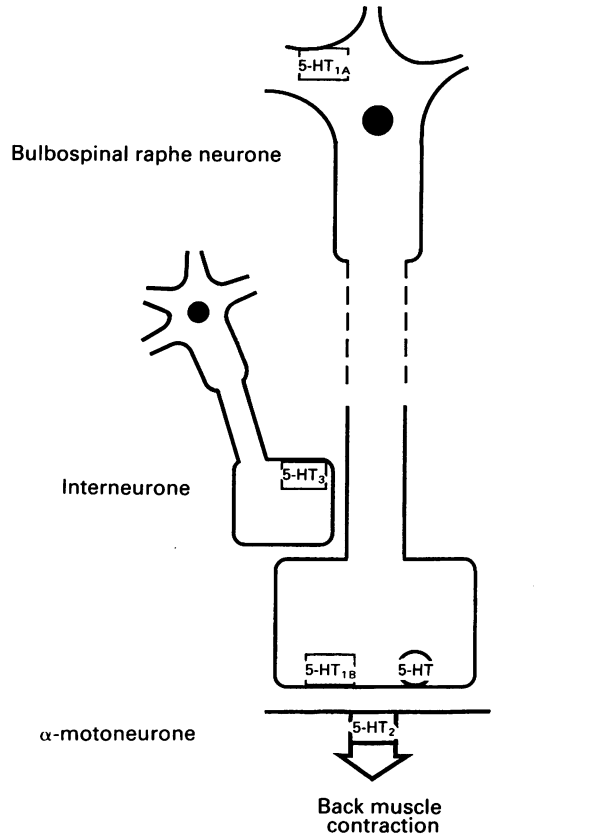

Figure 6 Diagrammatic representation of the location of the 5-HT receptors involved in the production of back-muscle contractions (BMC). From the current data and Fone *et al.* (1989), BMC are elicited by the activation of 5-HT_2 receptors located postsynaptic to bulbospinal raphe neurones in the ventral horn of the spinal cord, possibly on thoracic α-motoneurones. Activation of 5-HT_{1A} receptors, probably located on the brainstem raphe soma, potentiates this motor behaviour. In contrast, the apparently non-competitive inhibition of BMC by a 5-HT_3 agonist, suggests that the receptors involved in this effect are not located on motoneurones but possible on spinal interneurones. The effect of activation of terminal autoreceptors remains to be determined.

This work was funded by Action Research. We wish to thank Ian Topham for technical assistance and Glaxo and Janssen Pharmaceuticals for the supplying 5-CT and DOM, respectively.

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(Received August 3, 1990
Revised January 23, 1991
Accepted February 8, 1991)

Br. J. Pharmacol. (1991), **103**, 1556–1560 © Macmillan Press Ltd, 1991

Effects of midaglizole, a new hypoglycaemic drug on the electrophysiological properties of guinea-pig papillary muscle

Noriyasu Noguchi, [1]Junichi Hasegawa & Hiroto Mashiba

Department of Internal Medicine, Tottori University School of Medicine, Yonago 683 Japan

1 The electrophysiological effects of midaglizole, a new oral hypoglycaemic agent which is an α_2-adrenoceptor antagonist, were assessed in the reserpinized ventricular myocardium of the guinea-pig.

2 Midaglizole suppressed the maximal rate of rise ($\dot{V}_{max}$) and the amplitude of action potentials (APA) of the fast and the slow responses in a concentration-dependent manner without influencing the resting potentials.

3 Voltage-dependency of the $\dot{V}_{max}$ block of the action potentials by midaglizole (10 μM) did not appear in solutions containing various concentrations of KCl.

4 It is concluded that midaglizole has inhibitory effects on the fast Na^+ and slow Ca^{2+} currents of the membrane independent of the blockade of myocardial α-adrenoceptors, and that these effects may be significant in some clinical uses of the drug.

Keywords: α-adrenoceptor antagonist; cardiac muscle; action potentials; voltage-dependent block

Introduction

Several α_1- and α_2-adrenoceptor antagonists, such as prazosin, AR-C 239, yohimbine and bunazosin have a suppressive effect on various experimentally induced arrhythmias (Sheridan *et al.*, 1980; Lechat & Schmitt, 1982; Thandroyen *et al.*, 1983; Kimura *et al.*, 1984; Thomas & Tripathi, 1986; Mugelli *et al.*, 1986). Northover (1983) considered the antiarrhythmic effects of prazosin, yohimbine, tolazoline, dibenamine and phenoxybenzamine to be produced by interaction with the cell membrane and not by direct inhibition of the α-adrenoceptors. Hasegawa *et al.* (1988) described the suppressive effect of α-adrenoceptor antagonists on transient depolarization and triggered activity, and its protective effect against further deterioration of abnormal automatic activity during hypoxia. They also suggested that bunazosin and yohimbine exert not only α_1- or α_2-adrenoceptor antagonistic effects but also a blocking effect on the fast Na^+ and slow Ca^{2+} channels in a manner similar to slow kinetic antiarrhythmic drugs used in the case of *in vitro* arrhythmia. On the other hand, the inhibitory effects on membrane currents of antiarrhythmic drugs occasionally exert a proarrhythmic effect in clinical use (Levine *et al.*, 1989; Stanton *et al.*, 1989), and produce potentially suppressive effects on cardiac muscle contraction (Hammermeister *et al.*, 1972; Sheu & Lederer, 1985; Hoffmeister *et al.*, 1987).

Midaglizole is a newly synthesized hypoglycaemic drug (Kameda *et al.*, 1981; 1982a) which antagonizes the α_2-adrenoceptors (Yamanaka *et al.*, 1984, 1985) of pancreatic β-cells (Kameda *et al.*, 1982b). The present experiments were performed in order to determine whether midaglizole inhibits the fast Na^+ and slow Ca^{2+} channels in guinea-pig cardiac muscle and if so, whether the cardiac action of the drug represents a potential side-effect in its clinical use as a hypoglycaemic drug.

Methods

Guinea-pigs (200–300 g) were killed by a blow on the head. The papillary muscles (2.5–5 mm in length and 0.8–1.0 mm in diameter) were rapidly excised from the right ventricle and mounted in a tissue bath perfused with oxygenated (95% O_2, 5% CO_2) Tyrode solution (pH 7.4 ± 0.05). The bath temperature was continuously monitored with an electric thermometer (Nihon Kohden MGA-III) and maintained at 36.0 ± 0.2°C. The flow rate was kept constant at a rate of 3 ml min^{-1} with a micro-tube pump (Tokyo Rikakikai, MP-3). The preparations were fixed on a silicon rubber floor in the bath by means of stainless steel pins. Muscles were driven by rectangular current pulses through a pair of Ag-AgCl electrodes placed parallel with the preparations and connected to a stimulator (Nihon Kohden SEN-3201) through an isolator (Nihon Kohden SS-102J).

Membrane potentials (E_m) were measured differentially with two microelectrodes filled with 3 M KCl (resistance 10–15 MΩ) which led to an amplifier (Nihon Kohden MEZ-7101). Analog differentiation of the action potentials was obtained with an electric differentiator that was linear from 0 to 500 V s^{-1} (time constant usually, 10 μs, and 50 μs for slow action potentials). Action potentials and the maximum rate of rise ($\dot{V}_{max}$) of the action potentials were monitored on an oscilloscope (Nihon Kohden VC-10), photographed by a camera (Nihon Kohden RLG-6201), and simultaneously recorded on a chart recorder (Nihon Kohden RJG-4122). The muscles were stimulated at a rate of 1 Hz (voltage 1.5 times threshold, duration 2 ms) during the period of equilibration after mounting and during analysis of the steady-state effect of the drugs. Stimulation rate and mode were varied for each protocol.

Normal Tyrode solution had the following composition in mM: NaCl 132, KCl 4, $NaHCO_3$ 12, NaH_2PO_4 0.4, $CaCl_2$ 1.8, $MgCl_2$ 1 and glucose 10. The high K^+ solution for induction of the slow response contained 27 instead of 4 mM KCl and 0.2 mM $BaCl_2$ in the normal Tyrode solution.

To study the relationship between membrane potential (E_m) and $\dot{V}_{max}$, the membrane was depolarized by increasing the concentration of KCl in Tyrode solution from 2.7 to 5.4, 8.1, 10.8, 13.5, and 14.9 mM. A single stimulation was applied with each new solution to avoid contamination of use-dependent drug effects on $\dot{V}_{max}$ (Kohlhardt & Seifert, 1980; Gintant *et al.*, 1983). The following equation proposed by Windisch & Tritthart (1981) was used to obtain the experimental values for curve fitting

$$\dot{V}_{max} = V_s/(1 + \exp[(E_m - E_f)/S])^2$$

where V_s represents the saturation value (maximum value) of $\dot{V}_{max}$, E_f the membrane potential at which $\dot{V}_{max}$ is one quarter of V_s and S the slope factor. These values were obtained by the least squares method. The validity of this method to show the $\dot{V}_{max}$–E_m relationship of guinea-pig ventricular muscle has been verified previously (Kiyosue & Arita, 1982; Hisatome *et al.*, 1985; Hasegawa *et al.*, 1988).

[1] Author for correspondence.

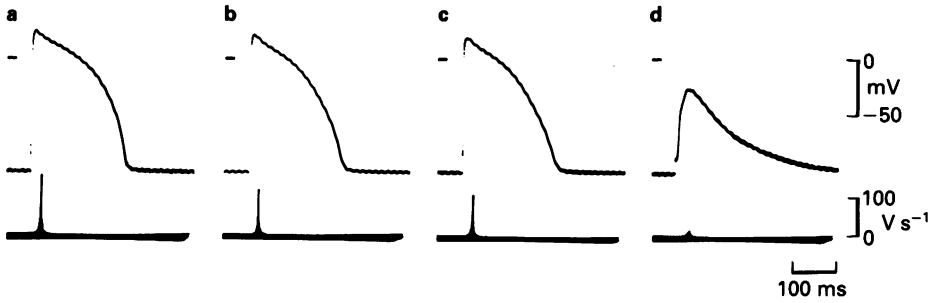

Figure 1 Depressant effect of midaglizole on the steady-state fast action potential. Action potentials during stimulation at a constant rate of 1 Hz in control (a), in the presence of 10 (b), 100 (c), and 1000 μM (d) midaglizole are shown. The bottom line in each panel shows the maximal rate of rise of action potentials ($\dot{V}_{max}$). In this and the following figures, the short horizontal lines preceding the action potential traces indicate the 0 mV level.

Midaglizole, (2-[2-(4,5-dihydro-1H-imadazol-2-yl)-1-phenylethyl] pyridine dihydrochloride sesquihydrate) was added to the solution just before each experiment. To avoid the interference of endogenous noradrenaline, the catecholamine content in the heart was lowered by pretreating the guinea-pigs subcutaneously with reserpine (Daiichi) 2.5 mg kg^{-1} one day before they were killed.

Statistical analysis

All experimental results were obtained from a single continuous impalement throughout the experiment. Values are given as mean ± s.e.mean. Statistical analysis was by Student's *t* test. Results were considered significant at the level $P < 0.05$.

Results

Effects of midaglizole on the steady-state normal action potentials stimulated at 1 Hz

Figure 1 shows typical experimental results of the effects of midaglizole on the steady-state action potentials. Midaglizole decreased $\dot{V}_{max}$ and the amplitude of the action potentials (APA) in a dose-related manner. The dose-response relationships for the effects of midaglizole on the parameters of the action potentials are illustrated in Figure 2. $\dot{V}_{max}$ and APA were reduced from the control values of 215 ± 14 V s^{-1} and 131 ± 1 mV, respectively, without any changes in the resting potential (−87.8 mV in control). The action potential duration measured at a 90% repolarization level (APD_{90}) was prolonged from the control value of 200 ± 12 ms.

Voltage-dependent effect of midaglizole on $\dot{V}_{max}$

The effect of 10 μM midaglizole on the $\dot{V}_{max}$–E_m relation was examined by changing the K^+ concentration of Tyrode solution to alter the E_m. $\dot{V}_{max}$ and E_m were measured by a single action potential at each K^+ concentration in 5 preparations (Figure 3a), and the mean value was normalized (see Figure 3b). The smooth curves were drawn by computer according to the equation of Windisch & Tritthart (1981). As shown in Figure 3b, midaglizole did not shift h_∞ in contrast to the significant reduction of $\dot{V}_{max}$ at each K^+ concentration (therefore, in each membrane potential) in the presence of midaglizole (Figure 3a).

Effects of midaglizole on the slow action potentials

Figures 4 and 5 show the inhibitory effects of midaglizole on the Ca^{2+}-dependent slow action potentials. At concentrations higher than 10 μM, midaglizole reduce the $\dot{V}_{max}$, overshoot of the slow action potentials and prolonged the APD_{90} compared to each of the control values of 6.9 ± 0.6 V s^{-1}, 67.8 ± 1.7 mV and 186 ± 19 ms, respectively. The resting

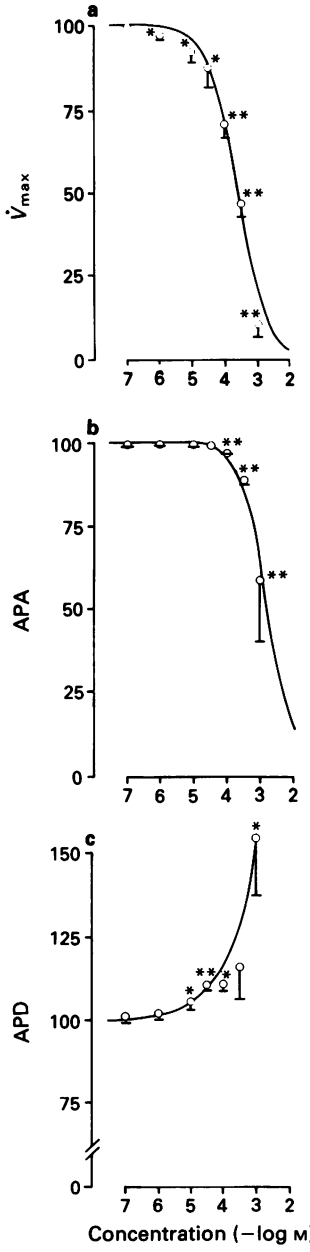

Figure 2 Dose-response relationships of the effects of midaglizole on the parameters of the steady state action potentials. The ordinates are the maximal rate of rise of action potentials ($\dot{V}_{max}$, a), the action potential amplitude (APA, b), and the action potential duration at 90% repolarization level (APD, c). The abscissa scale indicates the concentration of midaglizole. Each value is plotted as % of control value. Each point represents the mean value, and vertical bars are s.e.mean of 5 experiments. * $P < 0.05$; ** $P < 0.01$.

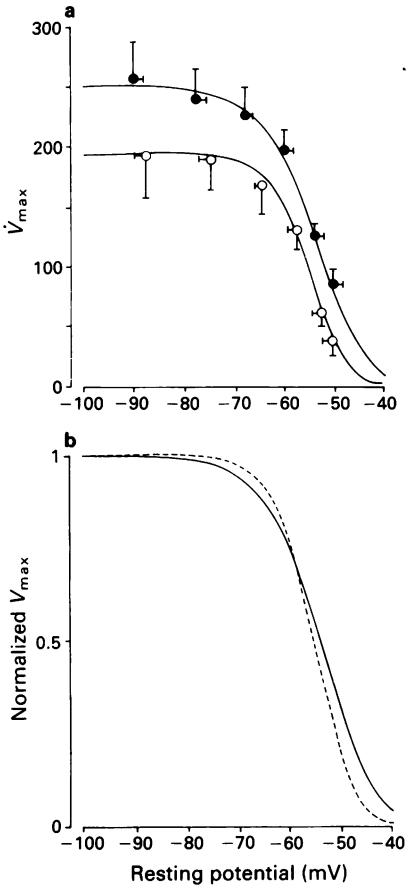

Figure 3 Effect of midaglizole on the relationship between $\dot{V}_{max}$ and resting membrane potential (E_m) at different K^+ concentrations: (●) control values; (○) values in the presence of 10 μM midaglizole in (a). Each measurement was made during a single stimulus at each K^+ concentration. Each value represents the mean of 4 experiments, and horizontal and vertical bars show s.e.mean. In (b) are shown the normalized curves for the values in (a). Broken line represents the curve in the presence of midaglizole. Value of 1.0 on the ordinate scale corresponds to the maximum value of $\dot{V}_{max}$ in each condition.

potential in the high K^+ solution was not influenced by midaglizole (−41.5 mV in control).

Discussion

Previous studies showed that the $\dot{V}_{max}$ of steady-state action potentials of Purkinje fibres, sinoatrial node or ventricular muscle cells is suppressed by prazosin (Dukes & Vaughan Williams, 1984; Rosen *et al.*, 1984), phentolamine (Ledda & Marchetti, 1971; Northover, 1983), yohimbine (Azuma *et al.*, 1978; Rosen *et al.*, 1984; Briggs & Meier, 1986) and by urapidil, a new α_1-adrenoceptor antagonist (Gulch *et al.*, 1986; Kotake *et al.*, 1988). Hasegawa *et al.* (1988) demonstrated that α-adrenoceptor antagonists produce inhibitory effects on the

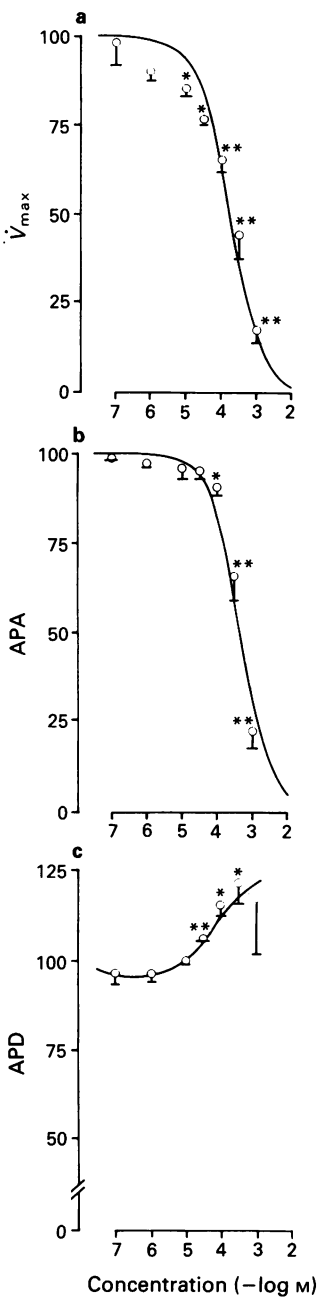

Figure 5 Dose-response relationships of the effects of midaglizole on parameters of the slow action potential: (a) $\dot{V}_{max}$; (b) APA: action potential amplitude; (c) APD: action potential duration measured at 90% repolarization. Each point represents the mean value, and vertical bars are s.e.mean of 5 experiments. $^*P < 0.05$; $^{**}P < 0.01$.

fast Na^+ and slow Ca^{2+} currents in exerting their antiarrhythmic effects, in particular on the arrhythmias induced by abnormal automaticity or triggered activity.

Midaglizole has been reported to have an antagonistic effect

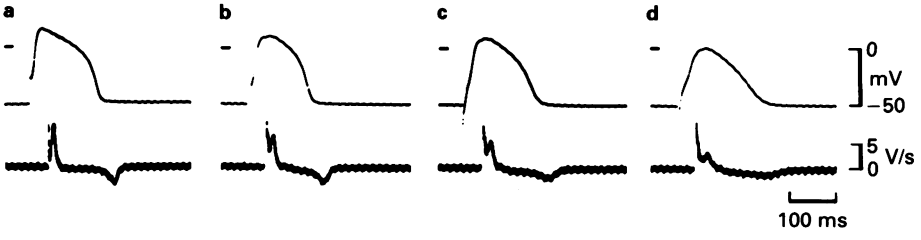

Figure 4 Effect of midaglizole on the slow action potential. Ca^{2+}-dependent slow action potential and $\dot{V}_{max}$ of partially depolarized muscle in control (a), in the presence of 30 (b), 100 (c) and 300 μM (d) midaglizole are shown.

against α_2-adrenoceptors (Kameda *et al.*, 1982a,b; Yamanaka *et al.*, 1984) or the low affinity state of α_2-adrenoceptors (Yamanaka *et al.*, 1985) and to reverse the α-adrenergic inhibition of insulin release from the pancreatic islet cells (Kameda *et al.*, 1982b). This effect on insulin secretion from the pancreas which in turn reduces the serum glucose level has been recognized in basic (Kameda *et al.*, 1981; 1982a,b) and clinical studies (Kashiwagi *et al.*, 1986; Kawazu *et al.*, 1987a,b).

In the present study, suppression of $\dot{V}_{max}$ of the fast and slow action potentials measured as indices of the fast Na^+ and slow Ca^{2+} (Ehara & Inazawa, 1980; Inazawa *et al.*, 1982) currents of the membrane by midaglizole were shown. Since the experiments were performed on the papillary muscles of guinea-pigs pretreated with reserpine without the application of catecholamines even for the induction of the slow responses, the inhibitory effects on the membrane currents by midaglizole might be independent of the blockade of cardiac α-adrenoceptors. As for the fast Na^+ channel inhibition, the lack of voltage-dependent block of $\dot{V}_{max}$ of the action potential may indicate that midaglizole is able to block the Na^+ channel in a manner that is not voltage-dependent. In this it differs from yohimbine, bunazosin and other antiarrhythmic drugs which decreased the $\dot{V}_{max}$ of the action potential in a use-dependent and voltage-dependent manner. This unique property of midaglizole requires further investigation with a voltage clamp technique.

Kawazu *et al.* (1987b) reported the mean plasma levels of midaglizole (approximately 6.0 μM) at the time of the maximum hypoglycaemic effect of the drug 1.0–1.5 h after oral administration at doses of 200 mg three times daily. This suggests the possibility that midaglizole may show a depressant effect on the membrane activities not only in the presence of extraordinary conditions, such as during the suppression of electrical activity by ischaemia or drugs, but also under normal conditions. However, the lack of dependency on the membrane potential of the inhibitory effect of midaglizole on $\dot{V}_{max}$ of the fast action potentials, indicates the lack of enhancement of the inhibitory effect by various kinds of damage-induced small depolarizations.

These effects of midaglizole on the cardiac membrane currents, may not necessarily indicate a lack of clinical usefulness. Other useful hypoglycaemic drugs, such as the sulphonylurea drugs, have been shown to have significant effects on cardiac activities in clinical concentrations without causing significant clinical problems (Lasseter *et al.*, 1972; Scientific Advisory Panel of the Executive Committee, 1979).

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(Received May 29, 1990
Revised December 17, 1990
Accepted February 8, 1991)

Br. J. Pharmacol. (1991), **103**, 1561–1567

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Modulation of the structure-binding relationships of antagonists for muscarinic acetylcholine receptor subtypes

E. Kristina Pedder, Phike Eveleigh, [1]David Poyner, Edward C. Hulme & [2]Nigel J.M. Birdsall

Division of Physical Biochemistry, National Institute for Medical Research, Mill Hill, London, NW7 1AA

1 Membranes from rat cerebral cortex, myocardium and extraorbital lacrimal gland were used as sources of M_1, M_2 and M_3 muscarinic acetylcholine receptors respectively and the affinities of seven antagonists for the three subtypes were examined under different experimental conditions.

2 The affinities for the membrane-bound receptors were measured at different ionic strengths and temperatures and compared with those determined on the receptor solubilised in the neutral detergent digitonin or the zwitterionic detergent, CHAPSO.

3 The range of measured affinity constants of a given antagonist for a specific subtype varied from 2 (atropine at M_1 receptors) to 1000 (AF-DX 116 at M_2 receptors).

4 As a consequence of these changes in affinity, which were dependent on the drug, the subtype and the experimental conditions, both the structure-binding relationships of a given subtype can be markedly changed as well as the selectivity of a drug for the different subtypes. For example it is possible to change the relative affinities of AF-DX 116 and gallamine at membrane-bound M_1 receptors from 50:1 to 1:60.

5 Experimental conditions for the observation of high selectivity of pirenzepine, AF-DX 116, gallamine and hexahydrosiladiphenidol for the three subtypes are given.

6 When the receptors are removed from their membrane environment by solubilisation in detergent, antagonist affinities are changed but the subtypes still retain different structure-binding relationships.

7 In general, AF-DX 116 and the allosteric antagonist, gallamine, behave differently from the other antagonists, suggesting that they bind in different ways to muscarinic receptors.

8 Careful attention should therefore be paid to the experimental conditions in binding assays used to assess the affinities and selectivities of new muscarinic antagonists in order to avoid misleading results.

9 The ability to produce enhanced or attenuated affinities and selectivities of antagonists, resulting from the induction of different conformations of the receptor by a variety of physical, chemical or molecular biological perturbations may lead to a better understanding of the structural basis of drug receptor interactions.

Keywords: Muscarinic receptors; muscarinic receptor subtypes; pirenzepine; gallamine; ligand binding; modulation; receptor modulation; structure-binding relationships

Introduction

Muscarinic receptor subtypes may be defined on molecular, pharmacological and functional criteria. To date, five molecular species of muscarinic acetylcholine receptors have been cloned and expressed but only three pharmacological subtypes have been identified with a degree of confidence, (for a review see Hulme *et al.*, 1990). A basic problem is the assignment of a well-defined pharmacological profile to each of the molecular species. This is complicated by the fact that the binding properties of muscarinic receptor subtypes are very similar and no single drug can be used to define a specific subtype. In addition, the binding properties of muscarinic receptors are sensitive to their environment so that the binding properties of a cloned receptor, transfected into an unusual cell line, such as a fibroblast, may be different from that found *in vivo* or *in vitro*. Indeed, there is a considerable variability between laboratories in the reported binding properties of expressed muscarinic receptor subtypes (reviewed in Hulme *et al.*, 1990).

We have previously determined the binding properties of muscarinic receptors in rat cerebral cortex, heart and lacrimal gland, after solubilisation in the zwitterionic detergent 3-(3-cholamidopropyl)dimethylammonio-2-hydroxy-1-propane-sulphonate (CHAPSO) (Poyner *et al.*, 1989a). In this paper we describe the antagonist binding properties of muscarinic receptors from the three tissues, when the receptors are solubilised in the neutral detergent digitonin, and when the membrane-bound receptors are perturbed by changes in ionic strength or temperature.

The structure-binding relationships for the receptors present in the three tissues can be altered such that drug selectivity may be enhanced considerably or attenuated. Some of the data have been published in preliminary form (Berrie *et al.*, 1986; Birdsall *et al.*, 1986b; 1987).

Methods

EDTA-washed membranes from rat cerebral cortex were prepared as previously described (Hulme *et al.*, 1983). The preparation of EDTA-washed membranes from extraorbital lacrimal glands and myocardium was as described by Berrie *et al.* (1984) for myocardial membranes except the KCl/sodium pyrophosphate treatment was omitted. The rapidly frozen membranes were stored at −20°C or −80°C. Solubilization of the membranes (4–6 mg ml^{-1}) in digitonin (1%) at 4°C was carried out as described previously (Berrie *et al.*, 1985). The yield of solubilized receptor varied from 20%–45% depending on the tissue/subtype solubilized.

The membrane binding studies were carried out in a high ionic strength buffer of 100 mM NaCl/10 mM $MgCl_2$/20 mM Na HEPES, pH 7.5 (in some experiments the magnesium chloride was omitted but this did not affect the measured antagonist affinities) or a low ionic strength buffer, 1 mM $MgCl_2$/20 mM Na HEPES, pH 7.5.

[1] Present address: MRC Molecular Neurobiology Unit, University of Cambridge Medical School, Cambridge, CB2 2QH.

[2] Author for correspondence.

The routine membrane binding assays were carried out by a microcentrifugation assay (Eveleigh *et al.*, 1989), with non-specific binding being defined as the radioactivity measured in the pellet when the radioligand was coincubated with 1 μM 3-quinuclidinylbenzilate. Incubation times at 30°C were, in general, 45 min (M_2 receptors) and 2 h (M_1 and M_3 receptors) but were extended to 3–4 h for inhibition experiments involving gallamine and telenzepine in which there are slow kinetic binding processes. For competition experiments carried out at 4°C, the routine procedure was to preincubate the membranes with the nonradioactive antagonist for 24 h (4 days in the case of telenzepine) prior to adding the ^{3}H-ligand. Incubation at 4°C continued for 24 h (4–7 days in the case of gallamine) before assaying for bound radioactivity.

The soluble receptor binding assays used a similar protocol to that for the membrane binding assays except the separation of free and bound radioligand was accomplished by gel filtration on 2 ml columns of Sephadex G50M (Berrie *et al.*, 1984). All assays were carried out at 4°C in a 1 mM Mg^{2+}/20 mM Na HEPES buffer, pH 7.5 containing 1% digitonin. Reconstitution experiments were carried out as previously described (Poyner *et al.*, 1989b).

As a check of whether the binding processes in competition experiments were in equilibrium at 4°C, the receptors (soluble or membrane bound) were incubated with two concentrations of (−)-[^{3}H]-N-methylscopolamine ([^{3}H]-NMS, generally 0.3 and 3 nM) and the ratio of the two observed IC_{50} values for the antagonist compared with the predicted ratio of $(1 + K_N[N]_1)/(1 + K_N[N]_2)$ where K_N is the affinity constant of [^{3}H]-NMS and $[N]_1$ and $[N]_2$ are the two concentrations of [^{3}H]-NMS. For most antagonists, there was a good agreement between observed and predicted ratios. The exceptions were gallamine (because of its allosteric interactions) and telenzepine, where the observed ratio was ~1 for M_1 and M_3 receptors. In the case of telenzepine, the low ratio was caused by the slow off-rate of telenzepine. That is, the [^{3}H]-NMS was just monitoring the binding to the unliganded receptor.

For competition assays, the concentration of [^{3}H]-NMS, the radioligand used in all experiments except those on M_1 receptors in membranes, was ca 0.3 nM and that of [^{3}H]-pirenzepine, 1 nM. In the [^{3}H]-NMS saturation binding assays, the concentrations of radioligand (7–12) generally covered a 1,000 fold range about the dissociation constant.

All data were analysed by non-linear least squares analysis using an appropriate model (saturation, competition, allosteric) (Hulme *et al.*, 1978; Stockton *et al.*, 1983). With the exception of gallamine, all the inhibition curves fitted well to a simple 1-site model. The Hill coefficients for individual curves were generally in the range 0.9–1.1. In no data set for an antagonist binding under a specific condition was the fit improved significantly and consistently by analysis of each individual curve using a 2-site model. Values for log affinity constants generally had estimated errors of ±0.02–0.07 log unit, these low values confirming the quality of the fit and appropriateness of the model. The gallamine ^{3}H-radioligand inhibition curves were analysed by use of the equation:

$$I = \frac{[G](K_1 - K_2)}{1 + K_N[N] + [G](K_1 + K_2 \cdot K_N[N])}$$

where I is the fractional inhibition of ^{3}H-radioligand binding, [G] and [N] the concentrations of gallamine and radioligand, K_N, the known affinity constant of the radioligand for the unliganded receptor and K_1 and K_2 are the affinity constants of gallamine for the unliganded and radioligand-occupied receptors respectively. Non-linear least squares analysis provided estimates of K_1 and K_2, the ratio K_1/K_2 being the negative cooperativity of gallamine (and radioligand). In competition assays with [^{3}H]-NMS as radioligand, the apparent affinity constants (except for telenzepine at 4°C) were corrected for occupancy of the receptors by [^{3}H]-NMS, to give the true affinity constant.

(−)-[^{3}H]-N-methylscopolamine ([^{3}H]-NMS) and [^{3}H]-pirenzepine were obtained from Amersham International and NEN respectively. The following antagonists were generous gifts: pirenzepine (Dr R. Hammer, Boehringer, Ingelheim), AF-DX 116 (11[[2-[(diethylamino)methyl]-1-piperidinyl]-acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine (Dr W. Eberlein, Dr Karl Thomas GmbH), hexahydrosiladiphenidol (Prof. G. Lambrecht, University of Frankfurt) and (±)-telenzepine (Dr C. Schudt, Byk Gulden GmbH). All other materials were of the highest possible grade.

Results

Three tissues from the rat, the cerebral cortex, myocardium and extraorbital lacrimal gland, were chosen as sources of the M_1, M_2 and M_3 muscarinic receptor subtypes respectively (for the nomenclature, see Levine & Birdsall, 1989 and Watson & Abbott, 1990). In the case of the cerebral cortex, muscarinic receptors other than M_1 are also present (Hammer *et al.*, 1980). However, M_1 receptors can be labelled selectively by low concentrations of [^{3}H]-pirenzepine and hence the properties of these receptors were examined in competition studies with this radioligand. The other two tissues are relatively pure sources of the M_2 and M_3 subtypes. In these cases, a selective labelling strategy was not required and [^{3}H]-NMS was used as the radioligand.

The incubation conditions were chosen so as to examine the effects of temperature and change in ionic composition on antagonist binding, as well as to facilitate comparison of the binding properties of the membrane-bound and soluble receptors. In soluble preparations, we found that the receptors in a 1 mM Mg^{2+}/20 mM HEPES buffer at 4°C are stable for the long incubation periods of 24–96 h which are sometimes necessary for equilibrium binding to be attained (Berrie *et al.*, 1985; Birdsall *et al.*, 1986a; Poyner *et al.*, 1989a). In order to 'bridge' the differences between the measurement of the binding properties of the membrane bound receptors in a buffer of approximately physiological ionic strength (100 mM NaCl/10 mM Mg^{2+}) at 30°C and the measurement on soluble receptors at 4°C, the antagonist binding properties of the membrane-bound receptors were also measured at 4°C in the high and low ionic strength buffers and in the case of M_2 receptors at 30°C in the 1 mM Mg^{2+} buffer. The results are summarized in Tables 1–3. Some additional experiments were carried out in a 100 mM NaCl/20 mM HEPES buffer at 30°C and 4°C but the omission of Mg^{2+} produced no detectable differences in antagonist affinities.

The antagonists examined were the 'non-selective' antagonists, atropine and N-methylscopolamine, the M_1 selective antagonists pirenzepine and (±)-telenzepine, the M_2 selective antagonists, AF-DX 116 and gallamine, and hexahydrosiladiphenidol which shows an element of M_3 selectivity. In the case of gallamine, attention was given to its known allosteric interactions with 'conventional' competitive antagonists (Stockton *et al.*, 1983) in both the design and analysis of the binding experiments.

The affinity constants for these antagonists measured at 30°C agree very closely with the log mean values collated by Hulme *et al.* (1990) and with published values for (±)-telenzepine and gallamine (Schudt *et al.*, 1989; Stockton *et al.*, 1983) [difference 0.04 ± 0.11 log unit, mean ± s.d., $n = 21$].

A number of the antagonists examined are racemates. Where it is known that there is a high enantioselectivity, the data are expressed in terms of the affinity of the more potent enantiomer ((−)-atropine, (+)-telenzepine) but not when the stereoselectivity is low (AF-DX 116, unpublished results) or not known (hexahydrosiladiphenidol).

Antagonist binding to cortical M_1 receptors (Table 1)

The membrane binding studies were carried out as competition experiments with [^{3}H]-pirenzepine to label selectively

Table 1 The binding of antagonists to rat cortical M_1 receptors

	100 mM NaCl/10 mM Mg^{2+} 30°C	100 mM NaCl/10 mM Mg^{2+} 4°C	1 mM Mg^{2+} 4°C		
Antagonist	*Membranes*	*Membranes*	*Membranes*	*Digitonin*	*CHAPSO*[1]
N-methylscopolamine	10.0	10.3	10.5	10.0	9.6[2]
(−)-Atropine	9.4	+9.5	+9.6	9.5	9.5
Pirenzepine	8.0	8.0	8.2	7.7	7.3[2,3]
(+)-Telenzepine	9.5	9.4	9.6	ND	ND
AF-DX 116	6.5	5.8	5.6	5.5	4.6
Hexahydrosiladiphenidol	7.8	7.9	8.2	7.9[2]	6.9
Gallamine[4]	4.8[5]	6.3[5]	7.4[5]	6.8[6]	5.0

Values are log affinity constants.
[1] Data from Poyner *et al.* (1989b).
[2] 0.1 < s.e.mean < 0.2, s.e.mean < 0.1 for other data in table (n = 2–8). For n = 2 (data prefixed with +), the range of estimated values was less than 0.2.
[3] High affinity site.
[4] Affinity of gallamine for the unliganded receptor.
[5] Negative cooperativity with pirenzepine, 40–60.
[6] Negative cooperativity with NMS, 26–30.
ND = not determined.

the M_1 receptors. In accord with this premise, inhibition curves for all the antagonists except gallamine approximated closely to simple binding isotherms.

The effect of lowering the temperature of incubation in the higher ionic strength medium was in general to produce a zero or small increase in affinity. However the affinity of AF-DX 116 decreased strongly (five fold) whereas the affinity of gallamine for the unliganded receptor increased 30 fold. A further 12 fold increase in gallamine affinity was produced by changing to the 1 mM Mg^{2+} buffer system (Figure 1a). In contrast to the large changes in gallamine affinity for the unliganded receptor, the estimates of the negative heterotropic cooperativity between pirenzepine and gallamine did not vary (range 40–60). This suggests that the changes in the gallamine binding site do not affect the binding of pirenzepine to the gallamine-receptor complex or to the unliganded receptor. Conversely, the changes at the gallamine binding site produced by the different incubation conditions are not affected by the binding of pirenzepine to the receptor.

When cortical receptors were solubilized in digitonin, the [^{3}H]-NMS-pirenzepine competition curve approximated to a mass action curve with a pirenzepine affinity of $(5 \pm 1) \times 10^7\,M^{-1}$. This value is close to that found in previous studies when pirenzepine binding to soluble M_1 receptors was measured directly with the ^{3}H-ligand (Berrie *et al.*, 1985). It therefore appeared that, in these experiments, most of the receptor sites solubilized (≥80%) were M_1 receptors which is on the high side of the 40–80% range of values estimated by Berrie *et al.* (1985). Therefore [^{3}H]-NMS, because of its 200 fold higher affinity than [^{3}H]-pirenzepine, was used as the radioligand in the competition experiments and it was assumed that it was monitoring predominantly the binding of antagonists to M_1 receptors.

The effect of digitonin solubilization was in general to produce a slight decrease (up to three fold) in antagonist affinity compared to values measured under the same conditions but in the absence of digitonin. The exception was the almost 10 fold decrease in the affinity of gallamine for the unliganded receptor (Figure 2). The value for (+)-telenzepine could not be determined because of its very slow on and off-rates under these conditions (data not shown). A minimum log K_A value of 8.5 could be estimated.

Solubilization in CHAPSO in general produced approximately 10 fold decreases in affinity of most antagonists. The affinity of gallamine decreased 250 fold but that of atropine was unchanged. As in membranes from the cerebral cortex, pirenzepine exhibited heterogeneous binding characteristics and the high affinity binding component was assumed to arise from M_1 receptors.

Antagonist binding to heart M_2 receptors (Table 2)

In the higher ionic strength buffer, the effect of changing the incubation temperature from 30°C to 4°C was to produce only small changes in affinity, generally an increase. As with M_1 receptors, there was an approximately three fold increase in atropine affinity and a very considerable increase in galla-

Table 2 The binding of antagonists to rat myocardial (M_2) receptors

	100 mM NaCl/10 mM Mg^{2+} 30°C	100 mM NaCl/10 mM Mg^{2+} 4°C	1 mM Mg^{2+} 30°C	1 mM Mg^{2+} 4°C		
Antagonist	*Membranes*	*Membranes*	*Membranes*	*Membranes*	*Digitonin*	*CHAPSO*[1]
N-methylscopolamine	9.3	9.7	9.6	9.9	9.7	9.4
(−)-Atropine	9.1	+9.3	ND	+9.4	9.2[2]	8.9
Pirenzepine	6.1	6.1	6.3	6.1	7.8	5.7[2]
(+)-Telenzepine	7.8	8.0	8.2	7.7	8.9	ND
AF-DX 116	7.3	7.2	6.8	6.5	5.8	4.3[2]
Hexahydrosiladiphenidol	6.7	6.6	6.9	6.4	7.8	6.3[2]
Gallamine[3]	6.1[4]	7.3[4]	7.4[4]	8.0[4]	7.6[4]	6.0[2,4]

Values are log affinity constants.
[1] Data from Poyner *et al.* (1989b).
[2] 0.1 < s.e.mean < 0.2, s.e.mean < 0.1 for other data in table (n = 2–7). For n = 2 (data prefixed with +), the range of estimates values was less than 0.1.
[3] Affinity of gallamine for the unliganded receptor.
[4] Negative co-operativity with NMS, 10–30.
ND = not determined.

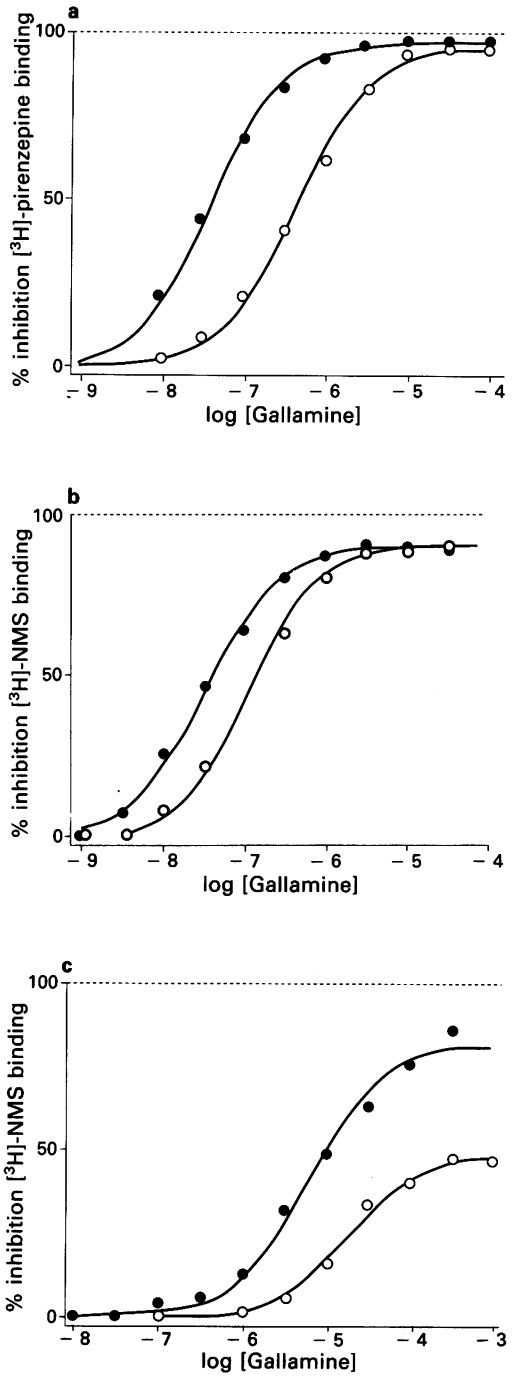

Figure 1 The effect of change in ionic conditions on the binding of gallamine to muscarinic receptor subtypes. Gallamine binding was measured in inhibition experiments with [^{3}H]-pirenzepine (M_1 receptors, cortex, a) or (−)-[^{3}H]-N-methylscopolamine ([^{3}H]-NMS) (M_2 receptors, heart, b and M_3 receptors, lacrimal gland, c) at 4°C in a 1 mM Mg^{2+} buffer, (●) or a 100 mM NaCl buffer (○). The incubation time was extended to 7 days in order that the part of the inhibition curve at high concentrations of gallamine be in equilibrium. The curve at less than 75% maximal inhibition was in equilibrium at 24 h and essentially identical to that found at 7 days. The data are from single experiments but are representative of 3–5 repeats. In all cases, high concentrations of gallamine failed to inhibit 100% of the specific radioligand binding (dashed line) as predicted by the allosteric model and equation used to analyse the data (see Experimental section). Non-linear least squares analyses of these data gave the following best fit parameters:

(a) M_1 receptors

1 mM Mg^{2+}: log $K_1 = 7.44 \pm 0.12$,
log $K_2 = 5.80 \pm 0.08$, $K_1/K_2 = 44$
100 mM NaCl: log $K_1 = 6.43 \pm 0.04$,
log $K_2 = 4.99 \pm 0.19$, $K_1/K_2 = 28$

The concentration of [^{3}H]-pirenzepine was 1.0 nM and its log affinity constant for the unliganded receptor 8.0 (○) and 8.2 (●).

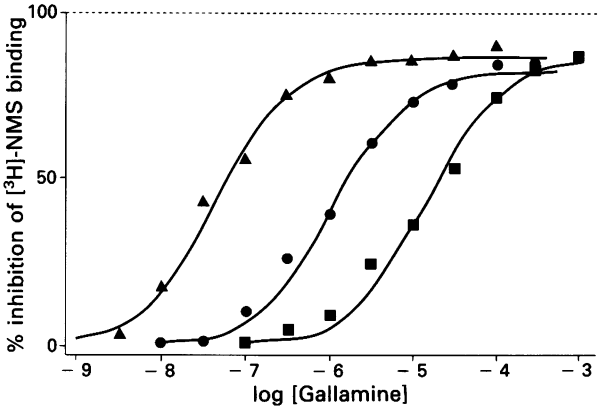

Figure 2 The binding of gallamine to muscarinic receptor subtypes solubilized in digitonin. Gallamine binding was measured at 4°C in the 1 mM Mg^{2+} buffer in inhibition experiments with (−)-[^{3}H]- N-methylscopolamine ([^{3}H]-NMS, 0.26–0.28 nM) as radioligand using digitonin solubilized muscarinic receptors from rat cerebral cortex (●), myocardium (▲) and lacrimal gland (■). The incubation time was extended to 7 days in order that the part of the inhibition curve at high concentrations of gallamine be in equilibrium. The data are from single experiments but are representative of 2–5 repeat experiments. The inhibition curves failed to reach 100% inhibition at high concentrations of gallamine (dashed line) and the data were analysed by the simple allosteric model and equation (Methods section) with log $K_N = 10.0$ (M_1), 9.7 (M_2) and 9.4 (M_3).

The best fit parameters are:

M_1 (●) log $K_1 = 6.54 \pm 0.05$,
log $K_2 = 5.27 \pm 0.08$, $K_1/K_2 = 17$
M_2 (▲) log $K_1 = 7.72 \pm 0.05$,
log $K_2 = 6.53 \pm 0.07$, $K_1/K_2 = 15$
M_3 (■) log $K_1 = 5.05 \pm 0.07$,
log $K_2 = 4.03 \pm 0.14$, $K_1/K_2 = 10$

mine potency. However the five fold decrease in AF-DX 116 affinity, found for M_1 receptors, was not observed.

The effect of lowering the ionic strength at 30°C or 4°C was in general a small increase in affinity with the exception of a 3–5 fold decrease in AF-DX 116 affinity and a substantial (5–20 fold) increase in gallamine potency (Figure 1b).

Solubilization of M_2 receptors produced some large changes in the structure-binding relationships of the antagonists examined. In particular, digitonin solubilization increased pirenzepine affinity 50 fold, as reported previously (Berrie *et al.*, 1985). The affinity of (+)-telenzepine and hexahydrosiladiphenidol were also increased over 10 fold, whereas AF-DX 116 affinity decreased 5 fold. These increases in affinity were not observed on solubilization in CHAPSO, dramatic decreases in the affinity of AF-DX 116 and gallamine (100 fold) being observed. In fact all antagonists, except hexahydrosiladiphenidol, bound more weakly in this detergent.

The increase in pirenzepine affinity on solubilization in digitonin could be reversed by reconstitution of the soluble recep-

(b) M_2 receptors

1 mM Mg^{2+}: log $K_1 = 8.04 \pm 0.03$,
log $K_2 = 6.57 \pm 0.06$, $K_1/K_2 = 30$
100 mM NaCl: log $K_1 = 7.33 \pm 0.03$,
log $K_2 = 5.97 \pm 0.04$, $K_1/K_2 = 23$

The concentration of [^{3}H]-NMS was 0.31 nM and its log affinity constant for the unliganded receptor was 9.7 (○) and 9.9 (●).

(c) M_3 receptors

1 mM Mg^{2+}: log $K_1 = 5.85 \pm 0.06$,
log $K_2 = 4.47 \pm 0.11$, $K_1/K_2 = 24$
100 mM NaCl: log $K_1 = 5.09 \pm 0.04$,
log $K_2 = 4.45 \pm 0.05$, $K_1/K_2 = 4$

The concentration of [^{3}H]-NMS was 0.26 nM and its log affinity constant for the unliganded receptor was 10.0 (○) and 10.2 (●).

Table 3 The binding of antagonists to rat lacrimal (M_3) receptors

	100 mM NaCl/10 mM Mg^{2+} 30°C	100 mM NaCl/10 mM Mg^{2+} 4°C	1 mM Mg^{2+} 4°C		
Antagonist	*Membranes*	*Membranes*	*Membranes*	*Digitonin*	*CHAPSO*[1]
N-methylscopolamine	9.8	10.0	10.2	9.4	8.9
(−)-Atropine	9.5	9.9	10.0	+8.9	8.7
Pirenzepine	6.6	6.9	7.1	5.9[2]	6.0
(+)-Telenzepine	8.1	8.4	8.6	7.8	ND
AF-DX 116	6.0	5.5	5.5	4.5[2]	4.2
Hexahydrosiladifenidol	8.1	8.6	8.8	7.6[2]	7.7[2]
Gallamine[3]	4.5[4]	5.0[4]	5.7[5]	5.1[2,5]	3.5

Values are log affinity constants.
[1] Data from Poyner *et al.* (1989b).
[2] 0.1 < s.e.mean < 0.2, s.e.mean < 0.1 for other data in the table (n = 2–4). For n = 2 (data prefixed with +), the range of estimates was <0.1.
[3] Affinity of gallamine for the unliganded receptor.
[4] Negative co-operativity with NMS, 2–4.
[5] Negative co-operativity with NMS, 15–30.
ND = not determined.

tor into lipids and removal of detergent by gel filtration. The log affinity constant decreased from 7.6 ± 0.1 (n = 3) to 5.6 ± 0.1 (n = 3), a value which is in fact somewhat lower than the affinity measured in membranes at 4°C.

Antagonist binding to lacrimal gland (M_3) receptors (Table 3)

As was found for M_1 receptors, the affinity of AF-DX 116 decreased on lowering the incubation temperature in the high ionic strength media whereas the affinity of the other antagonists increased 2–5 fold. The lowering of the ionic strength at 4°C produced small changes (0–50% increases) in antagonist affinity, the exception being gallamine the affinity of which for the unliganded receptor increased 5 fold. This is in line with the result found for M_1 and M_2 receptors. However, the negative co-operativity between NMS and gallamine increased from ~3 in the high ionic strength medium to 20 in 1 mM Mg^{2+} at 4°C (Figure 1c). This implies that the binding of gallamine to the NMS-occupied M_3 receptor (K_2) is not very sensitive to ionic strength changes, and is in contrast to M_1 and M_2 receptors where the negative co-operativity, which is the ratio of the affinity constants for the unliganded and the NMS-occupied-receptors, was not affected by ionic strength (see legend to Figure 1).

The binding properties of the receptors from the lacrimal gland were more strongly affected by digitonin solubilization than M_1 and M_2 receptors: all the antagonists bound approximately 10 fold weaker. A similar binding pattern was found for the M_3 receptors dissolved in CHAPSO, with the exception of the affinity of gallamine which was profoundly depressed.

Discussion

We have previously shown that for some antagonists, the lowering of ionic strength (Birdsall *et al.*, 1979) and temperature (Barlow *et al.*, 1979) of the incubation produces increases in their affinity for the muscarinic receptors in rat cerebral cortex (now known to be a mixture of receptor subtypes). In addition, antagonist binding to heart receptors has been shown to be decreased by the ability of the receptors under certain conditions to be coupled to G-proteins in the absence of ligand (Hulme *et al.*, 1981; Burgisser *et al.*, 1982). This latter effect is in fact attenuated by the EDTA pretreatment of the membranes used in this paper (Hulme *et al.*, 1983).

The results described in this paper give us an indication of how much the affinity constants of antagonists for a muscarinic receptor subtype can vary, depending on the conditions of the binding assay. The magnitude and direction of the perturbation depends on both the structure of the antagonist and the subtype with which it is interacting. For example, the smallest range of affinity change is less than two fold (atropine at M_1 receptors) whereas the affinity of AF-DX 116 for M_2 receptors can vary by 1000 fold. As a consequence, both the structure-binding relationships of a given subtype may be dramatically changed as well as the selectivity of a drug for the different subtypes.

Considering structure-binding relationships, AF-DX 116 at M_1 receptors is 50 times more potent than gallamine at 30°C in the high ionic strength medium whereas gallamine is 60 times more potent than AF-DX 116 if the binding is measured at 4°C in a 1 mM Mg^{2+} buffer. In other words the selectivity has been changed by a factor of 3000. This extreme variation is caused in part by gallamine binding to an allosteric site on muscarinic receptors (Stockton *et al.*, 1983) which has different properties, but also by the contribution of the anomalous decrease in AF-DX 116 affinity with decrease in temperature and ionic strength. At M_2 and M_3 receptors the relative affinities of AF-DX 116 and gallamine change by smaller but nevertheless substantial factors (500 and 50 fold respectively) such that the rank order of potencies is still reversed. A similar reversal of order of potency is found for pirenzepine and AF-DX 116 at M_2 receptors. In this case, the membrane binding studies show AF-DX 116 to be 3–15 fold more potent than pirenzepine whereas pirenzepine is 25–100 times more

Table 4 Affinity of selective antagonists for M_1, M_2 and M_3 receptors solubilized in digitonin

	M_1	M_2	M_3
Pirenzepine	**7.7**	**7.8**	5.9
AF-DX 116	5.5	**5.8**	4.5
Gallamine	6.5	**7.6**	5.1
Hexahydrosiladiphenidol	7.9	7.8	7.6

The values shown are log (affinity constants). The numbers in bold type denote the subtype for which a selective antagonist has its highest affinity.

Table 5 Affinity of selective antagonists for M_1, M_2 and M_3 receptors solubilized in CHAPSO

	M_1	M_2	M_3
Pirenzepine	**7.3**	5.7	6.0
AF-DX 116	4.6	4.3	4.2
Gallamine	5.0	**6.0**	3.5
Hexahydrosiladiphenidol	**7.9**	6.3	**7.7**

The values (log affinity constants) are from Poyner *et al.* (1989b). The numbers in bold type denote the subtype for which a selective antagonist has its highest affinity.

potent than AF-DX 116 when the receptor is solubilized in digitonin or CHAPSO. These two examples show that the rank order of potencies of antagonists may not be sufficient for a robust definition of a receptor subtype if the experiments are carried out under 'non-physiological' conditions.

The different receptor subtypes, solubilized in detergents, still bind certain antagonists with different affinities. The selectivity of four antagonists for M_1, M_2, and M_3 receptors solubilized in digitonin and CHAPSO are shown in Tables 4 and 5 respectively. The values in bold type show the subtype for which a specific antagonist has the highest affinity. In digitonin, pirenzepine exhibits its highest affinity for M_1 and M_2 receptors and gallamine for M_2 receptors. In contrast, there is no selectivity found for hexahydrosiladiphenidol and the M_2 selectivity of AF-DX 116 is attenuated. In CHAPSO, pirenzepine exhibits a high affinity for only M_1 receptors, gallamine for M_2 receptors and the low affinity of hexahydrosiladiphenidol for M_2 receptors is retained. However, AF-DX 116 binds with essentially identical (and very low) affinity to the three subtypes. It seems as if the different detergents can cause the receptor subtypes to adopt different conformations. Indeed it is possible to switch between these conformations by detergent exchange (Poyner et al., 1989b) and in this study, the increase in pirenzepine affinity found on solubilization of M_2 receptors with digitonin can be reversed by reconstitution of the soluble receptors into lipid, whereas little change in pirenzepine affinity for M_1 receptors is found.

In a study of the reconstitution of purified cerebral and atrial porcine receptors into lipids, Berstein et al. (1989), also found that the M_1/M_2 selectivity of pirenzepine was largely absent in digitonin but could be regenerated by reconstitution if the lipids contained cholesteryl hemisuccinate. The affinity of M_1 and M_2 receptors for pirenzepine was relatively low in the purified preparation in digitonin but was increased at M_1 (but not M_2) receptors on reconstitution, the opposite behaviour to that found in this paper. However the apparent discrepancies may be reconciled by assuming that the interaction of cholesterol (or cholesterol analogue) with M_1 or M_2 receptors generates a conformational state which results in high affinity pirenzepine binding. In membranes, only M_1 receptors are in this state but when the receptors are solubilized in digitonin, M_1 and M_2 receptors initially form the state but as purification proceeds the 'cholesterol' is stripped from the M_1 and M_2 receptors and results in the decreased pirenzepine affinity for pure M_1 and M_2 receptors which has been described (Berstein et al., 1989; Schimerlik et al., 1986).

The data on the membrane-bound receptors in Tables 1–3 can be rearranged to show the conditions under which maximal selectivity can be observed (Table 6). Pirenzepine and gallamine can both show a clear discrimination (at least 10 fold) between all three subtypes, whereas AF-DX 116 and hexahydrosiladiphenidol show only very substantial differences in affinity for M_2 and M_3 receptors, a lower selectivity (4–6 fold) between M_2/M_3 receptors and M_1 receptors being observed. It is notable that a 250 fold selectivity for gallamine and hexahydrosiladiphenidol at M_2/M_3 receptors can be created as well as a 10 fold M_2/M_3 selectivity for pirenzepine. These selectivities are considerably larger than the corresponding values found when binding studies are carried out under 'physiological' conditions. The most robust selectivity for an antagonist which we have found is the $M_2 > M_1 > M_3$ selectivity of gallamine, present under all our incubation conditions.

The explanation of why there are such variations in affinity both within and between subtypes is not known. The binding site for competitive antagonists and agonists is thought to lie in a pocket formed by seven hydrophobic membrane-spanning α-helical domains of the receptor. Peptide mapping studies (Curtis et al., 1989; Kurtenbach et al., 1990) and site directed mutagenesis studies have pinpointed an aspartate residue in the middle of the third transmembrane segment (Asp^{105} in the m1 sequence) as being important for the binding of antagonists.

However, within the putative transmembrane domains there is a very strong sequence conservation between the subtypes. This conservation is accentuated when a helical wheel model of muscarinic receptors is considered (Hulme et al., 1990). Conserved amino acid residues containing most of the hydrophilic side chains point towards the central 'pore' whereas the hydrophobic residues (containing essentially all of the non-conserved residues) are on the outside and facing the lipid bilayer. If this model is valid, the muscarinic receptor binding site may be considered as a conserved hydrophilic pocket within the membrane. The model may well explain the difficulties in designing very selective competitive antagonists as well as the relative insensitivity of the affinity constants of most antagonists to the ionic environment.

The exceptions to this behaviour are gallamine and AF-DX 116. Gallamine is a very polar molecule and can form a ternary complex with the receptor-antagonist binary complex. Hence the structure and sequence determinants for gallamine binding must be quite different from those of competitive antagonists. It may be that gallamine does not bind in the hydrophilic pocket but rather binds to several acidic residues on the extracellular loops on the receptor which are accessible to the cations in the buffer, hence explaining the sensitivity of gallamine binding to ionic strength. The extracellular loops may be more sensitive to detergent-induced conformational changes than the hydrophobic core α-helices and thus explain the decreases in gallamine affinity found on solubilization. AF-DX 116 presents an enigma. In most assays it behaves as a competitive antagonist but at high concentrations it may slow down the off-rate of [^{3}H]-NMS (Roffel et al., 1989) but also see Hammer et al. (1986). Its affinity shows a similar subtype selectivity profile to that of gallamine and a similar high sensitivity to detergent solubilization. It may be that gallamine and AF-DX 116 are binding in similar ways to muscarinic receptors but AF-DX 116 exhibits a very high negative heterotropic cooperativity with the muscarinic ligands, thus behaving effectively as a competitive antagonist. The high, but opposite, sensitivities of the affinities of AF-DX 116 and gallamine to ionic strength and temperature could also be explained by opposing effects of the changes in conformation of the extracellular acidic residues or the thermodynamics of the interaction with the two antagonists, which have very different structures and charge distributions.

We have already shown from pH studies that AF-DX 116 and gallamine bind to M_2 receptors in a manner similar to each other and different from the other antagonists examined here (Birdsall et al., 1989). In addition, AF-DX 116 exhibits a unique pH binding profile at M_1 receptors because only its binding is affected by protonation of a low pK residue on

Table 6 Affinity of selective antagonists for M_1, M_2 and M_3 receptors in membrane preparations

	M_1	M_2	M_3	*Condition*
Pirenzepine	**8.2**	6.1	7.1	4°C, 1 mM Mg^{2+}
AF-DX 116	6.5	**7.3**	6.0	30°C, 100 mM NaCl/10 mM Mg^{2+}
Gallamine	6.3	**7.4**	5.0	4°C, 100 mM NaCl/10 mM Mg^{2+}
Hexahydrosiladiphenidol	8.2	6.4	**8.8**	4°C, 1 mM Mg^{2+}

The values are log (affinity constants). The numbers in bold type denote the subtype for which a selective antagonist has its highest affinity.

these receptors (Palazzi *et al.*, unpublished results). Therefore there is direct evidence that AF-DX 116 does not bind to muscarinic receptors in the same way as other antagonists, and this may be linked to some of the effects described in this paper.

In conclusion, we have documented changes in antagonist affinity constants for muscarinic receptor subtypes when the binding assays have been carried out under different conditions. It has been possible to produce enhanced or attenuated affinities and selectivities resulting from different conformational states of the receptor. In order to understand the molecular and structural basis of drug-receptor interactions, it will be necessary not just to compare affinity constants measured under one condition but also to use the additional information of how the binding constants are affected by a variety of perturbations such as the chemical or molecular biological modification of the receptor, the protonation of different groups on the receptor and the perturbations described in this paper.

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(Received November 1, 1990
Revised January 25, 1991
Accepted February 8, 1991)

Br. J. Pharmacol. (1991), **103**, 1568–1572

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Rate and concentration-dependent effects of UK-68,798, a potent new class III antiarrhythmic, on canine Purkinje fibre action potential duration and $\dot{V}_{max}$

Timothy K. Knilans, [1]†David A. Lathrop, *Péter P. Nánási, †Arnold Schwartz & *†András Varró

Departments of *Pediatrics and of †Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, and Children's Hospital Research Foundation, Division of Cardiology, Cincinnati, Ohio 45229 U.S.A.

1 The frequency-dependent electrophysiological effects of UK-68,798 in concentrations of 1, 3, 10 and 30 nM were examined in isolated cardiac Purkinje fibres of the dog at both a number of constant rates of stimulation and following abrupt changes in pacing cycle length.

2 In all concentrations evaluated, UK-68,798 lengthened action potential duration in a concentration- and rate-dependent manner (e.g., at a cycle length = 500 ms, control APD_{90} = 234.0 ± 3.3 ms, while after 10 nM UK-68,798, APD_{90} = 315.0 ± 5.9 ms).

3 The duration of action potentials evoked following abrupt changes in pacing rate were also increased in a concentration-dependent manner at all diastolic intervals tested.

4 The fast and slow time constants for restitution of APD were not altered by UK-68,798. However, the amplitude terms for this relation were increased.

5 In addition, the maximum upstroke velocity ($\dot{V}_{max}$) was not significantly affected by exposure to UK-68,798 at any concentration or diastolic interval. The kinetics for recovery of $\dot{V}_{max}$ were thus unaffected.

6 These findings are similar to those previously reported for recognized class III antiarrhythmic agents (e.g., bretylium, clofilium, and sotalol); however, UK-68,798 was 1,000 to 10,000 times more potent.

7 The combined potency and selectivity of this agent seem to make it an ideal tool for the investigation of cardiac potassium channels believed responsible for controlling the duration of the action potential.

8 This potent and highly selective compound may prove extremely useful in the control of cardiac arrhythmias.

Keywords: UK-68,798; antiarrhythmic drugs; APD restitution; potassium channel blockers; recovery of $\dot{V}_{max}$; electrophysiology; Purkinje fibres

Introduction

Antiarrhythmic agents which are categorized as Class III (Vaughan Williams, 1975) are widely recognized as being effective in the clinical treatment of potentially lethal cardiac arrhythmias (Singh & Nademanee, 1985). Vaughan Williams (1975) noted that the only recognized drugs that produced acute Class III actions also demonstrated Class II effects (as do sotalol and bretylium). He commented that, 'in the future, it is probable that drugs with an acute (solely) Class III action will become available'.

UK-68,798 (see Figure 1) is a recently described novel, potent and selective Class III antiarrhythmic which is believed to act by blocking a time-dependent potassium current (Gwilt *et al.*, 1989a; 1990). This compound has been reported (Gwilt *et al.*, 1989a; 1990) to prolong action potential duration and effective refractory period in nanomolar concentrations in canine ventricular muscle and Purkinje fibres *in vitro* (paced at 1 Hz) without slowing final repolarization or significantly affecting diastolic potential, action potential amplitude or maximum upstroke velocity. In guinea-pig isolated papillary muscle, the increase in effective refractory period was demonstrated to be rate-dependent (Gwilt *et al.*, 1989a; 1990). In addition, this newly synthesized compound has also been demonstrated to increase ventricular fibrillation threshold in dog heart *in situ* without decreasing blood pressure (Gwilt *et al.*, 1989b).

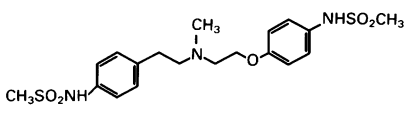

Figure 1 Chemical structure of UK-68,798.

Some antiarrhythmic drugs exert their effects in a frequency-dependent manner (Strauss *et al.*, 1970; Lathrop, 1985; Varró *et al.*, 1986). We have, therefore, examined the frequency-dependent characteristics of UK-68,798 in greater detail. Specifically, we have investigated the effects of UK-68,798 at various constant pacing cycle lengths and described its influence on restitution of action potential duration and recovery of maximum rising velocity in isolated cardiac Purkinje fibres and ventricular muscle of the dog.

Methods

Hearts from adult mongrel dogs of either sex were used for these studies. The dogs were first anaesthetized with sodium pentobarbitone (30 mg kg^{-1}) and the hearts were removed rapidly through a right lateral thoracotomy. The hearts were placed in cooled (0°C) oxygenated Tyrode solution containing (in mM): Na^+ 153.0, K^+ 5.4, Cl^- 162.9, Ca^{2+} 2.7, Mg^{2+} 1.05, HEPES buffer 5.04 and glucose 11.0. The solution had a pH of 7.35 to 7.45 when gassed with 100% oxygen at 37°C. Free running Purkinje strands and trabecular muscle fibres from either ventricle were dissected and mounted in a small volume (0.5 ml) tissue superfusion chamber. Each preparation was continuously superfused with oxygenated Tyrode solution at a rate of 5 ml min^{-1} at 37°C. The preparations were stimulated at a constant basic cycle length of 500 ms. Rectangular

[1] Author for correspondence at: Department of Medical Physiology, IMB, University of Tromsø, N-9000 Tromsø, Norway.

constant current stimuli were delivered from a MECA (Indianapolis, IN, USA) model STM-2 stimulator while isolated from ground by a WP-Instruments (New Haven, CT, USA) model 830 stimulus isolator. Stimuli were 1 ms in duration and were delivered to two platinum wire electrodes placed in the superfusion chamber at either end of the fibre. Each preparation was allowed to equilibrate in the bath for a period of 1 h.

Transmembrane potentials were recorded with conventional microelectrode techniques. Microelectrodes were filled with 3 M KCl and had tip resistances of 10–25 MΩ. These electrodes were connected to the input of a high impedance 10^{12} Ω electrometer (WP-Instruments, Model 750) having capacitance neutralization. The first derivative of transmembrane voltage with respect to time ($\dot{V}_{max}$) was electronically derived by use of an amplifier (MECA, Model EP 0.3) which was linear over a range of 20 to 1000 V s^{-1}.

The outputs of each of these amplifiers were displayed on a single beam storage oscilloscope (Tektronix, Beaverton, OR, USA; model R-5115) with dual sweep speed capability (Tektronix, model 5B12N). Oscilloscope displays were photographed on Polaroid film (type 667) with a Tektronix camera (model C-59).

Protocol for determining response to pacing at different basic cycle lengths

In these experiments, both canine Purkinje strands and ventricular muscle preparations were continuously stimulated at four different cycle lengths (500, 700, 1000, 3000 ms). After equilibration to the new pacing frequency, action potential amplitude, maximum diastolic potential, APD_{50}, APD_{90} and $\dot{V}_{max}$ were determined. Following this procedure and after superfusing the preparation for 30 min in successively higher concentrations of UK-68,798 (1, 3, 10 and 30 nM), the procedure was repeated. Cumulative concentration-response relations were thus developed. Exposure to UK-68,798 was followed by a 30 min period of washout in drug-free normal Tyrode solution and the measurements were repeated.

Protocol for establishing concentration-response relationship: restitution of APD and recovery of $\dot{V}_{max}$

In another group of Purkinje strand preparations, following a 60 min equilibration period while each preparation was stimulated at constant frequency (2 Hz), control action potential parameters were again measured. To determine the characteristics of restitution of APD and recovery of $\dot{V}_{max}$ a single extra stimulus was introduced at diastolic intervals varying from 0 to 4 s following a train of 30 stimuli delivered at a cycle length of 500 ms. Diastolic intervals were measured from the point corresponding to 90% repolarization of the preceding basic action potential to the delivery of the extra stimulus. The effective refractory period, defined as the shortest interstimulus interval at which an extra stimulus could be introduced while still evoking an action potential, was also determined. Following this procedure the preparations were sequentially exposed to increasing concentrations of UK-68, 798 (1, 3, 10 and 30 nM) for 30 min and the measurements were repeated at each concentration.

Drug preparation

UK-68,798 (Pfizer Central Laboratories, Sandwich, England) was prepared daily from a 10 mM stock solution by dilution in normal Tyrode solution.

Data analysis

The concentration-response relations were fit to the equation (DeLean *et al.*, 1978):

$$y = (a - d)/(1 + (x/c)^b) + d$$

where x was the concentration, y the estimated response, a the estimated minimum response, b the estimated slope factor, c the estimated 50% effective concentration and d the estimated maximum response.

The relationship between the diastolic interval and APD_{90} of the action potential evoked by the extra stimulus defined the relation for restitution of APD. This relation was resolved into two exponential components by normalizing APD_{90} at each diastolic interval (APD_t) to a duration 5% greater than the APD measured at 4 s (APD_{pl}). The fast and slow time constants and amplitude terms for this relation were estimated using standard exponential curve fitting procedures. The curve was thus fit to the equation (Elharrar & Surawicz, 1983):

$$APD_t = APD_{pl} \cdot \{1 - [A_f \cdot e^{(-t/\tau_f)}] - [A_s \cdot e^{(-t/\tau_s)}]\} \quad (2)$$

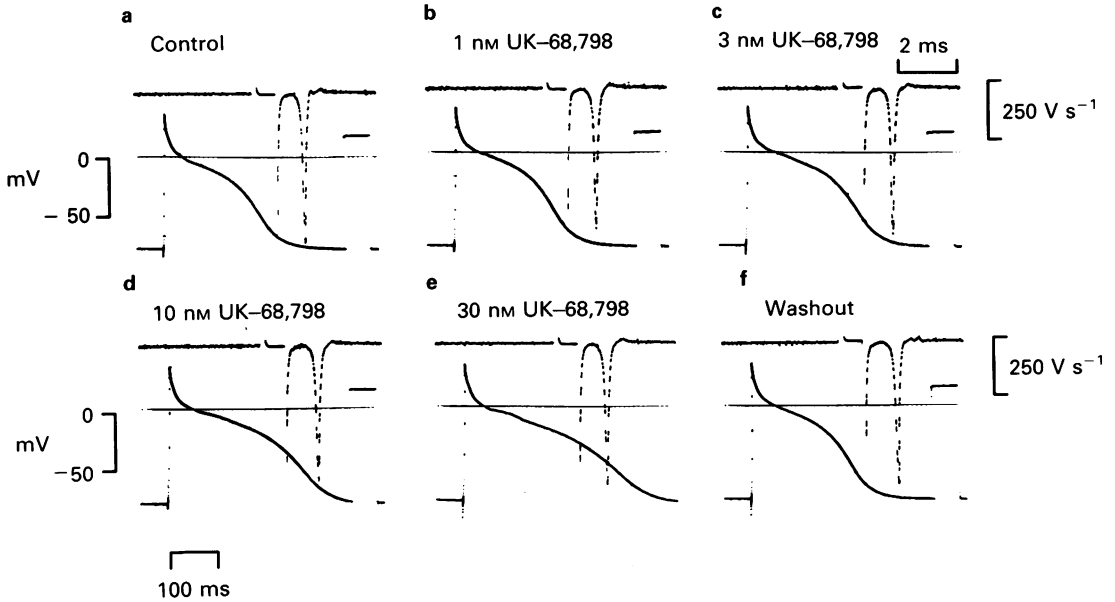

Figure 2 Concentration-dependent effects of UK-68,798 on the action potential characteristics of dog isolated Purkinje fibres stimulated at a constant basic cycle length of 500 ms. Calibration pulses (100 mV in amplitude and 50 ms in duration) follow each action potential. Voltage scale and time scale for action potentials are seen to the left. Amplitude and time scales for the recording of the first derivative of transmembrane voltage with respect to the time are seen to the right. The derivative traces have been inverted in this illustration.

where t represented the diastolic interval preceding APD_t, and A and τ the amplitudes and the time constants of the fast (A_f and τ_f) and the slow (A_s, τ_s) exponential components.

The relations for recovery of $\dot{V}_{max}$ were similarly fit to a single exponential relation utilizing a plateau value equal to the highest measured $\dot{V}_{max}$.

Student's t tests (Sokal & Rohlf, 1969) for either paired or unpaired data were used to determine statistical significance of the results. Results of these tests were considered significant when $P < 0.05$.

Results

Effect of UK-68,798 at basic cycle length (500 ms)

The effect of increasing concentrations of UK-68,798 (1, 3, 10 and 30 nM) on the electrophysiological characteristics of dog Purkinje fibres paced at a basic cycle length of 500 ms are summarized in Table 1 and illustrated in Figure 2. At all concentrations applied, UK-68,798 did not significantly alter the MDP, APA or $\dot{V}_{max}$. The rate of the initial rapid repolarization (phase I), observed at rapid sweep speeds, was not affected by UK-68,798 (Figure 3). UK-68,798 did, however, increase APD_{90}, APD_{50} and ERP in a concentration-dependent manner. The estimated 50% effective concentration for increasing APD_{90} (EC_{50}) was 5.5 ± 0.9 nM (Figure 4).

Effect of UK-68,798 at different constant pacing cycle lengths: lack of effect at all concentrations applied, of UK-68,798 on MDP, APA or $\dot{V}_{max}$

The influence of 10 nM UK-68,798 on electrophysiological parameters of dog Purkinje fibres was evaluated at four dif-

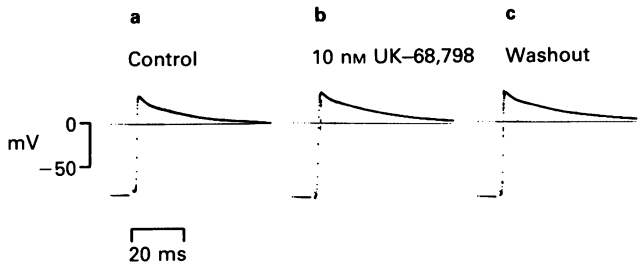

Figure 3 Effect of UK-68,798 (10 nM) on initial action potential repolarization (phase I). The preparation was stimulated at a constant basic cycle length of 500 ms. Action potential in (a) was recorded prior to exposure to drug; (b) was recorded after 30 min exposure to 10 nM UK-68,798 and (c) after 30 min washout of the drug.

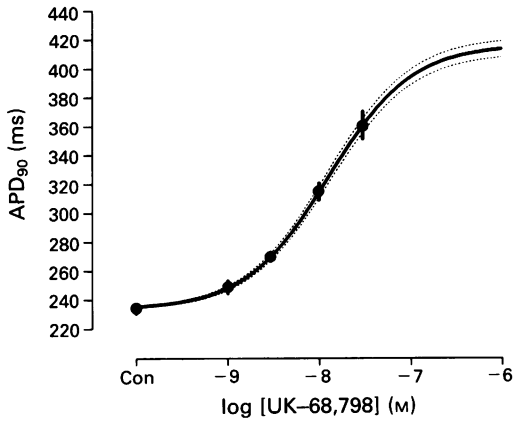

Figure 4 Concentration-response relation for the effect of UK-68,798 on the duration of the action potential at 90% of repolarization (APD_{90}) in dog Purkinje strands stimulated at a constant basic cycle length of 500 ms. Five preparations, each obtained from a different dog, were used. Error bars and dashed lines represent mean ± s.e.

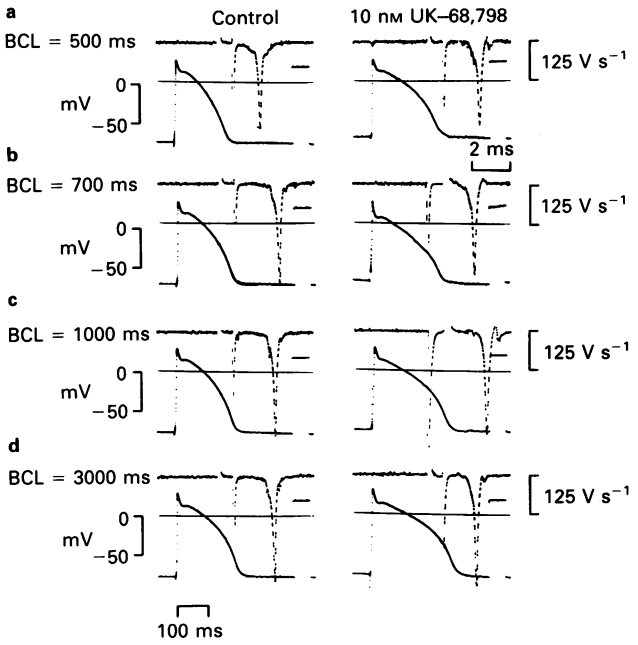

Figure 5 Effect of UK-68,798 (10 nM) on the action potential characteristics of dog ventricular muscle stimulated at four different basic cycle lengths. Calibration pulses (100 mV in amplitude and 50 ms in duration) follow each action potential. Voltage and time scales for action potentials are given to the left, amplitude and time scales for the first derivative of the transmembrane voltage with respect to time are to the right. Derivatives have been inverted in this illustration.

Table 1 The effects of UK-68,798 on the electrophysiological characteristics of dog Purkinje fibres at a basic cycle length of 500 ms

		UK-68,798			
	Control	1 nM	3 nM	10 nM	30 nM
MDP (mV)	−82.8 ± 0.5 (5)	−83.6 ± 0.8 (5)	−82.4 ± 0.8 (5)	−82.8 ± 0.5 (5)	−82.4 ± 0.4 (5)
APA (mV)	120.0 ± 0.6 (5)	122.0 ± 0.6 (5)	122.0 ± 0.6 (5)	121.1 ± 0.5 (5)	120.0 ± 0.6 (5)
APD_{50} (ms)	157.0 ± 4.6 (5)	172.0 ± 4.1 (5)*	180.0 ± 2.7 (5)*	199.0 ± 2.4 (5)*	203.0 ± 4.4 (5)*
APD_{90} (ms)	234.0 ± 3.3 (5)	249.0 ± 4.3 (5)*	270.0 ± 3.2 (5)*	315.0 ± 5.9 (5)*	361.0 ± 9.5 (5)*
$\dot{V}_{max}$ ($V s^{-1}$)	598.0 ± 18.3 (5)	604.0 ± 18.6 (5)	614.0 ± 23.2 (5)	596.0 ± 25.6 (5)	574.0 ± 32.8 (5)
ERP (ms)	225.8 ± 4.3 (4)	239.5 ± 0.9 (4)*	258.5 ± 2.1 (4)*	304.5 ± 5.8 (4)*	353.0 (2)
ERP/APD_{90}	0.96 ± 0.01 (4)	0.96 ± 0.02 (4)	0.95 ± 0.01 (4)	0.95 ± 0.02 (4)	0.97 (2)

* $P \leq 0.01$.

Mean ± standard error (number of observations).

MDP, maximum diastolic potential; APA, action potential amplitude; APD_{50}, action potential duration at 50% of repolarization; APD_{90}, action potential duration at 90% of repolarization, $\dot{V}_{max}$, maximum rising velocity of the action potential upstroke; ERP, effective refractory period.

Table 2 The effects of UK-68,798 on premature action potentials (diastolic interval = 40 ms) in dog Purkinje fibre

		UK-68,798			
	Control	1 nM	3 nM	10 nM	30 nM
TOP (mV)	−79.6 ± 0.8	−80.4 ± 1.0	−79.2 ± 1.0	−78.8 ± 0.8	−78.0 ± 1.4
APD_{90} (ms)	173.0 ± 4.1	185.0 ± 5.2*	199.0 ± 5.8*	241.0 ± 9.1*	290.0 ± 14.6*
$\dot{V}_{max}$ ($V s^{-1}$)	576.0 ± 12.9	586.0 ± 21.6	570.0 ± 35.6	558.0 ± 25.0	498.0 ± 46.2

* $P \leq 0.01$.
Mean ± standard error, number of preparations (n) = 5.
TOP, take off potential; APD_{90}, action potential duration at 90% of repolarization; $\dot{V}_{max}$ maximum rising velocity of the action potential upstroke.

ferent constant pacing cycle lengths (500, 700, 1000 and 3000 ms). UK-68,798 did not significantly affect $\dot{V}_{max}$ or MDP. However APD_{90} was increased in a rate-dependent manner such that at long cycle lengths the increase was proportionately greater than at short cycle lengths. Similar changes were observed in ventricular muscle fibres, although the absolute magnitude of the increase in APD_{90} was less than that observed in Purkinje fibres (Figure 5).

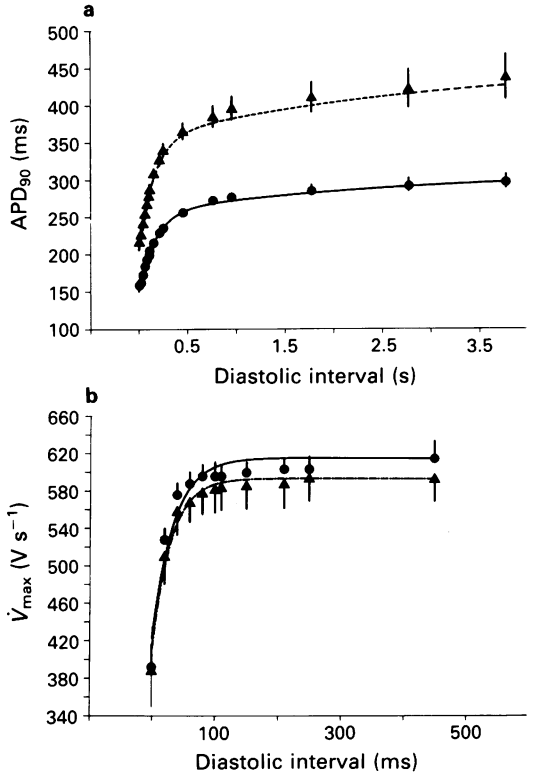

Figure 6 (a) Effect of UK-68,798 (10 nM) on dog Purkinje fibre restitution of action potential duration measured at 90% of repolarization (APD_{90}). (b) Effect of UK-68,798 (10 nM) on dog Purkinje fibre recovery of $\dot{V}_{max}$: (●) represent the mean control values obtained from five separate Purkinje strand preparations, (▲) represent mean values obtained after 30 min exposure to 10 nM UK-68,798. Both of the APD restitution curves were fit to a double exponential relation (equation 2). There was no significant difference between the time constants of these relations. The curves for recovery of $\dot{V}_{max}$ were fitted to a single exponential relation. There was no significant change in the time constant of these relations before or after UK-68,798.

Effects of UK-68,798 following abrupt changes in pacing cycle length

The effects of UK-68,798 on action potentials evoked at a short diastolic interval (40 ms) are summarized in Table 2. The only significant effect produced by UK-68,798 at this diastolic interval was lengthening of APD_{90}, as noted at the basic cycle length of 500 ms.

The response of action potential duration to UK-68,798 over a wide range of diastolic intervals is illustrated in Figure 6a. Analysis of the restitution curve was undertaken as described in the Methods section. Slow and fast time constants for restitution of APD were not significantly changed following exposure to any concentrations of UK-68,798 examined (Table 3) and the only significant effect was to increase the magnitude of the amplitude terms of the relation.

In addition, UK-68,798 did not affect the time constant for the recovery of $\dot{V}_{max}$ at any concentration examined (Figure 6b, Table 4).

Discussion and conclusions

The cardiac electrophysiological effects of UK-68,798 were studied in dog isolated Purkinje strands and ventricular muscle preparations at several constant rates of stimulation and in dog Purkinje strands following abrupt changes in the diastolic interval. The only significant effect produced was a lengthening of action potential duration and effective refractory period. These effects occurred in a frequency-dependent manner at all examined concentrations and were observed after both changing constant pacing cycle lengths and abrupt changes in diastolic intervals. There was no significant effect on $\dot{V}_{max}$, APA or MDP. These findings are similar to those previously reported to be produced by class III antiarrhythmic agents (e.g., bretylium, clofilium, and sotalol: Strauss *et al.*, 1970; Steinberg *et al.*, 1981; Nattel & Zeng, 1984; Lathrop, 1985; Varró *et al.*, 1986). However, as a marked difference, UK-68,798 exerted its effects at concentrations which are 1,000 to 10,000 times less than those produced by these previously recognized class III antiarrhythmics.

Table 3 The effects of UK-68,798 on the kinetics of restitution of action potential duration

		UK-68,798			
	Control	1 nM	2 nM	10 nM	30 nM
τ_f	176.5 ± 22.5	157.7 ± 16.1	167.1 ± 10.6	163.0 ± 15.4	218.0 ± 38.6
A_f	107.1 ± 5.6	113.9 ± 6.2	131.7 ± 6.6[a]	151.2 ± 13.2[a]	188.2 ± 7.2[b]
τ_s	2904 ± 237	2794 ± 188	3008 ± 134	3265 ± 611	3134 ± 564
A_s	55.7 ± 8.5	61.8 ± 6.4	58.2 ± 3.6	104.1 ± 34.4[a]	132.7 ± 34.4[a]
n	5	5	5	5	5

[a] $P \leq 0.05$; [b] $P \leq 0.01$.
Mean ± standard error, number of preparations (n) = 5.
τ_f, time constant of fast exponential component; A_f, amplitude of fast exponential component; τ_s, time constant of the slow exponential component; A_s, amplitude of the slow exponential component.

Table 4 The effects of UK-68,798 on the kinetics of recovery of $\dot{V}_{max}$

		UK-68,798			
	Control	1 nM	3 nM	10 nM	30 nM
τ	33.7 ± 7.1	38.4 ± 4.3	40.6 ± 8.1	30.0 ± 5.0	45.6 ± 5.7
A	193.2 ± 22.8	119.4 ± 10.6	156.5 ± 37.1	181.8 ± 27.3	156.8 ± 27.3
Plateau $\dot{V}_{max}$	616.0 ± 17.2	622.0 ± 17.7	618.0 ± 25.0	594.0 ± 24.2	515.0 ± 45.0
n	5	5	5	5	4

Mean ± standard error; n, number of observations.
τ, time constant of exponential; A, amplitude of exponential; Plateau $\dot{V}_{max}$, maximum value of $\dot{V}_{max}$.

The effects on lengthening of APD at a basic cycle length (500 ms) confirm the findings of Gwilt *et al.* (1989a, 1990). The frequency-dependent effects extend those previously reported in guinea-pig isolated papillary muscle preparations (Gwilt *et al.*, 1989a; 1990) and further support the classification of UK-68,798 as a specific class III antiarrhythmic.

In agreement with Gwilt *et al.* (1989a; 1990), our demonstration of a lack of effect on MDP supports the conclusion that UK-68,798 does not affect the inward rectifier current. In addition, we have demonstrated that UK-68,798 (10 nM) does not alter the slope of phase I repolarization at a basic cycle length of 500 ms suggesting that UK-68,798 also does not affect the transient outward current.

The high potency of UK-68,798 (EC_{50} for lengthening of APD_{90} = 5.5 nM) might be of benefit in clinical application as the lower concentrations which may be necessary to achieve the desired cardiac effects will provide fewer adverse extracardiac effects. In addition, the apparently selective effect of UK-68,798 on inhibition of a time-dependent potassium current suggests that this compound also may have great potential as a research tool to study and isolate specific potassium channels within the cardiac cell membrane.

This study was supported, in part, by a grant from the National Heart, Lung and Blood Institute (R01 HL37034-01A1). The authors thank Dr Ilona Bødi as well as Peggy Farmer, Barbara Cincush, and Steven E. Hysell for their assistance in the performance of the studies described. UK-68,798 was a gift from Pfizer Central Laboratories, Sandwich, England.

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(Received August 8, 1990
Accepted February 8, 1991)

Br. J. Pharmacol. (1991), **103**, 1573–1579 © Macmillan Press Ltd, 1991

Identification and characterization of histamine H_1- and H_2-receptors in guinea-pig left atrial membranes by [^{3}H]-mepyramine and [^{3}H]-tiotidine binding

[1]Yuichi Hattori, Masayuki Endou, Satoshi Gando & Morio Kanno

Department of Pharmacology, Hokkaido University School of Medicine, Sapporo 060, Japan

1 Histamine receptors in the membranes prepared from guinea-pig left atria were characterized with [^{3}H]-mepyramine and [^{3}H]-tiotidine binding.

2 The binding of the H_1-antagonist, [^{3}H]-mepyramine, was saturable and of high affinity with a maximum binding capacity of 307 ± 27 fmol mg^{-1} protein ($n = 14$) and with an equilibrium dissociation constant (K_D) of 1.5 ± 0.2 nM ($n = 14$). The binding was rapid and readily reversible.

3 The competition curve for [^{3}H]-mepyramine binding by histamine was biphasic and revealed high and low affinity states of binding. The addition of 5'-guanylylimidodiphosphate (GppNHp) (100 μM) converted this heterogeneous binding into homogeneous binding of low affinity.

4 The competition curves of H_1-antagonists with [^{3}H]-mepyramine had Hill coefficients not significantly different from unity, consistent with competition with [^{3}H]-mepyramine at a single site. GppNHp did not shift the competition curves.

5 Dissociation constants for H_1-antagonists determined from inhibition of [^{3}H]-mepyramine binding correlated well with the constants derived from inhibition of the positive inotropic response of guinea-pig left atria to histamine.

6 The H_2-antagonist, [^{3}H]-tiotidine, labelled an apparently homogeneous population of recognition sites with a maximum binding capacity of 41 ± 8 fmol mg^{-1} protein ($n = 6$) and a K_D of 10.8 ± 1.2 nM ($n = 6$).

7 Although histamine competed for [^{3}H]-tiotidine binding in a concentration-dependent manner, the curve was monophasic and was not shifted by GppNHp.

8 It is concluded that both H_1- and H_2-receptors exist in guinea-pig left atria. H_1-receptors probably couple to intracellular effector(s) through a guanine nucleotide-dependent transducing mechanism. On the other hand, H_2-receptors seem unlikely to be linked to guanine nucleotide regulatory proteins in guinea-pig left atria, which may explain the failure of histamine to cause an increase in cyclic AMP in spite of the presence of H_2-receptors.

Keywords: Histamine; H_1-receptors; H_2-receptors; radioligand binding; guinea-pig left atria; guanine nucleotide regulatory proteins

Introduction

The pharmacological actions of histamine on the heart have been shown to be mediated by H_1- and H_2-receptors. The slowing of the atrioventricular conduction induced by histamine is due to activation of H_1-receptors, while the positive chronotropic and inotropic effects of histamine are mainly due to activation of H_2-receptors (see review by Levi *et al.*, 1982). However, in guinea-pig left atria H_1-receptors are responsible for the positive inotropic effect of histamine (Reinhardt *et al.*, 1974; Steinberg & Holland, 1975; Hattori & Kanno, 1985). The predominant involvement of H_1-receptors in the positive inotropic response to histamine has also been found in rabbit papillary muscle (Hattori *et al.*, 1988b). Nevertheless, in this tissue histamine causes a decrease in action potential duration and a relatively small increase in adenosine 3':5'-cyclic monophosphate (cyclic AMP), both effects which are antagonized by cimetidine, suggesting the coexistence of H_1- and H_2-receptors in rabbit papillary muscle (Hattori *et al.*, 1990).

In guinea-pig left atria histamine not only produces a positive inotropic effect, but also increases action potential duration (Borchard & Hafner, 1986; Hattori *et al.*, 1988a) as well as hydrolysis of phosphoinositides (Sakuma *et al.*, 1988; Hattori *et al.*, 1989). These responses are blocked by specific H_1-antagonists, such as chlorpheniramine and mepyramine, but not by H_2-antagonists. These data provide pharmacological evidence for the presence of H_1-receptors in guinea-pig left atria. On the other hand, the presence of H_2-receptors in guinea-pig left atria has previously been denied because of the failure of histamine to cause an increase in cyclic AMP (Reinhardt *et al.*, 1977; Verma & McNeil, 1977). However, these pharmacological studies do not exclude the possibility that H_2-receptors exist which are not linked to adenylate cyclase in guinea-pig left atria.

The first aim of the present study was to demonstrate and characterize H_1-receptors in membrane fractions derived from guinea-pig left atria by means of the radioligand binding technique using [^{3}H]-mepyramine. The radioligand, [^{3}H]-mepyramine, has been used to identify binding sites that have the characteristics of H_1-receptors in membrane fractions derived from brain and peripheral tissues of various species (Hill *et al.*, 1977; 1978; Tran *et al.*, 1978; Chang *et al.*, 1978; 1979). We aimed to determine from these experiments whether guanine nucleotide regulatory proteins are involved in the modulation by H_1-receptors of cardiac function. The second aim of the present study was to ascertain whether H_2-receptors are present in guinea-pig left atria by using a radiolabelled and highly potent H_2-receptor antagonist, [^{3}H]-tiotidine (Gajtkowski *et al.*, 1983).

Methods

Membrane preparation

Guinea-pigs of either sex weighing 250–500 g were killed by a blow on the head. The hearts were removed and rinsed in

[1] Author for correspondence.

ice-cold Tris-HCl buffer. Left atria were carefully dissected from the hearts. The tissues were minced with scissors and homogenized in 5 volumes of ice-cold Tris-HCl buffer by the use of a polytron for 15 s. The buffer composition (pH 7.4, 4°C) was (mM): Tris-HCl 50 and EDTA 5. The homogenates were centrifuged at 1,000g for 10 min at 4°C. The supernatant was filtered through a single layer of cheesecloth and retained. The pellet was suspended in 5 volumes of cold Tris-HCl buffer and centrifuged again. Membrane fractions in the supernatant were concentrated by centrifugation at 100,000g for 30 min at 4°C. The final pellets were resuspended in ice-cold Tris-HCl buffer and stored at −80°C until used. Protein content was determined by the method of Lowry *et al.* (1951), with bovine serum albumin used as standard.

[^3H]-*mepyramine binding*

The binding of [^{3}H]-mepyramine (23.9–29.0 Ci mmol^{-1}, New England Nuclear, Boston, MA, U.S.A.) was evaluated by a filtration method (Engel *et al.*, 1981). The membrane suspensions were diluted further in an incubation medium (Tris-HCl 50 mM, EDTA 0.5 mM, pH 7.4) to give a final protein concentration of 0.2 to 0.7 mg ml^{-1}. [^{3}H]-mepyramine and all drugs except mequitazine (see later) were prepared in the incubation medium. An aliquot of the membrane suspension (100 μl) was incubated with various concentrations of [^{3}H]-mepyramine in a final volume of 200 μl. Incubations were carried out for 60 min at 25°C and terminated by adding 5 ml of the cold incubation medium (4°C) to the entire incubation mixture, followed by rapid filtration over Whatman GF/C glass fibre filters. As [^{3}H]-mepyramine is reported to show antagonist-displaceable binding to the glass fibre filters (Hill & Young, 1981), the filters had been rinsed with the incubation medium containing 10 μM unlabelled mepyramine. Each filter was washed three times with 5 ml of the incubation medium (4°C) and then air dried overnight. The radioactivity trapped on a filter was counted in 15 ml of Scintisol EX-H (Dojin, Osaka, Japan) in a liquid scintillation counter. All values in binding experiments are the average of triplicates. Non-specific binding was defined as binding in the presence of 10 μM unlabelled mepyramine or 1 mM histamine. The equilibrium dissociation constant (K_D) and the maximum binding capacity (B_{max}) were calculated from plots according to the method of Scatchard (1949).

In experiments designed to study the association of [^{3}H]-mepyramine binding, 100 μl of the membrane suspension was incubated in triplicate with 2.4 nM [^{3}H]-mepyramine in the absence or presence of 1 mM histamine in a total volume of 200 μl. Incubations were carried out at 25°C for various times between 0 and 90 min. The reaction was terminated and the radioactivity in each sample was determined by the methods described above. The dissociation of [^{3}H]-mepyramine binding was examined by incubating 100 μl of the membrane suspension at 25°C for 60 min with 2.4 nM [^{3}H]-mepyramine in a total volume of 200 μl. Histamine at 1 mM was then added to each tube and the radioactivity was determined at various times between 0 and 30 min thereafter by the methods described above. Kinetic data were analyzed by linear transformation of binding data to a natural logarithm plot:

$$\ln([B_{eq} - B_t]/[B_{eq}]) = -k_{obs}(t)$$

where B_{eq} and B_t are the amounts of [^{3}H]-mepyramine specifically bound at equilibrium and time (t), respectively. Fitting kinetic data to this equation generates a plot with slope corresponding to $-k_{obs}$. The association rate constant (k_{+1}) was calculated from the equation:

$$k_{obs} = k_{+1}(L) + k_{-1}$$

where (L) is the concentration of [^{3}H]-mepyramine used in the experiment. The dissociation rate constant (k_{-1}) is equal to the slope of the regression line of the following plot:

$$\ln([B_t]/[B_{eq}]) = k_{-1}(t)$$

In binding competition experiments, the membrane suspension (100 μl) was incubated in triplicate with 2.4 nM [^{3}H]-mepyramine and various concentrations of displacing compounds in the absence or presence of 100 μM GppNHp. The incubations were carried out for 60 min at 25°C and terminated as described above. The data were analyzed by the iterative least-squares curve fitting programme described by Munson & Rodbard (1980). Results were analyzed to identify the presence of one or two classes of binding sites by comparing the sum of squares of residuals for each model. The most appropriate model was determined by statistical analysis using an F test of the sum of squares of the residuals. K_i values were calculated by the method of Cheng & Prusoff (1973).

[^3H]-*tiotidine binding*

All assays were run in triplicate in a final volume of 200 μl containing various concentrations of [^{3}H]-tiotidine (78.6–80.9 Ci mmol^{-1}, New England Nuclear). In experiments designed to study the inhibition of [^{3}H]-tiotidine binding by histamine, [^{3}H]-tiotidine was used at a concentration of 10 nM. Non-specific binding was determined in the presence of 100 μM cimetidine or 1 mM histamine. Reactions were initiated by the addition of 100 μl of the membrane suspension, and incubations were carried out at 25°C for 30 min. Then, bound radioactivity was isolated and measured by the same procedure as described for the [^{3}H]-mepyramine binding assay.

Organ bath experiments

Guinea-pig left atria were prepared and then mounted vertically in a water-jacketed bath containing 50 ml of Krebs-Henseleit solution bubbled with 95% O_2 and 5% CO_2 at a temperature of 30°C as previously described (Hattori & Kanno, 1985). The composition of the solution was (mM): NaCl 119, $CaCl_2$ 2.5, KCl 4.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24.9 and glucose 10.0. The lower end of the preparation was pinned on a pair of hook electrodes for stimulation and the other was connected by a silk thread to a force transducer (Nihon Kohden, TB-612T, Tokyo, Japan). Isometric tension developed in the preparation was recorded on a pen recorder (Nihon Kohden, RJG-3026) through a preamplifier (Nihon Kohden, RP-5). A resting tension of 20 mN was placed on each preparation. The atria were electrically driven by rectangular pulses of 1 ms duration and an intensity of 1.5 times threshold voltage of 0.5 Hz frequency. The pulses were delivered from an electronic stimulator (Sanei-Sokki, 3F46, Tokyo, Japan) through an isolation unit (Sanei-Sokki, 5361). The preparations were allowed to equilibrate for at least 60 min before the experiments.

The concentration-response curves for the positive inotropic effect of histamine were determined in a cumulative manner by increasing the concentration of histamine in steps of 0.5 log units (van Rossum, 1963). On completion of the concentration-response curve, the preparations were thoroughly washed. After the force of contraction had returned to predrug level, one of the H_1-antagonists was added. The incubation time for the H_1-antagonists was 60 min. The concentration-response curve for histamine was then obtained in the presence of the H_1-antagonists. The EC_{50} value, i.e., the concentration required to produce 50% of the maximal response induced by histamine, was determined from log-probit plots of the individual response *vs.* concentrations, and the ratio of the EC_{50} values in the presence and absence of each H_1-antagonist was calculated (DR, dose-ratio). The K_B values for the H_1-antagonists were determined according to the Schild equation (Arunlakshana & Schild, 1959):

$$\log(DR - 1) = n \log[B] + \log K_B$$

where [B] refers to the molar concentration of antagonist and n the slope of the regression line. For a simple competitive antagonism, the resulting Schild regression should be linear and have a slope not significantly different from unity. Thus,

the K_B values were calculated with a constrained slope of unity. We confirmed that repetitive determinations did not affect the concentration-response relation for the positive inotropic effect of histamine. Thus, the pD_2 values, i.e., $-\log EC_{50}$, for histamine in the first and second concentration-response curves were 6.69 ± 0.19 and 6.60 ± 0.16, respectively ($n = 5$).

Drugs

The following compounds were used: histamine dihydrochloride (Merck, Darmstadt, F.R.G.), mepyramine maleate (Sigma, St. Louis, MO, U.S.A.), (+)-chlorpheniramine maleate (Schering, Osaka, Japan), diphenhydramine hydrochloride (Kowa, Nagoya, Japan), promethazine hydrochloride (Yoshitomi, Osaka, Japan), mequitazine (Nihon Shoji, Osaka, Japan), cimetidine (Fujisawa, Osaka, Japan), metiamide (Smith Kline and French, Welwyn, Hertfordshire) and 5'-guanylylimidodiphosphate (GppNHp) (Boehringer Mannheim, Mannheim, F.R.G.). Mequitazine and metiamide were dissolved in methanol and dimethyl sulphoxide, respectively. Further dilutions were made with Tris-HCl buffer solution or Krebs-Henseleit solution. All other compounds were dissolved in Tris-HCl solution or distilled water.

Statistics

The experimental data given in text, table and figures are presented as means ± s.e.mean. For Hill coefficients, a statistical significance indicated a coefficient significantly different from unity. A P value less than 0.05 was considered an indication of statistical significance.

Results

Binding of [^{3}H]-mepyramine

When guinea-pig left atrial membrane fractions were incubated with increasing amounts of [^{3}H]-mepyramine in the absence or presence of 10 μM unlabelled mepyramine to define non-specific binding, the specific binding obtained was a saturable process of high affinity (Figure 1). The same results were obtained when non-specific binding was determined in the presence of 1 mM histamine. Non-specific binding was less than 60% of total binding at the highest concentration of [^{3}H]-mepyramine. Scatchard analysis of the data (Figure 1c) revealed that [^{3}H]-mepyramine bound to a single population of binding sites. Mean B_{max} and K_D values from 14 separate experiments were 307 ± 27 fmol mg^{-1} protein and 1.5 ± 0.2 nM. The Hill coefficient was calculated as 0.99 ± 0.01 from Hill plots of the data.

Kinetic analysis of the binding of [^{3}H]-mepyramine to guinea-pig left atrial membranes showed that the specific binding of 2.4 nM [^{3}H]-mepyramine occurred rapidly with half-maximal binding within 1 min and achievement of equilibrium after 20 min (Figure 2). The observed forward rate constant (k_{obs}) was calculated to be 0.146 min^{-1}. The binding of [^{3}H]-mepyramine was reversible as demonstrated by the addition of histamine (final concentration 1 mM) at equilibrium (Figure 2). The dissociation of [^{3}H]-mepyramine initiated by histamine was very rapid and yielded a dissociation rate constant (k_{-1}) of 0.0331 min^{-1}. From the k_{-1} and the k_{obs}, an association rate constant (k_{+1}) of 0.0470 nM^{-1} min^{-1} was calculated. Thus, the equilibrium dissociation constant (K_D) estimated from the ratio k_{-1}/k_{+1} was 0.70 nM, which is comparable with the value of K_D determined from the saturation experiments.

Figure 3 shows the curves of competition by histamine for [^{3}H]-mepyramine binding in the absence and presence of GppNHp. In the absence of nucleotides the displacement curve had a Hill coefficient significantly different from unity ($n_H = 0.84 \pm 0.02$, $n = 4$, $P < 0.01$) and was best fit by a model

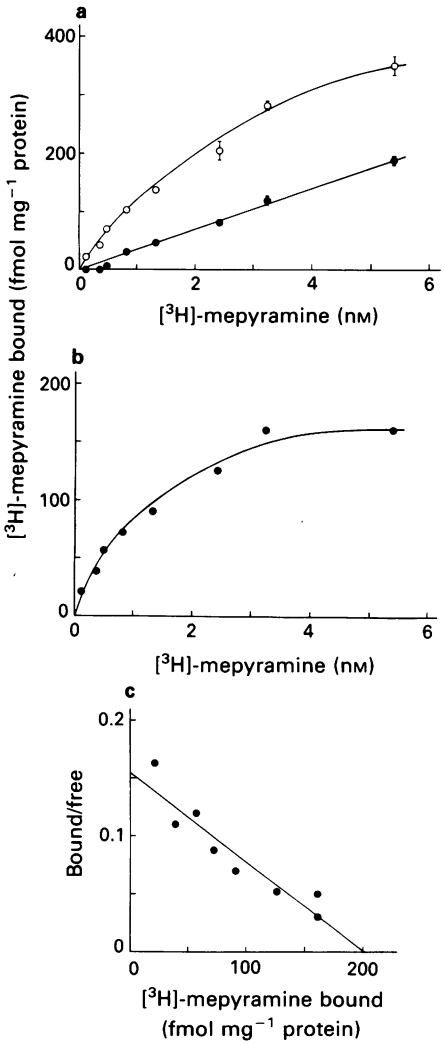

Figure 1 Binding of [^{3}H]-mepyramine to the membranes prepared from guinea-pig left atria. (a) Binding of [^{3}H]-mepyramine in the absence of (○) or presence (●) of 10 μM unlabelled mepyramine. Points are means of triplicate determinations made on a single membrane preparation. Bars represent s.e.mean. Where no bars are shown the error was within the size of the symbol. (b) Receptor-specific binding; obtained by subtracting the binding of [^{3}H]-mepyramine in the presence of 10 μM unlabelled mepyramine from the uninhibited binding. (c) Scatchard transformation of the specific binding. In this experiment B_{max} and K_D values were 203 fmol mg^{-1} protein and 1.3 nM, respectively.

for two classes of binding sites that have high affinity and low affinity for histamine. In four separate experiments, 69 ± 5% of the total receptor population was of high affinity (R_H) with a dissociation constant (K_H) of 2.1 ± 0.2 μM; 31 ± 5% of the receptor was of low affinity (R_L) with a dissociation constant of 28 ± 8 μM. In the presence of 100 μM GppNHp, the curve was shifted to the right and its slope was not significantly different from unity ($n_H = 1.02 \pm 0.02$, $n = 4$), consistent with histamine binding to a single low affinity form of the receptors in the presence of GppNHp (Figure 3).

In contrast, unlabelled mepyramine inhibited [^{3}H]-mepyramine binding with a monophasic displacement curve (Figure 4). The data were fit best by a model of one binding site for unlabelled mepyramine ($n_H = 1.01 \pm 0.09$). GppNHp (100 μM) did not shift the curve to the right. Similarly, the binding data from displacement of [^{3}H]-mepyramine by other H_1-antagonists ((+)-chlorpheniramine, diphenhydramine, promethazine and mequitazine) were best fit by a single-site model (data not shown). The K_i values for these H_1-antagonists are listed in Table 1.

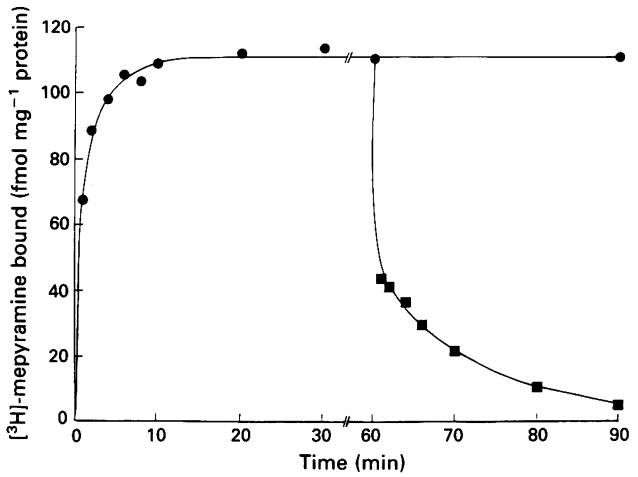

Figure 2 Kinetics of [^{3}H]-mepyramine binding to the membranes from guinea-pig left atria. The association reaction (●) was started by the addition of the membranes and terminated at individual time points. The dissociation reaction (■) was initiated by the addition of histamine (1 mM) after 60 min. The specific binding at each time point is given as mean of triplicate determinations.

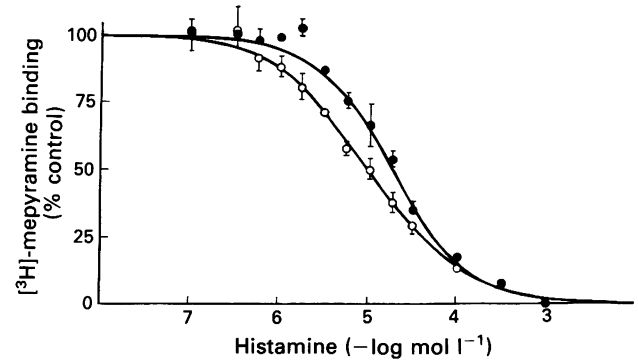

Figure 3 Inhibition of 2.4 nM [^{3}H]-mepyramine binding to guinea-pig left atrial membranes by histamine in the absence (○) or presence (●) of 100 μM GppNHp. Each curve represents a single experiment performed in triplicate and points are means with s.e.mean shown by vertical bars. Analysis of the curves as described in Methods gave the following estimates: histamine without GppNHp: pK_H 5.8, pK_L 4.8, R_H 70% and R_L 30%; with GppNHp: pK_L 4.8 and R_L 100%.

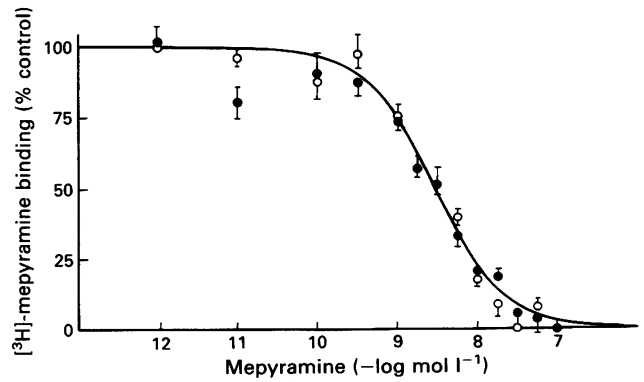

Figure 4 Inhibition of 2.4 nM [^{3}H]-mepyramine binding to guinea-pig left atrial membranes by unlabelled mepyramine in the absence (○) or presence (●) of 100 μM GppNHp. Each curve represents a single experiment performed in triplicate and points are means with s.e.mean shown by vertical bars. Analysis of the curves as described in Methods gave the following estimates: mepyramine without GppNHp: pK_i 9.08; with GppNHp: pK_i 9.09.

Table 1 Inhibitory potencies of H_1-antagonists on [^{3}H]-mepyramine binding and histamine-induced positive inotropic effect in guinea-pig left atria

Drugs	*pK_i values for inhibition of [^{3}H]-mepyramine binding*	*pK_B values for inhibition of the positive inotropic effect of histamine*
Mepyramine	8.89 ± 0.11 (5)	8.63 ± 0.12 (6)
(+)-Chlorpheniramine	9.05 ± 0.11 (4)	8.68 ± 0.04 (5)
Diphenhydramine	7.93 ± 0.25 (5)	7.75 ± 0.19 (6)
Promethazine	8.40 ± 0.31 (4)	7.91 ± 0.10 (5)
Mequitazine	8.32 ± 0.06 (4)	7.49 ± 0.13 (4)

pK_i and pK_B values were determined as described in Methods. Values indicate the means ± s.e.mean. Numerals in parentheses indicate the number of experiments.

The K_B values for H_1-antagonists were calculated from the inhibition of the positive inotropic effect of histamine in guinea-pig left atria (Table 1) and compared with their potencies in inhibiting [^{3}H]-mepyramine binding to guinea-pig left atrial membranes. The K_i values for H_1-antagonists

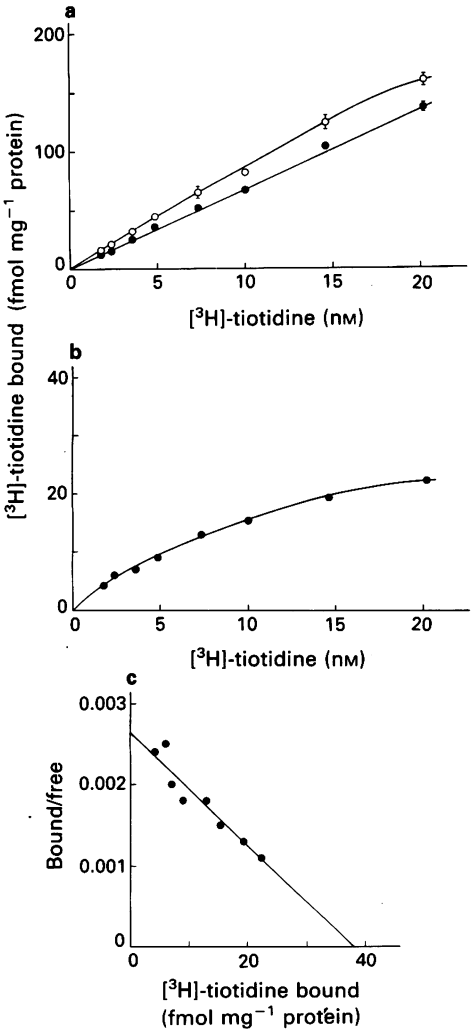

Figure 5 Binding of [^{3}H]-tiotidine to the membranes prepared from guinea-pig left atria. (a) Binding of [^{3}H]-tiotidine in the absence (○) or presence (●) of 100 μM cimetidine. Points are means of triplicate determinations made on a single membrane preparation. Bars represent s.e.mean. (b) Receptor-specific binding; obtained by subtracting binding of [^{3}H]-tiotidine in the presence of 100 μM cimetidine from the uninhibited binding. (c) Scatchard transformation of the specific binding. In this experiment B_{max} and K_D values were 38 fmol mg^{-1} protein and 14.6 nM, respectively.

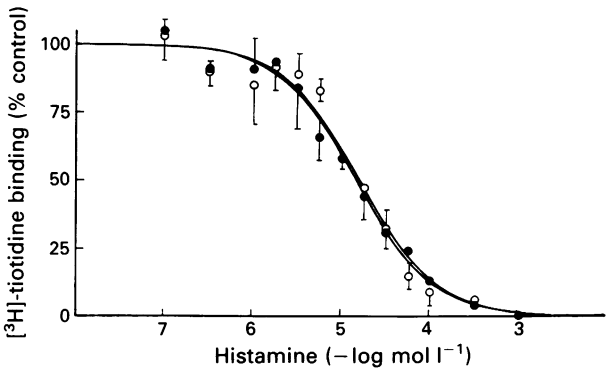

Figure 6 Inhibition of 10 nM [^{3}H]-tiotidine binding to guinea-pig left atrial membranes by histamine in the absence (○) or presence (●) of 100 μM GppNHp. Each curve represents a single experiment performed in triplicate and points are means with s.e. mean shown by vertical lines. Analysis of the curves as described in 'Methods' gave the following estimates: histamine without GppNHp: pK_i 5.11; with GppNHp: pK_i 5.15.

obtained in the [^{3}H]-mepyramine binding experiments correlated well with their K_B values obtained in the functional experiments.

Binding of [^{3}H]-tiotidine

The binding of [^{3}H]-tiotidine to guinea-pig left atrial membrane fractions is shown in Figure 5. The binding not displaced by 100 μM cimetidine increased linearly with the concentration of [^{3}H]-tiotidine. This portion of binding probably represents the non-specific binding of [^{3}H]-tiotidine. The specific binding defined as the difference between the total binding and the non-specific binding appeared to saturate over a concentration range of 1.875–20 nM and to be of high affinity. The non-specific binding accounted for 70–85% of the total binding. The relatively large portion of the non-specific component for this ligand has been reported in previous studies on guinea-pig cerebral cortex (Gajtkowski *et al.*, 1983) and guinea-pig lung parenchyma (Foreman *et al.*, 1985). Scatchard analysis of the specific binding (Figure 5c) resulted in a linear plot suggesting a single class of binding sites. Mean B_{max} and K_D values, calculated from the Scatchard analysis of six experiments, were 41 ± 8 fmol mg^{-1} protein and 10.8 ± 1.2 nM. The Hill coefficient was calculated as 1.05 ± 0.08 from Hill plots of the data. Thus, the maximum binding capacity for [^{3}H]-tiotidine was significantly less than that for [^{3}H]-mepyramine. The ratio of [^{3}H]-mepyramine binding sites to [^{3}H]-tiotidine binding sites was 7.5.

Histamine inhibited the specific [^{3}H]-tiotidine binding in a concentration-dependent fashion (Figure 6). The curve for the inhibition by histamine of [^{3}H]-tiotidine binding showed that the data were best fit by assuming the presence of one binding site for histamine. The addition of 100 μM GppNHp had no effect on the binding of histamine (Figure 6). In four separate experiments, the K_i values for histamine in the absence and presence of GppNHp were 8.5 ± 1.6 μM and 8.6 ± 1.2 μM, respectively. The Hill coefficients were calculated as 1.02 ± 0.04 and 1.03 ± 0.04 from Hill plots of the data without and with GppNHp, respectively.

The H_2-antagonists cimetidine and metiamide also inhibited the specific [^{3}H]-tiotidine binding. In three separate experiments, the K_i values for cimetidine and metiamide were 343 ± 143 nM and 674 ± 155 nM, respectively.

Discussion

In the present study we used the potent H_1-antagonist [^{3}H]-mepyramine to label H_1-receptors in a particulate membrane fraction of guinea-pig left atrium. The specific binding of [^{3}H]-mepyramine to this membrane fraction was saturable, of high affinity and readily reversible. Histamine and a series of H_1-antagonists inhibited the specific [^{3}H]-mepyramine binding in a concentration-dependent manner. Moreover, there was a good correlation between the potencies of the H_1-antagonists in blocking the positive inotropic effect of histamine (K_B) and their potencies of displacing [^{3}H]-mepyramine binding (K_i). Thus, these binding sites detected by [^{3}H]-mepyramine appear to fulfil the criteria which are required for the identification of H_1-receptors.

In contrast to our results, Bennardini *et al.* (1984) failed to detect specific [^{3}H]-mepyramine binding to H_1-receptors in guinea-pig left atrial membranes. This apparent discrepancy could be explained in terms of differences in membrane isolation techniques and assay conditions. The K_D of 1.5 nM calculated for [^{3}H]-mepyramine from the saturation experiments in our study is within a range of the reported values (0.5–7 nM) obtained in peripheral tissues (Chang *et al.*, 1979; Kondo *et al.*, 1985; Hide *et al.*, 1988) and in brain (Hill *et al.*, 1978; Chang *et al.*, 1979).

Uzan *et al.* (1979) have reported that mequitazine possesses a greater affinity for peripheral H_1-receptors than for central H_1-receptors and proposed the idea of subdividing H_1-receptors to a central type and a peripheral type. However, the present study showed that the K_i of mequitazine for the specific [^{3}H]-mepyramine binding in guinea-pig left atrium was 4.8 nM, a value which is in good agreement with the value obtained in guinea-pig cerebellar homogenate (1.7 nM; Wallace & Young, 1983). Thus, it seems unlikely that mequitazine has an affinity for peripheral H_1-receptors different from that for central H_1-receptors. A much more extensive study will be necessary to clarify whether there is a difference between central and peripheral H_1-receptors.

The concentration-response curves for the inhibition by histamine of [^{3}H]-mepyramine binding in guinea-pig left atrial membranes revealed the existence of two binding sites having different affinities for histamine, i.e. high and low affinity sites. This heterogeneous binding was converted into homogeneous binding of low affinity in the presence of GppNHp. High-affinity, guanine nucleotide-sensitive agonist binding is thought to reflect the agonist binding to receptor-guanine nucleotide regulatory protein (G protein) complexes (Gilman, 1987); in the presence of guanine nucleotides, receptor-G protein complexes tend to dissociate, leaving the receptor to a low affinity, uncoupled state. Thus, our data suggest that a considerable portion of H_1-receptors is present in a state coupled to G proteins in guinea-pig left atria. Hence, functional consequences of H_1-receptor activation in guinea-pig left atria might be explained by activation of G proteins coupled to H_1-receptors. Stimulation of H_1-receptors induces phosphoinositide hydrolysis in guinea-pig left atria (Sakuma *et al.*, 1988; Hattori *et al.*, 1989) that is known to be mediated *via* an interaction with G proteins (Berridge, 1987). In fact, the dissociation constant for the guanine nucleotide-sensitive, high agonist affinity site (K_H: 2.1 μM) is in close agreement with the concentration of histamine required to elicit a half-maximal effect on phosphoinositide hydrolysis in guinea-pig left atria (1.4 μM) (Hattori *et al.*, 1989). On the other hand, the half-maximal positive inotropic effect of histamine in guinea-pig left atria is obtained at about 10 times lower concentration than the K_H value. However it should be borne in mind that the half-maximal concentration of an agonist for a certain receptor-mediated response is dependent not only on the efficacy of the agonist but also on the extent of the receptor reserve. It remains unknown whether the G proteins related to hydrolysis of phosphoinositides are also responsible for the positive inotropic effect of H_1-receptor stimulation. It has been reported that G protein-coupled receptors, such as cloned M_2 muscarinic receptors, are able to interact with different G proteins, related to inositol phosphate metabolism and inhibition of adenylate cyclase activity (Ashkenazi *et al.*, 1987).

In radioligand binding studies of H_2-receptors, [^{3}H]-tiotidine has been reported to be a valuable tool as a radiolabel (Gajtkowski *et al.*, 1983). We have demonstrated specific binding of [^{3}H]-tiotidine to membranes from guinea-pig left atria. The K_D of 10.8 nM calculated for [^{3}H]-tiotidine from saturation experiments in this study is in close agreement with those for other guinea-pig tissues; cerebral cortex (17 nM; Gajtkowski *et al.*, 1983) and lung parenchyma (8.5 nM; Foreman *et al.*, 1985). The binding of [^{3}H]-tiotidine to guinea-pig left atrial membranes was displaced by histamine and by the H_2-antagonists, cimetidine and metiamide. Furthermore, the K_i values for cimetidine and metiamide determined in this study are similar to those reported previously (Gajtkowski *et al.*, 1983; Foreman *et al.*, 1985). These results suggest that the specific [^{3}H]-tiotidine binding in this study represents binding to H_2-receptors, that is, H_2-receptors are present in guinea-pig left atria. Stimulation of H_2-receptors is known to result in activation of adenylate cyclase *via* a stimulatory G protein (Gs) in a variety of tissues including the heart (Johnson, 1982). In guinea-pig left atria, however, histamine causes no detectable elevation of cyclic AMP production (Reinhardt *et al.*, 1977; Verma & McNeill, 1977). In the present study we found that the inhibition of the [^{3}H]-tiotidine binding to guinea-pig left atrial membranes by histamine remained unaltered by the addition of 100 μM GppNHp, indicating that the binding of histamine to H_2-receptors in guinea-pig left atria does not induce interaction of the receptor with Gs. However, this does not imply a lack of functional Gs in guinea-pig left atria, because stimulation of β-adrenoceptors, which are well known to be Gs-coupled receptors (Stiles *et al.*, 1984), produces an increase in cyclic AMP and elicits a positive inotropic effect in the same tissue (Hattori *et al.*, 1987). We have observed that the concentration-response curve for the inhibition by histamine of [^{3}H]-tiotidine binding to the membranes from guinea-pig ventricles is shifted to the right in the presence of 100 μM GppNHp (unpublished data). In guinea-pig ventricular muscles histamine is capable of increasing cyclic AMP, an effect mediated by H_2-receptors (Reinhardt *et al.*, 1977; Verma & McNeill, 1977). These results suggest that, in spite of the presence of H_2-receptors in guinea-pig left atria, uncoupling of the receptors from Gs may result in the failure of histamine to stimulate adenylate cyclase activity.

In conclusion, the present binding experiments utilizing [^{3}H]-mepyramine and [^{3}H]-tiotidine provide evidence for the presence of both H_1- and H_2-receptors in guinea-pig left atrium. Whereas H_1-receptors could interact with G proteins, H_2-receptors seem unlikely to be linked to G proteins. The former may raise the possibility that the pharmacological response to H_1-receptor stimulation may be mediated through G proteins. The latter is consistent with results showing that histamine fails to induce any change in cyclic AMP level in this tissue (Reinhardt *et al.*, 1977; Verma & McNeill, 1977).

The authors thank Miss M. Yuzaki for her excellent technical assistance and Miss M. Kobayashi and Miss Y. Yonezawa for secretarial assistance.

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(Received August 29, 1990
Revised January 17, 1991
Accepted February 11, 1991)

Br. J. Pharmacol. (1991), **103**, 1580–1584 © Macmillan Press Ltd, 1991

Functional changes in $GABA_A$ receptor stimulation during the oestrous cycle of the rat

[1]Peter Westerling, Silvana Lindgren & Bengt Meyerson

Department of Medical Pharmacology, University of Uppsala, Uppsala, Sweden

1 Slices of rat cuneate nucleus were used to study whether or not gonadal steroids influence the γ-aminobutyric acid (GABA) system *in vivo*. Females in different stages of the oestrous cycle as well as steroid-treated (oestrogen, progesterone or both) ovariectomized animals were used.

2 Functional changes in the $GABA_A$ receptors were assayed using the effects of potentiators (benzodiazepine, barbiturate) and antagonists (picrotoxin) on the muscimol control dose-response curves.

3 The potentiating effect of the benzodiazepine, flurazepam was unchanged during the oestrous cycle, and the hormone treatments did not alter this effect.

4 During oestrus, an increase was seen in the potentiating effect of the barbiturate (pentobarbitone). This suggests a synergistic effect between barbiturates and gonadal steroids. Progesterone treatment also increased the effect of pentobarbitone.

5 The antagonistic action of picrotoxin was unaffected during the oestrous cycle. However, progesterone (or progesterone and oestrogen) treatment reduced the potency of picrotoxin.

6 This study supports the idea that endogenous steroids (presumably progesterone) affect the $GABA_A$ receptors during the oestrous cycle by a mechanism associated with the barbiturate site of the $GABA_A$ receptor complex.

Keywords: $GABA_A$-receptors; muscimol; flurazepam; pentobarbitone; oestrous cycle of the rat; progesterone; oestrogen

Introduction

The inhibitory neurotransmitter, γ-aminobutyric acid (GABA) is recognised by at least two different receptors ($GABA_A$ and $GABA_B$ receptors). Stimulation of the $GABA_A$ receptor evokes an increase in membrane chloride conductance. This effect generally leads to membrane hyperpolarization. However, presynaptic inhibition is associated with a depolarizing phenomenon, primary afferent depolarization (PAD) (see Levy, 1980 for review).

The $GABA_A$ receptor forms a complex with different binding sites for benzodiazepines, barbiturates and other related compounds (Olsen, 1982). Numerous candidates with different chemical structures have been proposed as endogenous modulators of the $GABA_A$ receptor complex, for instance polypeptides (diazepam binding inhibitor, Guidotti *et al.*, 1983) and β-carbolines (Braestrup *et al.*, 1983). Steroid modulation of the $GABA_A$ receptor complex has also been shown (Simmonds & Turner, 1987; Majewska, 1987; Lambert *et al.*, 1987; Gee *et al.*, 1988).

Progesterone, because of its anticonvulsive (Bäckström, 1976; Landgren *et al.*, 1987) and anaesthetic (Seyle, 1942; Meyerson, 1967; Norberg *et al.*, 1987) properties, is of particular interest in this context. The central nervous system depression brought about by progesterone is thought to be mediated by metabolites, e.g. 3α-hydroxy pregnane-20-one (Majewska *et al.*, 1986; Simmonds & Turner, 1987; Norberg *et al.*, 1987) acting on $GABA_A$ receptors. It has been shown by *in vitro* studies that the effect of steroids on the $GABA_A$ receptor complex is similar to that of barbiturate stimulation (Harrison & Simmonds, 1984; Majewska *et al.*, 1986; Simmonds & Turner, 1987; Lambert *et al.*, 1987; Turner & Simmonds, 1989). Picrotoxin antagonizes the depolarization by blocking the barbiturate site (Simmonds, 1981). Steroids have the ability to reduce the inhibition induced by picrotoxin (Simmonds *et al.*, 1984). The effect of certain pregnane steroids may also involve the GABA/benzodiazepine Cl^- ionophore (Simmonds & Turner, 1987; Majewska, 1987; Lambert *et al.*, 1987). The mechanism by which steroids interact with the $GABA_A$ receptor complex is not fully understood. However, taken together the *in vitro* studies, the documentation of progesterone-related changes in mood and altered central neuronal excitability, all suggest that central nervous GABAergic transmission is likely to be influenced by steroids under physiological and pathophysiological conditions. To explore this question further under controlled experimental conditions, we used female rats at different stages of the oestrous cycle. Hormonal treatment (oestrogen, progesterone or both) was also given to ovariectomized animals to investigate the role of progesterone alone and after oestrogen priming. Functional changes in the $GABA_A$ receptor complex were investigated by the cuneate-slice method previously described by Simmonds (1978; 1980; 1981). GABA and $GABA_A$ receptor agonists (e.g. muscimol) depolarize afferent nerves (PAD) in the cuneate nucleus slice. This effect is potentiated by benzodiazepines (e.g. flurazepam) and barbiturates (e.g. pentobarbitone).

Methods

Female Sprague-Dawley rats (specified pathogen-free, purchased from A-lab, Sollentuna, Sweden) weighing 336 ± 6.9 g were kept under a reversed day-night cycle (12/12 h, with the light off between 09 h 00 min and 21 h 00 min). They had free access to water and commercial food pellets.

One group of animals was ovariectomized under pentobarbitone anaesthesia (about 60 mg kg^{-1}, i.p.). The other group consisted of unoperated females. The animals were used in the experiment 1 to 2 months after surgery.

The cuneate slice method

To obtain a functional measurement of the $GABA_A$ receptor complex, the cuneate nucleus slice preparation described by Simmonds (1978; 1980; 1981) was used. The animals were stunned and decapitated at a low cervical level with a guillotine. An incision was made at the level of obex, the medulla oblongata was then placed in ice-cold Krebs medium. A razor

[1] Author for correspondence.

blade was used to obtain two slices (≈0.6–0.8 mm thick) containing the dorsal funiculus and cuneate nucleus of each side. The slices were placed in a two-compartment bath so that the cuneate nucleus was in one compartment and the dorsal funiculus projected through a greased slot to the other compartment. The direct current between the two compartments was recorded continuously on a chart recorder with Ag/AgCl electrodes. Both compartments were perfused with Krebs medium, but drugs were added only to the cuneate nucleus compartment.

Two slices were taken from each animal, and the effects of flurazepam and pentobarbitone were tested on one slice (slice a) and the effects of two doses of picrotoxin on the other slice (slice b) (Lindgren & Simmonds, 1987). The drug-induced peaks were measured at their highest amplitude.

Muscimol was used routinely as an agonist (Simmonds, 1980), dissolved in the Krebs medium and the cuneate nucleus was perfused for 2 min. The control doses were 2.5 μM, 5 μM and 10 μM muscimol, these doses having been shown to be in the lower part of the dose-response curve in this preparation by Simmonds (1980), thus reducing desensitization of the $GABA_A$ receptor complex. During picrotoxin treatment the doses of muscimol were increased up to 20 μM (3 μM picrotoxin) and 80 μM (30 μM picrotoxin), to obtain approximately the same response as the control values. Picrotoxin (3 μM and 30 μM), flurazepam (1 μM) and pentobarbitone (10 μM) were all dissolved in the Krebs medium and perfused for 30 min before and during the redetermination of the muscimol dose-response curves. During flurazepam or pentobarbitone perfusion the doses of muscimol were decreased to 1.25 μM, 2.5 μM and 5 μM. All these doses gave responses in the lower part of the dose-response curves. A recovery time of about 20 min was allowed between each dose given. To assess any non specific changes in the responsiveness of the slices, responses to superfusion with an additional 3.9 mM KCl (for 5 min) were monitored regularly. All experiments were performed at room temperature (≈18–22°C).

The potentiation or inhibition by drug-perfusion was measured as the leftward or rightward shift of the log dose-response curves. Parallel shifts of the log dose-response curves (Figure 2) allowed dose-ratios (log units) to be calculated. Dose-ratios were calculated from the intersections of the dose-response curves with a horizontal line (Simmonds, 1980). Only those experiments where the dose-curves were shifted in an approximately parallel fashion were used.

Steroid treatment

Vaginal smears were taken daily by the lavage technique (between 08 h 00 min and 09 h 00 min) to determine the various stages of the oestrous cycle (dioestrus, prooestrus, oestrus) in intact females.

Ovariectomized rats were either not treated or treated with progesterone (1 mg per animal, s.c. 3–3.5 h before they were killed), oestradiol benzoate (25 $\mu g\,kg^{-1}$, s.c. 52 h before they were killed) or oestradiol benzoate followed 48 h later by progesterone (dose as above). The hormonal treatments were chosen with regard to doses which are known to induce a central nervous response, e.g. to activate female copulatory behaviour (lordosis response) (Meyerson, 1967).

Drugs

The following drugs were used: muscimol (Sigma, St. Louis, U.S.A.), pentobarbitone sodium salt (Apoteksbolaget, Sweden), flurazepam (Roche), picrotoxin (EGA-Chemie) isethionic acid, oestradiol benzoate and progesterone (Sigma, St. Louis, U.S.A.). The steroids were dissolved in olive oil. The Krebs medium contained (mM): NaCl 118, KCl 2.1, KH_2PO_4 0.93, $CaCl_2$ 2.5, $MgSO_4$ 2.2, $NaHCO_3$ 25 and glucose 11, and a mixture of 93.5% O_2 and 6.5% CO_2 was bubbled through it.

Statistical methods

All values are means ± s.e.mean; statistical comparisons were made by analysis of variance (ANOVA) followed by Tukey HSD multiple comparisons test. The levels of significance were set at $P < 0.05$ and $P < 0.01$.

Results

Cl^- dependence

In order to assess the Cl^- dependence of the method in our hands, Cl^- in the Krebs medium was replaced by isethionate. The depolarization induced by 10 μM muscimol was determined after 40%, 60% and 80% reduction of the Cl^- concentration. When 80% of the Cl^- was substituted, a significant decrease in depolarization was seen (Figure 1), thus establishing the Cl^- dependence of the cuneate method.

Response to K^+

Regularly, or at least at the beginning and end of an experiment, the response to K^+ was measured (raised to 6 mM in the Krebs medium). This K^+-induced depolarization was used to test the condition of the slice. Only preparations which gave at least a 0.3 mV response to the first application of KCl were used and subsequent experimental data were accepted only if the response to KCl at the end of the experiment (6–8 h) was not less than 80% of the initial response. The KCl-induced depolarizations were in slice (a); before experiment: 0.42 mV ± 0.028 ($n = 56$), after experiment: 0.39 mV ± 0.028 ($n = 56$), and in slice (b); before experiment: 0.37 mV ± 0.033 ($n = 46$), after experiment: 0.36 mV ± 0.027 ($n = 46$).

Effect of oestrous cycle on the $GABA_A$ system

Muscimol produced a concentration-related depolarization of the dorsal funiculus fibres in the cuneate slice (10 μM muscimol: 0.47 mV ± 0.06, 5 μM muscimol: 0.32 mV ± 0.04, 2.5 μM muscimol: 0.20 mV ± 0.03). The recovery time was about 20 min, with a slower recovery for the highest dose (Figure 2).

Flurazepam When flurazepam (1 μM) was perfused, a parallel shift to the left in the muscimol dose-response curve was seen (Figure 2).

During the oestrous cycle, no significant change (Table 1) (ANOVA; $F = 2.43$, $P > 0.11$) was seen of the potentiation of muscimol by flurazepam.

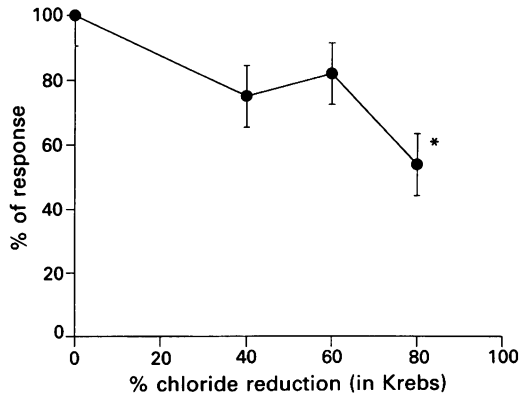

Figure 1 Reduction of responses to 10 μM muscimol in rat cuneate nucleus slice by replacing 20%, 40% and 80% of the Cl^- concentration with isethionate. The values are expressed as a percentage (of muscimol-induced depolarization) with s.e.mean shown by vertical bars ($n = 3$–5). Statistical significance (compared with the 100% value) was calculated by Student's t test (*$P < 0.05$).

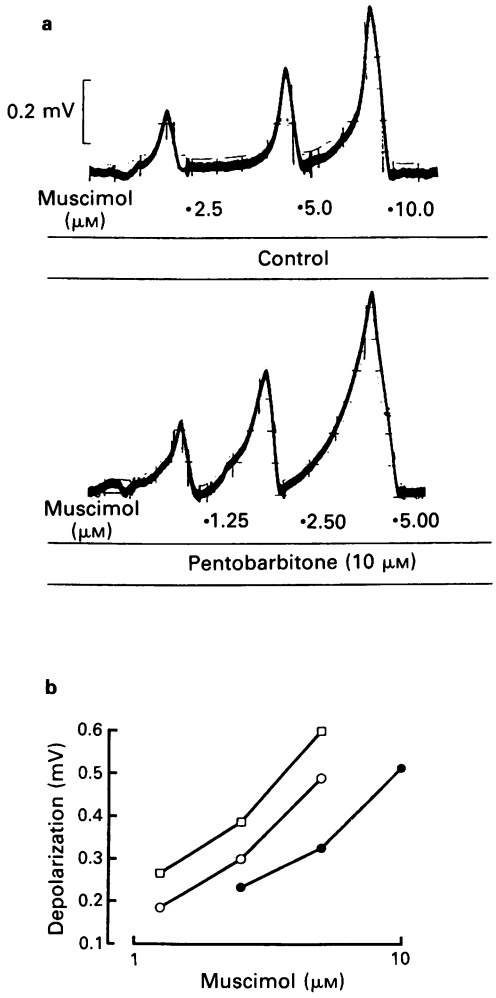

Figure 2 Original tracings of responses to 2 min applications of muscimol and potentiation by pentobarbitone 10 μM. (a) Note that, the tracings run from the right to the left. The recovery time was about 20 min after each given dose. (b) Effects of flurazepam 1 μM (○) and pentobarbitone 10 μM (□) on log dose-response curves for muscimol (data from a); control (●). The results represents a single experiment from one animal and each point is a single response. The approximately parallel shift represents a 0.27 log unit shift (flurazepam) and a 0.40 log unit shift (pentobarbitone).

Pentobarbitone Pentobarbitone (10 μM) caused a greater leftward shift of the muscimol dose-response curve than flurazepam (Figure 2).

The pentobarbitone stimulation was significantly increased during the oestrous cycle (ANOVA; $F = 6.23$, $P < 0.01$). When animals were in oestrus, pentobarbitone caused a significantly greater potentiation of muscimol than when they were in dioestrus (Table 1).

Picrotoxin Upon exposure to picrotoxin, an approximately parallel shift to the right in the muscimol dose-response curve was obtained, this antagonism was dose-dependent (Table 1).

Animals in different stages of the oestrous cycle showed no changes in the antagonism of muscimol by picrotoxin (ANOVA; $F = 0.28$. $P > 0.76$ and $F = 0.39$, $P > 0.68$ for 3 μM and 30 μM).

Effects of hormone treatment on the $GABA_A$ system

In order to establish the role of different hormones in the potentiation of muscimol, female rats were ovariectomized and some were given oestrogen, progesterone or both. The hormones were injected 1 to 2 months after surgery in a single dose.

Flurazepam The addition of flurazepam to the medium perfusing slices from ovariectomized, untreated rats caused a leftward shift of the muscimol dose-response curve, similar to that in slices from unoperated rats (Table 2).

The hormone treatments did not significantly alter the effect of flurazepam (ANOVA; $F = 0.85$, $P > 0.48$). Comparison between the oestrous cycle and hormone treatments showed a difference between oestrus and ovariectomized animals (0.25 ± 0.029 log unit vs 0.14 ± 0.026 log unit, respectively, $t = 2.40$, $P < 0.05$).

Table 1 Potentiation of effects of muscimol by flurazepam pentobarbitone and antagonism by picrotoxin on cuneate slices during the oestrous cycle in rats

Oestrous cycle	*Fluraz* (1 μM)	*Pentobarb* (10 μM)	*Picrot* (3 μM)	*Picrot* (30 μM)
Dioestrous	0.17 (8) ± 0.027	0.26 (8) ± 0.031	0.54 (7) ± 0.089	1.03 (7) ± 0.068
Prooestrous	0.24 (7) ± 0.036	0.30 (7) ± 0.038	0.51 (7) ± 0.028	1.09 (7) ± 0.027
Oestrous	0.26 (8) ± 0.029	0.41 (8)** ± 0.037	0.57 (6) ± 0.080	1.04 (6) ± 0.089

The potentiation or antagonism of muscimol was measured as lateral shifts (log units) of the muscimol dose-response curve (see Methods). The values are means ± s.e.mean, (*n*). Statistical significance (compared to dioestrous) was calculated by ANOVA followed by Tukey HSD test (** $P < 0.01$).

Table 2 Potentiation of muscimol by flurazepam, pentobarbitone and antagonism by picrotoxin in cuneate slices from ovariectomized and ovariectomized and hormone-treated rats

Hormone treatment	*Fluraz* (1 μM)	*Pentobarb* (10 μM)	*Picrot* (3 μM)	*Picrot* (30 μM)
Ovariectomized	0.14 (9) ± 0.026	0.22 (9) ± 0.040	0.62 (6) ± 0.039	1.21 (6) ± 0.036
Ovariectomized + oestrogen	0.17 (7) ± 0.027	0.29 (7) ± 0.033	0.50 (5) ± 0.082	1.11 (5) ± 0.074
Ovariectomized + progesterone	0.20 (9) ± 0.037	0.36 (9)* ± 0.052	0.37 (8)** ± 0.053	0.93 (8)* ± 0.084
Ovariectomized + oest and prog	0.20 (8) ± 0.033	0.34 (8)* ± 0.025	0.46 (7)* ± 0.048	0.98 (7)* ± 0.063

Effects of oestrogen (oest; 25 μg kg^{-1}, s.c., 3–3.5 hours before rats were killed), progesterone (prog; 1 mg/animal, s.c., 48 h before rats were killed) or oestrogen and progesterone on the potentiation of muscimol by flurazepam (Fluraz; 1 μM), pentobarbitone (Pentobarb; 10 μM) or antagonism of muscimol by picrotoxin (Picrot; 3 μM, 30 μM) in cuneate nucleus slices from ovariectomized rats. The potentiation or antagonism was measured as lateral shifts (log units) of the muscimol dose-response curve (see Methods). The values are means ± s.e.mean, (*n*). Statistical significances (compared to ovariectomized) were calculated by ANOVA followed by Tukey HSD test (* $P < 0.05$, ** $P < 0.01$).

Pentobarbitone In slices from ovariectomized, untreated rats, pentobarbitone shifted the muscimol dose-response curve to the left (Table 2). The potentiation induced by pentobarbitone was greater than that of flurazepam.

In comparing the hormone treatments, there was a tendency towards a difference (ANOVA; $F = 2.81$, $P < 0.057$). Treatment with progesterone or oestrogen combined with progesterone significantly increased the potentiation due to pentobarbitone of the muscimol-induced depolarization of the dorsal funiculus.

Comparison between the oestrous cycle and hormone treatments showed a difference between oestrus and ovariectomized animals (0.41 ± 0.037 log unit vs 0.22 ± 0.040 log unit, respectively, $t = 3.21$, $P < 0.01$).

Picrotoxin Antagonism of the muscimol response by picrotoxin at 3 μM and 30 μM differed with the different hormone treatments (ANOVA; $F = 3.48$, $P < 0.03$, $F = 3.20$, $P < 0.04$ for 3 μM and 30 μM, respectively).

Progesterone reduced the antagonism of picrotoxin; this was seen with both doses of picrotoxin (Table 2). The combined treatment with oestrogen and progesterone also diminished the antagonism of picrotoxin on muscimol-induced depolarization.

Discussion

The present study addresses the question of whether or not gonadal steroids influence the GABA system *in vivo*. In order to measure functional $GABA_A$ receptors, the cuneate slice method was used (Simmonds, 1978; 1980; 1981). When GABA or GABA agonists are perfused over the cuneate nucleus, a depolarization is produced which is associated with presynaptic inhibition (Schmidt, 1971).

An important aspect of this study was to establish the cuneate slice method in female rats. It was shown that flurazepam, pentobarbitone and picrotoxin had the same modulatory effect in ovariectomized and dioestrus animals as in male rats.

The chloride-dependence of the method was determined by reducing the Cl^- concentration in the Krebs medium. The muscimol-induced depolarization decreased with lower (20%) concentrations of chloride. It has been shown by Simmonds (1978) that replacing 80% of the Cl^- in the medium reduced GABA depolarization. This was confirmed in our pre-experimental study. These results emphasise the role of chloride in the depolarization of fibres of the dorsal funiculus.

GABA is subject to uptake processes. This problem was overcome in the present study by use of the agonist muscimol which is not a good substrate for GABA uptake processes (Simmonds, 1980); also, muscimol is more potent than GABA in the cuneate nucleus slice (Pickles & Simmonds, 1980). In *in vitro* studies both steroids (Simmonds *et al.*, 1984) and barbiturates (Harrison & Simmonds, 1983) reduce the potency of picrotoxin as an antagonist at the $GABA_A$ receptor complex. It was therefore also considered important to study the inhibitory effect of picrotoxin in animals under different hormonal *in vivo* conditions.

When flurazepam was added to the perfusion medium there was a shift to the left of the muscimol dose-response curve (two fold) which corresponded well with the results obtained by Simmonds (1981). The potentiating effect of flurazepam was unchanged during the oestrous cycle, nor did the hormone treatments have any effect. This demonstrates that neither oestrogen nor progesterone plays a role in the potentiation of the $GABA_A$ receptor complex brought about by benzodiazepines.

Pentobarbitone caused a greater modulation than flurazepam, the effect being about a 3 fold shift to the left of the muscimol dose-response curve. During oestrus this effect increased to a 4 fold shift, suggesting a synergistic effect between pentobarbitone and the hormone levels during oestrus. In the female rat, serum levels of progesterone rise to a single peak (from about 25 ng ml^{-1} to about 150 ng ml^{-1}), whereas oestradiol levels in serum show a more irregular pattern with more numerous peaks (from about 50 pg ml^{-1} to about 250 pg ml^{-1}) during oestrus (Södersten *et al.*, 1981). It has been proposed that steroid hormones potentiate the $GABA_A$ receptor complex in a barbiturate-like manner *in vitro* (Harrison & Simmonds, 1984; Majewska *et al.*, 1986; Simmonds & Turner, 1987; Lambert *et al.*, 1987; Turner & Simmonds, 1989). Majewska and colleagues (1989) have recently shown that pregnancy and the postpartum period alter the binding sensitivity of brain $GABA_A$ receptors to muscimol. The authors suggest that this effect is a result of the action of certain endogenous steroids. By using voltage-clamp recordings from bovine isolated chromaffin cells, Cottrell *et al.* (1987) could show that the steroid anaesthetic, alphaxalone, activated $GABA_A$ receptors in a barbiturate-like manner. Gee *et al.* (1988) studying steroid structure-activity relationships suggest, however, that the steroids and anaesthetic barbiturates do not share a common site of action. Although we do not suggest identical mechanisms of action our results do indicate that the alterations in steroid levels during the oestrous cycle affect the $GABA_A$ receptor by a mechanism associated with the barbiturate site of the $GABA_A$ receptor complex.

The treatment with oestrogen, progesterone or a combination of both given to ovariectomized rats had no effect on the flurazepam-induced potentiation of muscimol. However, the pentobarbitone-induced potentiation of muscimol was significantly increased when progesterone or progesterone and oestrogen were given. This further supports the hypothesis that progesterone acts in a synergistic manner in association with a barbiturate-like mechanism. This investigation cannot answer the question of whether it is progesterone itself or metabolites of the hormone that are acting on the $GABA_A$ receptor complex. However, as the potentiation of the muscimol effect was seen 3 to 3.5 h after the steroid injection (but not after 5 h, Westerling *et al.* unpublished data) and the effect is presumably mediated through membrane mechanisms, it is reasonable to assume that progesterone metabolites are active and not the hormone itself. However, we cannot exclude genomic activation giving rise to products which are responsible for the potentiation of the muscimol stimulation. If progesterone acts directly at the membrane, the effect seems to be a long-lasting modulation of the $GABA_A$ receptor complex.

Pentobarbitone and picrotoxin have been shown to have reciprocal actions on single $GABA_A$ receptor channels (Twyman *et al.*, 1989). In our study picrotoxin inhibition became less pronounced with progesterone or progesterone and oestrogen treatments. This further supports the idea that progesterone acts at or close to the same site as barbiturates on the $GABA_A$ receptor complex. However, during the oestrous cycle no effect on the picrotoxin inhibition could be seen. This might be due to differences in efficiency between the effects of exogenous and endogenous hormones. However, endogenous hormones seem to have a greater effect on pentobarbitone dose-response shifts. This disparity needs further investigation.

In our study, effects of *in vivo* manipulation could be seen in an *in vitro* assay. Majewska and colleagues (1985) showed that adrenalectomy reduced muscimol binding in certain brain areas *in vitro*. The authors suggest that glucocorticoids act directly at the membrane level to alter the affinity of $GABA_A$ receptors.

We conclude that there is a functional change in the $GABA_A$ receptor complex during the oestrous cycle due to the actions of progesterone and/or its metabolites. The results further suggest an association of the steriod action with the barbiturate site of the $GABA_A$ receptor complex.

The authors thank Dr M.A. Simmonds for valuable help in establishing the cuneate slice method and for valuable comments about the manuscript. The work was supported by the Swedish Medical Research Council (04X-00064).

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(Received September 10, 1990
Revised February 11, 1991
Accepted February 12, 1991)

Br. J. Pharmacol. (1991), **103**, 1585–1591 © Macmillan Press Ltd, 1991

Cardiac haemodynamic effects of the non-peptide, angiotensin II-receptor antagonist, DuP 753, in conscious Long Evans and Brattleboro rats

*P. Batin, [1]S.M. Gardiner, A.M. Compton, P.A. Kemp & T. Bennett

Departments of *Medicine and Physiology and Pharmacology, Queen's Medical Centre, Nottingham, NG7 2UH

1 In previous experiments in conscious, water-replete Long Evans and Brattleboro rats the non-peptide angiotensin II-receptor antagonist, DuP 753, caused only slight hypotension and peripheral (particularly renal) vasodilatations. However in water-deprived (i.e. renin-dependent) Brattleboro rats, DuP 753 caused marked hypotension and regional vasodilatations. The major objective of the present study was to determine if the hypotensive effects of DuP 753 under any of the experimental conditions studied previously were contributed to by negative effects on cardiac haemodynamics.

2 Male, Long Evans and Brattleboro rats were chronically instrumented with electromagnetic flow probes on the ascending aorta and with intravascular catheters. Data were collected by use of a microcomputer-based system that provided digitised print-out of instantaneous heart rate, mean arterial blood pressure, cardiac output, stroke volume, peak aortic flow, maximum positive slope of the aortic flow signal ($+ dF/dt_{max}$), total peripheral conductance and central venous pressure.

3 Incremental i.v. bolus doses (0.1–10 mg kg^{-1}, at 15 min intervals) of DuP 753 were administered to water-replete Long Evans ($n = 8$) and Brattleboro ($n = 8$) rats, and to water-deprived (14 h) Brattleboro rats ($n = 9$) (the latter animals show marked activation of the renin-angiotensin system). In all groups, 15 min after the highest dose of DuP 753 had been given, a supramaximal dose of captopril (2 mg kg^{-1}) was injected to determine if it had any additional effects.

4 In water-replete, Long Evans and Brattleboro rats, DuP 753 (0.1–1 mg kg^{-1}) caused slight, transient hypotension, with rises in total peripheral conductance; increases in cardiac output, peak aortic flow, $+ dF/dt_{max}$ and stroke volume were inconsistent, and central venous pressure did not change. Higher doses of DuP 753 (3 and 10 mg kg^{-1}) caused modest, sustained hypotension that was due entirely to an increase in total peripheral conductance, since cardiac output, peak aortic flow and $+ dF/dt_{max}$ showed transient elevations; captopril had no additional hypotensive or vasodilator effects.

5 Under resting conditions, water-deprived Brattleboro rats showed an increase in mean arterial blood pressure, but there were reductions in total peripheral conductance, cardiac output and $+ dF/dt_{max}$. DuP 753 (0.1–10 mg kg^{-1}, i.v., boluses at 15 min intervals) caused incremental hypotension and tachycardia and dose-dependent rises in total peripheral conductance that were accompanied by transient increases in cardiac output, peak aortic flow and $+ dF/dt_{max}$; captopril had no additional hypotensive or vasodilator effects.

6 Under no conditions were there any negative effects of DuP 753 on myocardial function; moreover, it is likely the transient increases in cardiac function following DuP 753 were indirect and due to the reduction in afterload. Thus, it appears the haemodynamic effects of DuP 753 can be explained by its vasodilator action.

Keywords: Cardiac haemodynamics; non-peptide angiotensin II-receptor antagonist; DuP 753

Introduction

Our understanding of the involvement of the renin-angiotensin system (RAS) in cardiovascular regulation has been advanced greatly by the availability of angiotensin converting enzyme (ACE) inhibitors such as captopril, and antagonistic peptide analogues of angiotensin II (AII) such as saralasin. However, results obtained with these pharmacological tools *in vivo* may not be clear-cut since ACE inhibitors can affect the metabolism of several peptides in addition to inhibiting the generation of AII (Zusman, 1984; Skidgel *et al.*, 1987) and the antagonistic peptide analogues of the latter also have agonistic effects (see Tomlinson *et al.*, 1990).

Recently it has been demonstrated that there are at least two sub-types of AII receptors. The AII-receptor sub-type associated with vascular smooth muscle (originally described as AII-B (Whitebread *et al.*, 1989) or AII_1 (Chiu *et al.*, 1989)) is selectively blocked by compounds such as 2-n-butyl-4-chloro-5-hydroxymethyl-1-[2-(1H-tetrazole-5-yl)bi-phenyl-4-yl methyl]imidazole, potassium salt (DuP 753) (Chiu *et al.*, 1990; Wong *et al.*, 1990b,c,d,e,f). DuP 753 is totally lacking in agonistic effects and has no influence on vascular responses to noradrenaline, vasopressin or bradykinin, or on ACE activity (Chiu *et al.*, 1990; Wong *et al.*, 1990b,c,d,e,f).

In conscious, normotensive, Sprague-Dawley rats, or in rats with DOCA-salt hypertension, DuP 753 has no hypotensive effect, but in normotensive Sprague-Dawley rats treated with frusemide, in rats with hypertension due to renal artery ligation and in spontaneously hypertensive rats, DuP 753 lowers blood pressure (Wong *et al.*, 1990b,c,d,e,f). In those studies detailed haemodynamic measurements were not made, leaving open the possibility that DuP 753 might influence regional vascular conductances and/or cardiac output, even in conditions in which mean arterial blood pressure was unaffected. In order to cover the former possibility we examined the regional haemodynamic effects of DuP 753 in conscious, water-replete, Long Evans and Brattleboro rats (Batin *et al.*, 1991). We chose these animals because we have substantial background information on them and, more importantly, cardiovascular status in Brattleboro rats can be made dependent on the RAS

[1] Author for correspondence.

simply by depriving them of drinking water for a short time (Gardiner & Bennett, 1985; Bennett & Gardiner, 1987; Tomlinson et al., 1990; Muller et al., 1990).

In water-replete, Long Evans and Brattleboro rats, DuP 753 had little effect on mean arterial blood pressure, although it did cause mesenteric and renal vasodilatations. The absence of a change in mean arterial blood pressure when renal and mesenteric vasodilatations occurred, indicates that cardiac output might have risen along with vascular conductances, but no direct measurement of cardiac haemodynamics was possible in those animals since they were extensively instrumented for recording renal, mesenteric and hindquarters blood flows. Therefore, the first aim of the present study was to make direct measurements of cardiac haemodynamics in water-replete, Long Evans and Brattleboro rats and to assess changes in those variables in response to incremental i.v. bolus doses of DuP 753 to match our previous protocol (Batin et al., 1991). This protocol was chosen originally because DuP 753 has an extremely long duration of action (Wong et al., 1990c,d,e; Batin et al., 1991) and hence it is not possible to avoid cumulative effects with any reasonable dosing cycle.

In water-deprived Brattleboro rats with marked activation of the RAS, captopril causes substantial hypotension (Gardiner & Bennett, 1985; Tomlinson et al., 1990; Muller et al., 1990). Consistent with these findings, administration of DuP 753 to water-deprived Brattleboro rats caused renal, mesenteric and hindquarters vasodilatations together with a sizeable fall in mean arterial blood pressure (Batin et al., 1991). However, in those experiments we could not exclude the possibility that inhibition of the cardiotonic effects of AII (Urata et al., 1990; Hirakata et al., 1990; Lindpaintner et al., 1990) contributed to the hypotensive action of DuP 753. Therefore, the second aim of the present study was to measure changes in cardiac haemodynamics in water-deprived Brattleboro rats given DuP 753, following the same protocol as previously (Batin et al., 1991).

As mentioned above, it is feasible that the acute cardiovascular effects of captopril involve mechanisms in addition to inhibition of AII production. If this were the case then administration of captopril in the presence of a maximally effective dose of DuP 753 should cause additional cardiac haemodynamic changes. Our third aim was to investigate this possibility, as we had in our previous investigation involving measurement of regional haemodynamics (Batin et al., 1991).

Methods

Male, Long Evans and homozygous Brattleboro rats (350–450 g) were anaesthetized (sodium methohexitone, 60 mg kg^{-1} i.p., supplemented as required) and had an electromagnetic flow probe (Skalar, Delft, Netherlands) implanted around the ascending thoracic aorta (Smith & Hutchins, 1979; Smits et al., 1982; Gardiner et al., 1990a,b,c). Animals were given ampicillin (7 mg kg^{-1} i.m.; Penbritin, Beechams) after surgery and kept in their home cages for at least 7 days. Thereafter, under brief anaesthesia (sodium methohexitone 40 mg kg^{-1}, i.p.) intravascular catheters were implanted; the tip of an intraarterial catheter was positioned in the distal abdominal aorta (via the ventral caudal artery), and 3 catheters were implanted in the right jugular vein. One of the venous catheters was placed with its tip near the right atrial orifice to allow the measurement of central venous pressure; this catheter was infused continuously with saline (NaCl 154 mmol l^{-1}) to prevent emboli forming at its tip (Gardiner et al., 1990a,b,c). All catheters were tunnelled subcutaneously to emerge at the back of the neck close to the flow probe connector. The catheters were contained within a flexible spring connected to a harness worn by the rat. A short interconnecting cable from the flow probe was taped to the spring to allow connections to be made to the flowmeter (Skalar, MDL 1401) without disturbing the animal. The whole assembly was supported by a counterbalanced lever system (Gardiner et al., 1990a,b,c). Animals were returned to their home cages and left to recover for at least 48 h before experiments were begun. During this time Long Evans rats were infused with an additional 20 ml of isotonic saline each day (at 1 ml h^{-1}) in order to avoid any possible problems with post-surgical hypodipsia. The latter phenomenon is not observed in Brattleboro rats since their usual polydipsia is re-established very soon after surgery (unpublished observations).

During an experiment, the inputs from the thoracic aortic flow probe and intraarterial and central venous pressure transducers were fed into a custom-built haemodynamics microprocessor (University of Limburg Instrument Services and Pharmacology Departments) interfaced with a Tandon 386 microcomputer. This system digitized the input signals every 2 ms and stored data every 1 or 10 s to disc, as required. Off-line, selected portions of the experiment were analysed and average values for mean arterial blood pressure, heart rate, cardiac output, stroke volume, peak aortic flow, maximum rate of positive change of aortic flow ($+ dF/dt_{max}$) and central venous pressure were obtained. (Peak aortic flow and $+ dF/dt_{max}$ have been taken as indirect indices of contractility (Schoemaker, 1989)). The cardiac variables were not normalised for body weight since water deprivation in Brattleboro rats causes substantial loss of body weight (Gardiner & Bennett, 1985). However, in the water-replete state there were no differences in body weight in the different groups of animals.

All experiments began between 06 h 30 min and 07 h 00 min. The water-deprived Brattleboro rats had had their drinking water removed at 17 h 00 min the previous day and hence had been without water for 14 h. This protocol produces about 25% reduction in plasma volume and makes systemic arterial blood pressure very sensitive to the hypotensive effects of captopril (Gardiner & Bennett, 1985; Tomlinson et al., 1990; Muller et al., 1990).

As mentioned in the Introduction, DuP 753 has a very prolonged action (Wong et al., 1990c,d,e; Batin et al., 1991), and hence it is not possible to avoid cumulative effects. Therefore, in our previous study (Batin et al., 1991) we chose to give incremental i.v. bolus doses of DuP 753 (0.1–10 mg kg^{-1}) at 15 min intervals, as did Wong et al. (1990c,d,e). In the present experiments we used the same protocol as previously since we wanted to obtain cardiac haemodynamic data to match the regional haemodynamic data obtained earlier (Batin et al., 1991).

A dose of 10 mg kg^{-1} DuP 753 has a maximal effect (Wong et al., 1990d,e; Batin et al., 1991), that causes complete inhibition of the regional haemodynamic responses of exogenous AII (50 ng) for at least 9 h (Batin et al., 1991). Therefore 15 min after the 10 mg kg^{-1} dose of DuP 753 a supramaximal dose of captopril (2 mg kg^{-1}; Muller et al., 1990) was given to assess if it had any additional cardiac haemodynamic effects in the presence of DuP 753, as we had in our previous study when regional haemodynamics only were measured.

All injections were given in 0.1 ml and flushed in with 0.1 ml saline (dead space of the catheter). DuP 753 and captopril were dissolved in isotonic saline; injection of isotonic saline alone had no significant cardiovascular effects, and over the time course of the experiment (i.e. 90 min) there were no significant changes in any cardiovascular variable if DuP 753 was not administered. We did not carry out 'placebo' experiments since the purpose of the investigation was to determine if any negative changes in cardiac function contributed to the hypotensive action of DuP 753 under any condition.

Experiments were carried out in water-replete, Long Evans ($n = 8$) and Brattleboro ($n = 8$) rats and water-deprived ($n = 9$) Brattleboro rats.

Data analysis

Data were analysed by Friedman's test (Theodorsson Norheim, 1987) for within-group comparisons. In order to assess the acute effects of DuP 753, the changes over the first

5 min following each dose were compared to the immediate pre-injection value. In addition, over the time course of the experiment each pre-injection value was compared to the original pre-drug baseline. Between group comparisons were made with the Kruskal-Wallis test or Mann-Whitney U test, as appropriate.

Drugs

DuP 753 (gift from Dr R. Smith, DuPont, U.S.A.) and captopril (gift from Squibb, U.S.A.) were dissolved in isotonic saline.

Results

Resting cardiovascular variables

In the resting state there were no significant differences between cardiovascular variables in water-replete Long Evans and Brattleboro rats. However, following water deprivation, Brattleboro rats showed an increase in mean arterial blood pressure accompanied by decreases in cardiac output, $+ dF/dt_{max}$, and total peripheral conductance (Table 1).

Responses to DuP 753 in water-replete Long Evans rats

The lower doses of DuP 753 (0.1–1 mg kg^{-1}) caused slight, transient falls in mean arterial pressure accompanied by rises in total peripheral conductance, but no consistent changes in the other cardiovascular variables (Figure 1). Doses of 3 and 10 mg kg^{-1} DuP 753 caused small, but sustained, falls in mean arterial blood pressure, and increases in heart rate and total peripheral conductance (Figure 1). There were transient increases in cardiac output, peak aortic flow and $+ dF/dt_{max}$, but not in stroke volume or central venous pressure (Figure 1). Administration of captopril (2 mg kg^{-1}) 15 min after the highest dose of DuP 753 caused a slight tachycardia and a transient rise in mean arterial blood pressure, accompanied by brief increases in peak aortic flow and $+ dF/dt_{max}$ (Figure 1).

Responses to DuP 753 in water-replete Brattleboro rats

DuP 753 (0.1–10 mg kg^{-1}) caused effects similar to those seen in Long Evans rats. Thus, there were incremental reductions (albeit slight) in mean arterial blood pressure associated with rises in total peripheral conductances and heart rate (Figure 2). There were no sustained changes in cardiac output, stroke volume, peak aortic flow, $+ dF/dt_{max}$ or central venous pressure (Figure 2). Furthermore, after the 10 mg kg^{-1} dose of DuP 753, captopril had no additional effects (Figure 2).

Responses to DuP 753 in water-deprived Brattleboro rats

The lower doses of DuP 753 (0.1–1 mg kg^{-1}) caused transient falls in mean arterial blood pressure accompanied by brief increases in heart rate, cardiac output, stroke volume, peak aortic flow and $+ dF/dt_{max}$ (Figure 3). The higher doses of DuP 753 (3 and 10 mg kg^{-1}) caused sustained hypotension and tachycardia, together with transient increases in cardiac output, peak aortic flow, $+ dF/dt_{max}$ and total peripheral conductance (Figure 3). However, stroke volume fell relative to the pre-drug baseline and there were no consistent changes in central venous pressure (Figure 3). Administration of captopril (2 mg kg^{-1}) 15 min after DuP 753 (10 mg kg^{-1}) caused a slight tachycardia, but no other significant effects (Figure 3).

Relative to the pre-drug baseline values, 15 min after administration of DuP 753 at a dose of 10 mg kg^{-1}, the falls in mean arterial blood pressure and the rises in total peripheral conductance and peak aortic flow were significantly greater in water-deprived than in water-replete Brattleboro rats (Table 2).

Discussion

In the present work we investigated the cardiac haemodynamic effects of DuP 753 in conscious rats primarily to determine if, under any experimental condition in which we had previously studied DuP 753 (Batin *et al.*, 1991), there were signs of negative changes in myocardial function that could have contributed to the effects of DuP 753 on mean systemic arterial blood pressure. For this reason we employed the protocol used previously, both by us (Batin *et al.*, 1991) and Wong *et al.* (1990c,d,e), involving incremental i.v. bolus doses of DuP 753 at 15 min intervals. Although this protocol does not produce sustained cardiovascular effects with the lower doses of DuP 753, it is likely the recovery is due to increased activity of the RAS, rather than a waning of drug action, since the effects following administration of DuP 753 are rapid in onset and prolonged (Wong *et al.*, 1990c,d,e; Batin *et al.*, 1991). Recently Wong *et al.* (1990e) reported that, after administration of DuP 753 *in vivo*, an active metabolite (EXP 3174) was formed. EXP 3174 is more potent than DuP 753 and is a non-competitive antagonist at the AII_1 receptor (Wong *et al.*,

Table 1 Resting cardiovascular variables in conscious rats

	Water-replete		*Water-deprived*
	Long Evans ($n = 8$)	*Brattleboro* ($n = 8$)	*Brattleboro* ($n = 9$)
Heart rate (beats min^{-1})	381 ± 8	358 ± 11	330 ± 10†
Mean arterial blood pressure (mmHg)	100 ± 2	100 ± 4	113 ± 1†*
Cardiac output (ml min^{-1})	107 ± 6	109 ± 6	88 ± 3†*
Peak aortic flow (ml min^{-1})	411 ± 22	432 ± 26	387 ± 16
$+ dF/dt_{max}$ (1 min^{-2})	1773 ± 104	1784 ± 140	1605 ± 89†*
Stroke volume (μl beat^{-1})	283 ± 17	305 ± 19	269 ± 12
Total peripheral conductance (ml min^{-1} mmHg^{-1})	1083 ± 69	1102 ± 85	780 ± 28†*
Central venous pressure (cmH_2O)	3.3 ± 0.9	3.8 ± 0.6	4.9 ± 0.4

Values are mean ± s.e.mean.
† $P < 0.05$ versus water-replete Long Evans rats.
* $P < 0.05$ versus water-replete Brattleboro rats.

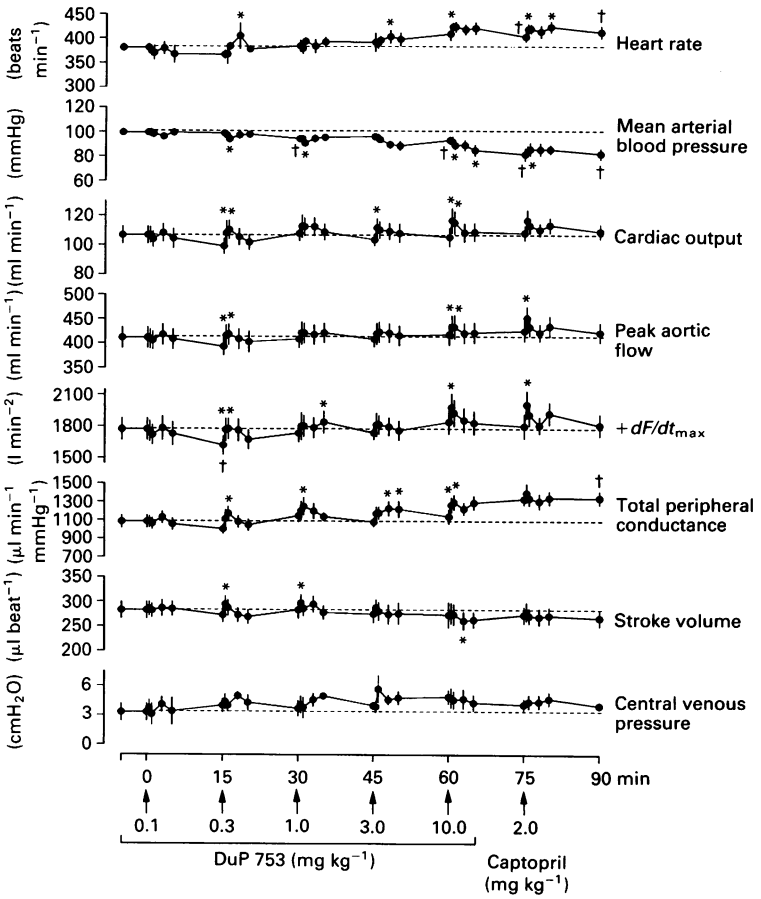

Figure 1 Cardiovascular variables in conscious, water-replete, Long Evans rats ($n = 8$). At the arrows between 0 and 60 min, increasing i.v. bolus doses (0.1–10 mg kg^{-1}) of DuP 753 were administered (i.e. at 15 min intervals). Fifteen min after the highest dose of DuP 753 a supramaximal dose of captopril (2 mg kg^{-1}) was administered. The values shown are the means, and the bars represent s.e.means; where the bars are not visible they lie within the symbol. * $P < 0.05$ versus pre-injection value for that dose; † $P < 0.05$ versus original pre-drug baseline. For clarity this latter comparison was made only for the 15 min post-injection values. The dashed line corresponds to the original baseline.

1990e), and hence could contribute to the effects following administration of DuP 753. While it could be argued that, in the present study, the changes in cardiac haemodynamics with increasing doses of DuP 753 were due to cumulative generation of EXP 3174, it is notable that the profiles of effect of DuP 753 and EXP 3174 on mean arterial blood pressure are similar (Wong *et al.*, 1990c,d,e,f). This observation lends support to the suggestion that the apparent recovery from the effects of the lower doses of DuP 753 was due to additional activation of the RAS. Although there is a theoretical possibility that AII could act on AII_2 receptors to influence cardiovascular function in the presence of DuP 753, Wong *et al.* (1990b) were unable to identify a functional role for such receptors. Moreover, in water-deprived Brattleboro rats, administration of PD 123177 (a selective antagonist for AII_2 receptors (Wong *et al.*, 1990b)) at doses up to 100 mg kg^{-1} had no haemodynamic effects (Gardiner, S.M. *et al.*, unpublished observations).

Cardiac haemodynamic changes following DuP 753 in water-replete rats

Consistent with our previous findings of renal and mesenteric vasodilator responses to the lower doses of DuP 753 in water-replete, Long Evans and Brattleboro rats, the present study showed transient increases in total peripheral conductance following the same intervention. As we suspected, (Batin *et al.*, 1991) there were transient rises in cardiac output coincident with the vasodilatation, and hence mean arterial blood pressure changed little. In both Long Evans and Brattleboro rats in the water-replete state there was no indication of a negative effect of DuP 753 on cardiac haemodynamics. Thus, the

Table 2 Changes (relative to pre-drug baseline) in cardiovascular variables in water-replete ($n = 8$) and water-deprived ($n = 9$) Brattleboro rats 15 min after DuP 753 (10 mg kg^{-1})

	Water-replete	*Water-deprived*
Heart rate (beats min^{-1})	32 ± 10*	58 ± 11*
Mean arterial blood pressure (mmHg)	−10 ± 4*	−39 ± 3*†
Cardiac output (ml min^{-1})	0 ± 4	5 ± 2
Peak aortic flow (ml min^{-1})	9 ± 9	39 ± 8†*
+ dF/dt_{max} (l min^{-2})	36 ± 35	144 ± 56
Stroke volume (μl beat^{-1})	−26 ± 14	−28 ± 5*
Total peripheral conductance (ml min^{-1} mmHg^{-1})	148 ± 104	482 ± 36*†

Values are mean ± s.e.mean.
* $P < 0.05$ versus baseline.
† $P < 0.05$ water-deprived versus water-replete.

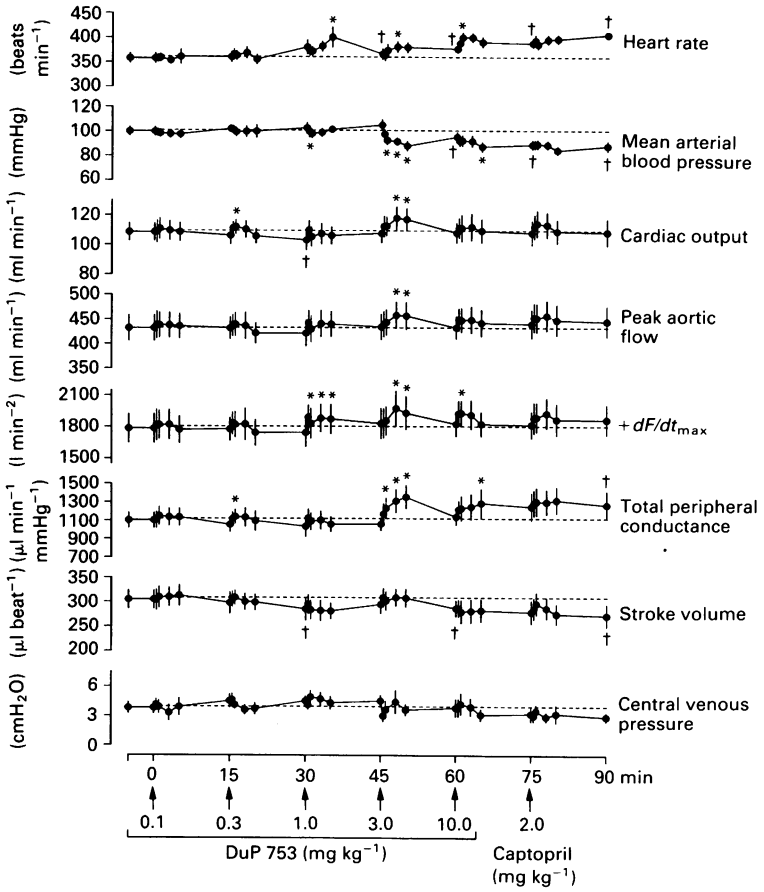

Figure 2 Cardiovascular variables in conscious, water-replete, Brattleboro rats ($n = 8$). At the arrows between 0 and 60 min, increasing i.v. bolus doses (0.1–10 mg kg^{-1}) of DuP 753 were administered (i.e. at 15 min intervals). Fifteen min after the highest dose of DuP 753 a supramaximal dose of captopril (2 mg kg^{-1}) was administered. The values shown are the means, and the bars represent s.e.means; where the bars are not visible they lie within the symbol. * $P < 0.05$ versus pre-injection value for that dose; † $P < 0.05$ versus original pre-drug baseline. For clarity this latter comparison was made only for the 15 min post-injection values. The dashed line represents the original baseline.

modest hypotensive effects of DuP 753, even at the highest dose employed, appeared to be entirely due to a rise in total peripheral conductance. It is likely the transient increases in cardiac output, peak aortic flow and + dF/dt_{max} were a consequence of this reduction in afterload, and possibly contributed to by reflex responses to the latter.

The vasodilator effect of DuP 753 in water-replete, Long Evans and Brattleboro rats indicates that endogenous AII is involved in the tonic control of total peripheral conductance in these animals, and our previous experiments indicate the renal and mesenteric vascular beds are important targets for this effect (Batin *et al.*, 1991). In the present study we were careful to ensure there was no post-operative hypovolaemia in the Long Evans rats; thus the results probably represent the normal condition of the animals studied. An involvement of endogenous AII in the tonic control of total peripheral conductance is consistent with our previous findings with captopril in water-replete Long Evans and Brattleboro rats (Gardiner & Bennett, 1985; Muller *et al.*, 1990). The present observation that a supramaximal dose of captopril was without an additional hypotensive effect after DuP 753 (10 mg kg^{-1}) had been administered indicates that the properties of captopril, other than its ability to inhibit AII generation, are not likely to have contributed to its acute hypotensive or vasodilator effects in previous experiments (Gardiner & Bennett, 1985; Tomlinson *et al.*, 1990; Muller *et al.*, 1990). We have no ready explanation for the small increases in heart rate, mean arterial blood pressure, peak aortic flow and + dF/dt_{max} seen following administration of captopril to water-replete Long Evans rats.

The lack of any signs of a negative inotropic effect of DuP 753 under conditions in which it caused clear vasodilatation indicates that, in the water-replete state, endogenous AII was not exerting an important influence on cardiac function (Urata *et al.*, 1990; Hirakata *et al.*, 1990; Lindpaintner *et al.*, 1990). It is likely the gradual reduction in stroke volume following DuP 753 in the water-replete condition (most marked in Brattleboro rats) was due to the tachycardia causing a shortening of cardiac filling time.

Cardiac haemodynamic changes following DuP 753 in water-deprived Brattleboro rats

In water-deprived Brattleboro rats, in the resting state, there was a clear-cut reduction in cardiac output. However, mean arterial blood pressure was raised because there was a disproportionately large reduction in total peripheral conductance (Table 1). While these findings could indicate abnormality of baroreflex control in water-deprived Brattleboro rats, we have been unable to show impairment of baroreflex modulation of pulse interval under these conditions (Gardiner & Bennett, 1988).

The marked reduction in total peripheral conductance in water-deprived Brattleboro rats was abolished following administration of the highest dose of DuP 753 (10 mg kg^{-1}) and although there was a significant difference in the changes of total peripheral conductance in water-replete and water-deprived Brattleboro rats in this circumstance (Table 2), the absolute values of total vascular conductance were not different (Figures 2 and 3). However, given that DuP 753 can influence both the pre- and postjunctional actions of AII (Chiu *et al.*, 1990; Wong *et al.*, 1990a,b,c,d,e,f) it is not possible to determine from the present experiments the extent to which

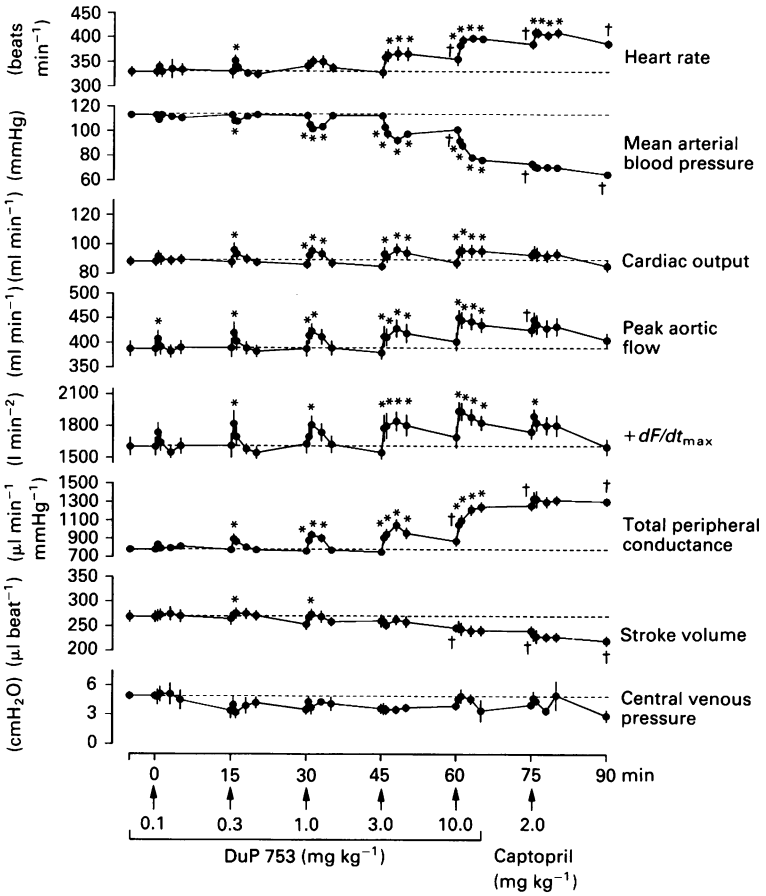

Figure 3 Cardiovascular variables in conscious water-deprived Brattleboro rats ($n = 9$). At the arrows between 0 and 60 min, increasing i.v. bolus doses (0.1–10 mg kg^{-1}) of DuP 753 were administered (i.e. at 15 min intervals). Fifteen min after the highest dose of captopril (2 mg kg^{-1}) was administered. The values shown are the means, and the bars represent s.e.means; where the bars are not visible they lie within the symbol. * $P < 0.05$ versus pre-injection value for that dose; † $P < 0.05$ versus original pre-drug baseline. For clarity this latter comparison was made only for the 15 min post-injection values. The dashed line represents the original baseline.

inhibition of these two processes contributed to the vasodilator effects of DuP 753.

It is notable that even in water-deprived Brattleboro rats with marked activation of the RAS the hypotensive effects of DuP 753 were not contributed to by reductions in cardiac output. On the contrary, administration of DuP 753 caused increases, (albeit generally transient), in cardiac output, peak aortic flow and + dF/dt_{max}. These changes went hand-in-hand with the increases in total peripheral conductance and hence, as in water-replete rats, were probably due to reflex changes and the reduction in afterload. Thus, even under conditions in which circulating levels of AII are known to be elevated chronically (Bennett & Gardiner, 1985) it did not appear that DuP 753 exerted cardiac effects that could be attributed to inhibition of a myocardial supportive action of endogenous AII (Urata *et al.*, 1990; Hirakata *et al.*, 1990; Lindpaintner *et al.*, 1990).

The absence of any additional hypotensive effects of captopril following administration of DuP 753 to water-deprived Brattleboro rats indicates that the profound hypotensive and vasodilator effects of captopril, alone, in this model (Gardiner & Bennett, 1985; Tomlinson *et al.*, 1990; Muller *et al.*, 1990) can also be explained by inhibition of the generation of AII and, furthermore, its hypotensive effects do not seem to involve suppression of cardiac output in this setting. Indeed, it is notable that captopril (and DuP 753) caused tachycardia in water-deprived rats, as we have noted previously (Gardiner & Bennett, 1985; Tomlinson *et al.*, 1990; Muller *et al.*, 1990; Batin *et al.*, 1991). This finding is at odds with the frequent assertion that reduction of blood pressure by inhibitors of the RAS is not accompanied by tachycardia. However, it may be that the hypotensive effects of inhibitors of the RAS are accompanied by less tachycardia than would be seen with other hypotensive agents (Batin *et al.*, 1991).

This work was supported in part by a grant from the Royal Society. We are grateful to Dr R. Smith (DuPont de Nemours) for the generous gift of DuP 753.

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(Received October 17, 1990
Revised February 1, 1991
Accepted February 12, 1991)

Br. J. Pharmacol. (1991), **103**, 1592–1596 © Macmillan Press Ltd, 1991

A quantitative investigation into the dependence of Ca^{2+} mobilisation on changes in inositol 1,4,5-trisphosphate levels in the stimulated neutrophil

[1]Neil T. Thompson, Robert W. Bonser, James E. Tateson, Graham D. Spacey, Roger W. Randall, *Harold F. Hodson & *Lawrence G. Garland

Biochemical Sciences and *Research Directorate, Wellcome Research Laboratories, Langley Court, Beckenham, Kent, BR3 3BS

1 The coupling of N-formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe) receptor stimulation to Ca^{2+} mobilisation has been investigated in the human neutrophil by measuring the concentration-effect curves for inositol 1,4,5-trisphosphate (IP_3) formation and Ca^{2+} mobilisation.

2 fMet-Leu-Phe-dependent mobilisation of intracellular Ca^{2+} has been monitored in fluo-3-loaded human neutrophils by measuring increases in the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in the presence of extracellular EGTA. Fluo-3 was used in preference to fura-2 because it was found to be more sensitive to the high Ca^{2+} levels seen in stimulated neutrophils.

3 fMet-Leu-Phe induced a rapid mobilisation of intracellular Ca^{2+} ($EC_{50} = 2.9 \pm 0.1$ nM) and increased $[Ca^{2+}]_i$ to a maximum of 1286 ± 184 nM.

4 The amount of IP_3 in fMet-Leu-Phe-stimulated neutrophils was determined by competition with [^{3}H]-IP_3 for a specific IP_3 binding protein isolated from bovine adrenocortical microsomes. Basal IP_3 levels of 13.3 ± 2.0 pmol per 10^7 cells were increased nearly 4 fold by maximally effective concentrations of fMet-Leu-Phe.

5 The EC_{50} for the IP_3 response (95 ± 18 nM) was much higher than that for mobilisation of intracellular Ca^{2+}, such that only a doubling in the concentration of IP_3 was required to fully mobilise intracellular Ca^{2+}.

6 As a result of this relationship IP_3 production was more sensitive than Ca^{2+} mobilisation to inhibition by demethoxyviridin, an inhibitor of phospholipase activation.

Keywords: Signal transduction; phospholipase C; phospholipase D; phospholipase inhibitor; demethoxyvirdin

Introduction

Hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by a specific phospholipase C (PLC) is a common step in the activation of many tissues by Ca^{2+}-mobilising ligands (Berridge, 1987; Berridge & Irvine, 1989). Both products of the reaction have second messenger roles. Inositol 1,4,5-trisphosphate (IP_3) mobilises intracellular Ca^{2+} from a non-mitochondrial pool (Berridge, 1987; Berridge & Irvine, 1989) whilst 1,2-diacyl-*sn*-glycerol represents the main physiological activator of the enzyme protein kinase C (Nishizuka, 1986; Berridge, 1987).

Ca^{2+} mobilisation by IP_3 has been studied extensively both in permeabilised cells and by micro-injection techniques and has been found to be mediated by a specific receptor associated with the endoplasmic reticulum (Streb *et al.*, 1984). Recent work has revealed that the IP_3 receptor and the IP_3-sensitive Ca^{2+} channel are part of the same intrinsic membrane protein (Ferris *et al.*, 1989).

The actions of IP_3 have been reported to be highly co-operative, suggesting that just a small change in the cytoplasmic concentration could lead to a large effect on Ca^{2+} stores (Meyer *et al.*, 1988). Analysis of the concentration-dependence of IP_3-mediated effects has identified a number of anomalies however. For example, in most permeabilised tissues exogenous IP_3 mobilises Ca^{2+} with concentrations for half-maximal effect (EC_{50}) being in the range 0.1–1.0 μM (Berridge, 1987). However, estimates of the affinity of IP_3 for its receptor are generally much higher than this e.g. 6 nM in bovine adrenocortical microsomes (Palmer & Wakelam, 1989). Furthermore, estimates of IP_3 concentrations in intact cells before and after activation have been found to vary considerably between tissues (Shears, 1989). In anterior pituitary cells, IP_3 levels were increased 3 fold to 56 nM following stimulation with gonadotrophin-releasing hormone (Morgan *et al.*, 1987) whilst in rabbit neutrophils basal levels of 0.1 μM were increased 10 fold following stimulation by the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe) (Bradford & Rubin, 1986). Although it is clear that IP_3 mobilises intracellular Ca^{2+} it is not apparent what increase in IP_3 concentration is required to achieve complete mobilisation of the Ca^{2+} pool.

To address this question we have analysed the relationship between Ca^{2+} mobilisation and the amount of IP_3 produced in fMet-Leu-Phe-stimulated human neutrophils. In a similar study on vasopressin-stimulated rat hepatocytes, only 15% of the maximal inositol trisphosphate that could be generated was needed to mobilise intracellular Ca^{2+} fully (Thomas *et al.*, 1984). However, this particular study had the shortcoming that no distinction was made between the different inositol trisphosphate isomers. In the human neutrophil the concentration-effect curve for fMet-Leu-Phe-stimulated Ca^{2+} mobilisation has been reported to lie to the left of those for other fMet-Leu-Phe-dependent effects (Korchak *et al.*, 1984; Rossi *et al.*, 1985). However, in these studies the relationship between the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and IP_3 levels was not investigated. Furthermore, previous studies have used either quin2 or fura-2 to monitor the $[Ca^{2+}]_i$. The disadvantage of both these fluorescent probes is that they have a relatively high affinity for Ca^{2+} (120 nM and 224 nM respectively) and are therefore insensitive to high $[Ca^{2+}]_i$ such as those generated by maximally effective concentrations of agonist. To circumvent this problem we have made use of a new probe, fluo-3, which is reported to have a

[1] Author for correspondence.

lower affinity for Ca^{2+} (864 nM) and therefore should measure such high $[Ca^{2+}]_i$ values more accurately (Minta *et al.*, 1989; Kao *et al.*, 1989; Merritt *et al.*, 1990).

Methods

Preparation of fura-2- and fluo-3-loaded human neutrophils

Human neutrophils were prepared as described previously (Tateson *et al.*, 1988) and suspended in N-[2-hydroxyethyl]-piperizine]N-[2-ethane sulphonic acid] (HEPES)-buffered (30 mM pH 7.2) Hank's balanced salt solution containing 1.6 mM Ca^{2+} (HBH). Neutrophils at 3×10^7 per ml were incubated with 0.5 μM fura-2AM or 2 μM fluo-3AM for 15 min at 37°C then diluted 5 fold with cold HBH before centrifugation at 300 *g* for 10 min. The cell pellet was then resuspended in HBH to 1×10^8 cells per ml and kept on ice until use.

Fluorescence measurements

Aliquots (0.1 ml) of loaded neutrophils were diluted 5 fold with HBH warmed to 37°C in fluorimeter cuvettes. Cytochalasin B (5 μM) was then added together with 1 μl of dimethylsulphoxide containing demethoxyviridin where indicated. After preincubation at 37°C for 4 min, 4 mM ethylene glycol-bis(β-aminoethylether) N,N,N',N'-tetraacetic acid (EGTA) was added and the mixture stirred continuously. This concentration of EGTA was calculated to reduce the free concentration of Ca^{2+} to <60 nM. fMet-Leu-Phe was then added and fluorescence followed until it reached a peak (after 10–20 s) at which point 100 μM digitonin, 10 μM diethylene- triaminepentacetic acid (DTPA) and 4 mM $CaCl_2$ were added to release the trapped probe into a saturating concentration of Ca^{2+}. The fluorescence under these conditions was designated F_{max}. EGTA (8.0 mM) was then added to reduce the free Ca^{2+} to <10 nM and obtain a minimum fluorescence value (F_{min}). NaOH (26 mM) was added at this stage to maintain the pH > 7. Fluorescence was monitored with a Perkin-Elmer MPF2 spectrofluorimeter and protocols for experiments with fluo-3 and fura-2 were identical except that the monochromators were set at 340 nm excitation, 490 nm emission for fura-2 and 506 nm excitation 526 nm emission for fluo-3. The fluorescence from fura-2- and fluo-3-loaded cells was quantitated as described by Pollock *et al.* (1986) using K_d values of 224 nM for fura-2 and 864 nM for fluo-3 (Merritt *et al.*, 1990). To determine accurately the position of the concentration-effect curve, it was necessary to have a reliable estimate of the maximal increase in $[Ca^{2+}]_i$. Stimulation of neutrophils with a maximally effective concentration of fMet-Leu-Phe (3 μM), in the absence of extracellular Ca^{2+}, increased $[Ca^{2+}]_i$ to $\geqslant 1.3$ μM (Figure 1). At such high free Ca^{2+} concentrations fura-2 is >85% saturated with Ca^{2+} whilst fluo-3 is only 60% saturated. Thus fluo-3 fluorescence is a more sensitive measure of $[Ca^{2+}]_i$ in this range and was the preferred dye for measuring concentration-effect curves under these conditions. In order to quantitate the peak increase in fluorescence the cells were lysed approximately 10–20 s after addition of the stimulus (see above). Over longer periods of stimulation however, (>5 min), some loss of Ca^{2+}-sensitive fluo-3 was observed, suggesting that caution should be used when experimental protocols require longer incubation periods than those used here.

Measurement of inositol 1,4,5-trisphosphate

Human neutrophils, prepared as described above were used at a concentration of 2.0×10^7 per ml in a total incubation volume of 0.2 ml. Cells were treated with 5 μM cytochalasin B for 5 min at 37°C before addition of demethoxyviridin where indicated. After a further 5 min cells were stimulated by addition of fMet-Leu-Phe and the reaction stopped 20 s later by addition of 50 μl ice-cold $HClO_4$ (10% v/v). The samples were then kept on ice and subsequent operations were carried out at 0–4°C. Precipitated protein was removed by centrifugation and 0.2 ml aliquots of the supernatants neutralised, in the presence of a pH indicator (BDH '678') with approximately 300 μl of KOH (22 mM) containing 50 mM-HEPES. The IP_3 content of the neutralised samples was measured with the adrenocortical binding protein assay as described by Palmer *et al.* (1988).

Curve fitting

The concentration-effect data for fMet-Leu-Phe was fitted to the equation $f(x) = (A*x^n)/(B^n + x^n)$ using commercially available software (RS-1, BBN Software Products Corporation), where x = concentration of fMet-Leu-Phe, A = maximal effect, B = concentration giving half-maximal effect and n = slope. Each experiment was fitted separately and the curves displayed in Figure 3 were derived from the mean values of the above parameters.

Materials

Fura-2AM and fluo-3AM were obtained from Molecular Probes Inc., Oregon U.S.A. Cytochalasin B, fMet-Leu-Phe and DTPA were obtained from Sigma, Poole, Dorset. EGTA (puriss p.a. grade) was obtained from Fluka Chemicals Ltd, Derbyshire, U.K. 1β-Hydroxy-18norandrosta-5,8,11,13-tetraeno[6,5,4-bc]furan-3,7,17-trione (demethoxyviridin, DMV) was the kind gift of Dr J. Hanson, Sussex University, Brighton, E. Sussex.

Results

Measurement of $[Ca^{2+}]_i$ with fluo-3

Stimulation of fluo-3-loaded cells by a maximally effective concentration of fMet-Leu-Phe (3 μM) in the presence of excess extracellular EGTA initiated a rapid but transient increase in fluorescence at 526 nm, indicating release of intracellular Ca^{2+} (Figure 1b). The $[Ca^{2+}]_i$ in resting cells was estimated to be approximately 120 nM and was seen to increase, following fMet-Leu-Phe stimulation, to 1300 nM. These values are similar to estimates made from changes in fura-2 fluorescence (Figure 1a). The increase in $[Ca^{2+}]_i$ was identical in neutrophils which had not been pretreated with 5 μM-cytochalasin B (result not shown).

Concentration-dependence of fMet-Leu-Phe-stimulated Ca^{2+} mobilisation and IP_3 production

We have shown previously that fMet-Leu-Phe induced a rapid and transient increase in the amount of IP_3 in human neutrophils (Thompson *et al.*, 1990). It can be seen from Figure 2 that this paralleled the mobilisation of intracellular Ca^{2+} measured in fluo-3 loaded cells. In the present studies fMet-Leu-Phe (3 μM) caused a maximal increase in IP_3 (measured at 20 s) from basal levels of 13.3 ± 2.0 pmol per 10^7 cells to 50.3 ± 8.1 pmol per 10^7 cells ($n = 13$). The EC_{50} for this response was estimated to be 95 ± 18 nM ($n = 3$) (Figure 3). The location of this concentration-effect curve was significantly to the right of that for mobilisation of Ca^{2+} by fMet-Leu-Phe (Figure 3). At concentrations of 30 nM and above

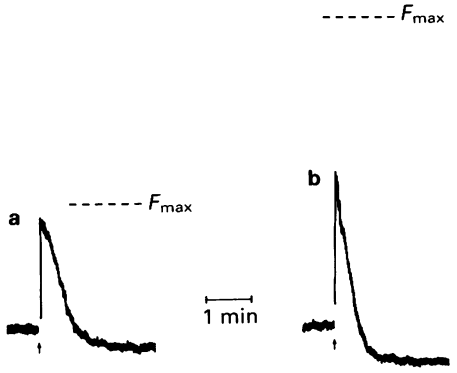

Figure 1 Fluorescence from fura-2-(a) or fluo-3-(b) loaded human neutrophils was measured in the presence of 4 mM EGTA as described in Methods. In both cases the neutrophils were pre-incubated for 5 min with 5 μM cytochalasin B before the addition of 0.3 μM fMet-Leu-Phe indicated by the arrow. F_{max} was determined in non-stimulated samples incubated alongside those shown. The results shown are representative of a large number of similar fluorescence traces from at least four different experiments.

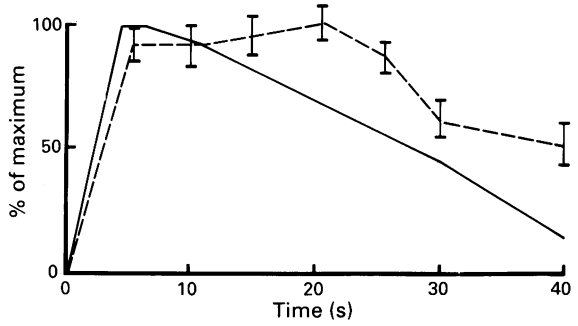

Figure 2 Fluorescence from fluo-3-loaded neutrophils stimulated by 0.3 μM fMet-Leu-Phe (solid line) was measured in the presence of 4 mM EGTA as described in Methods. Inositol 1,4,5-trisphosphate (IP_3) formed in fMet-Leu-Phe (0.3 μM)-stimulated neutrophils (broken line) was measured at 5 s intervals by the procedure described in Methods. In all cases neutrophils were preincubated for 5 min with cytochalasin B before addition of fMet-Leu-Phe. The fluorescence trace is a single determination representative of a number of others whilst the IP_3 data represent the mean from three different experiments.

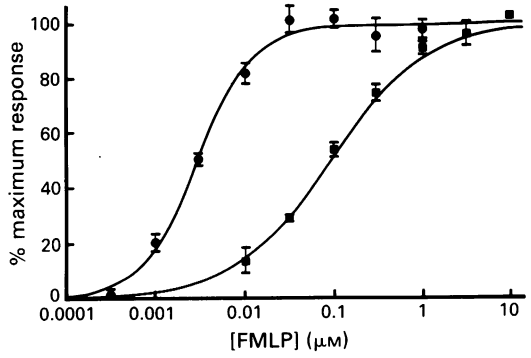

Figure 3 The peak increase in $[Ca^{2+}]_i$ in fluo-3-loaded neutrophils (●) was monitored in the presence of 4 mM EGTA as described in Methods. Inositol 1,4,5-trisphosphate (IP_3) formation (■) was measured 20 s after the addition of the indicated concentrations of fMet-Leu-Phe (FMLP). The neutrophils in both cases were pre-incubated with 5 μM cytochalasin B for 5 min before addition of fMet-Leu-Phe. The results are mean from three experiments (s.e.mean shown by vertical bars) in which the data were expressed as a percentage of the maximal observed response. The lines were fitted to the data as described in Methods. The slope parameters as determined by this fit were 1.42 ± 0.11 for $[Ca^{2+}]_i$ and 0.82 ± 0.13 for IP_3.

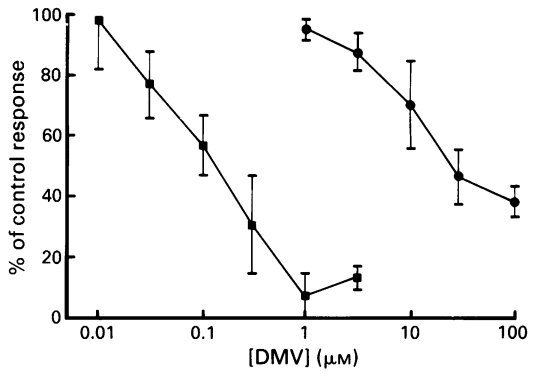

Figure 4 Neutrophils were pre-incubated with 5 μM cytochalasin B and the indicated concentration of demethoxyviridin (DMV) for 5 min at 37°C before addition of 3 μM fMet-Leu-Phe. The formation of inositol 1,4,5-trisphosphate (■) was determined 20 s after addition of fMet-Leu-Phe. The peak increase in $[Ca^{2+}]_i$ (●) was determined in fluo-3-loaded neutrophils in the presence of 4 μM extracellular EGTA.

fMet-Leu-Phe induced a maximal increase in $[Ca^{2+}]_i$ to 1286 ± 184 nM ($n = 3$) in the presence of extracellular EGTA. The EC_{50} for this response was estimated to be 2.9 ± 0.1 nM, i.e. approximately 30 times lower than that for IP_3 formation (Figure 3).

Inhibition of fMet-Leu-Phe-stimulated responses by the phospholipase inhibitor, demethoxyviridin

The above result demonstrated that only a small part of the maximal fMet-Leu-Phe-stimulated IP_3 formation was required to release all of the intracellular Ca^{2+}. In order to investigate this further we have made use of demethoxyviridin (DMV), a newly discovered and potent inhibitor of fMet-Leu-Phe-dependent activation of both PLC and phospholipase D (PLD) in human neutrophils (Bonser *et al.*, 1991). DMV inhibited fMet-Leu-Phe (3 μM)-stimulated IP_3 formation with half-maximal inhibition occurring at a concentration (IC_{50}) of 250 ± 130 nM (Figure 4). In contrast, DMV was much less effective as an inhibitor of the increase in $[Ca^{2+}]_i$ provoked by the same concentration of fMet-Leu-Phe (Figure 4). Significant inhibition of Ca^{2+} release was observed only at DMV concentrations (> 1 μM) that inhibited the IP_3 response by > 90%.

Discussion

Agonist-dependent formation of IP_3 and mobilisation of intracellular Ca^{2+} are part of the same signal transduction pathway linking receptor occupancy to increases in $[Ca^{2+}]_i$ (Berridge, 1987; Berridge & Irvine, 1989). In human neutrophils the EC_{50} for fMet-Leu-Phe-stimulated IP_3 formation is 30 times greater than that for Ca^{2+} mobilisation. From the location of the concentration-effect curves for these responses, it is apparent that only 30–40% of maximal IP_3 formed by fMet-Leu-Phe receptor stimulation is required to mobilise all the intracellular Ca^{2+}. This implies that < 40% of the fMet-Leu-Phe receptors need to be occupied to induce full Ca^{2+} mobilisation making this response very sensitive to low concentrations of fMet-Leu-Phe. A very low EC_{50} for fMet-Leu-Phe-stimulated Ca^{2+} mobilisation has been reported previously with quin2-loaded neutrophils (Korchak *et al.*, 1984; Rossi *et al.*, 1985).

A similar dissociation between [^{3}H]-inositol trisphosphate formation and peak $[Ca^{2+}]_i$ was reported in vasopressin-

stimulated hepatocytes (Thomas *et al.*, 1984). In this case however, the inositol 1,4,5-trisphosphate isomer was not distinguished from other inositol trisphosphates. Neutrophils clearly have IP_3-sensitive Ca^{2+} stores (Prentki *et al.*, 1984), but we cannot rule out the possibility that Ca^{2+} is also mobilised from an IP_3-insensitive pool responding to an unknown second messenger. However, this would not alter the fact that release of Ca^{2+} from the IP_3-sensitive stores is dependent upon lower fMet-Leu-Phe concentrations than is IP_3 formation itself.

Assuming a neutrophil volume of 4.5 μl per 10^7 cells (Tivey *et al.*, 1951), we have calculated that in fMet-Leu-Phe stimulated cells the IP_3 concentration increased from basal levels of approximately 3 μM to maximum levels of 10 μM, and that a doubling of the resting IP_3 concentration to only 6 μM was required to mobilise fully intracellular Ca^{2+}. Such a large effect resulting from a small change in IP_3 concentration is consistent with the highly co-operative nature of IP_3-induced Ca^{2+} mobilisation (Meyer *et al.*, 1988). This property is reflected in the steep slope of the Ca^{2+} curve in Figure 3. The calculated concentrations of IP_3 in human neutrophils reported here are very much greater than the average K_D of IP_3 for its receptor (approximately 0.05 μM e.g. Ferris *et al.*, 1989; Palmer & Wakelam, 1989) and 10 times greater than the EC_{50} for IP_3 dependent Ca^{2+} mobilisation in permeabilised human neutrophils (0.75 μM) (Prentki *et al.*, 1984). A similar discrepancy has been identified in a number of tissues and a satisfactory explanation is not available at present (for review, see Shears, 1989). The cellular IP_3 concentrations reported for other tissues using a similar assay for amounts of IP_3 are, in general, lower than those reported here for neutrophils (Palmer & Wakelam, 1989). The significance of this is also unclear.

The use of fluo-3 in human neutrophils has been characterized in detail recently (Merritt *et al.*, 1990) and we have found it to have a number of advantages over fura-2 as a measure of high levels of $[Ca^{2+}]_i$ (see Methods). The extent to which fluo-3 enters intracellular organelles has not been determined previously and was not assessed directly in the present study. However, release of fluo-3 from secretory granules does not contribute to the agonist-dependent fluorescence change observed in Figure 1 because this is unchanged in neutrophils which have not been treated with cytochalasin B which respond with a much reduced secretory granule release (result not shown).

We have found that DMV, an inhibitor of phospholipase activation, inhibited fMet-Leu-Phe-dependent IP_3 formation at much lower concentrations than were required to inhibit Ca^{2+} mobilisation. This further supports the suggestion that only a fraction of the maximal amount of IP_3 produced by receptor stimulation was required to mobilise intracellular Ca^{2+}. DMV inhibits fMet-Leu-Phe dependent activation of PLD in human neutrophils with an IC_{50} of 31 ± 10 nM (Bonser *et al.*, 1991), indicating that at the level of phospholipase activation, this compound has a 10 fold selectivity for PLD over PLC ($IC_{50} = 250 \pm 130$ nM, Figure 4). Furthermore, it is apparent from the results in Figure 4 that functional responses which are dependent upon release of intracellular Ca^{2+} will be much less sensitive to inhibition by DMV than would be expected from its potency as an inhibitor of PLC activation. Therefore, at the functional level DMV would be expected to be very much more effective against PLD-dependent responses (e.g. superoxide anion generation), than against PLC-dependent responses mediated by intracellular Ca^{2+} mobilisation.

These observations predict that the functional selectivity of DMV towards PLD-dependent with respect to Ca^{2+}-dependent responses results directly from the excess capacity in fMet-Leu-Phe-stimulated IP_3 production. The amount of IP_3 produced by agonist-dependent PLC activation will probably vary in different tissues and therefore the specificity of DMV towards PLD with respect to PLC-dependent responses is likely to be tissue-related. Thus the implication of this receptor reserve is that non-selective phospholipase inhibitors will have varying degrees of selectivity at the functional level depending on the tissue.

In summary, there are two main consequences of the ability of fMet-Leu-Phe to induce formation of IP_3 in excess of what is required to mobilise fully intracellular Ca^{2+}. Firstly, Ca^{2+} mobilisation is more sensitive than IP_3 formation to fMet-Leu-Phe receptor occupancy. Secondly, fMet-Leu-Phe-dependent Ca^{2+} mobilisation, and we would predict Ca^{2+}-dependent responses, are much less sensitive than PLC activation to the inhibitor of phospholipase activation, DMV. A further implication is that since the enzymic activity of PLC is not directly related to changes in $[Ca^{2+}]_i$, inhibition of this enzyme is likely to be an inefficient way of inhibiting this signal transduction pathway.

We are grateful to Mr C. O'Neil and Miss I. Acevedo for their excellent technical assistance. We also thank Dr J. Hanson for providing us with demethoxyviridin and Mrs V.A. Morant for preparing the typescript.

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(Received November 16, 1990
Revised January 28, 1991
Accepted February 12, 1991)

Br. J. Pharmacol. (1991), **103**, 1597–1601 © Macmillan Press Ltd, 1991

The effect of drugs acting on cholinoceptors and mucosal chloride on luminal bicarbonate transport by rat caecum under *in vitro* conditions

[1]Paul Canfield & Tarik Abdul-Ghaffar

Department of Physiology & Biophysics, St. Mary's Hospital Medical School, Norfolk Place, London W2 1PG

1 The transport of HCO_3^- (Jsm) from a HCO_3^--buffered serosal to an unbuffered mucosal saline solution has been studied in rat caecum *in vitro*.

2 Carbachol, bethanechol and acetylcholine (ACh) caused a concentration-dependent fall in Jsm with similar maximum effects. 1,1-Dimethyl-4-phenyl-piperazinium iodide (DMPP) also inhibited Jsm but the effect was less than with the other drugs. Maximum cholinoceptor inhibition was less than that obtained with anoxia.

3 Responses were blocked by atropine (10^{-5} M) but hexamethonium (2×10^{-4} M) significantly altered the response only to DMPP.

4 Physostigmine (10^{-5} M) shifted the ACh response curve to the left but physostigmine itself caused inhibition of Jsm which was blocked by atropine.

5 Substitution of mucosal Cl^- by NO_3^- reduced Jsm to a similar extent to maximum cholinoceptor effect and abolished responses to bethanecol. Anoxia further reduced Jsm in the presence of NO_3^-.

6 Mucosal SITS and DIDS (1 mM) reduced Jsm but this was less than the maximum inhibition seen with drugs acting on cholinoceptors or mucosal Cl^- removal. Serosal DIDS caused a similar inhibition.

7 We conclude that cholinoceptor agonists inhibit but do not abolish luminal bicarbonate transport by an action on muscarinic receptors.

Keywords: Bicarbonate transport; cholinoceptor agonists and antagonists; caecum *in vitro*; mucosal chloride substitution; anion transport inhibitors

Introduction

The rat caecum is a major site for absorption of water, salt and short chain fatty acids (Umesaki *et al.*, 1979; Edmonds & Thompson, 1980). Under *in vitro* conditions, it exhibits an alkaline flux from a bicarbonate-buffered serosal bathing solution to an unbuffered mucosal solution (Jsm) and this is sustained for several hours. Replacement of the bicarbonate/CO_2 buffer by HEPES/O_2 abolishes the flux whilst anoxia reduces it by about two thirds (Canfield, 1991). There have been several studies characterizing ion transport in rabbit caecum (Clauss *et al.*, 1985; 1989; Hatch, 1987; Hatch & Freel, 1988) but not in rat and none of the studies has looked at the effects of physiological mediators on ion transport in caecal tissue. This paper describes the effects of drugs acting on cholinoceptors on luminal bicarbonate secretion in the rat caecum *in vitro*. Some of these results have been presented to the Physiological Society (Abdul-Ghaffar & Canfield, 1990).

Methods

Tissue preparation

Normally fed, male Wistar rats (250–350 g) were killed by cervical dislocation. The abdomen was opened by a mid-line incision and the caecum exteriorised, dissected free of mesentery and removed. It was opened and the contents washed away with cold, isotonic saline. Portions of the tissue were tied over the end of a plastic tube (cross sectional area 1.13 cm^2) with the mucosa facing the tube lumen. Six pieces of tissue were set up in each experiment from 2 animals. This is essentially the same apparatus previously described for use with rat stomach (Canfield & Price, 1981). The lumen of the tube contained 5 ml of a solution of composition (in mM): NaCl 136, KCl 5, $MgSO_4$ 1.2, $CaCl_2$ 2.4 and glucose 11.7, gassed with 100% O_2. The tube was suspended in a bath containing 30 ml of a similar solution in which 26 mM $NaHCO_3$ replaced an equivalent amount of NaCl. This was gassed with 95% O_2: 5% CO_2 (pH 7.4) and maintained at 36°C.

Measurement of alkalinisation

The unbuffered solution was replaced every 15 min throughout the experiment for determination of HCO_3^- by back-titration with 5 mM HCl using an autotitrator system (ABU 80, Radiometer, Copenhagen). During titration at room temperature the solution was continuously gassed with 95% O_2: 5% CO_2. The titration end point was determined as that pH obtained when a sample of unbuffered saline taken directly from the stock reservoir was similarly gassed and this was redetermined several times during an experiment. This method of estimating alkalinisation was validated by adding known amounts of $NaHCO_3$ to 5 ml of unbuffered saline equivalent in amount to that produced by the tissues. A linear regression of 'recovered' bicarbonate by titration against added bicarbonate gave a correlation coefficient of 0.996 ($n = 24$) with a slope of 1.03. The advantage of titrating in this way is that titrations were sufficiently rapid to perform six in 15 min with one autotitrator, in contrast to the need to bubble with N_2 for 20 min when titrating to an alkaline end point as reported by Dietz & Field (1973) or the need for one autotitrator per tissue for the method of continuous pH-stat titration (Flemstrom, 1977; Sullivan & Smith, 1986).

Drugs

The following were used; acetylcholine chloride, hexamethonium chloride, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), physostigmine, bethanechol (carbamyl β-methyl choline chloride), carbachol (carbamylcholine chloride), atropine sulphate, tetrodotoxin (TTX), 4-acetamido-4′-isothiocyanatostilbene-2-2′-disulphonic acid (SITS), and

[1] Author for correspondence.

4,4'-diisothiocyanatostilbene-2-2'-disulphonic acid (DIDS). All drugs were obtained from Sigma, Poole, Dorset, and were made up on the day of the experiment and added to the serosal side unless otherwise indicated.

Expression of results

Measurements of alkaline flux from serosa to mucosa are designated as Jsm. Tissues were allowed to recover for 60 min before flux measurements began and the unbuffered solution was changed every 15 min during this period. Statistical comparisons were made between the mean steady basal value of the 15 min period immediately preceding the experimental procedure and the final 15 min flux value at the end of the procedure period. Results are expressed as mean ± s.e.mean as μmol cm^{-2} h^{-1} of base. Statistical analysis was performed with either a paired or unpaired Student's t test as appropriate and P values less than 0.05 were taken as significant. The number of pieces of tissue used in each study is indicated by n.

Ion substitution

In some experiments Cl^- was excluded from the unbuffered saline in contact with the mucosa, being replaced with nitrate ($NaNO_3$, $Ca(NO_3)_2$). The other ions were unchanged. When solution changes were made the tissue was washed 3 times at 5 min intervals with the new mucosal solution before flux measurements recommenced and the titration end point with the new solution was determined.

Results

Cholinoceptor agonists

Acetylcholine (ACh), carbachol and bethanechol all inhibited Jsm and the maximum effect was not significantly different between the drugs. Figure 1 shows the decrease of Jsm from the preceding level of flux plotted against drug concentration for these drugs plus the effect of DMPP which was also inhibitory but had a significantly lower maximum effect. Maximum values were; ACh = 2.4 ± 0.5 (n = 6 at 5 × 10^{-4} M), bethanechol = 2.7 ± 0.4 (n = 11 at 2.5 × 10^{-4} M), carbachol = 2.6 ± 0.3 (n = 6 at 10^{-5} M) and DMPP

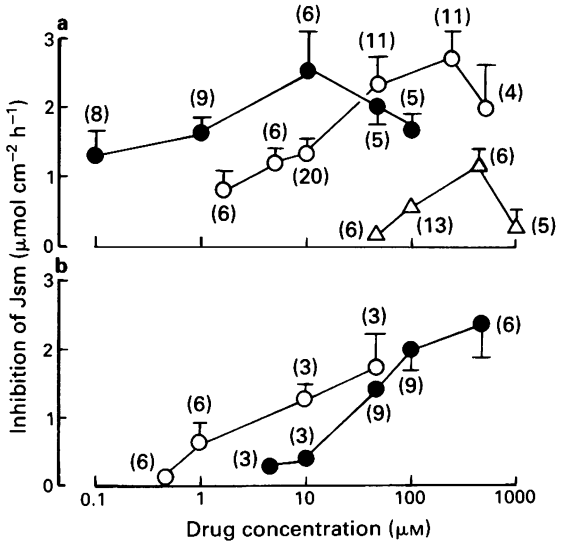

Figure 1 Reduction in rate of bicarbonate secretion from preceding basal value in each tissue (μmol cm^{-2} h^{-1}) against log drug concentration. In (a) carbachol (●), bethanechol (○) and 1,1-dimethyl-4-phenyl piperazinium (DMPP) (△). In (b) acetylcholine alone (●) and in the presence of physostigmine 10^{-5} M (○). All values are mean with s.e.mean (vertical bars) and number of tissues in parentheses.

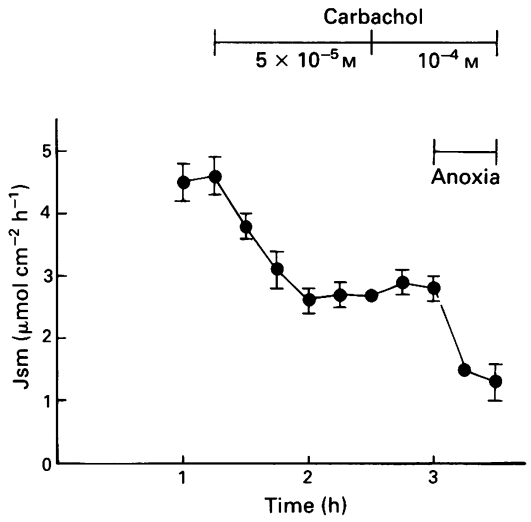

Figure 2 The effect of a maximum concentration of carbachol with subsequent application of anoxia on basal bicarbonate secretion (Jsm μmol cm^{-2} h^{-1}). Values are mean with s.e.mean (vertical bars) and n = 4.

1.2 ± 0.2 μmol cm^{-2} h^{-1} (n = 6 at 5 × 10^{-4} M). The maximum inhibition obtained was significantly less than that seen following 30 min of anoxia in the same tissues as shown in Figure 2 for bethanechol.

Cholinoceptor antagonists

Atropine (10^{-5} M) inhibited the response to all four agonists (Table 1). Hexamethonium (2 × 10^{-4} M) had no significant effect on responses to carbachol and acetylcholine but the response to DMPP was reduced to a value not significantly different from zero.

Effect of physostigmine

One possible explanation for the small response to DMPP is that it results from the release of endogenous ACh which is then rapidly broken down by the tissue, effectively truncating the response. The use of the choline esterase inhibitor, physostigmine (10^{-5} M) should allow testing of this hypothesis. We first tested physostigmine with exogenous ACh (Figure 1b) where it shifted the concentration-response curve to the left indicating that the tissue did indeed break down ACh. However, a complication was found in these experiments in

Table 1 Effect of hexamethonium (C6) and atropine (Atr) on the inhibitory action of cholinoceptor agonists on bicarbonate secretion

Agonist	*Control*	*C6*	*Atr*	n
Carbachol	2.1 ± 0.4*	2.2 ± 0.5*	0.5 ± 0.4	8
Bethanechol	1.9 ± 0.4*	not done	0.2 ± 0.1	6
ACh	1.4 ± 0.2*	1.2 ± 0.2*	0.2 ± 0.2	6
DMPP	0.5 ± 0.1*	0.1 ± 0.3	0.2 ± 0.4	6

Values are mean fall in rate of bicarbonate secretion from preceding basal values (in μmol cm^{-2} h^{-1}) ± s.e.mean. Tissues were either controls or were pre-incubated for 60 min with hexamethonium (2 × 10^{-4} M) or atropine (10^{-5} M). They were then exposed for a further 60 min to one of the following; carbachol 10^{-5} M, bethanechol 10^{-5} M, acetylcholine 10^{-4} M or the nicotinic agonist 1,1-dimethyl-4-phenyl piperazinium (DMPP) 10^{-4} M.
* indicates a significant (P < 0.05) fall in rate of secretion. All responses were blocked by atropine but only DMPP was affected by hexamethonium.

Table 2 Effect of physostigmine and atropine on basal bicarbonate secretion

	Jsm (μmol cm^{-2} h^{-1})	
Treatment	*Experiment A*	*Experiment B*
Initial rate	4.6 ± 0.3	4.3 ± 0.2
+ Physostigmine	4.0 ± 0.2*	
+ Physostigmine + atropine	4.5 ± 0.3	4.3 ± 0.4

Values are mean rate of bicarbonate secretion (Jsm) ± s.e.mean, $n = 6$ in both experiments. * indicates a significant difference from the initial value ($P < 0.05$) by paired t test. In experiment A, physostigmine (10^{-5} M) was added for 60 min after a steady initial value was obtained and then atropine (10^{-5} M) was also added for a further 60 min. In experiment B only atropine was added for 60 min. Atropine alone had no effect on basal Jsm but did reverse the effect of physostigmine.

that physostigmine alone resulted in an inhibition of Jsm. Overall, physostigmine (10^{-5} M) resulted in a mean decrease of 0.67 μmol cm^{-2} h^{-1} ($n = 65$, 95% confidence limits 0.45–0.88 μmol cm^{-2} h^{-1}). The effect of physostigmine was blocked by atropine indicating an action via muscarinic receptors and presumably due to endogenous ACh. Atropine had no effect on basal Jsm in the absence of physostigmine (Table 2). TTX (10^{-6} M) did not block the effect of physostigmine (Table 3)

Table 3 Effect of tetrodotoxin (TTX) on basal bicarbonate secretion and response to physostigmine

Experiment A	
Initial rate	3.9 ± 0.5
+ TTX	3.8 ± 0.6
+ TTX + physostigmine	3.6 ± 0.6*
Experiment B	
Initial	4.6 ± 0.4
+ Physostigmine	3.9 ± 0.3*
+ Physostigmine + TTX	4.0 ± 0.3*

Values are mean rate of bicarbonate secretion (Jsm in μmol cm^{-2} h^{-1}) ± s.e.mean and $n = 6$ for both experiments. * indicates a significant difference ($P < 0.05$) from initial value by paired t test. In experiment A, tetrodotoxin (TTX 10^{-6} M) was added for 45 min when an initial steady rate had been reached then physostigmine (10^{-5} M) was also added for a further 45 min. In experiment B the drugs were added in the reverse order. TTX alone did not alter basal Jsm nor did it prevent the effect of physostigmine

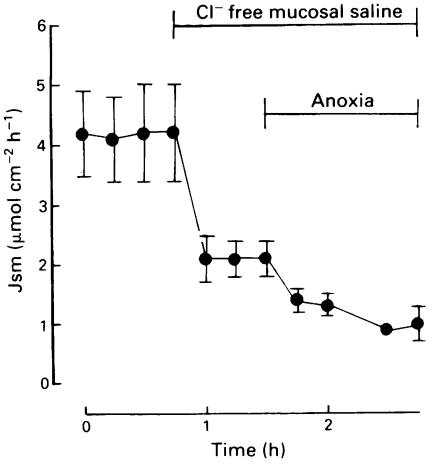

Figure 3 The effect of substituting mucosal chloride with nitrate and subsequent application of anoxia on basal bicarbonate secretion (Jsm μmol cm^{-2} h^{-1}). Values are mean with s.e.mean (vertical bars) and $n = 5$.

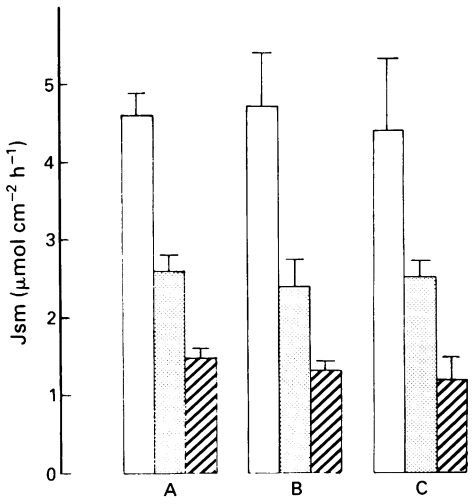

Figure 4 Comparison of the effect on basal bicarbonate secretion (Jsm μmol cm^{-2} h^{-1}) shown by open columns, of 3 treatments (A, B or C), shown by stippled columns and the effect of subsequent application of anoxia (hatched columns) on the treatment response. (A) Maximum concentration of carbachol (5×10^{-5} M); (B) mucosal Cl^- substituted with NO_3^- and (C) maximum concentration of bethanechol (5×10^{-4} M). Values are mean with s.e.mean (vertical bars) and in (A) $n = 6$; in (B) $n = 5$ and in (C) $n = 4$. There were no significant differences between the effects on Jsm of the initial treatments nor between the values following subsequent application of anoxia.

suggesting that release of endogenous ACh did not result from spontaneous activity in the intrinsic nervous system of the tissue. These results mean that concentration-response curves in the presence of physostigmine would have to be constructed against an uncontrolled background of ACh-induced inhibition and experiments with physostigmine were discontinued.

Replacement of mucosal chloride

Drugs acting on cholinoceptors are well established as stimulants of electrogenic chloride secretion and inhibitors of neutral NaCl uptake in the intestine (Donowitz & Welsh, 1987). Thus, they may inhibit bicarbonate secretion in the caecum by inhibiting a HCO_3^-/Cl^- exchange mechanism. We therefore, examined the effect of removing Cl^- from the unbuffered solution in contact with the mucosa, on basal Jsm and the response to bethanechol. The results are shown in

Table 4 Effect of 4,4'-diisothiocyanatostilbene-2-2'-disulphonic acid (DISDS), 4-acetamido-4'-isothiocyanatostilbene-2-2'-disulphonic acid (SITS) and anoxia on rate of bicarbonate secretion (Jsm)

	Jsm (μmol cm^{-2} h^{-1})			
Treatment	*Initial*	*+ Drug*	*Fall Jsm*	*Anoxia*
Mucosal DIDS 1 mM ($n = 15$)	4.7 ± 0.3	3.4 ± 0.2	1.3 ± 0.2*	1.4 ± 0.1
Mucosal SITS 1 mM ($n = 6$)	4.7 ± 0.6	4.1 ± 0.4	0.6 ± 0.2*	1.7 ± 0.1
Serosal DIDS 1 mM ($n = 6$)	4.3 ± 0.5	2.6 ± 0.3	1.7 ± 0.3*	1.1 ± 0.2

The drug (DIDS or SITS) was added when the initial values were steady. The drug value was after 60 min exposure. The anoxia value followed 45 min treatment in the presence of the drug used. The fall in Jsm column was calculated from the difference between initial and drug value for each tissue. The values are mean ± s.e.mean and all three drug treatments resulted in a significant (* $P < 0.05$) fall in Jsm. There was no significant difference between results with mucosal and serosal DIDS by unpaired t test.

Figure 3. Replacement with nitrate resulted in a decrease of Jsm which became steady after 30 min and was further reduced by anoxia. The results are quantitatively the same as the results obtained with maximal inhibition by bethanechol or carbachol with subsequent application of anoxia and are summarised in Figure 4 for comparison. Bethanechol (2.5×10^{-4} M) was without effect in NO_3^--substituted mucosal saline.

Action of anion transport inhibitors

To test further the relationship between Cl^- and HCO_3^-, we examined the effect of two putative anion exchange inhibitors; DIDS or SITS (1 mM) were applied to the mucosal side for 60 min and Jsm compared with the pre-drug value. Results are shown in Table 4. Both inhibitors caused a significant reduction in Jsm but this was less than the maximum inhibition obtained with cholinoceptor agonists or with mucosal Cl^- replacement. Application of 1 mM DIDS to the serosal side also inhibited Jsm and the effect was not significantly different from that obtained with the mucosal drug.

Discussion

Cholinoceptor agonists

The cholinoceptor agonists clearly have an inhibitory effect on the luminal bicarbonate flux (Jsm) in this preparation. The reversal of the effect by atropine and the very weak action of hexamethonium indicate that inhibition is via muscarinic receptors. Zimmerman & Binder (1982) have reported that rat colonic enterocytes have muscarinic receptors so that inhibition may be a direct effect on the mucosal cells but our results do not allow us to distinguish between this and an indirect effect mediated by the cholinoceptor-mediated release of another, unidentified agent from elsewhere in the tissue. The maximum level of inhibition obtained with bethanechol and carbachol was significantly less than that seen in the presence of anoxia showing that part of the oxygen-dependent flux was resistant to cholinoceptor drugs. Although the nicotinic agonist DMPP inhibited Jsm and the effect was prevented by hexamethonium, the maximum response was small. This may have been the result of a rapid breakdown of acetylcholine released postganglionically but attempts to test this with physostigmine were frustrated by the inhibitory effect of physostigmine itself which is discussed below.

Effect of physostigmine

Physostigmine alone caused a variable amount of inhibition of Jsm which could be prevented by atropine. The most likely explanation for this is that there is a spontaneous release of acetylcholine in the tissue and that this is normally broken down so rapidly that it does not effect the measured Jsm (atropine alone did not alter Jsm). In the presence of physostigmine, this acetylcholine would persist and inhibit Jsm; the variability of the effect between tissues reflecting differing rates of release of endogenous acetylcholine. The inability of TTX to prevent this action of physostigmine suggests that the acetylcholine release did not result from spontaneous activity in the intact intrinsic nerve system of the preparation. Similar results were reported by Cooke (1984) on the effect of physostigmine on short circuit current in guinea-pig ileum. Although physostigmine did result in a leftward shift of the acetylcholine concentration-response curve, this cannot be directly compared with the curve for acetylcholine alone because the concentration of endogenously released acetylcholine at the receptors cannot be determined. A possible alternative explanation for the weak inhibitory effect of DMPP is that non-selective stimulation of the ganglia results in activation of both an inhibitory and stimulatory pathway, each capable of separately influencing Jsm. Harris *et al.* (1989) have reported that distension of rat distal colon, both *in vivo* and *in vitro*, stimulated bicarbonate secretion via an intrinsic neural reflex but have not identified the mediator. The possible role of the intrinsic nervous system in regulating caecal bicarbonate secretion should be investigated further.

Ion substitution and anion transport inhibitors

Substitution of mucosal Cl^- with NO_3^- reduced Jsm by the same amount as maximal cholinoceptor inhibition (in the presence of mucosal Cl^-) and also abolished the effect of cholinoceptor drugs on Jsm. In neither situation was the oxygen-dependent part of Jsm completely inhibited. The inhibitory effect of mucosal Cl^- removal conflicts with the previously reported lack of effect of bilateral Cl^- removal in this preparation (Canfield, 1991) but it is likely that the solution changing in the earlier study was not adequate as the washes were made in rapid succession rather than at 5 min intervals. Interpretation of the results of Cl^- substitution is difficult without further studies. The removal of Cl^- from the mucosal solution will alter ionic concentration gradients across the tissue and its replacement with a less permeant ion is likely to hyperpolarize the luminal surface which would oppose the exit of anions (Feldman *et al.*, 1988). There is also the possibility that the substituting ion may be able to replace Cl^- on the exchanger to some extent and allow it to operate at a reduced rate of transport. Additionally, Cl^- may diffuse passively from the serosal to the mucosal side and thus become available to an exchanger. Such an effect would be limited by the replacement of the mucosal saline every 15 min but might allow a sufficient concentration of Cl^- to permit a reduced rate of operation.

In our experiments, mucosal SITS or DIDS significantly reduced Jsm but this reduction was much smaller than that produced by maximum cholinoceptor inhibition or replacement of mucosal Cl^-. As serosal DIDS was equally effective, it seems unlikely that this inhibition was the result of a specific action on an apical HCO_3^-/Cl^- exchanger. In the earlier study (Canfield, 1991) where lower concentrations were used, only serosal DIDS caused a significant fall in Jsm. Smith *et al.* (1986) have previously drawn attention to non-specific effects of disulphonic stilbenes in rabbit colon. As there are also reports of stilbene-resistant anion exchangers in rabbit kidney (Schuster, 1985), turtle bladder (Kohn *et al.*, 1990) and rat distal colon (Feldman & Stephenson, 1990) epithelia, it would seem that these drugs are of limited general value for investigation of epithelial anion exchange mechanisms; a conclusion reached by Smith *et al.* (1986) in respect of rabbit proximal colon.

The quantitative similarity between the results with drugs acting on cholinoceptors and mucosal Cl^- substitution may be a coincidence. Alternatively they could be explained if the oxygen-dependent part of Jsm resulted from the operation of at least two transport mechanisms; one which utilised an apical HCO_3^-/Cl^- exchange mechanism and was totally inhibited by either cholinoceptor agonists or Cl^- removal and the other which was Cl^--independent and unaffected by drugs acting on cholinoceptors. Previous studies of *in vitro* bicarbonate transport in mammalian large intestine have reported Cl^--independent secretion in rabbit proximal colon (Sullivan & Smith, 1986), total dependence on mucosal Cl^- in rat distal colon (Feldman *et al.*, 1990) and an electrogenic secretion in rabbit caecum (Hatch & Freel, 1988) and rat distal colon (Harris *et al.*, 1989). The proposed explanation for the present findings requires further investigation of the dependence of Jsm on mucosal Cl^- concentration, transmucosal p.d. and measurement of the relationship between HCO_3^- secretion and Cl^- uptake by tissues under various conditions. If these substantiate this explanation, then mucosal Cl^- substitution may offer the possibility of studying the regulation of these mechanisms separately. Two separately controlled mechanisms for luminal bicarbonate secretion have been proposed previously for amphibian duodenum (Flemstrom *et al.*, 1982).

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(Received November 27, 1990
Revised February 7, 1991
Accepted February 15, 1991)

Br. J. Pharmacol. (1991), **103**, 1602–1606 © Macmillan Press Ltd, 1991

Nitric oxide is the mediator of ATP-induced dilatation of the rabbit hepatic arterial vascular bed

[1]R.T. Mathie, *V. Ralevic, [2]B. Alexander & *G. Burnstock

Department of Surgery, Royal Postgraduate Medical School, London W12 0NN and *Department of Anatomy & Developmental Biology, University College London, London WC1E 6BT

1 Livers of 10 New Zealand White rabbits were perfused *in vitro* with Krebs-Bülbring buffer via the hepatic artery (HA) and portal vein (PV) at constant flows of 23 ± 1 and 77 ± 1 ml min^{-1} 100 g^{-1} respectively. The tone of the preparation was raised with noradrenaline (concentration: 10 μM).

2 Dose-response curves for the vasodilatation produced by adenosine 5'-triphosphate (ATP), acetylcholine (ACh), adenosine, and sodium nitroprusside (SNP) were obtained following injection into the HA supply. Injections were then repeated in the presence of the L-arginine to nitric oxide pathway inhibitors N-monomethyl-L-arginine (L-NMMA, $n = 6$) and N-nitro-L-arginine methyl ester (L-NAME, $n = 4$) at concentrations of 30 μM and 100 μM for each inhibitor.

3 Both L-NMMA and L-NAME antagonized the responses to ATP and ACh; L-NAME was 2–3 times more potent than L-NMMA as an inhibitor of these endothelium-dependent vasodilatations. Neither L-NMMA nor L-NAME attenuated responses of the endothelium-independent vasodilators, adenosine and SNP.

4 These results indicate that nitric oxide is the mediator of ATP-induced vasodilatation in the HA vascular bed of the rabbit and that the receptor responsible for the release of nitric oxide, the P_{2y}-purinoceptor, is located predominantly on the endothelium.

Keywords: Hepatic artery; portal vein; ATP; adenosine; purinoceptors; EDRF; nitric oxide; L-NAME; L-NMMA

Introduction

The importance of adenosine in the control of hepatic arterial (HA) blood flow has recently been established (Lautt *et al.*, 1985; Lautt & Legare, 1985; Ezzat & Lautt, 1987; Mathie & Alexander, 1990), and a regulatory role for adenosine 5'-triphosphate (ATP) has also been suggested (Mathie & Alexander, 1990). Work from our laboratory has demonstrated, in the HA vascular bed of the rabbit, the existence of purinoceptors which mediate adenosine-induced vasodilatation (A_2 receptors; Mathie *et al.*, 1991), and ATP-induced vasoconstriction (P_{2x} receptors) and vasodilatation (P_{2y} receptors) (Ralevic *et al.*, 1991a).

We concluded from these studies that the vasodilatation induced by ATP was likely to have been mediated by the release of the endothelium-derived relaxing factor (EDRF), nitric oxide (Palmer *et al.*, 1987; Ignarro *et al.*, 1987), following activation of endothelial P_{2y}-purinoceptors in the HA vascular bed; nitric oxide in turn elicits relaxation by activating guanylate cyclase in vascular smooth muscle cells (Murad, 1986; Ignarro, 1989). Our conclusion was based on the observation that methylene blue attenuated relaxant responses to ATP and to the endothelium-dependent vasodilator acetylcholine (ACh), but not those to the endothelium-independent dilator, sodium nitroprusside (SNP), consistent with a direct inactivation of EDRF by methylene blue (Martin *et al.*, 1985; Watanabe *et al.*, 1988). However, the action of methylene blue may, at least partly, proceed through inhibition of smooth muscle guanylate cyclase (Martin *et al*; 1985), thus leaving open the possibility that some of the relaxation to ATP could have taken place through direct action on a sub-population of P_{2y}-purinoceptors located on the smooth muscle; this, in fact, has recently been shown to be the predominant location of these receptors in the common hepatic artery of the rabbit (Brizzolara & Burnstock, 1991).

The present study was therefore conducted to clarify the role of nitric oxide in ATP-induced vasodilatation, and thereby establish the location of the P_{2y}-purinoceptors in the HA vascular bed. We have used the same isolated dual-perfused rabbit liver model described previously (Alexander *et al.*, 1991; Mathie *et al.*, 1991; Ralevic *et al.*, 1991a). Rabbit livers were perfused via both the HA and the portal vein (PV) at constant physiological flow rates from a common reservoir of Krebs-Bülbring buffered solution. Vasodilator responses were examined in the presence and in the absence of the specific inhibitors of the biosynthesis of nitric oxide from L-arginine, N-monomethyl-L-arginine (L-NMMA: Palmer *et al.*, 1988; Rees *et al.*, 1989a,b), and N-nitro-L-arginine methyl ester (Moore *et al.*, 1990) for which we have used the recently adopted abbreviation L-NAME.

Methods

Operative procedures

Experiments were carried out on a total of 10 New Zealand White rabbits of either sex, weighing 2.1–2.7 kg (mean 2.4 kg). The operative technique has been described previously (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 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

[1] Author for correspondence.
[2] Present address: Department of Surgery, King's College School of Medicine and Dentistry, Rayne Institute, Coldharbour Lane, London SE5 9NU.

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 with oxygenated (95% O_2/5% CO_2) Krebs-Bülbring buffer solution, 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. Each liver was perfused via the HA and PV at constant flow rates (mean values for all experiments: $23 \pm 1\,ml\,min^{-1}$ $100\,g^{-1}$ and $77 \pm 1\,ml\,min^{-1}$ $100\,g^{-1}$ respectively). 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.

After an equilibration period of 5–10 min, the tone of the preparation was raised by addition to the perfusate of noradrenaline to a final concentration of 10 μM.

Drug administration

Adenosine (hemisulphate), ATP, ACh, SNP, noradrenaline bitartrate and N^{ω}-nitro-L-arginine methyl ester (L-NAME) were obtained from Sigma. N^{G}-monomethyl-L-arginine (L-NMMA) was obtained from Wellcome Research Laboratories. (The 'ω' and 'G' designations are used here in order to retain the description of the compounds provided by the manufacturers. We would like to point out, however, that these superscripts are, in fact, synonymous; see also Discussion.) All the above were dissolved in distilled water, except noradrenaline which was made up as a 10 mM stock solution in 0.1 mM ascorbic acid (to prevent oxidation).

ATP, ACh, adenosine and SNP were injected in turn as 0.1 ml boluses into the HA, in the dose range 10^{-11}–10^{-6} mol. Injections of distilled water 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.

The above protocol was employed in 2 separate groups: in *Group I* (6 rabbits) the effect of the above series of agents was investigated before and after the addition to the perfusate of L-NMMA to final concentrations of 30 μM ($n = 6$) and 100 μM ($n = 3$); in *Group II* (4 rabbits) the effect of the same agents was studied before and after the addition of L-NAME to final concentrations of 30 μM ($n = 4$) and 100 μM ($n = 4$).

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 ACh and ATP were calculated by use of the pD_2, the negative logarithm of the number of mol required to elicit a half-maximal response.

Results

Perfusion indices

Group I (N-monomethyl-L-arginine, L-NMMA) Basal perfusion pressures in the HA and PV were 34 ± 10 mmHg and 4 ± 1 mmHg respectively. Pressures in the HA and PV increased to 134 ± 13 mmHg and 10 ± 2 mmHg respectively following the addition of noradrenaline to the perfusate. The addition of 30 μM L-NMMA to the perfusate produced a further, non-significant, 5 mmHg mean increase in HA tone. The total volume of bile collected was 6 ± 1 ml over the 225 ± 20 min perfusion period, approximating to an hourly mean output of 1.6 ml.

Group II (N-nitro-L-arginine methyl ester, L-NAME) Basal perfusion pressures in the HA and PV were 65 ± 13 mmHg and 7 ± 1 mmHg respectively; neither was significantly different from the corresponding value in Group I. Pressures in the HA and PV increased to 149 ± 28 mmHg and 11 ± 1 mmHg respectively following the addition of noradrenaline to the perfusate; these values were not significantly different from those of Group I. The addition of 30 μM L-NAME to the perfusate produced a further, non-significant, 6 mmHg mean increase in HA tone. The total volume of bile collected was 13 ± 1 ml over the 250 ± 14 min perfusion period, equivalent to an hourly mean output of 3.1 ml.

Responses to adenosine 5'-triphosphate, acetylcholine, adenosine and sodium nitroprusside

Adenosine 5'-triphosphate

Group I (L-NMMA): Bolus injections of ATP produced dose-dependent vasodilator responses in the HA (Figure 1a). L-NMMA at a concentration of 30 μM had no effect on the responses, but at 100 μM there was a significant attenuation of the responses to the three highest doses of ATP. There was no significant change in pD_2 value with either concentration of L-NMMA (8.4 ± 0.3 to 8.1 ± 0.2 with 30 μM L-NMMA).

Group II (L-NAME): Control injections of ATP produced a greater maximum vasodilatation in this group than in Group

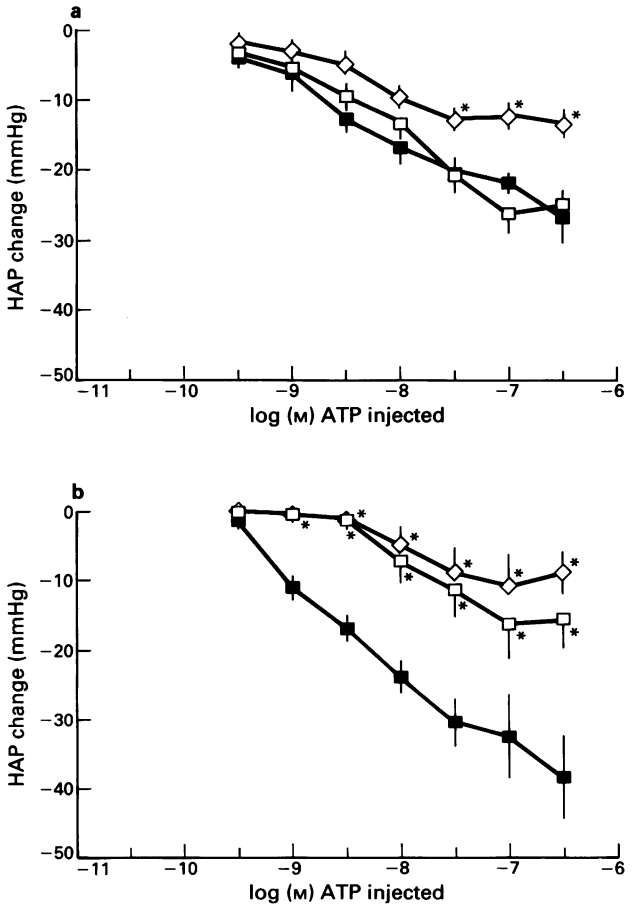

Figure 1 Responses of hepatic arterial pressure (HAP) to increasing doses of adenosine 5'-triphosphate (ATP), before and during perfusion with (a) N-monomethyl-L-arginine (L-NMMA), and (b) N-nitro-L-arginine methyl ester (L-NAME): (□) 30 μM; (◇) 100 μM L-NMMA/L-NAME. * indicates significant difference from control response (■) ($P < 0.05$); $n = 6$ for (a) and $n = 4$ for (b).

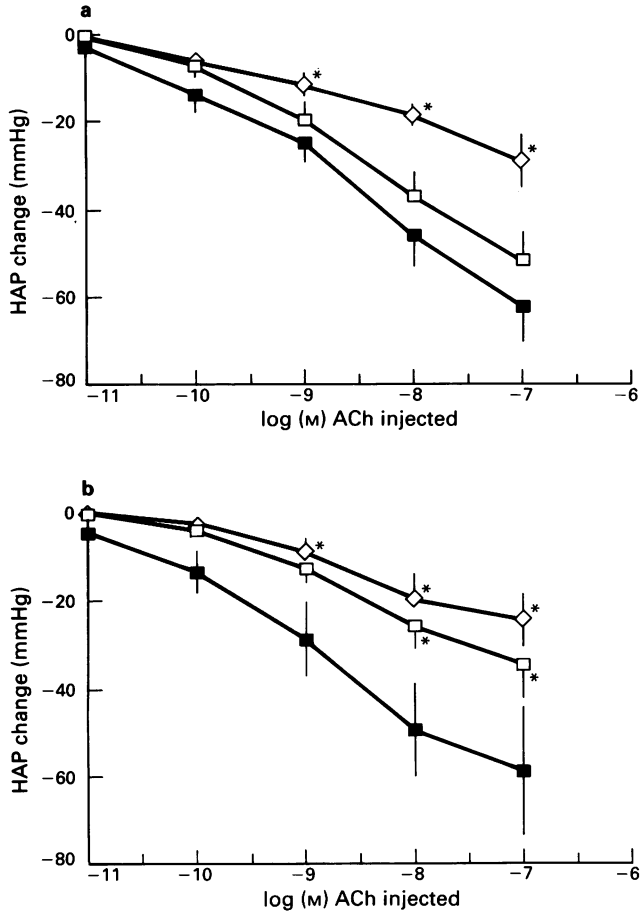

Figure 2 Responses of hepatic arterial pressure (HAP) to increasing doses of acetylcholine (ACh), before and during perfusion with (a) N-monomethyl-L-arginine (L-NMMA), and (b) N-nitro-L-arginine methyl ester (L-NAME): (□) 30 μM; (◇) 100 μM L-NMMA/L-NAME. * indicates significant difference from control response (■) ($P < 0.05$); $n = 6$ for (a) and $n = 4$ for (b).

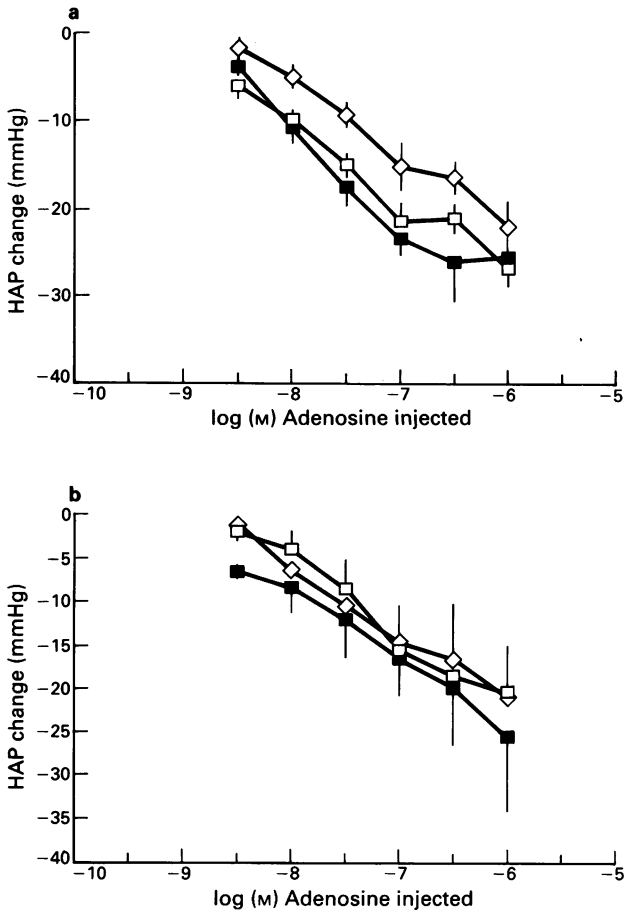

Figure 3 Responses of hepatic arterial pressure (HAP) to increasing doses of adenosine, before and during perfusion with (a) N-monomethyl-L-arginine (L-NMMA), and (b) N-nitro-L-arginine methyl ester (L-NAME): (■) control response; (□) 30 μM L-NMMA/L-NAME; (◇) 100 μM L-NMMA/L-NAME. In (a) $n = 6$, in (b) $n = 4$.

I (Figure 1b). L-NAME at both 30 μM and 100 μM caused significant inhibition of ATP-induced vasodilatation at all doses of ATP except the smallest. L-NAME at 30 μM produced a statistically significant change in pD_2 from 8.4 ± 0.2 to 7.6 ± 0.2; the 100 μM concentration caused no further change in pD_2. For the 30 μM concentration, therefore, L-NAME had approximately 3 times the inhibitory potency of L-NMMA.

Acetylcholine

Group I (L-NMMA): Bolus injections of ACh produced dose-dependent vasodilator responses in the HA (Figure 2a). L-NMMA at a concentration of 30 μM had no significant effect on the responses, but at 100 μM there was significant inhibition of the responses at the three highest doses of ACh. The control pD_2 (8.7 ± 0.1) was not altered by either concentration of L-NMMA.

Group II (L-NAME): L-NAME at 30 μM caused significant attenuation of ACh-induced vasodilatation at the highest two doses of ACh, while at 100 μM there was significant inhibition at the highest three doses of ACh (Figure 2b). The control pD_2 (9.0 ± 0.2) was significantly reduced to 8.6 ± 0.2 by the 30 μM concentration of L-NAME; the 100 μM concentration produced no further change in pD_2. For the 30 μM concentration, therefore, L-NAME had approximately twice the inhibitory potency of L-NMMA.

Adenosine Bolus injections of adenosine produced dose-dependent vasodilatation of the HA, which was not significantly affected by L-NMMA (Group I) or L-NAME (Group II) at either 30 μM or 100 μM (Figure 3), confirming that the mechanism of adenosine-induced vasodilatation is independent of nitric oxide.

Sodium nitroprusside Relaxations to SNP were not inhibited, but at some doses were enhanced, both by L-NMMA (Group I) and by L-NAME (Group II), indicating a retained ability of the smooth muscle to relax (Figure 4).

Discussion

These studies have provided direct evidence that ATP-induced vasodilatation of the HA vascular bed is predominantly mediated by an endothelial receptor, stimulation of which results in smooth muscle relaxation through the action of the EDRF, nitric oxide (Palmer *et al.*, 1987; Ignarro *et al.*, 1987). This conclusion arises from the observation that the vasodilatations caused by ATP and ACh were significantly attenuated by the inhibitors of the L-arginine to nitric oxide pathway, L-NMMA and L-NAME. The present experiments therefore confirm the main conclusion of our earlier work, in which methylene blue was also found to inhibit the vasodilatation caused by both ATP and ACh (Ralevic *et al.*, 1991a). Since at least part of the action of methylene blue can be attributed to direct inhibition of smooth muscle guanylate cyclase (Martin *et al.*, 1985), results obtained with it may be somewhat inconclusive. L-NMMA and L-NAME, however, are specific inhibitors of nitric oxide biosynthesis (Palmer *et al.*, 1988; Rees *et*

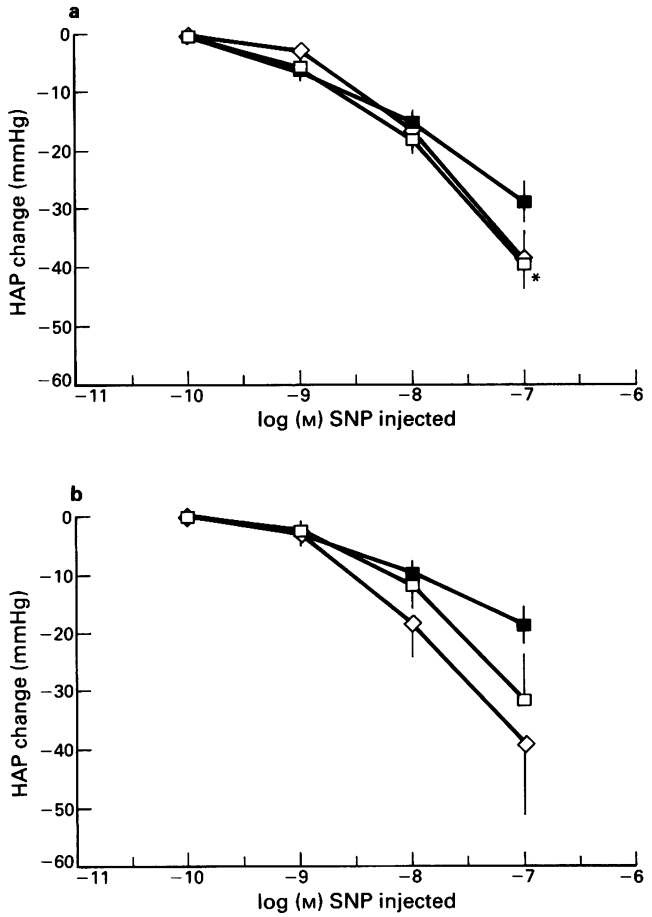

Figure 4 Responses of hepatic arterial pressure (HAP) to increasing doses of sodium nitroprusside (SNP), before and during perfusion with (a) N-monomethyl-L-arginine (L-NMMA), and (b) N-nitro-L-arginine methyl ester (L-NAME): (□) 30 μM; (◇) 100 μM L-NMMA/L-NAME. * indicates significant difference from control response (■) ($P < 0.05$): $n = 6$ in (a), $n = 4$ in (b).

al., 1989a,b; Moore *et al.*, 1990), and their use therefore provides a greater degree of certainty in the interpretation of data. It is implicit from the above that our results are consistent with the identification of EDRF as nitric oxide, formed from L-arginine (Moncada *et al.*, 1989).

L-NMMA was the compound originally used to establish the role of L-arginine as the precursor for the formation of endothelial nitric oxide (Palmer *et al.*, 1988). Since then, a number of additional structural analogues of L-arginine have been employed, some of which are more potent than L-NMMA. One of these is L-NAME, also used in the present investigation. The pD_2 values we have obtained for ATP and ACh support the view that L-NAME is up to 5 times more potent than L-NMMA at inhibiting endothelially-mediated vasodilatation (Moore *et al.*, 1990).

A problem in this field of study is the in consistency in the nomenclature with which the analogues of L-arginine are described; the use of different abbreviations to describe identical compounds has led to even more confusion. It is important to be clear about the terminology describing the 'ω' end of the L-arginine molecule, in particular to note the frequent use of the alternative 'G' (guanidino) designation specifying the *same* nitrogen atom (to which is attached a nitro group in the case of L-NAME, or a methyl group in the case of L-NMMA). Thus, the L-NMMA we have used in this study is identical to the N^{ω}-monomethyl-L-arginine used by Rees *et al.* (1989a); the L-NAME we have employed is identical to the N^{G}-nitro-L-arginine methyl ester used by Moore *et al.* (1990).

It was necessary in this investigation to ensure that the introduction of L-NMMA or L-NAME resulted in no change in the tone of the preparation. We found no need to alter the concentration of noradrenaline in the perfusate to retain unchanged tone, unlike a recent study using the isolated mesenteric bed in which the concentration of noradrenaline had to be reduced to compensate for a substantial rise in perfusion pressure induced by L-NAME (Ralevic *et al.*, 1991b). The unchanged vascular tone seen in the present study may imply that the HA vascular bed exhibits less synthesis or basal release of nitric oxide than the mesenteric bed (Ignarro, 1989), although the former bed is evidently capable of producing a quantity similar to the latter following appropriate stimulation; it is also possible that the HA bed possesses relatively less smooth muscle guanylate cyclase.

Vasodilator responses to adenosine and SNP, which act directly on the smooth muscle through the production of adenosine 3',5'-cyclic monophosphate (cyclic AMP) and cyclic GMP respectively (Burnstock & Kennedy, 1986; Ignarro, 1989), were not antagonized by either L-NMMA or L-NAME, showing that neither of the L-arginine analogues produced inhibition due to non-specific effects on the vascular smooth muscle. The results also confirm the independence of adenosine-induced vasodilatation both from the endothelium and from nitric oxide. A previous study has shown that the vasodilator action of ATP is not due indirectly to the action of adenosine produced by ectoenzymatic breakdown of ATP (Ralevic *et al.*, 1991a).

It was interesting to note that the vasodilator respones to SNP appeared to be somewhat enhanced by both L-NMMA and L-NAME. Such an augmentation of responses to SNP has previously been noted after the application of 10 μM L-NMMA to the canine isolated basilar artery (Katusic *et al.*, 1990). A more complete investigation and discussion of this phenomenon is contained in a separate paper (Ralevic *et al.*, 1991b).

Our current results therefore reinforce our previous findings that, in the rabbit HA bed, vasodilatation due to ATP is mediated through P_{2y}-purinoceptors located predominantly on the vascular endothelium (Ralevic *et al.*, 1991a). Furthermore, they are consistent with the view that endothelium-derived nitric oxide is the second messenger in the chain of events that results in HA smooth muscle relaxation following the activation of these receptors. This investigation thus provides further evidence for an important contribution by purinergic vasoactive mechanisms in the control of the hepatic circulation, and demonstrates for the first time that the resistance of the HA vascular bed, in common with many other vascular beds and blood vessels (Rees *et al.*, 1989a; Moncada & Higgs, 1990), is subject to control by locally produced nitric oxide.

The work was supported in part by the British Heart Foundation. L-NMMA was the generous gift of Dr S. Moncada, Wellcome Research Laboratories, Beckenham, Kent, England. We thank Sigma Chemical Company Ltd., Poole, Dorset, England, for providing detailed chemical description of L-NAME.

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(Received November 15, 1990
Revised February 8, 1991
Accepted February 15, 1991)

Br. J. Pharmacol. (1991), **103**, 1607–1613

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Kinetics of (+)-tubocurarine blockade at the neuromuscular junction

Alexander C. Le Dain, [1]Barry W. Madsen & *Robert O. Edeson

Department of Pharmacology, University of Western Australia and *Department of Anaesthesia, Sir Charles Gairdner Hospital, Nedlands, WA 6009, Australia

1 Although (+)-tubocurarine (Tc) is classically considered to be a competitive antagonist at the neuromuscular junction, kinetic details of the interaction remain unclear.

2 We studied the competitive action of Tc on the nicotinic receptor at the frog neuromuscular junction using a quantitative analysis of the generation phase of miniature endplate currents (m.e.p.cs) recorded in Ringer solution (20°C) under voltage clamp (−90 mV) in the absence or presence of 1–5 μM Tc. Under control conditions four neurotransmission parameters were estimated by non-linear regression using a mathematical model of synaptic transmission incorporating transmitter release, diffusion, hydrolysis, receptor binding and channel gating. These parameters were then used in a further regression to estimate binding rate constants for Tc at the same endplate. Allowance was made for open channel block by Tc, which under the conditions of this study was only a small component of total blockade.

3 The results suggest that Tc binds to the two agonist recognition sites on the nicotinic receptor with equal affinity (stoichiometric K_Ds of 2.2 and 8.8 μM), and that most of the functional blockade at concentrations up to 5 μM is due to occupancy of only one site.

4 The association rate constant for Tc binding to sites on the nicotinic acetylcholine receptor appears to be very fast ($k_{+D} = 8.9 \times 10^8$ M^{-1} s^{-1}) and comparable to that for acetylcholine (ACh).

5 In the brief time during which an m.e.p.c. is generated ($\approx$200 μs), reversal of Tc blockade by transiently high concentrations of ACh seems to be kinetically limited.

Keywords: Nicotinic receptors; competitive antagonism; miniature endplate current generation; mechanism of action; quantitative modelling; kinetic parameters; non-linear regression

Introduction

(+)-Tubocurarine (Tc) is a potent neuromuscular blocker used to provide muscle relaxation during general anaesthesia. Despite the clinical importance of this drug and several decades of study, there is still incomplete understanding and some controversy regarding its many pharmacological effects at the neuromuscular junction. Tc is conventionally classified as a competitive antagonist to the binding of acetylcholine (ACh) at postsynaptic nicotinic receptors (Jenkinson, 1960), and more recently it has become generally recognised that it can also cause noncompetitive ion channel blockade (e.g. Lambert *et al.*, 1980). In many studies concerned with the mechanism of Tc-induced fade it has also been concluded that there is a presynaptic site of action (reviewed by Bowman *et al.*, 1988; with a contrary viewpoint by Bradley *et al.*, 1990), with some dissension over whether there is positive or negative feedback control of transmitter release. Furthermore, in some systems Tc can act as an agonist, albeit a very weak one, on nicotinic receptors (Takeda & Trautmann, 1984; Morris *et al.*, 1989). Although the competitive blocking action probably remains central to much of the inhibition seen *in vivo*, there is still uncertainty regarding antagonist binding affinity and the number of receptor sites involved in functional blockade. One biochemical study using a *Torpedo* preparation reported that binding was competitive at each of the two agonist recognition sites, with an approximate 230 fold difference in equilibrium dissociation constants (K_D = 0.033 and 7.7 μM, Neubig & Cohen, 1979). In another using ion flux data in *Electrophorus receptors* (Karpen & Hess, 1986), it was concluded that competitive blockade could be explained by Tc occupancy of one of these sites with K_D = 0.12 μM, although the possibility that both sites contributed could not be excluded. Sine (1988) examined the effects of dimethyltubocurarine on receptors from a human cell line and found that the two sites had almost identical binding affinities (K_D = 0.58 and 0.20 μM), but inhibition of the permeability response over the range 0.01–10 μM was due to occupancy of a single site. Electrophysiological examination of these questions has also not been straightforward. Colquhoun & Sheridan (1982) used a voltage-jump technique to study the rate of competitive blockade by Tc and concluded that the association rate constant for binding seemed to be very much faster (>10^9 M^{-1} s^{-1}) than previously thought. However, they were aware that several assumptions underlying their estimation were open to question, and finally commented that perhaps alternative experimental techniques needed to be developed to provide more definitive information.

Several authors (Land *et al.*, 1980; 1981; 1984; Salpeter, 1987; Jaramillo & Schuetze, 1988; Parnas *et al.*, 1989), including ourselves (Madsen *et al.*, 1984; 1987), have been exploring how much quantitative information regarding the process of neurotransmission can be obtained from the generation phase of quantal events, in this case miniature endplate currents (m.e.p.cs) at the neuromuscular junction. Though the models subserving these analyses are complex (compared, say, with that used to estimate the channel closing rate constant from m.e.p.c. decay) and the numerical computations are more time consuming, considerable progress has been made. Similar work in estimation of Markov reaction scheme rate constants from macroscopic current data has been done by Balser *et al.* (1990), Benveniste *et al.* (1990) and Chen & Hess (1990). Given the abovementioned differences of opinion and uncertainty regarding the kinetic effects of Tc, we decided to investigate whether this alternative approach could provide some complementary insight into one important component of the mechanism of Tc action.

Methods

Isolated sartorius muscles of the frog *Litoria moorei* were used as described previously (Madsen *et al.*, 1985). Briefly, muscles were bathed in carbogenated Ringer solution (pH 7.2, 20°C) and endplate regions of individual fibres were voltage clamped

[1] Author for correspondence.

(−90 mV, double electrode, Axon Instruments), with the rise time of a 1 mV step response better than 25 μs.

M.e.p.cs ($n \approx 250$) were low pass filtered at 3 kHz, captured by digital oscilloscope (Tektronix 5223) at a sampling rate of 50 kHz, and stored on computer (PDP 11/23) disk for offline analysis. Waveforms had to satisfy several criteria for acceptance (Madsen *et al.*, 1985) and in addition, those with a 20–80% rise time greater than 250 μs or without a mono-exponential decay phase were excluded. From the accepted m.e.p.cs ($n \approx 80$–120), representatives ($n = 3$) within one standard deviation of both the sample mean amplitude and mean rise time were randomly selected for further analysis using a numerical model of m.e.p.c. generation. In these analyses the channel closing rate constant (α) for each m.e.p.c. was estimated from the decay phase.

Details of the model have been published (Madsen *et al.*, 1984; 1987). In brief, the pre- and postsynaptic membranes of the neuromuscular junction were represented by two parallel disks of radius $r_m = 1.25\,\mu m$ (Land *et al.*, 1984) separated by a cleft height of $h = 60$ nm (Steinbach & Stevens, 1976). An origin was defined as the centre of the presynaptic disk, and synaptic events were assumed to be radially symmetric. Activation of nicotinic receptors was described by the following:

$$2A + R \underset{k_{-A}}{\overset{2k_{+A}}{\rightleftharpoons}} A + AR \underset{2k_{-A}}{\overset{k_{+A}}{\rightleftharpoons}} A_2R \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} A_2R^*$$

(u_1) (u_2) (u_3) (u_4)

Scheme 1

where A is ACh, R is the nicotinic receptor, A_iR are various bound states of the receptor, * denotes conducting species, α, β and the various ks are rate constants, and the u_i (x, r, t) are molar concentrations of the corresponding species as functions of position (distance x from the presynaptic membrane and radius r from the origin) and time (t, after instantaneous release of a quantum of ACh). ACh was assumed to be initially contained in a small cylinder of height 5 nm and radius 20 nm (Meshul & Pappas, 1984) located at the origin. Further assumptions were that receptor and acetylcholinesterase concentrations were exponentially distributed across the cleft (with parameters $p = 200\,\mu m^{-1}$ and $q = 50\,\mu m^{-1}$ respectively, see Madsen *et al.*, 1984), hydrolysis of ACh by acetylcholinesterase followed Michaelis-Menten kinetics, and diffusion of free ACh was given by

$$\frac{\partial u_1}{\partial t} = D_A\left[\frac{\partial^2 u_1}{\partial x^2} + \frac{\partial^2 u_1}{\partial r^2} + \frac{1}{r}\frac{\partial u_1}{\partial r}\right].$$

Transmitter diffusivity in the cleft (D_A) and the ACh binding association rate constant (k_{+A}) were fixed at literature values of $3 \times 10^{-6}\,cm^2\,s^{-1}$ and $10 \times 10^8\,M^{-1}\,s^{-1}$ respectively (Land *et al.*, 1981; 1984; Prinz & Maelicke, 1983b; Krouse *et al.*, 1985; Madsen *et al.*, 1987).

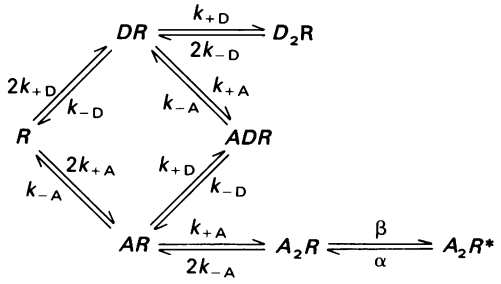
With these assumptions a set of partial differential equations can be solved numerically to give profiles of the various species in both space and time. The number $M(t)$ of open channels over the whole endplate at any particular time was computed as

$$M(t) = 2\pi N \int_0^h \int_0^{r_m} u_4(x, r, t) r\, dr\, dx$$

where N is Avogadro's number. Thus the time course of the m.e.p.c. is given by $iM(t)$ where i is the single channel current. Estimation of i and the Michaelis-Menten parameters V_m and K_m is described in Madsen *et al.* (1987), and for the present preparation the following values were obtained: $i = 1.72$ pA, $V_m = 8.2\,Ms^{-1}$, and $K_m = 11.9\,\mu M$.

The model then required four parameters to be estimated from experimental data under control conditions: the number of ACh molecules in a quantum (quantal size, q_s), the channel opening rate constant (β), the ACh equilibrium dissociation constant (K_A) and nicotinic receptor density (C). We used an adaptive simplex algorithm and a least squares criterion for this optimization process based on individual waveforms; the average processing time on a MicroVAX II computer was about 10 h per m.e.p.c. Regressions were considered acceptable when the fitted residual variance was less than the baseline noise; approximately 15% of m.e.p.cs initially chosen for analysis did not achieve this level, and these were replaced with additional waveforms selected as described above. Theoretical m.e.p.cs generated by the model during the fitting process were digitally filtered at 3 kHz to match the analogue filter bandwidth used with the experimental data (see Edeson *et al.* (1988) for previous use). Initial parameter estimates were systematically varied to counter potential problems of false convergence.

The neuromuscular blocking effects of Tc were investigated by collecting m.e.p.cs under similar conditions to controls. Tc (1–5 μM, Sigma) was dissolved in Ringer solution and perfused into the bath for 10 min, with a further 15 min allowed for equilibration. All experiments were paired, with Tc data being related to control at the same endplate. A competitive antagonist reaction scheme was used to model the postsynaptic blocking action of Tc as follows:



Scheme 2

where D_2R is the biliganded Tc-bound receptor. The method for selecting waveforms in the presence of Tc was identical to that for controls. Estimated parameters in these regressions were the Tc binding association rate constant (k_{+D}) and equilibrium dissociation constant (K_D); fixed parameters were the mean values for q_s, β, K_A and C from the control series at the same endplate. Assumed values for D_A and k_{+A} were the same as for control. The estimate for k_{-D} at a given endplate was calculated as the average product of k_{+D} and K_D from each of three waveforms. Open channel blockade by Tc, as measured by m.e.p.c. decay rate, was allowed for in the regression through the use of individual values of α for each m.e.p.c.

Results

Mean values for the four quantal transmission parameters estimated from the m.e.p.c. generation phase under control conditions are given in Table 1. They supersede our earlier estimates (Madsen *et al.*, 1987) since they were derived from a substantial database (36 m.e.p.cs from 12 muscles), they allow for the effects of a 3 kHz filter on the experimental rise time, and parameters D_A (ACh diffusion coefficient) and k_{+A} (ACh binding association rate constant) were fixed at literature values. Under these new conditions none of the four parameters varied by more than 23% from our earlier estimates. Figures A1–A3 in the Appendix give some information on the reliability of the iterative non-linear regression procedure used to obtain these estimates.

The right inset in Figure 1 shows the effect of 3 μM Tc on a representative m.e.p.c., and the main figure shows the average concentration-dependent reduction in m.e.p.c. amplitude in

Table 1 Quantal transmission parameter estimates under control conditions at the frog neuromuscular junction

Parameter	*Best estimate*
Quantal size (q_s, number of molecules)	30,600 ± 1200
Nicotinic receptor channel opening rate constant (β)	9140 ± 410 s^{-1}
ACh equilibrium dissociation constant (K_A)	44.9 ± 2.5 μM
Nicotinic receptor density (C)	9650 ± 360 μm^{-2}

Data given as mean ± s.e.mean for 36 m.e.p.cs from 12 different muscles. Membrane potential −90 mV, temperature 20°C. Values for the acetylcholine (ACh) diffusion coefficient in the synaptic cleft (D_A) and the ACh forward binding rate constant (k_{+A}) were fixed at 3×10^{-6} $cm^2 s^{-1}$ and 10×10^8 $M^{-1} s^{-1}$ respectively in these regressions. The mean channel closing rate constant (α) for all 12 muscles was 656 ± 25 s^{-1}.

the presence of 1–5 μM Tc. The Hill coefficient for this blockade was 1.23 ± 0.17 (mean ± s.e.mean, $n = 12$). In addition to this blockade which is predominantly due to competitive antagonism at the ACh sites on the nicotinic receptor and is known to be voltage-independent, recent studies (for example Colquhoun *et al.*, 1979) have established that there is (usually) a smaller voltage-dependent component involving noncompetitive blockade of the nicotinic receptor channel. This channel blockade, manifested as a decreased m.e.p.c. decay time constant, was observed in the present study (Figure 1). The 20–80% rise time of control m.e.p.cs was 150 ± 3 μs ($n = 12$ muscles), and this was not significantly altered by 1–5 μM Tc (linear regression, $r = -0.071$, $n = 15$, $P > 0.80$).

Model fits to the rising phase of typical control and Tc (3 μM) m.e.p.cs are shown in Figure 2. Figure 3 summarizes some of the key features of the competitive blockade induced by Tc, as predicted by the model. Inspection of these plots shows that the free ACh concentration within the cleft is not reduced in the presence of Tc ((a) and (d)); in fact in many instances it is marginally increased. This is because the dominant factor responsible for the reduction of ACh concentration in the first 100 μs following release is binding to available receptor sites (rather than acetylcholinesterase activity, see also Wathey *et al.*, 1979), and in the presence of 3 μM Tc approximately 40% of these binding sites (two per receptor) are occupied. Fewer functional receptors then generally translates to fewer open channels ((c) and (f)). Plots (g) and (h) show that most of the blockade by 3 μM Tc is due to receptors which have only one Tc molecule bound; in this case at the time of release, DR and D_2R accounted for 49% and 18% of receptors respectively. During the brief m.e.p.c. rising phase ($\approx$200 μs) there is insufficient time for dissociation of all of the Tc from the single site, and this species is also buffered to some degree by dissociation of the biliganded species D_2R. Plots (b) and (e) show that for the parameter values used in this study, binding of ACh to R occurs very rapidly with the most extreme case opposite the ACh release site ($r_1 = 0.02$ μm). At r_1, comparison of plots (b) with (c) and (e) with (f) shows that opening of channels from A_2R is somewhat delayed. Plot (i) shows that for a finite region around the point of ACh release there is receptor saturation (R approaches zero) and plots (b), (c), and (e)–(i) further reinforce the concept that m.e.p.cs are confined to a small area ($r \leqslant 0.4$ μm). This is in agreement with the prediction by Land *et al.* (1981) that 95% of the biliganded receptors lie within a disk of radius 0.3 μm. Plots (c) and (f) at $r_3 = 0.4$ μm show a very small increase in the number of open channels (A_2R^*) in the presence of Tc compared to control. Although this offsets

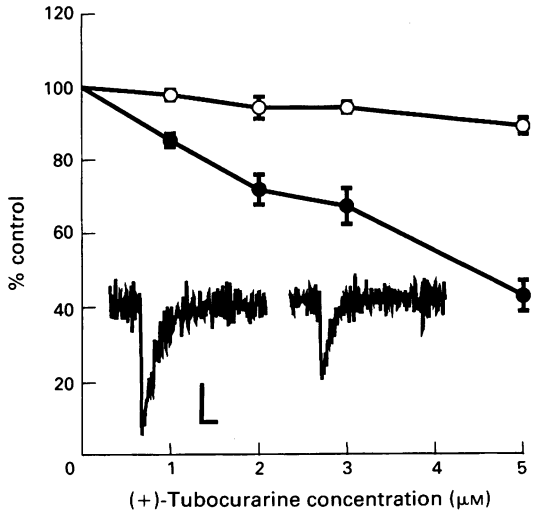


Figure 1 Effect of (+)-tubocurarine (Tc) on miniature endplate current (m.e.p.c.) amplitude (●) and decay time constant (○) as a percentage of control. Mean values ($n = 80$–120) were obtained for Tc and control m.e.p.cs from the same endplate and then expressed as a percentage of control. Values shown are mean of these percentages from 3–4 endplates at each Tc concentration; s.e.mean shown by vertical bars. Insets show typical m.e.p.cs from the same endplate under control conditions (left) and in the presence of 3 μM Tc (right). Recording bandwidth 3 kHz; calibration 1 nA (vertical), 2 ms (horizontal). The generation phases of these two particular m.e.p.cs are shown inverted and on an expanded time scale in Figure 2.

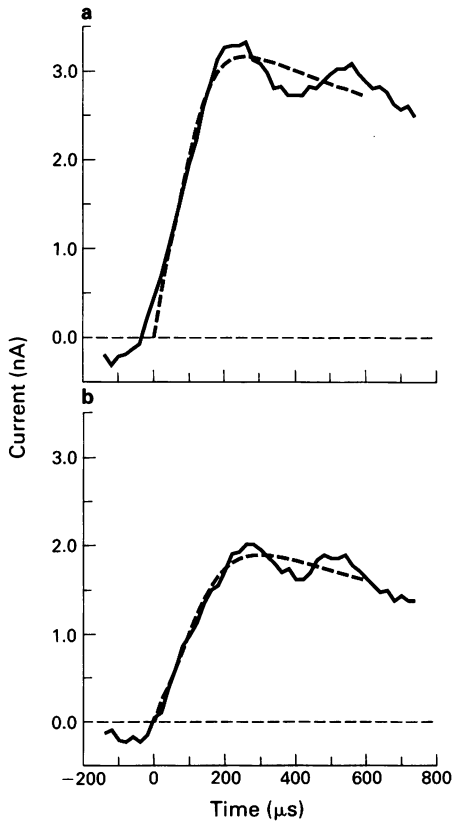


Figure 2 (a) Control m.e.p.c. (solid line) with fitted baseline (thin dashed line) and predicted curve from non-linear regression of the generation phase (thick dashed line). The best parameter estimates for this waveform were $q_s = 25{,}300$, $\beta = 10{,}900$ s^{-1}, $K_A = 50.2$ μM and $C = 11{,}400$ μm^{-2}. (b) M.e.p.c. from the same endplate in the presence of 3 μM (+)-tubocurarine (solid line). Best estimates for this waveform were $k_{+D} = 9.74 \times 10^8$ $M^{-1} s^{-1}$ and $K_D = 4.02$ μM.

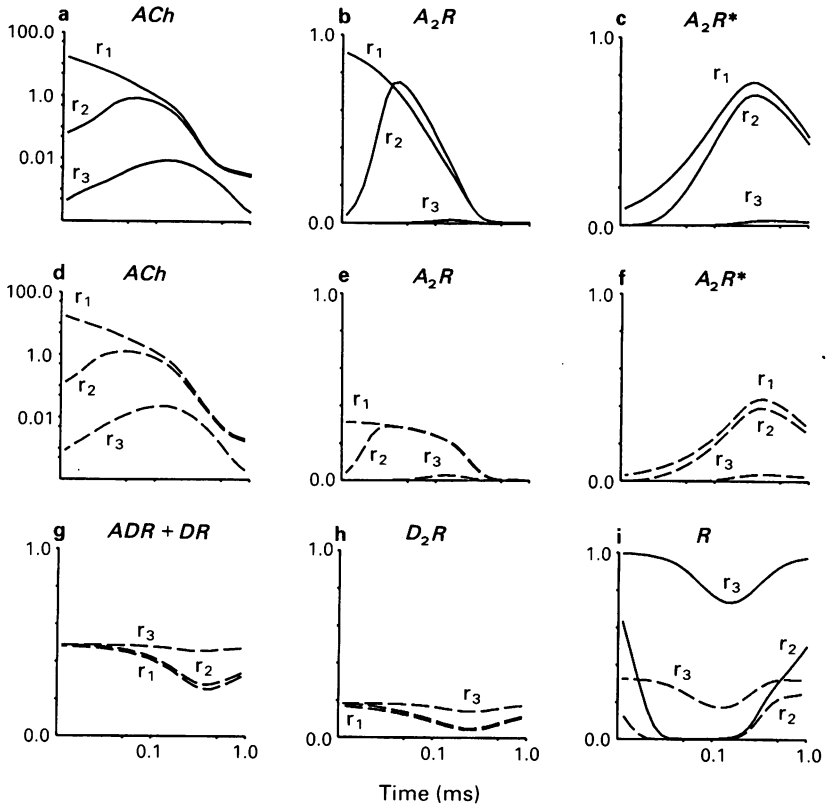


Figure 3 (a)–(i). Model-predicted behaviour of acetylcholine (ACh) and the various receptor species (see Schemes 1 and 2) on the postsynaptic membrane at several radii from the origin ($r_1 = 0.02$, $r_2 = 0.2$, and $r_3 = 0.4\,\mu$m) for an m.e.p.c. in the absence (solid lines) and presence (dashed lines) of $3\,\mu$M (+)-tubocurarine (Tc). The mean best fit parameters from three control waveforms and the best fit parameters from the waveform in Figure 2b, all from the same endplate, were used in these simulations. In (a) and (d) the ordinates show ACh concentration (mM) on a logarithmic scale, whereas in all other plots they show the relative proportion (range 0.0–1.0) of the various species on a linear scale. Plot (g) shows the relative proportion of those receptors with one molecule of Tc bound, whether or not ACh is bound to the other site; these receptors are nonfunctional in terms of channel opening. Note the logarithmic time scale in all plots.

to some degree the reduction in number of open channels seen at smaller radii, the total current integrated over the whole endplate is still smaller in the presence of Tc. Thus under control conditions a quantum of ACh binds to a particular number of functional channels (R) distributed over a certain area, and in the presence of Tc the smaller number of available sites means that ACh is bound over a slightly greater area.

Mean ($\pm$s.e.mean, 4 endplates) values for k_{+D} at Tc concentrations of 1 and $5\,\mu$M were 11.0 ± 3.1 and 11.1 ± 4.5 ($\times 10^8\,M^{-1}s^{-1}$) respectively, and for k_{-D} at the same concentrations these were 4.43 ± 1.22 and 3.56 ± 1.50 ($\times 10^3\,s^{-1}$) respectively. M.e.p.c. amplitude over this concentration-range decreased from 83% to 43% of control. Neither k_{+D} nor k_{-D} showed any significant dependence on Tc concentration (linear regression of k_{+D} on Tc concentrations of 1, 2, 3, and $5\,\mu$M, $r = 0.493$, $n = 14$, $P > 0.65$; and for k_{-D}, $r = -0.076$, $n = 14$, $P > 0.80$), representing a valuable test of the adequacy of the proposed mechanism of competitive antagonism in explaining the data. The mean values of k_{+D}, K_D and k_{-D} over all endplates and Tc concentrations studied (1–5 μM) were $(8.90 \pm 1.66) \times 10^8\,M^{-1}s^{-1}$, $4.39 \pm 0.55\,\mu$M and $(3.52 \pm 0.57) \times 10^3\,s^{-1}$ respectively. These are estimates of the intrinsic or per site binding constants; the corresponding stoichiometric constants (Klotz, 1985) are calculated by incorporating the statistical consideration of two binding sites per receptor, giving $2.18\,\mu$M and $8.78\,\mu$M for K_D.

Discussion

In our analysis of the competitive antagonist effect of Tc we assumed that it could bind to both agonist recognition sites on the nicotinic receptor with equal affinity. This seems reasonable *a priori* given molecular biological data showing that the two agonist sites reside on identical α subunits differing only in their interaction with other subunits. However, Neubig & Cohen (1979) reported widely disparate K_D values of 0.033 and $7.7\,\mu$M in a *Torpedo* preparation and so we began our study using a model with two separate site affinities. In all waveforms examined these invariably converged to a common value in the micromolar range. Further support for this finding was provided by the m.e.p.c. amplitude data (Figure 1) which had a Hill coefficient of 1.23, as it has been calculated that binding of a competitive antagonist to two identical binding sites should give a Hill coefficient for the functional response of approximately 1.2 (Taylor & Sine, 1982). Identical affinities for the two sites have also been observed in *Electrophorus* receptors (Prinz & Maelicke, 1983a). The high affinity K_D of $0.033\,\mu$M found by Neubig & Cohen (1979) could reflect differences between species, but is more probably due to receptor desensitization during the biochemical preparation process or assay because (i) it is much less than the Tc concentration normally required for blockade of activity ($\approx 0.43\,\mu$M, Jenkinson, 1960) and (ii) the ACh affinity in their system ($K_A \approx 0.015\,\mu$M) was much higher than that required for physiological activation ($K_A \approx 30\,\mu$M, Adams, 1981). Blount & Merlie (1989) studied the binding of Tc to various subunit pairs of the mouse muscle nicotinic receptor expressed in fibroblasts and found different intrinsic binding affinities for the $\alpha\gamma$ and $\alpha\delta$ dimers. However, as they acknowledged, interpretation of their data is complicated because of the likelihood of detergent-induced desensitization under their experimental conditions. In most systems investigated it appears that Tc by itself does not induce desensitization of nicotinic receptors (e.g. Karpen & Hess, 1986) but in *Torpedo* preparations mixed

results have been obtained, with Quast *et al.* (1978) and Weiland & Taylor (1979) reporting limited and no such capability respectively.

We also assumed in the present analysis that the four transmission parameters q_s, β, C and K_A estimated under control conditions at a given endplate were not altered by Tc. Considering each of these in turn: much less is known about presynaptic than postsynaptic effects of Tc. Current understanding of Tc-induced reduction in a transmitter release focuses on decreased quantal content following nerve-evoked release rather than a change in quantal size q_s (Bowman *et al.*, 1988). The assumption of no change in β follows implicitly from the use of a competitive antagonist model (Scheme 2); for channels to open, both agonist sites must be occupied by ACh with no Tc bound at all. If Tc is a reversible rather than an irreversible antagonist at the agonist recognition sites, then the number of receptors which can be activated at a given endplate in the presence of Tc should remain constant; those that are blocked are still available for activation if there is sufficient time and ACh for displacement. The absence of any significant Tc concentration effect on k_{+D} and k_{-D} provided a check on this assumption. Probably the assumption most open to question (but of less central importance, see Scheme 2) is that with Tc bound to one site, K_A for ACh binding to the other site does not change. Tc does induce some conformational changes in the nicotinic receptor (Covarrubias *et al.*, 1986; McCarthy & Stroud, 1989) which could result in allosteric coupling of the two sites, but like others (Colquhoun & Sheridan, 1982) we made this simplifying assumption in the absence of more specific quantitative information.

Whatever the approach for a quantitative study of Tc kinetics it is inevitable that several such assumptions will be necessary, and obviously the reliability of results will be dependent on the validity or accuracy of these assumptions in a manner determined by the model. In order to obtain estimates of the Tc association and dissociation rate constants using a voltage-jump technique, Colquhoun & Sheridan (1982) assumed that ACh binding was much faster than channel opening, free agonist concentration remained constant in the endplate region during their experimental protocol, membrane potential did not affect ACh or Tc binding, and β was $\approx 1000\,s^{-1}$. Compared with the present technique, many of these particular assumptions were either unnecessary or the variable concerned was directly estimated. Their value of β was probably at least an order of magnitude too small (Madsen & Edeson, 1988).

Many studies on the mechanism of Tc action which use electrophysiological or particularly the slower biochemical techniques are difficult to interpret because of concurrent agonist-induced receptor desensitization (see some discussion of this point by Karpen & Hess (1986)). If ACh is present for any length of time then there will be decreased channel activity because of desensitization, and some of this decreased activity may be difficult to separate from blockade due to competitive antagonism. Desensitization has usually been described as a slow process with time constants of seconds to minutes, but new high speed patch clamp perfusion techniques have now revealed components with faster time scales of only a few milliseconds for many receptor-channel systems (Brett *et al.*, 1986; Dudel *et al.*, 1990). Our method would seem to have a particular advantage in this respect. During an m.e.p.c., ACh is present in the synaptic cleft for less than 1 ms, whereas with other electrophysiological techniques where the agonist is either present in the bath or is released by nonfocal ionophoresis, there will inevitably be a time-dependent alteration in the relative proportions of active and desensitized receptor species.

The present results for Tc add to earlier findings in fish and mammalian preparations that functional competitive blockade by low micromolar concentrations of curariform antagonists is caused primarily by occupancy of only one site (Sine & Taylor, 1981; Sine, 1988). This was suggested by inspection of the model-predicted proportions of the different receptor species during an m.e.p.c. in the presence of Tc. An important feature of this blockade is that though ACh and Tc interact competitively and ACh concentration in the m.e.p.c. generation phase is very high, Tc must first dissociate from an occupied site before ACh can then bind to it. A k_{-D} of $3.5 \times 10^3\,s^{-1}$ represents a half life of 198 μs for dissociation of *DR*, so that within the time to peak of an m.e.p.c. ($\approx 200\,\mu s$) not all of the *DR* species will have had time to dissociate no matter how great the concentration of ACh. There would of course be more displacement if high ACh concentration were maintained for longer than this time, but this does not occur under normal physiological conditions where muscle contraction is dependent on an endplate potential reaching a peak in less than 1 ms (Kuba *et al.*, 1974). Presumably it is this kinetic limitation (which has been discussed by others; see Bowman, 1990) which largely explains why occupancy of only one site by Tc under normal neurotransmission conditions is sufficient to prevent channel activation. Consequently it would seem that experimental protocols with a long ACh exposure time (i.e. greater than 1 ms) will generate results closer to steady state than might be expected for ACh released during a normal nerve impulse, making it more difficult to interpret correctly key features of the interaction.

Our estimate of k_{+D} ($8.9 \times 10^8\,M^{-1}\,s^{-1}$) is very fast, in agreement with the tentative conclusions reached by Colquhoun & Sheridan (1982). Although this seems contrary to the usual pharmacological observation of antagonists having a slow onset of action, it has been appreciated for some time that this phenomenon is mainly due to an access problem (buffered diffusion) rather than necessarily a slow intrinsic reaction rate (Waud, 1968). Through use of photo-activatable agonists and antagonists of the nicotinic receptor, Krouse *et al.* (1985) concluded that antagonist binding rate constants were probably about an order of magnitude slower than those for agonists.

We wish to thank Paul Chan for technical electronic assistance, and the UWA Faculty of Medicine Research Committee and the Faculty of Anaesthetists, Royal Australasian College of Surgeons for financial support.

Appendix

Non-linear regression convergence

The ability of the optimization algorithm (see Madsen *et al.*, 1987) to estimate correctly neurotransmission parameters q_s, β, K_A, and C in the model (Madsen *et al.*, 1984) was examined by simulation under two conditions, without and with noise. Figure A1 shows the generation phase of an m.e.p.c. using the global values for the four parameters, and then four other

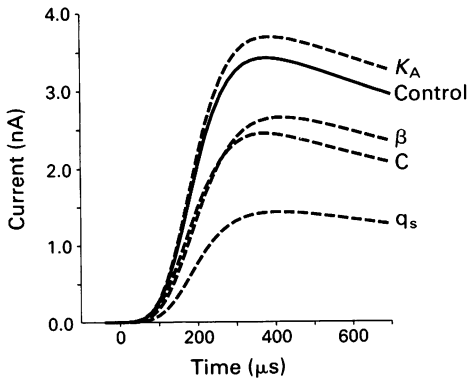


Figure A1 Generation phase of m.e.p.cs predicted by the model under standard conditions using global mean control values for the four parameters ($q_s = 30{,}600$, $\beta = 9140\,s^{-1}$, $K_A = 44.9\,\mu M$ and $C = 9650\,\mu m^{-2}$), and test conditions where each parameter was individually decreased by 40%. These simulated m.e.p.cs were digitally filtered at 3 kHz.

waveforms where each parameter was individually decreased by 40%. Under these conditions, it can be seen that m.e.p.cs are most sensitive to changes in q_s and least sensitive to changes in K_A.

Figure A2 summarizes the estimation problem for simulated m.e.p.cs without any added noise. Essentially in this situation the parameters can be determined to a nominated precision by appropriate tightening of the convergence criterion, at the cost of increased processing time. The level used in these results (sum of squares $\leqslant 0.001\,nA^2$, average run time 70 h on a MicroVAX II computer) was still greater than the maximum root mean square error allowed in the integration of the model partial differential equations (Madsen *et al.*, 1984).

A similar set of results is presented in Figure A3 for the case where theoretical m.e.p.cs with known q_s, β, K_A, and C had computer-generated noise added and were then digitally filtered at 3 kHz. In parallel with the experimental situation,

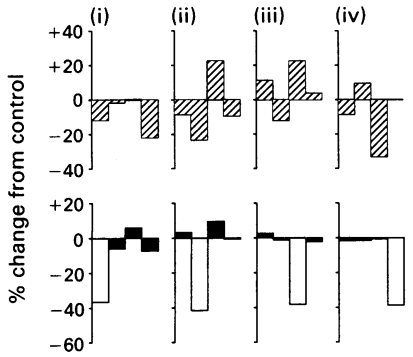


Figure A2 Reliability of non-linear regression convergence using m.e.p.cs generated without any random noise under the four test conditions (i–iv) described in Figure A1. Upper and lower panels for each condition show respectively the initial and best parameter estimates given in the order q_s, β, K_A and C. Best estimates for the parameter which had been decreased by 40% in the simulations are shown as open columns, while those which had not been changed from control values are shown as closed columns. Regression sum of squares convergence criterion (30 data points) was $\leqslant 0.001\,nA^2$.

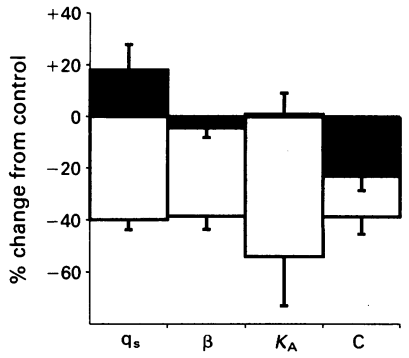


Figure A3 Reliability of non-linear regression convergence with simulated m.e.p.cs in which Gaussian noise had been added. M.e.p.cs with noise (mean = 0.0, s.d. = 0.6 nA) were digitally filtered at 3 kHz; this reduced the noise standard deviation to about 0.2 nA. The decrease in parameters q_s, β, and C from control was 40% while that for K_A was 80%. For each of the four conditions, three m.e.p.cs were produced with different sets of noise. An iteration limit of 24 ($\approx$10 h MicroVAX II processor time) was set for each m.e.p.c., and if the regression error standard deviation was less than the added noise standard deviation after filtering, the regression was accepted. Open columns show best estimates (mean with s.e.mean shown by vertical bars; $n = 3$) for the parameter which had been decreased; closed columns show best estimates ($n = 9$) from the remaining m.e.p.cs where the given parameter had not been changed from its control value.

three waveforms from three different sets of noise were used for each condition. Regressions were considered acceptable if the regression error standard deviation was less than the standard deviation of the noise which was added. It would appear that with a signal to noise ratio of about 15, changes of less than 40% in q_s, β, and C and 80% in K_A can be detected. Integrating these results with those of Figure A2 then suggests that these four parameters are identifiable, with the precision and accuracy of the estimation process for real m.e.p.cs being dependent on the quality of the data; the better the signal to noise ratio the more reliable the inference.

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(Received June 26, 1990
Revised December 24, 1990
Accepted February 8, 1991)

Br. J. Pharmacol. (1991), **103**, 1614–1620 © Macmillan Press Ltd, 1991

Inhibitory and excitatory effects of adenosine receptor agonists on evoked transmitter release from phrenic nerve endings of the rat

* P. Correia-de-Sá, A.M. Sebastião & [1]J.A. Ribeiro

Laboratory of Pharmacology, Gulbenkian Institute of Science, 2781 Oeiras, and *Laboratory of Pharmacology, ICBAS, University of Oporto, 4000 Oporto, Portugal

1 The effects of the adenosine analogues, 5'-N-ethyl-carboxamide adenosine (NECA), **R**-N^6-phenylisopropyladenosine (**R**-PIA), 2-chloroadenosine (CADO), and CGS 21680C on electrically evoked tritium outflow from preparations loaded with [^{3}H]-choline and on evoked endplate potentials (e.p.ps), as well as the ability of the xanthines, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) and PD 115,199 to antagonize the effects of the adenosine analogues, were investigated in phrenic nerve-diaphragm preparations.

2 NECA, **R**-PIA and CADO decreased, in a concentration-dependent manner, the evoked tritium outflow from preparations loaded with [^{3}H]-choline. NECA and **R**-PIA were about equipotent and more potent than CADO.

3 DPCPX shifted to the right in a near parallel fashion the concentration-response curve for the inhibitory effect of **R**-PIA on evoked tritium outflow.

4 In the presence of DPCPX, NECA increased, rather than decreased, evoked tritium outflow. PD 115,199 antagonized, in a concentration-dependent manner, this excitatory effect of NECA.

5 CGS 21680C, in low nanomolar concentrations, increased evoked tritium outflow, an effect also antagonized by PD 115,199.

6 CGS 21680C increased, and **R**-PIA decreased, the amplitude of e.p.ps recorded from preparations paralysed with tubocurarine. Both effects could be observed in the same endplate.

7 It is concluded that both inhibitory (probably A_1) and excitatory (probably A_2) adenosine receptors coexist at the rat neuromuscular junction, modulating the evoked release of acetylcholine.

Keywords: **R**-N^6 phenylisopropyladenosine; 5'-N-ethylcarboxamide adenosine; 2-chloroadenosine; CGS 21680C, PD 115,199; 1,3-dipropyl-8-cyclopentylxanthine; [^{3}H]-acetylcholine release; endplate potentials; neuromuscular transmission

Introduction

Adenosine inhibits neuromuscular transmission at the rat diaphragm neuromuscular junction, decreasing the amplitude and the quantal content of evoked endplate potentials (Ginsborg & Hirst, 1972; Ribeiro & Walker, 1975) and the amplitude of nerve-evoked twitch responses (Sebastião & Ribeiro, 1988).

The inhibitory effect of adenosine at the rat diaphragm neuromuscular junction is mediated by a xanthine-sensitive adenosine receptor with an agonist profile with **R**-N^6-phenylisopropyladenosine (**R**-PIA) and 5'-N-ethylcarboxamide adenosine (NECA) about equipotent and more potent than 2-chloroadenosine (CADO) (Sebastião & Ribeiro, 1988). This agonist profile was determined in preparations where the margin of safety of neuromuscular transmission was reduced with tubocurarine or with high magnesium concentrations in the bath. Both treatments affect transmitter release (e.g. Ginsborg & Jenkinson, 1976; Wessler *et al.*, 1986).

The present work was designed to investigate the potency of adenosine receptor agonists in inhibiting the evoked release of acetylcholine from phrenic nerve hemidiaphragm preparations where the safety margin of neuromuscular transmission has not been previously inhibited. Whilst in the process of investigating the antagonism of the inhibitory effect of NECA on evoked transmitter release with the A_1-selective antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), we made the incidental observation that in the presence of this xanthine, NECA increased transmitter release from the phrenic nerve endings. The pharmacological characteristics of this excitatory effect have been investigated.

[1] Author for correspondence.

Preliminary accounts of some of the results have already been published (Correia-de-Sá *et al.*, 1990; Ribeiro *et al.*, 1990).

Methods

The experiments were performed on left phrenic nerve-hemidiaphragm preparations from male Wistar rats of about 200 g in weight. Innervated muscle strips of about 1 cm width were used.

Isotope experiments

Evoked transmitter release was assessed by measuring evoked tritium outflow from preparations labelled with [^{3}H]-choline by the method of Wessler & Kilbinger (1986). The preparations were superfused (3 ml min^{-1}) in 3 ml organ baths at 37°C with Tyrode solution continuously gassed with 95% O_2 and 5% CO_2, containing (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, glucose 11.2 and choline 0.001. After a 30 min equilibration period the perfusion was stopped and the nerve endings were labelled during 40 min with 1 μM [^{3}H]-choline (specific activity 2.5 μCi nmol^{-1}). During this labelling period the phrenic nerve was electrically stimulated at 1 Hz with supramaximal rectangular pulses of 40 μs duration. Immediately after the end of the labelling period the preparations were again superfused (15 ml min^{-1}) and the nerve stimulation stopped. From this time onwards hemicholinium-3 (10 μM) was present to prevent uptake of choline. After a 60 min period of washout the perfusion was stopped and 3 ml bath samples collected every 3 min. The times indicated on the figures illustrating isotope

experiments indicate the times at which the samples were collected. Tritium content of the samples was measured by liquid scintillation spectrometry.

Transmitter release was evoked by phrenic nerve stimulation at 5 Hz during 3 min with supramaximal rectangular pulses of 40 μs duration. Three stimulation periods were used at 12–15 min (S_1), 39–42 min (S_2) and 66–69 min (S_3). Evoked tritium outflow was calculated by subtracting the basal tritium outflow from the total tritium outflow during the stimulation period. The basal outflow of tritium declined slightly during the whole collecting period (cf. Wessler & Kilbinger, 1986). Therefore, basal outflow during the stimulation period was obtained by calculating the arithmetic mean of two samples before stimulation and two samples reaching the pre-stimulation level after stimulation.

Test drugs were usually added 15 min before the end of S_3 and were present up to the end of the experiment; their effects were expressed by the ratio S_3/S_2 as compared with the ratio S_2/S_1 obtained in the same experiment. Occasionally the test drug was added 15 min before the end of S_2 and removed 15 min before the end of S_3; in this case the effect of the drug was expressed as the S_2/S_1 ratio as compared with the S_3/S_1 ratio obtained in the same experiments. When testing the ability of a xanthine to modify the effect of an adenosine receptor agonist, the xanthine was added 15 min before the end of S_1 and was present up to the end of the experiment. When testing the effects of the xanthine *per se* in the absence of agonists the xanthines were applied as any other trust drug, i.e. 15 min before the end of S_3. The test drugs were added to the bath by emptying the bath and refilling it with 3 ml of the drug solution at the concentration to be tested.

Endplate potentials recording

To record evoked endplate potentials (e.p.ps) the preparations were mounted at room temperature (22–25°C) in a Perspex chamber of 3 ml capacity through which the solutions flowed continuously at a rate of 3 ml min^{-1}. Because of the slow rate of flow it was not possible to determine rates of onset of the effects of the drugs. The changeover times in the figures corresponding to e.p.p. recordings indicate the times at which the inlet tube of the pump for perfusion was transferred to a new solution. The bathing solution (Tyrode) had the same composition as that used in the isotope experiments, except that choline was omitted. The solutions were gassed with 95% O_2 and 5% CO_2.

The nerve was continuously stimulated at 1 Hz with supramaximal rectangular pulses of 10 μs duration. The muscle action potentials and twitches in response to nerve stimulation were prevented by tubocurarine (1.1–1.2 μM). The necessary concentration of tubocurarine was adjusted in each experiment and was kept constant up to the end of the experiment.

The e.p.ps were recorded in the conventional way (Fatt & Katz, 1951) with intracellular microelectrodes filled with KCl (3 M) and of 10–20 MΩ resistance; the bath electrode was an Ag-AgCl pellet. Signals from the microelectrodes were fed into a conventional-pre-amplifier (WPI 750), the output of which was delivered in parallel to a storage oscilloscope (Tektronix 5103N) with a differential amplifier (5A22N) for monitoring individual e.p.ps, and to an electrometer (Keithley 610C) for monitoring the resting membrane potential. After amplification, series of 64 or 128 individual e.p.ps were averaged (20 MHz, ramp type) with a microprocessor (Datalab DL-4000 Signal Analysis System, 30 μs sampling interval), the input of which was coupled to the output of the oscilloscope. The analogic output of the microprocessor was slowly delivered (plot rate 80 s per channel) into a pen-recorder (Phillips PM 8252A, speed chart 60 mm min^{-1}). The output of the electrometer was fed into another channel of the pen-recorder, which was continuously recording the resting membrane potential (speed chart 60 mm h^{-1} when not displaying the averaged e.p.ps). The usual procedure was to continue to record averages of consecutive e.p.ps in the same solution until a stable value was obtained, i.e. until two or three averages differed by less than 2% in amplitude, and the resting membrane potential differed by less than 1%.

Drugs

Drugs used were: **R**-N^6-phenylisopropyladenosine (**R**-PIA), 5'-N-ethylcarboxamide adenosine (NECA), 1,3-dipropyl-8-cyclo-pentylxanthine (DPCPX) (Research Biochemicals Inc.); 2-chloroadenosine (CADO), hemicholinium-3, choline chloride; (+)-tubocurarine (Sigma); [methyl-^{3}H]-choline chloride (ethanol solution, 75–85 Ci $mmol^{-1}$) was from Amersham. CGS 21680C (2-[*p*-(2-carboxyethyl)phenethylamino]-5'-N-ethylcarboxamide adenosine) was a gift from Dr M. Williams of Ciba-Geigy (U.S.A.) and PD 115,199 (N-(2-(dimethylamino)-ethyl) - *N* - methyl - 4 - (2,3,6,7, - tetrahydro - 2,6 - dioxo - 1,3 - dipropyl - 1H - purin - 8 - yl) - benzenesulphonamide) was a gift from Dr R.F. Bruns of Parke-Davis (U.S.A.). **R**-PIA was made up as a 50 mM stock solution in dimethylsulphoxide (DMSO), PD 115,199 was made up as a 1 mM stock solution in 80% methanol/20% M NaOH (v/v); DPCPX was made up in a 5 mM stock solution in 99% DMSO/1% M NaOH (v/v). Aqueous dilutions of these stock solutions were used.

Statistics

Data are expressed as mean $\pm$ s.e.mean, and *n* represents the number of experiments. The significance of the difference between the means was calculated by Student's *t* test. *P* values of 0.05 or less were considered to represent significant differences.

Results

Inhibitory effects of adenosine analogues on evoked tritium outflow

During each period of nerve stimulation (S_1, S_2 and S_3) the outflow of tritium increased over the resting levels and returned to the pre-stimulation levels 3–6 min after stopping stimulation (Figure 1). Evoked [^{3}H]-acetylcholine release was measured from the increase in tritium outflow above the resting levels during the stimulation periods. In control conditions, i.e., in the absence of test drugs, the averaged ratio between the evoked tritium outflow during S_2 and the evoked tritium outflow during S_1 (S_2/S_1 ratio) was 0.81 $\pm$ 0.03 and the averaged S_3/S_2 ratio was 0.78 $\pm$ 0.03 (six experiments). No statistically significant differences ($P > 0.05$) were found between the ratios S_3/S_2 and the ratios S_2/S_1 obtained in control conditions.

In Figure 2 is illustrated the time course of tritium outflow in experiments where 5'-N-ethylcarboxamide adenosine

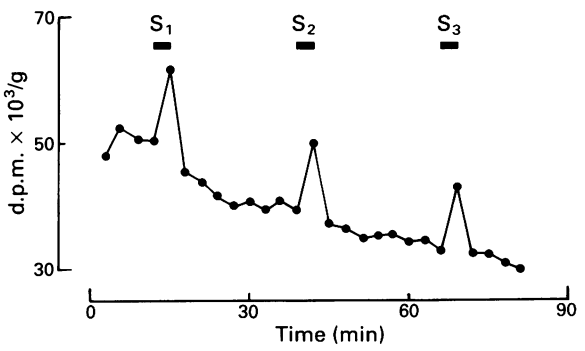

Figure 1 Time course of tritium outflow from a phrenic nerve-diaphragm preparation loaded with [^{3}H]-choline. Tritium outflow (ordinates) was measured in bath samples collected every 3 min and is expressed in d.p.m. per g wet wt. of the preparation. The abscissae indicate the times at which the samples were collected. The phrenic nerve was electrically stimulated (5 Hz, 3 min) during the periods indicated (S_1, S_2 and S_3).

(NECA, 100 nM) (Figure 2a) or R-N^6-phenylisopropyladenosine (R-PIA, 100 nM) (Figure 2b) were present during S_3. As can be seen, evoked tritium outflow during S_3 was lower than the evoked tritium outflow during S_2 or S_1. Similar experiments were carried out in different preparations and the results are summarized in Table 1. NECA (30–300 nM) and R-PIA (30–300 nM), when present during S_3 decreased in a concentration-dependent manner the evoked tritium outflow during S_3. These two adenosine analogues were about equipotent; nearly 50% reduction of the S_3/S_2 ratio as compared with the S_2/S_1 ratio was obtained with 100 nM of either NECA or R-PIA. The results obtained in five experiments where 2-chloroadenosine (CADO, 1–3 μM) was present during S_3 are also summarized in Table 1. This adenosine analogue also decreased in a concentration-dependent manner the evoked tritium outflow during S_3, but the concentrations of CADO needed to produce an inhibition similar to that observed with R-PIA or NECA were more than 30 times higher.

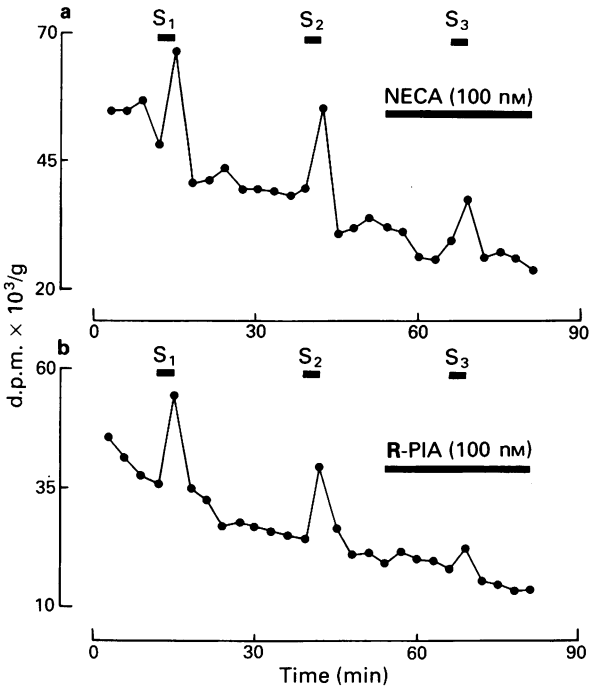

Figure 2 Effect of (a) 5'-N-ethylcarboxamide adenosine (NECA, 100 nM) and (b) R-N^6-phenylisopropyladenosine (R-PIA, 100 nM) on electrically-evoked tritium outflow from phrenic-diaphragm preparations loaded with [^{3}H]-choline. Tritium outflow (ordinates) was measured in bath samples collected every 3 min and is expressed in d.p.m. per g wet wt. of the preparation. The abscissae indicate the times at which the samples were collected. The phrenic nerve was electrically stimulated (5 Hz, 3 min) during the periods indicated (S_1, S_2 and S_3). NECA and R-PIA were applied 15 min before the end of S_3, as represented by the horizontal bar.

The inhibitory effect of NECA on evoked tritium outflow was reversible. Thus, when NECA (100 nM) was present during S_2 and removed before S_3, the tritium outflow during S_2 was lower than that obtained during S_3. In three of such experiments the averaged S_2/S_1 ratio was 0.42 ± 0.04 whereas the averaged S_3/S_1 ratio (considered as the control for these experiments) was 0.73 ± 0.01. The difference between these two ratios is statistically significant ($P < 0.05$), and the effect of NECA (100 nM) is of the same order (42% inhibition) as that observed in the experiments where NECA (100 nM) was present during S_3.

In order to know whether the inhibitory effect of adenosine analogues on evoked tritium outflow was mediated by a xanthine-sensitive adenosine receptor, experiments were designed where the adenosine receptor antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 2.5 nM) was present from the beginning of the release period and the adenosine analogues, R-PIA or NECA were present during S_3. DPCPX (2.5 nM) shifted to the right in a near parallel way the concentration-response curve for the inhibitory effect of R-PIA on evoked tritium outflow (Figure 3). The ratio between equiactive concentrations of R-PIA in the presence and in the absence of DPCPX (2.5 nM), calculated at the level of 50% inhibition in tritium outflow from the data shown in Figure 3 was 4.1.

DPCPX (2.5 nM) also antagonized, in a statistically significant manner ($P < 0.05$), the inhibition of evoked tritium outflow caused by NECA (100 nM), which inhibited outflow by 47 ± 4% ($n = 3$) in the absence of DPCPX and by only 10 ± 9% ($n = 3$) in the presence of DPCPX (2.5 nM).

In the concentration (2.5 nM) used to antagonize the inhibitory effect of the adenosine receptor agonists, DPCPX caused little or no effect on evoked tritium outflow; thus, when DPCPX (2.5 nM) was added only 15 min before the end of S_3, the S_3/S_2 ratio (0.93) was similar to the S_2/S_1 ratio (0.84) obtained in the same experiment. In a higher concentration (5 nM) DPCPX increased evoked tritium outflow by 42% (one experiment).

Enhancement of evoked tritium outflow by adenosine analogues

5'-N-ethylcarboxamide adenosine Figure 4 illustrates the time course of tritium outflow in one experiment where DPCPX (2.5 nM) was present from the beginning of the release period and NECA (300 nM) was added before S_3. Under these conditions, i.e., in the presence of DPCPX, NECA (300 nM) increased, rather than decreased, tritium outflow during S_3. In four similar experiments the averaged S_3/S_2 ratio was 1.30 ± 0.09 and the S_2/S_1 ratio was 0.79 ± 0.04, giving an

Table 1 Inhibitory effect of adenosine analogues on evoked tritium outflow from phrenic-diaphragm preparations loaded with [^{3}H]-choline

Drug present during S_3	*Concentration*	S_2/S_1	S_3/S_2	*% decrease*	n
NECA	30 nM	0.86 ± 0.03	0.73 ± 0.04	15 ± 8	2
	100 nM	0.99 ± 0.01	0.52 ± 0.05*	47 ± 4**	3
	300 nM	0.98 ± 0.01	0.35 ± 0.06*	64 ± 8**	3
R-PIA	30 nM	0.90 ± 0.05	0.68 ± 0.06*	24 ± 2**	3
	100 nM	0.74 ± 0.04	0.37 ± 0.01*	49 ± 4**	3
	300 nM	0.77 ± 0.12	0.31 ± 0.09*	61 ± 8**	3
CADO	1 μM	0.73 ± 0.08	0.64 ± 0.11	13 ± 5	2
	3 μM	0.87 ± 0.11	0.55 ± 0.10*	37 ± 7**	3

NECA: 5'-N-ethylcarboxamide adenosine; R-PIA: R-N^6-phenylisopropyladenosine; CADO: 2-chloroadenosine. Drugs were applied 15 min before the end of S_3 and were present up to the end of the experiment. Percentage decrease in evoked tritium outflow was calculated by comparing the S_3/S_2 ratio with the S_2/S_1 ratio obtained in the same experiment. Zero percent represents identity between the two ratios and 100% represents absence of evoked tritium outflow during S_3. Averaged tritium outflow during S_1: 60361 ± 8765 d.p.m. g^{-1} of wet weight of the preparation. * $P < 0.05$ (Student's t test) as compared with the S_2/S_1 ratio obtained in the same experiments. ** $P < 0.05$ (Student's t test) as compared with zero percent decrease.

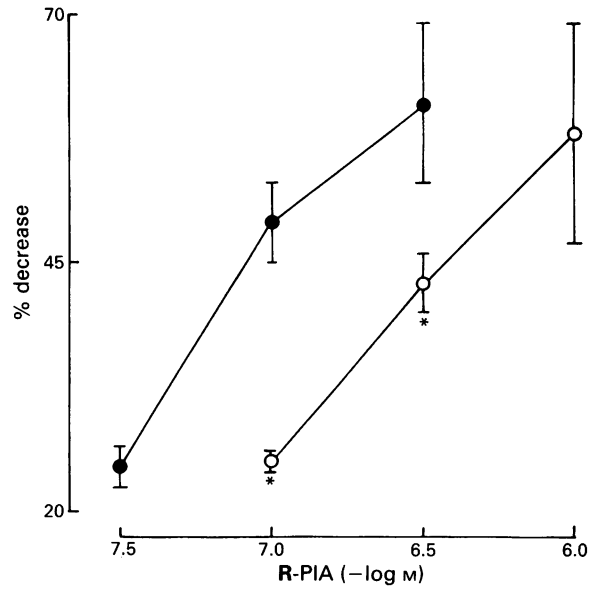

Figure 3 Concentration-response curves for the inhibitory effect of R-N^6-phenylisopropyladenosine (R-PIA) on electrically-evoked tritium outflow from phrenic nerve-diaphragm preparations in the absence (●) and in the presence (○) of 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 2.6 nM). R-PIA was added 15 min before the end of S_3 and remained in the bath until the end of the experiments. DPCPX was present from the beginning of the release period. The ordinates are percentage decrease of the S_3/S_2 ratio as compared with the S_2/S_1 ratio in the same experiments. Zero percent represents identity between the two ratios and 100% represents absence of evoked tritium outflow during S_3. Averaged S_2/S_1 ratio: 0.77 ± 0.03. Averaged tritium outflow during S_1: 63490 ± 2403 d.p.m. per g wet wt. of the preparation. Each point is the mean of three experiments. The vertical bars represent ± s.e.mean. * $P < 0.05$ (Student's t test) as compared with the effect of the same concentration of R-PIA in the absence of DPCPX.

increase of 65.3 ± 6.9% ($P < 0.05$) in evoked tritium outflow caused by 300 nM NECA (Figure 5). In the presence of a smaller concentration (1 nM) of DPCPX the excitatory effect of NECA (300 nM) was much smaller, causing only 6% increase in tritium outflow (one experiment). Concentrations of 1 μM

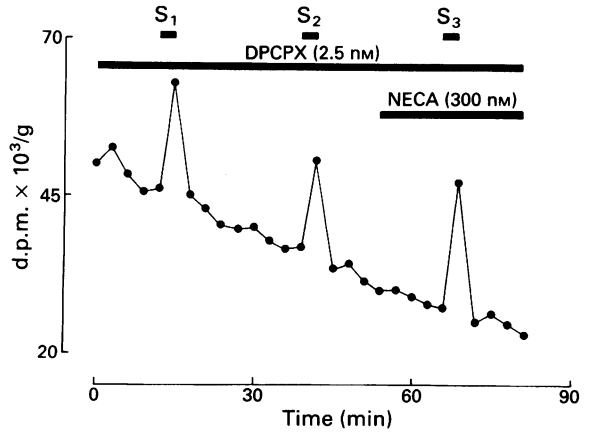

Figure 4 Effect of 5'-N-ethylcarboxamide adenosine (NECA, 300 nM) on electrically-evoked tritium outflow from a phrenic nerve-diaphragm preparation in the presence of 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 2.5 nM). Tritium outflow (ordinates) was measured in bath samples collected every 3 min and is expressed in d.p.m. per g wet wt. of the preparation. The abscissae indicate the times at which the samples were collected. The phrenic nerve was electrically stimulated (5 Hz, 3 min) during the periods indicated (S_1, S_2 and S_3). NECA was applied 15 min before the end of S_3 and DPCPX was present from the beginning of the release period, as represented by the horizontal bars.

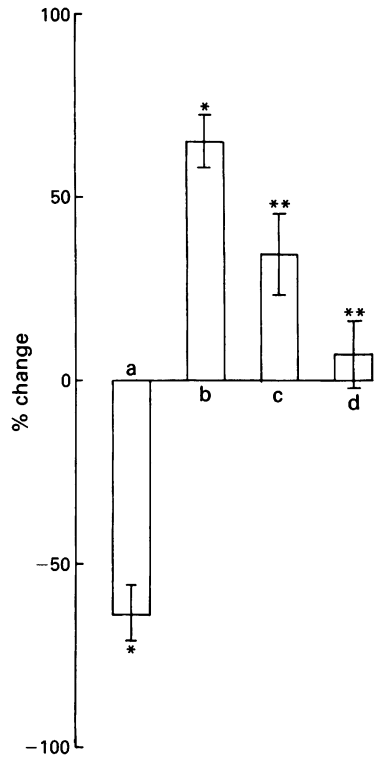

Figure 5 Action of PD 115,199 (5–25 nM) on the excitatory effect of 5'-N-ethylcarboxamide adenosine (NECA) on evoked tritium outflow from phrenic-diaphragm preparations in the presence of 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) (b–d). The inhibitory effect of NECA on evoked tritium outflow in the absence of DPCPX (a) is also shown for comparison: (a) NECA (300 nM); (b) NECA (300 nM) + DPCPX (2.5 nM); (c) NECA (300 nM) + DPCPX (2.5 nM) + PD 115,199 (5 nM); (d) NECA (300 nM) + DPCPX (2.5 nM) + PD 115,199 (25 nM). The ordinates are percentage change in the S_3/S_2 ratio as compared with the S_2/S_1 ratio in the same experiments. Zero percent represents identity between the two ratios, −100% the absence of evoked tritium outflow during S_3, and 100% a S_3/S_2 ratio two times higher than the S_2/S_1 ratio. Averaged S_2/S_1 ratio: 0.79 ± 0.02. Averaged tritium outflow during S_1: 51519 ± 8573 d.p.m. per g wet wt. of the preparation. PD 115,199 and DPCPX were present from the beginning of the release period and NECA was applied 15 min before the end of S_3 and remained in the bath up to the end of the experiments. Each column represents pooled data from 2–3 experiments. The vertical bars represent ± s.e.mean. * $P < 0.05$ (Student's t test) as compared with zero percent change. ** $P < 0.05$ (Student's t test) when compared with (b).

and 2 μM of NECA applied to preparations in the presence of DPCPX (2.5 nM) inhibited evoked tritium outflow by 14% ($n = 1$) and 46% ($n = 1$), respectively. This indicates that in micromolar concentrations NECA overcomes the antagonism of the inhibitory adenosine receptors by DPCPX.

To discover whether the excitatory effect of NECA on evoked tritium outflow, observed in the presence of DPCPX was mediated by a xanthine-sensitive adenosine receptor, experiments were designed to test the ability of PD 115,199 to antagonize this effect of NECA. PD 115,199 was applied at the beginning of the release period together with DPCPX (2.5 nM) and remained in the bath up to the end of the experiment; NECA was added 15 min before the end of S_3. As illustrated in Figure 5, PD 115,199 (5–25 nM) antagonized in a concentration-dependent manner the excitatory effect of NECA (300 nM) on evoked tritium outflow.

In the highest concentration (25 nM) used to antagonize the excitatory effect of adenosine receptor agonists, PD 115,199 was virtually devoid of effect on evoked tritium outflow, as assessed by the comparison between the S_3/S_2 (0.70) and S_2/S_1 (0.77) ratios in one experiment were PD 115,199 was only added before S_3 in the absence of adenosine receptor agonists. Used in concentrations of 50 nM and 100 nM PD 115,199

decreased evoked tritium outflow by 25% ($n = 1$) and 46% ($n = 1$), respectively.

CGS 21680C The effect of the A_2 adenosine receptor agonist, CGS 21680C (Hutchison *et al.*, 1989), on evoked tritium outflow is shown in Figure 6. CGS 21680C (2–3 nM) increased tritium outflow in a concentration-dependent manner. A higher concentration (10 nM) of CGS 21680C also increased evoked tritium outflow ($P < 0.05$), but the increase was smaller than that observed with 3 nM CGS 21680C. An inhibition of evoked tritium outflow was observed with 0.3 and 3 μM CGS 21680C. The excitatory effect of CGS 21680C (3 nM) on evoked tritium outflow was abolished in the presence of PD 115,199 (25 nM) (Figure 6).

Excitatory and inhibitory effects of adenosine analogues on endplate potential amplitude

Figure 7 illustrates the effect of CGS 21680C on the amplitude of e.p.ps recorded from phrenic nerve-diaphragm preparations paralyzed with tubocurarine (1.2 μM). CGS 21680C (3–10 nM) increased e.p.p. amplitude in a concentration-dependent manner, without modifying appreciably the decay phase of the e.p.ps (Figure 7b–c). The full effect of CGS 21680C was observed within 30–40 min after its application to the preparations and was hardly washed out, i.e. 45 min after returning the preparations to the control bathing solution the amplitude of the e.p.ps did not return to its value before applying CGS 21680C (Figure 7a). A small hyperpolarization (6 mV) of the muscle fibre was observed with 10 nM (Figure 7b) but not with 3 nM (Figure 7c) CGS 21680C. The averaged increase in e.p.ps amplitude caused by 3 nM GS 21680C was $15 \pm 0.5\%$ ($n = 2$) and that caused by 10 nM CGS 21680C was $28 \pm 0.5\%$ ($n = 4$, $P < 0.05$). The averaged hyperpolarization of the muscle fibre caused by 10 nM CGS 21680C was 6 ± 0.9 mV ($8 \pm 2\%$ increase, $P < 0.05$) from a resting potential of -71 ± 6 mV. To estimate the effect of CGS 21680C (10 nM) on e.p.p. amplitude independently of its effect on the resting membrane potential, the e.p.p. amplitude recorded in CGS 21680C (10 nM) at the observed resting membrane potential (V_{actual}) was corrected (Hubbard *et al.*, 1969) for its value at the resting membrane potential recorded in control conditions before applying CGS 21680C ($V_{standard}$) by multiplying the e.p.p. amplitude by $(V_{standard} - V_{tr})/(V_{actual} - V_{tr})$, where V_{tr} is the reversal potential for acetylcholine at the rat diaphragm neuromuscular junction (−15 mV, Cook & Quastel, 1973). After this correction the increase in e.p.p. amplitude caused by CGS 21680C (10 nM) was $17 \pm 0.4\%$ ($n = 4$, $P < 0.05$).

In order to know whether both excitatory and inhibitory effects of adenosine analogues could be observed in the same endplate, experiments were designed to compare the effects of R-PIA and CGS 21680C on the amplitude of e.p.ps recorded from the same endplate. Six endplates from different preparations were used. In three endplates where R-PIA (100 nM) reversibly decreased e.p.p. amplitude by $26 \pm 5\%$, CGS 21680C (10 nM) increased e.p.p. amplitude by $28 \pm 0.6\%$. One

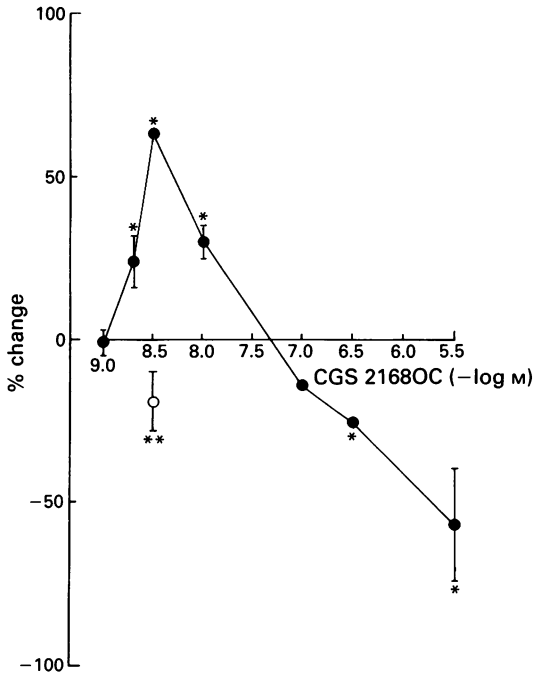

Figure 6 Concentration-response curve for the effect of CGS 21680C (●) on electrically-evoked tritium outflow from phrenic nerve-diaphragm preparations loaded with [^{3}H]-choline. The action of PD 115,199 (25 nM) on the effect of 3 nM CGS 21680C (○) is also shown. The ordinates are percentage change in the S_3/S_2 ratio as compared with the S_2/S_1 ratio in the same experiments. Zero percent represents identity between the two ratios, −100% the absence of evoked tritium outflow during S_3, and 100% a S_3/S_2 ratio two times higher than the S_2/S_1 ratio. Averaged S_2/S_1 ratio: 0.79 ± 0.02. Averaged tritium outflow during S_1: 58234 ± 2778 d.p.m. per g wet wt. of the preparation. CGS 21680C was applied 15 min before the end of S_3 and remained in the bath until the end of the experiments. PD 115,199 was present from the beginning of the release period. Each point is the mean of 2–4 experiments. The vertical bars represent ± s.e.mean, and are shown when they exceed the symbols in size. * $P < 0.05$ (Student's t test) when compared with zero percent. ** $P < 0.05$ (Student's t test) when compared with the effect of the same concentration of CGS 21680C in the absence of PD 115,199.

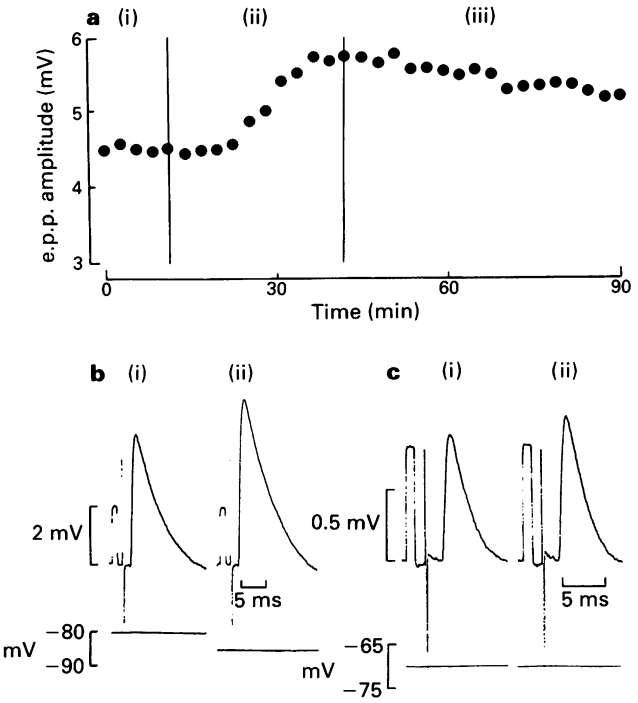

Figure 7 Effect of CGS 21680C on the averaged amplitude of evoked endplate potentials (e.p.ps) recorded from phrenic nerve-diaphragm endplates. Solutions contained tubocurarine (1.2 μM), which prevented muscle action potentials and twitches in response to nerve stimulation. (a) Time course of the effect of CGS 21680C (10 nM). The ordinates are the computed averages of 64 successive e.p.ps and the abscissae the times averaging began: (i) and (iii) control bathing solution; (ii) CGS 21680C (10 nM). (b) Pen-recorder traces of averaged e.p.ps corresponding to (i) 10 min and (ii) 40 min in (a). Each response is preceded by a calibration pulse of 2 mV amplitude and 1 ms duration. Lower part: membrane resting potential. (c) Pen-recorder traces of averaged e.p.ps recorded from another experiment where a lower concentration (3 nM) of CGS 21680C was used: (i) control bathing solution; (ii) CGS 21680C (3 nM). The full effect of CGS 21680C (3 nM), obtained 30 min after starting its perfusion, is shown. Each response is the computed average of 128 successive e.p.ps and is preceded by a calibration pulse of 0.8 mV amplitude and 1 ms duration. Lower section: membrane resting potential.

endplate was insensitive either to **R**-PIA (0.1–1 μM) or CGS 21680C (3–30 nM), and in two endplates **R**-PIA (100 nM) decreased in the usual way (27 $\pm$ 2%) e.p.p. amplitude but CGS 21680C (3–100 nM) was virtually devoid of effect on e.p.p. amplitude. The sensitivity to either submaximal (100 nM) or supramaximal (1–10 μM) concentrations of **R**-PIA was not appreciably different in endplates sensitive or not sensitive to CGS 21680C.

The averaged decrease in e.p.p. amplitude (26 $\pm$ 3%) caused by **R**-PIA (100 nM) in the five endplates (out of six) sensitive to this adenosine analogue corresponds to about 50% of its maximal effect since the effect of supramaximal concentrations (1–10 μM) of **R**-PIA was 55 $\pm$ 0.5% decrease in e.p.p. amplitude.

The full effect of **R**-PIA on e.p.p. amplitude was usually observed within 15 min after its application to the preparations and was washed out in about 30 min after returning to the control bathing solution. **R**-PIA (0.1–10 μM) did not cause appreciable changes in the decay phase of the e.p.p. or in the resting membrane potential of the muscle fibre.

Discussion

The present results show that the adenosine analogues **R**-PIA, NECA and CADO inhibit evoked tritium outflow and that CGS 21680C enhances evoked tritium outflow and increases the amplitude of e.p.ps recorded from phrenic nerve-diaphragm preparations. NECA also enhances evoked tritium outflow when applied to the preparations in the presence of the antagonist of its inhibitory effect, DPCPX. The excitatory effect of the adenosine analogues on evoked tritium outflow was antagonized by PD 115,199.

Using the same method as that used in the present work to label the transmitter stores of the phrenic nerve endings, Wessler & Kilbinger (1986) have shown that electrical stimulation of the phrenic nerve increases only the release of [^{3}H]-acetylcholine while the output of [^{3}H]-choline remains unchanged. Thus, the evoked outflow of tritium, defined as the difference between the total tritium outflow during stimulation and the basal tritium outflow, should be attributed to evoked release of [^{3}H]-acetylcholine from the phrenic nerve endings.

In the present work it was found that **R**-PIA and NECA are about equipotent in inhibiting evoked [^{3}H]-acetylcholine release and more potent than CADO. This order of potency of the adenosine analogues is similar to that found for the ability of **R**-PIA, NECA and CADO to inhibit nerve-evoked twitches in phrenic nerve-diaphragm preparations where the safety margin of neuromuscular transmission was decreased by high magnesium concentrations in the bath (Sebastião & Ribeiro, 1988). The concentrations of **R**-PIA used in the present work to decrease the evoked release of [^{3}H]-acetylcholine were of the same order as those needed to decrease the amplitude of e.p.ps recorded from tubocurarine-paralyzed phrenic nerve-diaphragm preparations. Taken together, these observations indicate that the adenosine analogues can inhibit neuromuscular transmission either in preparations where the safety margin of neuromuscular transmission had not been reduced, i.e. in isotope experiments, or in preparations where the safety margin of neuromuscular transmission had been reduced either presynaptically with high magnesium concentrations or mainly post-synaptically with tubocurarine; the degree of inhibition caused by the adenosine analogues does not appear to be dependent upon the method used to assess neuromuscular transmission.

The inhibition caused by **R**-PIA on evoked release of [^{3}H]-acetylcholine was antagonized by DPCPX. The shift (4.1) in the concentration-response curve for **R**-PIA caused by DPCPX (2.5 nM) is consistent with a K_i value for the xanthine of about 0.8 nM, which is of the same order as the K_i value (0.54 nM) obtained for DPCPX in experiments recording nerve-evoked twitch responses from phrenic nerve-diaphragm preparations partially inhibited with tubocurarine (Sebastião *et al.*, 1990) and is also of the same order as the K_i value for this xanthine at A_1 adenosine receptors (Bruns *et al.*, 1987; Lohse *et al.*, 1987).

The finding that NECA decreased evoked release of [^{3}H]-acetylcholine in the absence of DPCPX but increased [^{3}H]-acetylcholine release in the presence of DPCPX indicates that this adenosine analogue can act on both inhibitory and excitatory adenosine receptors in the phrenic nerve endings, its excitatory effect being observed when the inhibitory receptors are blocked. No excitatory effect of **R**-PIA was detected in the presence of DPCPX, suggesting that the affinity of this adenosine analogue for the inhibitory receptors is much greater than for the excitatory receptors. CGS 21680C increased evoked [^{3}H]-acetylcholine release even in the absence of DPCPX, indicating that the affinity of this adenosine analogue for the excitatory receptors is greater than its affinity for the inhibitory receptors. Since CGS 21680C has selectivity for the A_2 adenosine receptors (Hutchison *et al.*, 1989) it seems likely that the excitatory effect of the adenosine analogues on [^{3}H]-acetylcholine release is mediated by an A_2 adenosine receptor. This is supported by the observations that the excitatory effects of NECA and CGS 21680C were antagonized by PD 115,199. Though this xanthine is poorly selective for A_2 or A_1 adenosine receptors, it is a potent antagonist of the A_2 adenosine receptors when the A_1 adenosine receptors are blocked by DPCPX (Bruns *et al.*, 1987). Excitatory effects of adenosine analogues on acetylcholine release, probably mediated by A_2 adenosine receptors were reported recently (Barry, 1990; Brown *et al.*, 1990).

Since both inhibitory and excitatory adenosine receptors appear to be present at the phrenic nerve endings and both receptors can be activated by similar concentrations of NECA, it is possible that the observed inhibitory effect of NECA is smaller than that occurring if its excitatory action did not exist, and under these circumstances NECA would be more potent in inhibiting acetylcholine release.

That the excitatory adenosine receptors at the rat neuromuscular junction have an electrophysiological expression is supported by the observation that CGS 21680C increases the amplitude of intracellularly recorded e.p.ps. CGS 21680C (3 nM) increased e.p.p. amplitude without affecting the resting membrane potential of the endplate, and in a higher concentration (10 nM) CGS 21680C caused a greater percentage increase in e.p.p. amplitude than in the resting membrane potential. Since there is almost a one to one proportionality between the percentage increases in the e.p.p. amplitude and in the resting membrane potential (Fatt & Katz, 1951), the increase in e.p.p. amplitude caused by CGS 21680C (10 nM) cannot be solely attributed to the hyperpolarization of the endplate membrane. Furthermore, when the e.p.p. amplitudes recorded in CGS 21680C (10 nM) were corrected (Hubbard *et al.*, 1969) in relation to the control resting membrane potential, CGS 21680C also increased e.p.p. amplitude.

Muscle action potentials and twitches in response to nerve stimulation were prevented with tubocurarine, instead of low Ca^{2+}/high Mg^{2+}, to avoid changes in the ionic composition of the bathing solution that cause marked reductions in the quantal content of the e.p.ps. The use of tubocurarine prevented miniature endplate potential (m.e.p.ps) recordings and calculation of the quantal content of the e.p.ps., so that it was impossible to determine whether the increase in e.p.p. amplitude caused by CGS 21680C resulted from a pre- or post-synaptic action. However, the finding that CGS 21680C increased evoked tritium outflow from preparations loaded with [^{3}H]-choline is a strong indication that CGS 21680C has presynaptic excitatory effects. Additional changes in the post-synaptic sensitivity caused by CGS 21680C cannot be ruled out from the present results. **R**-PIA decreased e.p.p. amplitude without affecting the resting potential of the endplate. This adenosine analogue decreased [^{3}H]-acetylcholine release and does not modify the amplitude of m.e.p.ps (Barry, 1990), which indicates that the decrease in e.p.p. amplitude caused by **R**-PIA is due to a decrease in the quantal content of the e.p.ps.

Since R-PIA decreased e.p.p. amplitude and CGS 21680C increased e.p.p. amplitude in the same endplate, both inhibitory and excitatory adenosine receptors may coexist in the same nerve terminal. However, some nerve terminals might possess only inhibitory adenosine receptors since we found that in two endplates, R-PIA decreased e.p.p. amplitude in the usual way, whereas CGS 21680C was devoid of effect on e.p.p. amplitude. Insensitivity to both R-PIA and CGS 21680C was found in another endplate, suggesting that some nerve endings are devoid of both inhibitory and excitatory adenosine receptors. Some endplates of the phrenic nerve-diaphragm preparation are insensitive to adenosine as shown by Ginsborg & Hirst (1972) and Ribeiro & Walker (1975).

In summary, the present results suggest that both inhibitory and excitatory adenosine receptors are present at the rat phrenic nerve terminals, modulating the evoked release of the transmitter. Since the effect of the endogenous agonist, adenosine, is inhibitory (Ginsborg & Hirst, 1972; Ribeiro & Walker, 1975) and enhancement of transmission occurs when the presence of extracellular endogenous adenosine is prevented (Ribeiro & Sebastião, 1987; Sebastião & Ribeiro, 1988), the inhibitory adenosine receptors may have a preponderant role in relation to the modulation of transmitter release by adenosine. The physiological meaning of the excitatory adenosine receptors at the motor nerve endings remains to be elucidated.

We thank Warner-Lambert/Parke-Davis for the gifts of PD 115,199 and Ciba-Geigy for the gift of CGS 21680C. We also thank Dr A. Timóteo and Dr J.L. Fougo for collaboration in some experiments. The work performed in ICBAS was supported by JNICT.

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(Received December 12, 1990
Revised February 12, 1991
Accepted February 18, 1991)

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The *British Journal of Pharmacology* is published monthly by the Scientific & Medical Division, Macmillan Press Ltd.

The journal is covered by *Current Contents*, *Excerpta Medica* and *Index Medicus*.

All business correspondence and reprint requests should be addressed to the Scientific & Medical Division, Macmillan Press Ltd., Houndmills, Basingstoke, Hampshire RG21 2XS, UK. Telephone: (0256) 29242; Fax: (0256) 810526.

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ISSN 0007–1188

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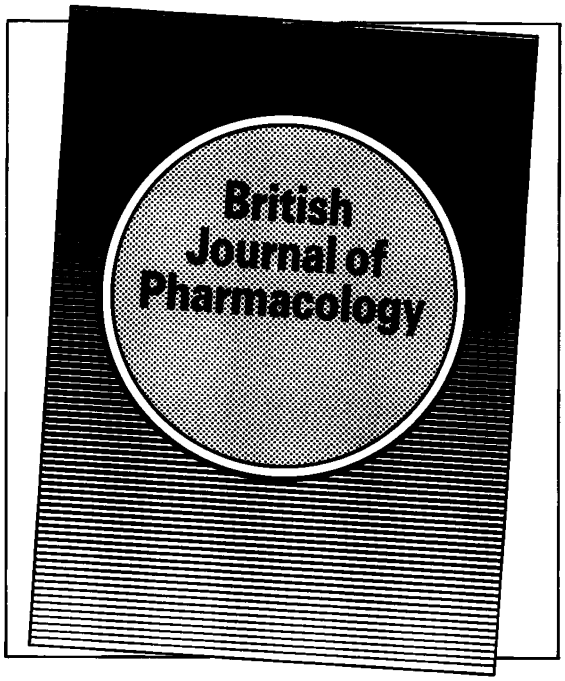

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and printed in Great Britain by Unwin Brothers Ltd., The Gresham Press, Old Woking, Surrey

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