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Paradoxical reversion of the inhibitory effects of dihydropyridine enantiomers on the calcium current in frog heart by CGP 28861

Alexander Filippov, [1]Evgeny Kobrinsky, Vladimir Porotikov & Maya Saxon

Institute of Biological Physics of the USSR Acad. Sci., Pushchino, Moscow Region, 142292, USSR

A dihydropyridine CGP 28861 (5×10^{-6} M) did not change slow inward Ca current as measured by the double sucrose gap method in frog atrial fibres but decreased the agonist effects of Bay K 8644, CGP 28392, (+)-(S)-202-791 and the antagonist effects of nifedipine and (+)-(PN)-200-110. Paradoxically, the weak antagonists (−)-(R)-202-791, (−)-(PN)-200-110 and (+)-Bay K 8644 increased Ca-current after washout of CGP 28861. These data suggest that CGP 28861 can convert the dihydropyridine Ca-channel receptor from an antagonistic site into an agonistic one.

Introduction Competition between dihydropyridine Ca channel antagonists and agonists was shown in binding and pharmacological experiments (see for example review by Glossmann *et al.*, 1985; Williams *et al.*, 1985) supporting the idea of one high affinity binding site for agonists and antagonists. On the other hand, cooperative interaction between dihydropyridine agonists and antagonists was demonstrated recently in cardiac cells, supporting the idea of two sites, one for agonists and another for antagonists (Kokubun *et al.*, 1986). Hence the questions of the number of sites and of the interaction between effects of different dihydropyridines remain open. Another question is whether a dihydropyridine receptor can be shielded from the action of agonists or antagonists by related compounds which themselves do not influence Ca current?

Compound CGP 28861, the derivative of the calcium agonist CGP 28392 ($OCHF_2$ substituent in the *para*-instead of the *ortho*-position on the phenyl ring), binds with low affinity to the dihydropyridine site in cardiac membranes (Erne *et al.*, 1984). However, its electrophysiological effect in unknown. We studied the influence of this compound on the calcium channel current and on the effects of dihydropyridine agonists and antagonists on frog atrial fibres.

Methods Frogs (*Rana ridibunda*) weighing 50–100 g of either sex were killed by destroying the spinal cord. Trabeculae 0.07–0.15 mm in diameter were voltage clamped by the double sucrose gap method as described previously (Filippov *et al.*, 1983). Fast sodium channels were blocked by 2×10^{-6} M tetrodotoxin (Sankyo, Japan). Slow inward calcium current was measured from the peak to the steady value which was reached at the end of 100 ms voltage pulse. Pulses were measured relative to the resting potential. Stimulation of the preparation and data recording were performed automatically with a computer system SM-3 and CAMAC-modules (Polon, Poland). Sampling interval was 0.05 ms.

Dihydropyridines used were: (+)-or-(−)-(PN)-200-110, (isopropyl 4-(2,1,3-benzoxadiasol-4-yl)-1.4-dihydro-5-methoxycarbonyl-2,6-dimethyl-3-pyridinecarboxylate); (+)-(S)-or-(−)-(R)-202-791, (isopropyl 4-(2,1,3,-benzoxadiasol-4-yl)-1,4-dihydro-2,6-dimethyl-5-nitro-3-pyridinecarboxylate) (Sandoz Ltd); (±)-Bay K 8644, (+)-Bay K 8644, (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) (Bayer); CGP 28392, (4-(2-difluoromethoxy)-phenyl)-1,4,5,7-tetrahydro-2-methyl-5-oxo-fluoro(3,4-b)pyridine-3-carboxylic acid ethylester); CGP 28861 (Ciba Geigy). Drugs were dissolved in ethanol or dimethylsulphoxide (DMSO) to prepare 1×10^{-2} M stock solutions which were diluted with the appropriate Ringer solution. Ringer solution of the following composition (mM) was used: NaCl 110, KCl 2.5, $CaCl_2$ 1.8, $MgCl_2$ 1, $NaHCO_3$ 2.4, glucose 5.5; isotonic sucrose solution (sucrose dissolved in twice distilled water); pH = 7.5 at 18 ± 2°C.

Results CGP 28861 (up to 5×10^{-6} M) did not influence the slow inward calcium channel current ($n = 8$) (Figure 1a as an example). In spite of this the compound decreased the effect of the dihydropyridine agonists, Bay K 8644, CGP 28392 and (+)-(S)-202-791 and of the antagonists, nifedipine and (+)-(PN)-200-110, on the calcium current (Figure 1b,c for example). An unexpected action was observed with enantiomers (−)-(R)-202-791, (−)-(PN)-200-110 and (+)-Bay K 8644. They have been reported to be weak antagonists (Williams *et al.*,

[1] Author for correspondence.

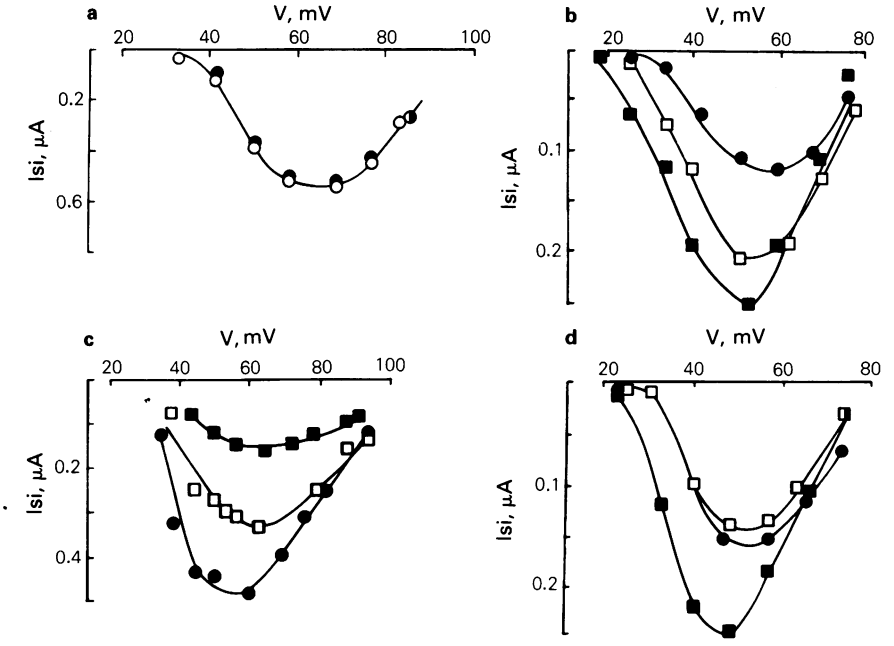

Figure 1 Current-voltage relations of the slow inward calcium channel current: (○) control (a); (●) CGP 28861, 5×10^{-6} M, 10 min (a-d); (□) CGP 28861 + CGP 28392, 5×10^{-6} M (b); (+)-(PN)-200-110, 5×10^{-8} M (c); (−)-(R) -202-791, 2×10^{-6} M (d), 10 min; (■) dihydropyridines 10 min after wash-out of CGP 28861 (b, c, d) V, mV, is the amplitude of the depolarizing voltage step used. Isi, μA, is the amplitude of the slow inward calcium channel current. In all these experiments there were no changes in resting potential.

1985; Franckowiak *et al.*, 1985). In our experiments ($n = 5$) they decreased Ca current no more than by 25% (at 2×10^{-6} M) compared with control. However in a series of experiments, when CGP 28861 was removed from the bathing solution, a paradoxical increase of calcium channel current was observed in spite of the fact that the antagonist still remained in the solution (e.g. Figure 1d). Thus antagonistic effects were converted into agonistic effects. This was seen in 3 experiments with (−)-(R)-202-791, and in 3 experiments with (−)-(PN)-200-110 and (+)-Bay K 8644. The effect was not related to the solvents used since it was not observed when in control experiments ethanol or DMSO were used instead of CGP 28861.

Discussion These experiments suggest that CGP 28861 interacts with dihydropyridine Ca channel receptors for agonists and antagonists without producing any effect on the slow inward calcium channel current, while influencing the effects of other dihydropyridines. The compound decreases the effects of the strong Ca channel agonists and antagonists. Moreover the compound seems to modify the dihydropyridine receptors so that weak antagonists become agonists after wash-out of CGP 28861. How can these results be interpreted? We think that they are in favour of the hypothesis for different binding sites for dihydropyridine agonists and antagonists. We suggest that CGP 28861 competes for binding sites with dihydropyridine agonists and antagonists. However, the compound probably increases the effects of Ca agonists even after washing out. Since dihydropyridines may have dual effects, agonistic and antagonistic (Brown *et al.*, 1986), one can suggest that the weak antagonists are converted into agonists if their binding to agonistic sites increases. However, the results do not exclude the possibility that the site on the calcium channel for dihydropyridine agonists and antagonists is the same and some dihydropyridine receptor modification can convert it from an antagonistic into an agonistic one.

The molecular mechanism of the effect observed is unknown. Recently, activation of G_1 protein of adenylate cyclase was shown to convert Ca antagonists into Ca agonists (Scott & Dolphin, 1987). Whether a similar mechanism occurred in our experiments remains to be shown.

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Tricyclic antidepressants block N-methyl-D-aspartic acid-induced lethality in mice

J. David Leander

Central Nervous System Research, Lilly Research Laboratories, Eli Lilly and Company, Lilly Corporate Center, Indianapolis, IN 46285, U.S.A.

It has been suggested on the basis of *in vitro* studies that tricyclic antidepressants interact with the N-methyl-D-aspartic acid (NMDA)-receptor complex to block the action of NMDA. The present study showed that tricyclic antidepressants prevented lethality produced by a large dose of NMDA. The potency of the drugs in preventing NMDA-induced lethality correlated with the inhibition of [^{3}H]-MK-801 binding at the NMDA receptor complex, and not with effects on amine uptake. These *in vivo* data support the *in vitro* data of Reynolds & Miller (1988a,b).

Introduction Excitatory amino acid receptors for glutamate have usually been divided into three groups based upon the prototypic agonists that preferentially activate each of these receptors: N-methyl-D-aspartic acid (NMDA), kainic acid and quisqualic acid (Watkins & Evans, 1981). Radioligand binding studies support the separation of these three receptors (Foster & Fagg, 1984). It has recently been shown that the NMDA receptor is part of a complex consisting of several different components. There is the recognition site at which NMDA is the agonist and at which competitive antagonists, such as 2-amino-5-phosphono-pentanoic acid (AP-5), 2-amino-7-phosphonheptanoic acid (AP-7), and 3-(($\pm$)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) (Watkins & Olverman, 1987), produce their effects. That site is coupled to an ion channel selective for cations within which phencyclidine-like drugs (Kemp *et al.*, 1987) and Mg^{2+} (Ascher & Nowak, 1987) appear to act at different sites as NMDA antagonists (Reynolds & Miller, 1988a). Besides those sites, there are two other superficial sites which modulate NMDA receptor activity. One of these sites is a novel, non-strychnine-sensitive glycine site (Johnson & Ascher, 1987). The second site has been identified by Zn^{2+} and related divalent cations and prevents activation of the NMDA-induced ion channel (Peters *et al.*, 1987; Westbrook & Mayer, 1987).

Recent studies using [^{3}H]-MK-801 binding have been able to distinguish between drug actions at the five different binding sites described above (Reynolds *et al.*, 1987; Reynolds & Miller, 1988a). By use of the appropriate biochemical challenge, the precise location of the site of interaction of drugs with the NMDA receptor can be determined. It has recently been shown that various tricyclic antidepressants, including desipramine and imipramine, inhibit [^{3}H]-MK-801 binding at modest concentrations in a Zn^{2+}-like manner (Reynolds & Miller, 1988b). The inhibition of binding for a series of tricyclic compounds was correlated with the ability of these drugs to inhibit NMDA-mediated Ca^{2+} fluxes into cultured neurones (Reynolds & Miller, 1988b).

In mice, NMDA produces a stereoisomer-selective, dose-responsive lethal effect after intraperitoneal injection (Leander *et al.*, 1988). This NMDA-induced toxic effect is blocked by phencyclidine-like drugs and high doses of competitive NMDA antagonists (Leander *et al.*, 1988). The purpose of the present investigation was to determine whether desipramine, imipramine and related tricyclic structures would protect mice against NMDA-induced lethality, as suggested by the recent *in vitro* studies of Reynolds & Miller (1988a,b).

Methods Male Charles River (Portage, MI) CF-1 mice (20–25 g) in groups of 10 or 15 were treated with various doses of tricyclic antidepressants or related drugs by the intraperitoneal route. Thirty minutes later, the mice were challenged with a lethal intraperitoneal dose (200 mg kg^{-1}) of NMDA. All drugs were administered in a volume of 1 ml per 100 g body weight. The vehicle was distilled water, and all doses were calculated as the total salt. The N-methyl-D-aspartic acid was purchased from Sigma Chemical Company (St. Louis, MO). The forms and sources of the other drugs were: desipramine HCl (Merrell Dow, Cincinnati, OH, U.S.A.); carbamazepine, imipramine HCl and chlorimipramine HCl (Ciba-Geigy, Summit, NJ, U.S.A.): amitriptyline HCl and protriptyline HCl (Merck Sharp & Dohme, West Point, PA, U.S.A.); promazine HCl (Weyth, Philadelphia, PA, U.S.A.); chlorpromazine HCl (Smith Kline & French, Philadelphia, PA, U.S.A.); nortriptyline HCl, nisoxetine HCl and fluoxetine HCl (Lilly Research Laboratories, Indianapolis, IN,

Table 1 The dose-response protective effects and ED_{50}'s against N-methyl-D-aspartic acid (NMDA)-induced lethality

Drugs	% *protected* 10	20	40	80 ($mg\,kg^{-1}$)	ED_{50} $mg\,kg^{-1}$
		Tricyclic antidepressants			
Desipramine	0	47	87	87	29
Imipramine			20	60	68
Chloripramine			0	80	66
Nortriptyline		7	40	85	45
Amitriptyline			20	50	80
Protriptyline	0	30	40	80	42
		Miscellaneous compounds			
Promazine		7	67	100	34.2
Chlorpromazine			10	50	80
Carbamazepine				0	—
Nisoxetine		10	40	*	—
Fluoxetine				30	—

* The $80\,mg\,kg^{-1}$ dose of nisoxetine was lethal in a majority of the 10 mice injected.

U.S.A.). All drugs were initially tested at a dose of $80\,mg\,kg^{-1}$. If 50% or more of the 10 mice which were dosed survived the NMDA, the dose of the pretreatment drug was successively halved until a dose-effect curve was generated with a low dose producing 0–20% protection against NMDA. From such quantal data, an ED_{50} was calculated by use of a computer fit, probit-based dose-response curve.

Results All of the tricyclic antidepressants tested produced 50% or greater protection against $200\,mg\,kg^{-1}$ of NMDA at the $80\,mg\,kg^{-1}$ dose. Table 1 shows the dose-response data and the ED_{50}'s calculated. With the tricyclic antidepressants, the desmethyl analogues, desipramine, nortriptyline and protriptyline, were most potent. Interestingly, the deschloro- analogue of chlorpromazine, promazine, was more potent than chlorpromazine.

Carbamazepine, an anticonvulsant with a tricyclic structure sharing some similarities with the tricyclic antidepressants, was ineffective in providing any protection. Likewise, the selective noradrenaline and 5-hydroxytryptamine uptake inhibitors, nisoxetine and fluoxetine, respectively, were without significant effects.

Discussion The present data show that various tricyclic antidepressants and at least two phenothiazines (promazine and chlorpromazine) are effective in protecting mice from a lethal dose of NMDA. The lethal effect of NMDA has been shown to be blocked previously only by competitive NMDA receptor antagonists and non-competitive NMDA antagonists interacting at the phencyclidine-like binding site (Leander *et al.*, 1987; 1988). Representatives of numerous classes of central nervous system drugs were ineffective in blocking NMDA-induced lethality (Leander *et al.*, 1988). The present results complement the *in vitro* studies of Reynolds & Miller (1988a,b) and suggest that there are at least three sites available *in vivo* to block the actions of NMDA. Those three sites are the recognition site for NMDA and the competitive antagonists, the site in the ion channel where phencyclidine-like drugs work and the zinc-like site where the tricyclic structures work.

This protective effect from the tricyclic structure cannot be due to their well-known actions of inhibition of noradrenaline or 5-hydroxytryptamine uptake in neurones since the specific inhibitors, nisoxetine and fluoxetine, were not effective in the present study. Also, the doses which protect in the present study do not correlate well with the *in vivo* doses that block noradrenaline and 5-hydroxytryptamine uptake in mice, as reported by Fuller & Wong (1977). However, the log of the *in vivo* ED_{50} doses in the present study correlate highly with the log of the IC_{50}'s reported by Reynolds & Miller (1988b) in Table 1 for inhibiting [^{3}H]-MK-801 binding. That Pearson product moment correlation coefficient is $r = 0.902$ ($n = 7$, $P < 0.01$). This very strong correlation supports the conclusion that the NMDA protection is due to the binding effects reported by Reynolds & Miller (1988b).

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Expression of functional postjunctional α_2-adrenoceptors in rabbit isolated distal saphenous artery—a permissive role for angiotensin II?

W.R. Dunn, J.C. McGrath & V.G. Wilson

Autonomic Physiology Unit, Institute of Physiology, University of Glasgow, Glasgow, G12 8QQ

In the rabbit isolated distal saphenous artery, the population of postjunctional adrenoceptors is of the α_1 variety under normal *in vitro* experimental conditions, based on the potency order of selective agonists and on the effects of the antagonists prazosin and rauwolscine against responses to UK-14304. Angiotensin II (A II, 0.05 μM) however, without affecting resting baseline tension, markedly enhanced responses to UK-14304, particularly at low concentrations. This previously unseen component of the response to UK-14304 was resistant to prazosin (0.1 μM) but susceptible to rauwolscine (1 μM). A II would therefore appear to have a permissive role for the expression of a quiescent population of postjunctional α_2-adrenoceptors in the rabbit distal saphenous artery.

Introduction The demonstration of postjunctional α_2-adrenoceptors in isolated vascular smooth muscle preparations is difficult, particularly in arteries (McGrath, 1982). Recently Sulpizio & Hieble (1987) and Furuta (1988) have demonstrated an enhancement of responses to α_2-adrenoceptor agonists in isolated preparations in the presence of pharmacological stimulants. The presence of Bay K 8644 or inducing tone with prostaglandin $F_{2\alpha}$, enhanced responses to BHT-920 which were prazosin-resistant and rauwolscine-sensitive, in canine isolated saphenous artery and portal vein respectively. Furthermore, the physiological stimulant angiotensin II (A II), enhances postjunctional α_2-adrenoceptor function in some venous preparations (Schumann & Lües, 1983; Daly *et al.*, 1988a). In this study we have examined the influence of A II on responses to the selective α_2-adrenoceptor agonist UK-14304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline) in the rabbit distal saphenous artery.

Methods Male albino rabbits (2.5–2.7 kg) were killed by stunning followed by exsanguination. A length of distal saphenous artery from either leg was placed in physiological salt solution (for composition: see Daly *et al.*, 1988a) containing propranolol (1 μM) and cocaine (10 μM). The artery was then divided into six 'ring' segments (3–4 mm long) and prepared for recording of isometric tension.

After equilibration, each preparation was exposed to 3 μM (−)-noradrenaline (NA) and 10 min later washed until complete relaxation was effected. After a further 45 min cumulative concentration-response curves (CCRC) were obtained to either NA, phenylephrine (PE) or UK-14304. In experiments involving UK-14304, following complete washout, tissues were exposed to either saline, prazosin (0.1 μM) or rauwolscine (1 μM) 45 min before a second CCRC. In some tissues A II (0.05 μM) was added 15 min before UK-14304. Results are expressed as the percentage (mean ± s.e.mean) of the maximum response to NA or as the percentage of the maximum response of the first CCRC to UK-14304. Differences between means were considered statistically significant if $P < 0.05$: Student's t test (paired or unpaired).

Results All three agonists produced concentration-dependent contractions in the distal saphenous artery. Both NA and PE were full agonists while UK-14304 was a partial agonist producing approximately 65% of the NA maximum response. The relative potency of the three agonists was, NA > PE > UK-14304 (Figure 1a). The responses to UK-14304 were antagonized by both 1 μM rauwolscine (3 fold rightward displacement) and 0.1 μM prazosin (100 fold parallel rightward displacement) (Figure 1b).

A II (0.05 μM) produced a transient contraction (duration 10–12 min) which relaxed to baseline before the CCRC to UK-14304 was started. A II produced a marked increase in sensitivity to low concentrations of UK-14304, with no increase in the maximum response, resulting in a change in the slope of the CCRC to UK-14304 (Figure 1c). The magnitude of the displacement produced by A II for the threshold concentration of UK-14304 was

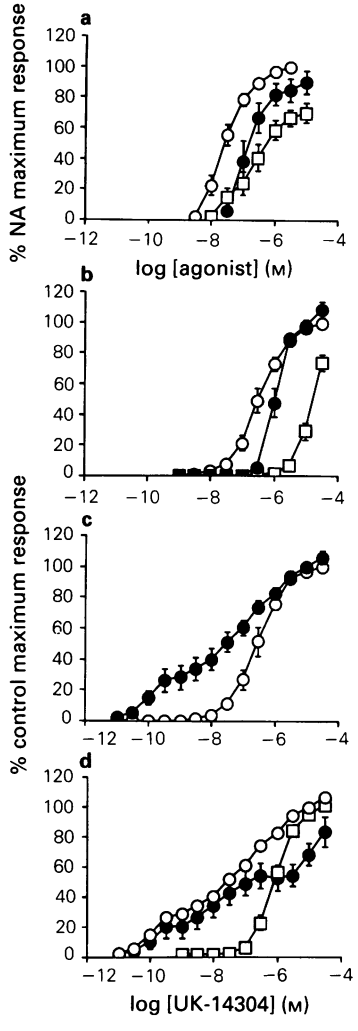

Figure 1 The effects of agonists and antagonists at α-adrenoceptors and the influence of angiotensin II (A II) in the rabbit isolated distal saphenous artery. (a) The contractile responses to the agonists (−)-noradrenaline (NA) (○), phenylephrine (PE) (●) and UK-14304 (□). (b) The effect of rauwolscine 1 μM (●) and prazosin 0.1 μM (□) on contractions elicited by UK-14304 (○). (c) The effect of A II 0.05 μM (●) on contractions elicited by UK-14304 (○). (d) The effect of prazosin 0.1 μM (●) and rauwolscine 1 μM (□) on contractions elicited to UK-14304 (○) in the presence of A II 0.05 μM. All responses are expressed as a percentage of either the maximum response to NA (a) or the maximum response in the control cumulative concentration-response curve to UK-14304 (b, c, d) and are the mean of 6–7 observations of different animals. The vertical lines indicate the s.e.mean.

approximately 300 fold: 10^{-8} M in the absence of A II compared to 10^{-11} M in the presence of A II. Prazosin (0.1 μM) in the presence of A II, was ineffective against the 'uncovered responses' to low concentrations of UK-14304 but displaced the upper portion of the CCRC. Rauwolscine (1 μM) prevented the potentiating effect of A II on responses to UK-14304 (Figure 1d).

Discussion Although pressor responses to postjunctional α_1- and α_2-adrenoceptor stimulation are easily demonstrated *in vivo* (McGrath, 1982), responses via postjunctional α_2-adrenoceptors in isolated vascular preparations have been difficult to show. This is particularly true in arterial vessels, the likely source of α_2-adrenoceptor-mediated pressor responses in whole animals, while even in veins only a few clear examples have been shown (e.g. Constantine *et al.*, 1982; Daly *et al.*, 1988b). There is, however, some evidence that the presence of tissue stimulants can enhance the expression of α_2-adrenoceptor-mediated vasoconstriction (see Introduction). We have now, for the first time in an isolated arterial preparation, shown that activation with a physiological stimulant, namely A II, reveals a quiescent population of α_2-adrenoceptors in a vessel whose response was mediated entirely by α_1-adrenoceptors in the absence of A II. This is associated with a marked increase in the sensitivity of the preparation (up to 300 fold) to the selective α_2-adrenoceptor agonist UK-14304.

The rabbit distal saphenous artery clearly responds to α-adrenoceptor agonists through α_1-adrenoceptors under normal, *in vitro*, experimental conditions. Firstly, the relative potency of agonists is NA > PE > UK-14304 and secondly, prazosin and rauwolscine produced antagonism against UK-14304 consistent with that shown previously at α_1-adrenoceptors in other rabbit blood vessels: prazosin (estimated $-\log K_b$ value 8.7), rauwolscine (estimated $-\log K_b$ value 6.3) (see Daly *et al.*, 1988c). A II, without altering the resting baseline tension, produced a marked increase in sensitivity to UK-14304. Thus, previously absent responses were 'uncovered' at low concentrations of UK-14304: these responses were unaffected by prazosin (0.1 μM: a concentration 50 times greater than its K_b value at α_1-adrenoceptors in this tissue). The CCRC at higher concentrations of UK-14304 however, was shifted to the right, indicating the continued expression of responses via α_1-adrenoceptors. In contrast, in the presence of A II, rauwolscine (1 μM) antagonized the response to UK-14304 more effectively than prazosin (0.1 μM), in effect preventing the increase in sensitivity produced by A II. This confirms that the 'uncovered' responses were mediated by postjunctional

α_2-adrenoceptors. The present study shows that a stimulating agent is required for expression of functional postjunctional α_2-adrenoceptors in the rabbit distal saphenous artery. The requirement for physiological stimulants, such as A II, could therefore explain the hitherto perplexing differences in the expression of postjunctional α_2-adrenoceptors between *in vivo* and *in vitro* preparations.

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The failure of endothelin to displace bound, radioactively-labelled, calcium antagonists (PN 200/110, D888 and diltiazem)

X.H. Gu, J.J. Liu, J.S. Dillon & Winifred G. Nayler

Department of Medicine, University of Melbourne, Austin Hospital, Heidelberg, Victoria, 3084, Australia

The effect of endothelin, a potent vasoconstrictor polypeptide, on three types of calcium antagonist binding sites was examined, in rat cardiac membrane fragments. Endothelin 10 nM affected neither the affinity nor density of dihydropyridine binding sites. At concentrations of 10^{-12}–10^{-7} M, endothelin failed to displace bound (+)-[^{3}H]-PN 200/110, (−)-[^{3}H]-D888 and (+)-*cis*-[^{3}H]-diltiazem. These results suggest that the calcium antagonist binding sites associated with L-type calcium channels are not the primary site of action of endothelin.

Introduction Endothelin is a novel and potent vasoconstrictor polypeptide derived from vascular endothelial cells (Yanagisawa *et al.*, 1988; Tomobe *et al.*, 1988). The vasoconstriction is dose-dependent, persistent and has been observed in a variety of vascular beds (coronary, renal and pulmonary) in different mammalian species (rats, guinea-pigs, rabbits and swine) (Tomobe *et al.*, 1988; O'Brien *et al.*, 1987; Gillespie *et al.*, 1986; Yanagisawa *et al.*, 1988). The potency of this peptide is such that its constrictor effect has an ED_{50} of 10^{-9} to 10^{-10} M (Yanagisawa *et al.*, 1988).

Yanagisawa *et al.* (1988) and O'Brien *et al.* (1987) have reported that the vasoconstrictor effect of endothelin is dependent upon the presence of extracellular Ca^{2+}, and is attenuated by the calcium antagonists nicardipine (10 nM) and verapamil (5 μM). In addition, the chemical structure of endothelin resembles that of other peptide toxins which interact with membrane-located ion-conducting channels (Yanagisawa *et al.*, 1988). These latter observations have caused speculation as to whether endothelin is a naturally occurring Ca^{2+}-agonist which interacts with the dihydropyridine (DHP)-sensitive L-type Ca^{2+} channels (Yanagisawa *et al.*, 1988). The following experiments were undertaken to investigate this possibility, using endothelin to displace (+)-[^{3}H]-PN 200/110, (−)-[^{3}H]-desmethoxyverapamil (D888) and (+)-*cis*-[^{3}H]-diltiazem bound to high affinity binding sites associated with L-type channels in cardiac membrane fragments.

Methods Cardiac membranes were isolated from female Sprague Dawley rats (250–300 g) by the method of Glossmann & Ferry (1985). Protein was measured by the procedure of Lowry *et al.* (1951) with bovine serum albumin used as standard.

(+)-[^{3}H]-PN 200/110 (84 Ci mmol^{-1}), (−)-[^{3}H]-D888 (79 Ci mmol^{-1}) and (+)-*cis*-[^{3}H]-diltiazem (170 Ci mmol^{-1}) binding was monitored as described by Glossmann & Ferry (1985). (+)-[^{3}H]-PN 200/110 0.015–0.9 nM was used for saturation binding. Non-specific binding was defined by adding 1 μM (−)-Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate). Incubation was at 25°C in 50 mM Tris-HCl buffer (containing 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 1 mM $CaCl_2$, pH 7.4), using a protein concentration of 0.1–0.5 mg ml^{-1} in a final volume of 0.25 ml. To test the effect of endothelin on DHP binding activity, 10 nM endothelin was added directly to the incubation medium. For displacement (competition) studies, 10^{-12}–10^{-7} M endothelin was incubated with 0.05 nM (+)-[^{3}H]-PN 200/110 (isopropyl-4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-methoxycarbonyl pyridine) in the same Tris-HCl medium. Two temperatures, 25°C and 37°C were used for the competition studies, to determine whether there was any temperature-dependent effect of endothelin on DHP binding sites. After 60 min incubation, bound and free (+)-[^{3}H]-PN 200/110 were separated by rapid filtration across Whatman GF/C filters and the radioactivity was counted (40% efficiency) with a liquid scintillation counter as described previously (Gu *et al.*, 1988). Because of the photolability of the DHP, (+)-[^{3}H]-PN 200/110 binding experiments were performed under sodium lighting.

For competition experiment with (−)-[^{3}H]-D888 or (+)-*cis*-[^{3}H]-diltiazem, 10^{-12}–10^{-7} M endothelin was used as displacer. The binding experiments were

[1] Author for correspondence.

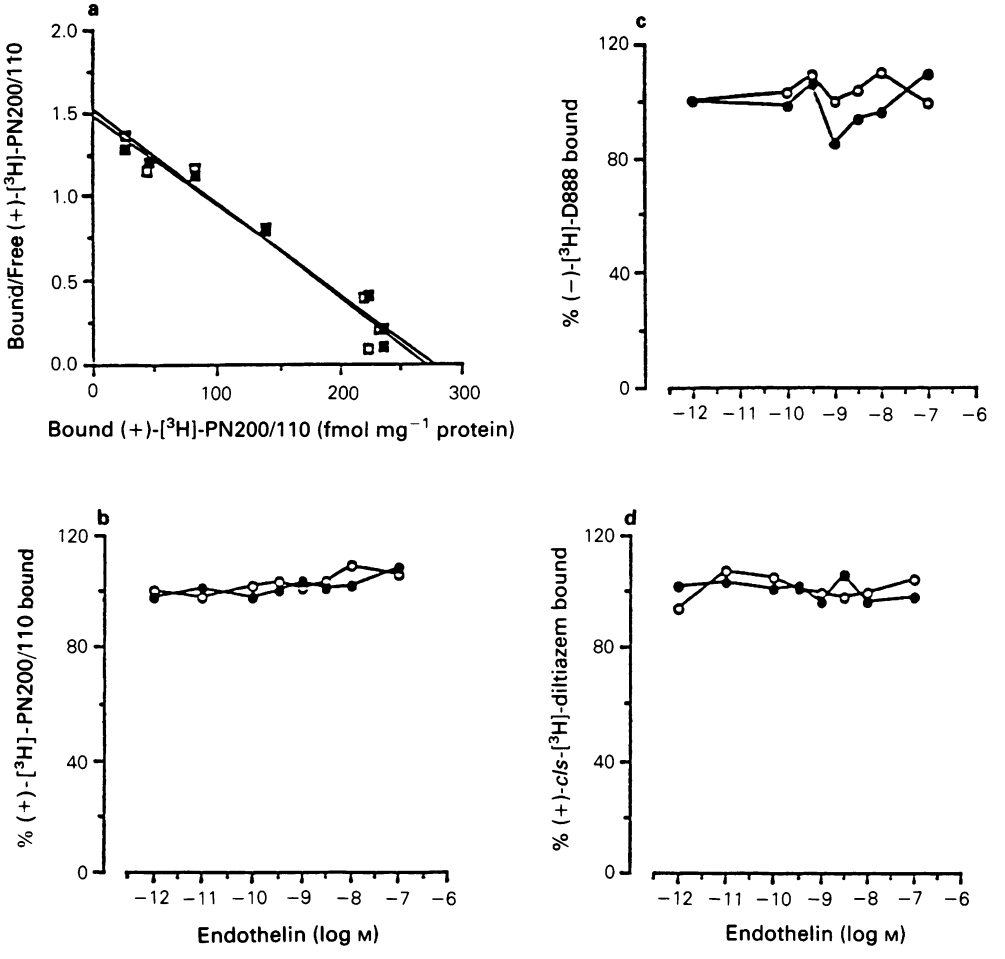

Figure 1 Effect of endothelin on tritium-labelled calcium antagonist binding. Representative Scatchard plots of (+)-[^{3}H]-PN 200/110 binding to rat cardiac membranes (a). The membranes were incubated in reaction buffer without (control) (□) and with 10 nM endothelin (■). The binding parameters are given in the text. Similar estimates were obtained for 6 separate experiments. Effect of 10^{-12}–10^{-7} M endothelin on (+)-[^{3}H]-PN 200/110 (b), (−)-[^{3}H]-D888 (c) and (+-*cis*-[^{3}H]-diltiazem (d) binding to cardiac membranes incubated at either 25°C (○) or 37°C (●). Each curve is the representative of 3 separate experiments for (b), (c) and (d).

carried out with 1 nM (−)-[^{3}H]-D888 or 2.6 nM (+)-*cis*-[^{3}H] diltiazem in a total volume of 0.25 ml of 50 mM Tris-HCl buffer (as described above). Non-specific binding was defined by adding 1 μM (−)-gallopamil ((−)-D600) or 20 μM unlabelled (+)-*cis*-diltiazem respectively. After 60 min incubation at 25°C and 37°C, bound ligand was collected by filtration.

Estimates of equilibrium binding data (K_D and B_{max}) were obtained from Scatchard, Hill and Hofstee analysis, using the 'EDBA' (McPherson, 1983) and 'Scafit' (Munson & Rodbard, 1980) programme. The binding data were subjected to Student's t test at the $P < 0.05$ level of significance.

Endothelin was supplied as a gift by Professor Gamamoto to Dr F. Mendelsohn. The peptide was synthesized at the Peptide Institute in Osaka, Japan. Preliminary experiments confirmed it to be active, as evident by its constrictor effect on rat coronary vasculature at 10^{10} M and a positive inotropic effect on the myocardium at 10^{-9} M.

Results Scatchard plots (figure 1a) show that 10 nM endothelin had no effect on either the affinity (K_D) or density (B_{max}) of (+)-[^{3}H]-PN 200/110 binding to

rat cardiac membrane fragments ($K_D = 0.052 \pm 0.005$ and 0.052 ± 0.05 nM; $B_{max} = 217.2 \pm 17.5$ and 216.0 ± 19.0 fmol mg^{-1} protein for endothelin and control groups, respectively). Figure 1b shows the effect of 10^{12}-10^{-7} M endothelin on the (+)-[^{3}H]-PN 200/110 binding activity of cardiac membranes when incubated at either 25°C or 37°C. Again, under these conditions and over the concentration-range used here, endothelin was without effect on DHP binding. Similar results were obtained for displacement studies with (−)-[^{3}H]-D888 and (+)-*cis*-[^{3}H]-diltiazem when the same concentration range 10^{-12}–10^{-7} M of endothelin was used, and at either 25°C or 37°C (figure 1c and 1d).

Discussion These results indicate that endothelin does not displace PN 200/110 (a dihydropyridine), D888 (a phenylalkylamine) or diltiazem (a benzothiazepine) from their specific binding sites in cardiac membrane fractions. As a corollary, it follows that this peptide, if it does interact with the L-type Ca^{2+} channel (Nayler, 1988) does so by a mechanism which does not involve the specific binding sites for dihydropyridine, phenylalkylamine or benzothiazepine-based calcium antagonists. Certainly these results do not exclude the possibility of the L-type channels providing a site of action for endothelin but they raise the possibility that other binding sites are involved. These other sites may be associated with the T-type of Ca^{2+} channels which lack binding sites for the 'classical' calcium antagonists.

There is no conflict between our results and those of the earlier investigators who used either nitrendipine or verapamil to attenuate the effect of endothelin, because both nitrendipine and verapamil interact with other membrane-located systems, particularly when used at relatively high dose-levels. The lack of effect of endothelin cannot be explained in terms of the nature of the test preparation, because the membrane fraction is a crude preparation containing membranes from cardiac and vascular smooth muscle cells.

In conclusion, these results suggest that the calcium antagonist binding sites associated with the L-type calcium channels are not the primary site of action of endothelin.

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Prostaglandin E_2 inhibits and indomethacin and aspirin enhance, A23187-stimulated leukotriene B_4 synthesis by rat peritoneal macrophages

[1]Graham R. Elliott, Adrianus P.M. Lauwen & Ivan L. Bonta

Department of Pharmacology, Medical Faculty, Erasmus University, P.O.B. 1738, 3000 DR, Rotterdam, The Netherlands.

1 The calcium ionophore, A23187, stimulated leukotriene B_4 (LTB_4), thromboxane B_2 (TxB_2) and prostaglandin E_2 (PGE_2) synthesis by 4 day carrageenin-elicited rat peritoneal macrophages.

2 At concentrations of 2×10^{-7}–2×10^{-5} M indomethacin and aspirin enhanced A23187-stimulated LTB_4 synthesis and inhibited PGE_2 and TXB_2 formation.

3 PGE_2 inhibited A23187-stimulated LTB_4 and TXB_2 formation as well as the augmentation of LTB_4 release caused by aspirin and indomethacin. However, PGE_2 was ineffective when the cells were challenged with arachidonic acid (AA).

4 Dibutyryl adenosine 3′ : 5′-cyclic monophosphate (db-cyclic AMP) partially inhibited A23187-stimulated LTB_4 production.

5 Our results suggest that PGE_2 inhibits macrophage LTB_4 synthesis by limiting the availability of AA. Indomethacin and aspirin, possibly by removing the regulatory effect of PGE_2, promote the synthesis of the pro-inflammatory LTB_4.

Introduction

It is becoming increasingly apparent that eicosanoid products of arachidonic acid (AA) metabolism are important modulators of macrophage cyclo-oxygenase and lipoxygenase pathways. For example, mouse resident peritoneal macrophage cyclo-oxygenase and 5′-lipoxygenase activities were inhibited by hydroperoxy- and hydroxy-eicosatetraenoic acid metabolites of the lipoxygenase pathway (Chang *et al.*, 1985; Humes *et al.*, 1986). Synthesis of a cyclo-oxygenase metabolite, prostaglandin E_2 (PGE_2) by rat peritoneal macrophages was stimulated by the lipoxygenase product, leukotriene C_4 (LTC_4) (Schenkelaars & Bonta, 1986), while PGE_2 inhibited synthesis of the cyclo-oxygenase metabolites, thromboxane B_2 (TXB_2) and 6-keto-$PGF_{1\alpha}$ (Elliott *et al.*, 1985). Such interactions between eicosanoids may be important for regulation of macrophage functions, as demonstrated by Schenkelaars & Bonta (1986) who found that LTC_4 stimulated the secretion of the lysosomal enzyme β-glucuronidase (GUR). This secretory response was enhanced by the non-steroidal anti-inflammatory drugs (NSAIDs) indomethacin and aspirin which possess cyclo-oxygenase inhibitory activity. Exogenously added PGE_2 prevented this stimulation of enzyme release. Of relevance to these interactions are reports showing that PGE_1 inhibited, and indomethacin stimulated, human neutrophil LTB_4 formation (Ham *et al.*, 1983; Docherty & Wilson, 1987). Therefore, in the light of published data, it appeared conceivable that indomethacin and aspirin stimulated macrophage GUR release by promoting synthesis of leukotrienes, as a consequence of the inhibition of PGE_2 synthesis. In order to investigate this possibility we examined the effect of PGE_2, indomethacin and aspirin on A23187-stimulated LTB_4 release from carrageenin-elicited rat peritoneal macrophages.

Methods

Experimental animals

Male Wistar rats (170–200 g) were injected with 2 ml carrageenin (1 mg ml^{-1}, i.p.) on day 1 and the elicited peritoneal macrophages were isolated on day 4. Animals were ordered 12 at a time and divided into

[1] Author for correspondence.

groups of 3 or 4. Experiments were repeated on animals from the same batch.

Isolation and incubation of macrophages

Carrageenin-elicited peritoneal macrophages were isolated by density-gradient centrifugation over Ficoll–Isopaque as previously described (Schenkelaars & Bonta, 1986). They were then suspended (2×10^6 cells ml^{-1}) in Dulbecco's modified Eagle's Minimum Essential Medium (DMEM), and kept on ice until needed. The cell preparation was more than 95% viable, as assayed by trypan blue exclusion and consisted of greater than 85% macrophages as judged by morphological criteria under the light microscope. The rest of the cells were mainly lymphocytes; the contribution of polymorphonuclear leukocytes and mast cells was always less than 1%. Lymphocytes do not release eicosanoids and this contamination was thus not a problem in the experimental design used (Kurland & Bockman, 1978; Poubelle *et al.*, 1987). Aliquots of the macrophage preparation (1 ml) were transferred to 1 ml polypropylene reaction vials on ice and then incubated for 30 min at 37°C in a water bath (Gemsa *et al.*, 1982; Williams *et al.*, 1984). The reaction vials were then centrifuged and the supernatants analysed for LTB_4, PGE_2 and TXB_2 by radioimmunoassay (RIA) (Zijlstra & Vincent, 1984). None of the compounds used interfered with the measurement of PGE_2, TXB_2 or LTB_4. PGE_2 was not analysed in those experiments where it was added to the incubations. The PGE_2 RIA had a 100% cross reaction with PGE_1. However rat macrophages do not synthesize PGE_1 unless incubated with dihomo-γ-linolenic acid (Elliott *et al.*, 1986), its direct precursor, so that we feel justified in giving concentrations of PGE_2 rather than of immunoreactive PGE. The cell pellet was heated for 5 min at 95°C in 150 μl Tris/EDTA buffer and the reaction vials centrifuged at 12000 *g* for 1 min. The concentration of adenosine 3′ : 5′-cyclic monophosphate (cyclic AMP) in the supernatants was assayed by a modification (Bonta *et al.*, 1984) of the protein binding method of Gilman (1970).

Chemicals

Carrageenin was dissolved in physiological saline. Stock solutions of AA, PGE_2, A23187 and indomethacin in ethanol were diluted with DMEM. Aspirin and db-cyclic AMP were dissolved directly in DMEM.

A23187, aspirin, indomethacin, AA, db-cyclic AMP and PGE_2, (10 μl volumes) were added to incubations at the beginning of the 30 min period. The final concentration of ethanol resulting from the addition of A23187, AA, PGE_2 and indomethacin was 0.01% or less and had no effect on any of the parameters assayed.

Carrageenin was obtained from Marine Colloids, Inc., Springfield, N.J., USA. Standard PGE_2, TXB_2, LTB_4, cyclic AMP, AA, db-cyclic AMP and A23187 were obtained from Sigma Chemical Co., St. Louis, USA. Antisera against TXB_2 and PGE_2 were purchased from Bio-Yeda, Rehovot, Israel and LTB_4 antiserum from Wellcome Diagnostics, Beckenham. Radiolabelled cyclic AMP, PGE_2 and TXB_2 were obtained from Amersham International plc, Aylesbury, Buckinghamshire, and radiolabelled LTB_4 from Wellcome Diagnostics. Indomethacin and aspirin were purchased from the Pharmacy Department, Dijkzigt Hospital, Rotterdam, The Netherlands.

Statistical analysis

Statistical analysis was carried out by use of the Mann–Whitney U-test.

Results

The effect of indomethacin and aspirin on A23187-stimulated macrophage eicosanoid synthesis

A23187 (10^{-6} M) stimulated macrophage PGE_2, TXB_2 and LTB_4 synthesis and release (Table 1). Indomethacin and aspirin enhanced A23187-stimulated LTB_4 synthesis and inhibited A23187-stimulated PGE_2 and TXB_2 formation (Table 2).

Table 1 The effect of A23187 on rat peritoneal macrophage eicosanoid synthesis and release

Treatment	*PGE_2*	*TXB_2*	*LTB_4*
Control	4.60 ± 1.30	0.95 ± 0.35	<0.02
A23187 (10^{-6} M)	20.80* ± 4.50	6.15* ± 2.16	0.24* ± 0.17

Results are expressed as eicosanoid release in ng per 2×10^6 nucleated cells and are the mean ± s.d. in 9–11 experiments. PGE_2, prostaglandin E_2; TXB_2, thromboxane B_2; LTB_4, leukotriene B_4.
* $P < 0.05$ with respect to appropriate control values.

Table 2 The effect of indomethacin and aspirin on A23187-stimulated eicosanoid synthesis and release from rat peritoneal macrophages

Net eicosanoid release	*Indomethacin/Aspirin* (M)				
	0	*2×10^{-8}*	*2×10^{-7}*	*2×10^{-6}*	*2×10^{-5}*
Indomethacin					
PGE_2	8.85 ± 1.00	6.68 ± 1.46	5.58* ± 1.56	2.68* ± 1.04	2.41* ± 1.56
TXB_2	10.12 ± 2.84	8.70 ± 1.34	4.50* ± 1.26	1.19* ± 0.19	0.82* ± 0.39
LTB_4	0.38 ± 0.08	0.48 ± 0.08	0.62* ± 0.06	0.66* ± 0.07	0.53* ± 0.04
Aspirin					
PGE_2	22.87 ± 3.15	13.95* ± 6.19	11.98* ± 5.54	9.11* ± 5.75	2.05* ± 0.67
TXB_2	5.39 ± 1.85	5.90 ± 3.04	2.17* ± 0.80	1.89* ± 0.64	0.92* ± 0.45
LTB_4	0.15 ± 0.05	0.16 ± 0.04	0.26* ± 0.05	0.47* ± 0.11	0.44* ± 0.11

Results are expressed as ng net eicosanoid release from 2×10^6 nucleated cells. Data shows the mean ± s.d. in 7–9 experiments.
* $P < 0.05$ with respect to appropriate control.

These NSAIDs also inhibited the basal formation of cyclo-oxygenase metabolites (data not shown), but had no detectable stimulatory effect on the basal synthesis of LTB_4, which was below the level of detection of the RIA (0.02 ng ml^{-1}).

The effect of PGE_2 and NSAIDs on A23187-stimulated macrophage eicosanoid synthesis

PGE_2 (2.8×10^{-10} M–2.8×10^{-5} M) inhibited the A23187-stimulated synthesis and release of TXB_2

Table 3 The effect of prostaglandin E_2 (PGE_2) on A23187-stimulated eicosanoid synthesis and release in rat peritoneal macrophages

Net eicosanoid release	*PGE_2* (M 2.8)				
	0	*10^{-10}*	*10^{-8}*	*10^{-6}*	*10^{-5}*
TXB_2	18.4 ± 4.2	18.2 ± 1.6	9.7* ± 4.3	4.8* ± 0.5	3.5* ± 0.3
LTB_4	0.20 ± 0.06	0.23 ± 0.03	0.10* ± 0.03	0.05* ± 0.03	0.04* ± 0.04

Results are expressed as net eicosanoid release from 2×10^6 nucleated cells. Data shows the mean ± s.d. in 6 experiments.
* $P < 0.05$ with respect to appropriate control values.

Table 4 The effect of prostaglandin E_2 (PGE_2) on the indomethacin- and aspirin-dependent enhancement of A23187-stimulated leukotriene B_4 (LTB_4) synthesis and release

Treatment	*Indomethacin/Aspirin* (M)				
	0	*2×10^{-8}*	*2×10^{-7}*	*2×10^{-6}*	*2×10^{-5}*
Indomethacin					
A23187 (10^{-6} M)	100	118 ± 32	177* ± 19	194* ± 28	153* ± 18
A23187 + PGE_2 (2.8×10^{-5} M)	100	106 ± 12	113† ± 6	77† ± 13	67† ± 34
Aspirin					
A23187 (10^{-6} M)	100	110 ± 40	190* ± 21	366* ± 120	339* ± 59
A23187 + PGE_2 (2.8×10^{-5} M)	100	124 ± 30	170* ± 26	161*† ± 4	218*† ± 39

Results are expressed as the percentage of values obtained without indomethacin or aspirin and were calculated from three separate experiments.
* $P < 0.05$ control vs indomethacin or aspirin treated.
† $P < 0.05$ A23187 vs A23187 + PGE_2
Absolute values for LTB_4 release, expressed as ng per 2×10^6 nucleated cells, in the absence of indomethacin or aspirin, were as follows for the indomethacin experiments: A23187, 0.27 ± 0.05. A23187 + PGE_2, 0.20 ± 0.02 ($P < 0.05$ vs A23187); and for the aspirin experiments: A23187, 0.19 ± 0.09. A23198 + PGE_2, 0.14 ± 0.03.

Table 5 The effect of prostaglandin E_2 (PGE_2) on arachidonic acid (AA)-stimulated eicosanoid synthesis by rat peritoneal macrophages

Treatment	*TXB_2*	*LTB_4*
Control	0.98 ± 0.37	<0.02
AA (8×10^{-6} M)	11.48* ± 3.60	0.21* ± 0.10
AA + PGE_2 (2.8×10^{-5} M)	12.48* ± 2.20	0.28* ± 0.11

Results are expressed as mediator release in ng per 2×10^6 nucleated cells. Data are the mean ± s.d. in 8 experiments.
* $P < 0.05$ control vs AA or AA + PGE_2.

and LTB_4 in a concentration-related manner (Table 3).

In addition to inhibiting the eicosanoid release elicited by A23187 PGE_2 (2.8×10^{-5} M) also reversed the enhancing effect of indomethacin and aspirin on A23187-stimulated macrophage LTB_4 formation (Table 4). However, it had no effect on the synthesis of LTB_4 and TXB_2 following challenge with 8×10^{-6} M AA (Table 5).

Challenge of the cells with A23187 resulted in an elevation in intracellular cyclic AMP concentration and this was inhibited in a concentration-dependent manner by indomethacin (2×10^{-7} M–2×10^{-5} M) (Table 6). When cells were incubated for 30 min with db-cyclic AMP (5×10^{-7} and 5×10^{-5} M) during activation with A23187 there was an inhibition of the release of TXB_2 and LTB_4 (Table 7).

Discussion

For our experiments we used a non-physiological agent, A23187, to stimulate calcium flux. A23187 was used primarily as a leukotriene releasing agent so that we could investigate regulatory events associ-

Table 6 The effect of indomethacin on basal and A23187-stimulated rat peritoneal macrophage cycle AMP concentrations

	Indomethacin (M)			
Treatment	*0*	*2×10^{-7}*	*2×10^{-6}*	*2×10^{-5}*
Control	1.42 ± 0.16	1.10* ± 0.14	0.83* ± 0.19	0.79* ± 0.21
A23187 (10^{-6} M)	2.81† ± 0.04	1.96† ± 0.60	1.69* ± 0.64	1.01* ± 0.26

Results are expressed as pmol cyclic AMP per 2×10^6 cells.
Data are the mean ± s.d. in 6 experiments.
* $P < 0.05$ control vs indomethacin treated.
† $P < 0.05$ A23187 treated vs appropriate control.

Table 7 The effect of db-cyclic AMP on basal and A23187-stimulated thromboxane B_2 (TXB_2) and leukotriene B_4 (LTB_4) release

	db-Cyclic AMP (M)		
Eicosanoid	*0*	*5×10^{-7}*	*5×10^{-5}*
TXB_2			
Basal	100	105 ± 10	81* ± 7
A23187 (10^{-6} M)	100	75* ± 15	64* ± 11
LTB_4			
Basal	N/D	N/D	N/D
A23187 (10^{-6} M)	100	84* ± 8	73* ± 4

Results are expressed as the percentage of release obtained in the absence of db-cyclic AMP and were calculated using the percentage changes measured in 3 separate experiments. Data are the mean ± s.d.
* $P < 0.05$ control vs db-cyclic AMP treated.
Control values, expressed in ng per 2×10^6 nucleated cells, for basal and A23187-stimulated eicosanoid release were for TXB_2: basal 0.65 ± 0.27; A23187 19.71 ± 8.2; and for LTB_4: basal not done (ND); A23187 1.06 ± 0.48.

ated with LTB_4 formation, specifically the role played by PGE_2 in the mobilization and subsequent metabolism of AA to LTB_4, events thought to be associated with an increase in calcium flux. We feel that it is reasonable to assume that PGE_2 and the NSAIDs used would also modify the effect of other mediators which similarly stimulated AA turnover.

In this article we have shown that added PGE_2 inhibited A23187-stimulated LTB_4 synthesis. The lowest effective concentration of PGE_2 used (2.8×10^{-8} M) inhibited A23187-stimulated LTB_4 formation by 50% (Table 3). Basal and ionophore-stimulated rat peritoneal macrophages released about 10^{-8} M and 4×10^{-8} M PGE_2 respectively (calculated from data given in Table 1) indicating that endogenously formed PGE_2 could also play a role in regulating leukotriene synthesis. This extends our previous finding that PGE_2 inhibits the synthesis and release of TXB_2 and 6-keto-$PGE_{1\alpha}$ induced by carrageenin (Elliott *et al.*, 1985). PGE_2 also inhibited the further increase in LTB_4 formation observed when cells were incubated with A23187 together with indomethacin or aspirin. The finding that the NSAIDs promoted A23187-stimulated LTB_4 synthesis supports our contention that endogenously formed PGE_2 could have a regulatory function. However, we cannot say to what extent the stimulatory effect of the cyclo-oxygenase inhibitors on LTB_4 formation was due to removal of the inhibitory PGE_2. A switching of AA from the cyclo-oxygenase to the lipoxygenase path i.e. 'substrate shunting', could also have contributed to the increase observed. Basal synthesis of LTB_4 was too low to assay, even in the presence of aspirin and indomethacin. It would appear therefore that cyclo-oxygenase inhibitors can influence leukotriene formation only if the lipoxygenase enzyme is stimulated by some other agent, i.e. they are not direct activators of the lipoxygenase.

Interestingly, Docherty & Wilson (1987) found that neither aspirin nor ibuprofen (a NSAID with cyclo-oxygenase inhibitory activity), had an effect on A23187-stimulated LTB_4 formation by human neutrophils, although indomethacin had a stimulatory action. Human neutrophil LTB_4 release is sensitive to the inhibitory action of PGE_1 (Ham *et al.*, 1983), so that the reason for the lack of effect of aspirin and ibuprofen on LTB_4 production is not clear.

In our experiments PGE_2 inhibited both lipoxygenase (LTB_4) and cyclo-oxygenase (TXB_2) metabolite release. Furthermore, PGE_2 had no effect on AA stimulated LTB_4 or TXB_2 synthesis. It is unlikely therefore that PGE_2 acted on specific enzymes within the AA cascade. A more likely explanation is that PGE_2 limited the availability of AA. PGE_2 is thought to exert its immunosuppressive effects by stimulating cyclic AMP synthesis (Bonta & Parnham, 1982) and we found that db-cyclic AMP partially inhibited A23187-stimulated LTB_4 and TXB_2 formation. In support of this interpretation, carrageenin-stimulated eicosanoid synthesis has also been shown to be inhibited by db-cyclic AMP (Elliott *et al.*, 1985). Furthermore, we show in this paper that both PGE_2 and cyclic AMP synthesis were decreased when macrophages were incubated with indomethacin. This finding is consistent with the proposal that endogenously formed PGE_2 is important for the maintenance of macrophage cyclic AMP concentrations (Lim *et al.*, 1983). There are two conceivable mechanisms by which cyclic AMP and db-cyclic AMP, could reduce the amount of AA available to the different enzymes, stimulation of AA reacylation and inhibition of phospholipase (PL) activity. Indeed, Lapetina *et al.* (1981) reported that cyclic AMP stimulated the reincorporation of AA into platelet phosphatidylinositol and Hirata *et al.* (1984) demonstrated that cyclic AMP blocked deactivation of the PLA_2 inhibitory polypeptide, lipocortin, by agents such as A23187 and phorbol esters.

Interestingly, most of the NSAIDs, such as aspirin and indomethacin, which are used to treat certain chronic inflammatory conditions are thought to act, at least in part, by inhibiting the cyclo-oxygenase pathway (Brune & Rainsford, 1979). Schenkelaars & Bonta (1986) demonstrated that both aspirin and indomethacin enhanced leukotriene-stimulated macrophage lysosomal enzyme release. We have now shown that these two NSAIDs also enhance A23187-stimulated LTB_4 synthesis and that this effect is reversed by added PGE_2. Indomethacin has also been shown to promote neutrophil superoxide production although the authors suggested that this was due to an inhibition of diacylglycerol lipase activity (Dale & Penfield, 1987). Our results, together with other findings (Docherty & Wilson, 1987; Schenkelaars & Bonta, 1986) provide experimental evidence for the theoretical proposal of Rang & Dale (1987) that NSAIDs could, by inhibiting PGE_2 synthesis and stimulating leukotriene production, exacerbate tissue damage in the long term.

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The human astrocytoma cell line 1321 N1 contains M_2-glandular type muscarinic receptors linked to phosphoinositide turnover

Elizabeth A. Kunysz, [1]Anton D. Michel, Roger L. Whiting & Kris Woods

Department of Pharmacology, Syntex Research Centre (R2-101), 3401 Hillview Avenue, Palo Alto, CA, 94304, U.S.A.

1 Muscarinic receptors present in the human astrocytoma cell line 1321 N1 were characterized in radioligand binding studies and in functional studies of carbachol-stimulated phosphatidylinositol (PI) turnover.

2 In radioligand binding studies the muscarinic receptor in intact cells could be labelled using [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) but not by [^{3}H]-pirenzepine. In the intact cells these receptors displayed low pirenzepine affinity (pKi = 6.83) indicating that they were not of the M_1 subtype. Furthermore, the 1321 N1 muscarinic receptors displayed low affinity for the two M_2-cardiac selective ligands methoctramine (pKi = 5.82) and AF-DX 116 (pKi = 6.29). This pharmacology was consistent with the 1321 N1 cells containing a single population of muscarinic receptors that displayed a similar pharmacology to the M_2-receptor present in exocrine gland tissue.

3 The M_2-gland nature of the receptors was further indicated in the functional studies where antagonist affinities were determined from their ability to antagonize carbachol-stimulated PI turnover in 1321 N1 cells. pA_2 values for pirenzepine (7.31), methoctramine (6.10) and AF-DX 116 (6.52) were similar to those determined in the binding studies.

4 From these studies we conclude that 1321 N1 astrocytoma cells contain an M_2-gland muscarinic receptor which mediates muscarinic receptor-mediated stimulation of PI turnover in these cells.

Introduction

Muscarinic receptors mediate a diverse range of physiological actions including regulation of cardiac, exocrine gland, smooth muscle and CNS functions. Pharmacological studies have provided evidence that these events are not mediated through just one type of muscarinic receptor, rather they are mediated by at least two muscarinic receptor subtypes (Hammer *et al.*, 1980; Hammer & Giachetti, 1982). In addition to this heterogeneity of the muscarinic receptor it has also become clear that the subtypes of the muscarinic receptor can interact with multiple effector systems such as the adenylate cyclase and inositol phospholipid second messenger systems (Harden *et al.*, 1986) and may also interact with ion channels (Yatani *et al.*, 1987).

A question which has remained unresolved in recent years concerns whether a single receptor subtype couples to a single effector system or whether each muscarinic receptor subtype can interact with multiple effectors.

Hammer & Giachetti (1982) initially proposed that the M_1 and M_2 receptors identified in binding studies, and differentiated on the basis of their affinity for pirenzepine, interacted with discrete effector systems. In their scheme, the M_1 receptor was coupled positively to phosphatidyl inositol (PI) turnover and the M_2 receptor was coupled negatively to adenylate cyclase. Harden *et al.* (1986) have favoured such a system and have even proposed that muscarinic receptors be classified on the basis of their effector system (Hughes & Harden 1986; Hepler *et al.*, 1987).

One major problem with these studies has been the finding that in chick heart a muscarinic receptor with high pirenzepine affinity and therefore an M_1 receptor under the classification scheme of Hammer & Giachetti (1982) is coupled to adenylate cyclase (Brown *et al.*, 1985) while in the human astrocytoma

[1] Author for correspondence.

cell line 1321 N1 a muscarinic receptor with low affinity for pirenzepine and therefore an M_2 receptor by definition (Hammer & Giachetti, 1982) is coupled to PI turnover (Evans *et al.*, 1984; Brown *et al.*, 1985). On the basis of these pharmacological studies it would appear that the M_1 and M_2 muscarinic receptors can couple to either the PI pathway or to the adenylate cyclase system.

In recent years it has become evident that muscarinic receptors can no longer be classified as belonging to only two subtypes as originally proposed by Hammer & Giachetti (1982). Indeed, considerable evidence from radioligand binding studies has accumulated to indicate that at least three muscarinic receptor subtypes exist (Waelbrook *et al.*, 1986; De Jonge *et al.*, 1986). These have been termed M_1, M_2 and M_3 (De Jonge *et al.*, 1986) and correspond, respectively, to the M_1, M_2-cardiac and the M_2-glandular receptors. The M_1 receptor displays high pirenzepine affinity while M_2-cardiac and M_2-gland receptors display low affinity for pirenzepine. The M_2-cardiac and M_2-gland receptors can be differentiated on the basis of their affinity for AF-DX 116 (Hammer *et al.*, 1986), methoctramine (Michel & Whiting, 1988) and himbacine (Delmendo *et al.*, 1988).

With these recent findings in mind we have undertaken studies to reevaluate the receptor identity of the muscarinic receptors found in the human astrocytoma cell line 1321 N1. The data obtained indicate that the muscarinic receptor subtype present in these cells is of the M_2-gland subtype.

Methods

Cell culture

Human astrocytoma cells (1321 N1) were provided by Dr J.H. Brown, San Diego, U.S.A. Cells were cultured at 37°C in a humidified atmosphere (5% CO_2) as a monolayer culture using a low glucose DMEM/5% foetal calf serum medium. Cells were seeded at a density of 4×10^4 cells ml^{-1} and grown until confluent (usually 5–7 days). After 3 days the medium was replaced with fresh medium. Cells were harvested by incubating with trypsin (0.025%) for 4 min at 37°C. When cells were grown in 150 cm^2 flasks the yield of cells was in the order of 20–30 $\times 10^6$ cells per flask which provided enough cells for between 10–30 assay tubes. In some studies cells were harvested with a cell scrapper. In these studies cell viability was only 65% compared to >95% when using trypsin. In two experiments using the non-trypsin treated cells the binding parameters for pirenzepine, methoctramine and AF-DX 116 were not significantly different from those obtained with cells that were treated with trypsin.

Membrane preparations

Membrane preparation was performed as described previously (Michel & Whiting, 1987; Kunysz *et al.*, 1988). Briefly EDTA washed (Cheung *et al.*, 1982) cerebrocortical, cardiac and submaxillary gland membranes were prepared from 200–300 g male Sprague-Dawley rats. For some experiments 1321 N1 cells, harvested as described above, were also used. Tissues were homogenized in 50 mM Tris, 5 mM Na_2EDTA buffer (pH 7.4 at 4°C) with a polytron P10 tissue disrupter (setting 10; 2 × 10 s bursts). The homogenate was centrifuged at 48,000 *g* for 15 min. The pellet obtained was washed, by resuspension and centrifugation, once in homogenizing buffer and twice in 50 mM Tris, 0.5 mM EDTA buffer (pH 7.4 at 4°C). Membranes were stored under liquid nitrogen until required.

Binding assays

Binding assays were conducted as described previously (Michel & Whiting, 1987) with minor modifications. In all studies a Tris-Krebs assay buffer of the following composition was used (mM): NaCl 144, KCl 4.7, KH_2PO_4 1.7, $CaCl_2:(H_2O)_6$ 2.5, $MgCl_2$ 1.1, glucose 10, Tris 10, pH 7.4 at 37°C. Assays were conducted at 37°C in a final volume of 1 ml at pH 7.4. In all studies atropine (1 μM) was used to define non-specific radioligand binding (NSB). In competition studies the radioligand [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) was present at a fixed concentration of 0.1 nM while in saturation studies the concentration of [^{3}H]-NMS was varied between 0.02 and 4 nM. In competition experiments with [^{3}H]-pirenzepine, a fixed concentration of 0.5 nM was employed while in saturation studies the concentration of [^{3}H]-pirenzepine was varied between 0.05 and 80 nM.

Incubations were for 2 h at 37°C and were terminated by vacuum filtration over Whatman GF/B glass fibre filters using a Brandel 48 well cell harvester. After filtration the filters were washed with 15 ml of room temperature water in those studies using [^{3}H]-NMS and with ice cold water in studies where [^{3}H]-pirenzepine was used. The filters were pretreated with 0.1% polyethyleneimine 18 h before use in order to reduce filter binding of the radioligands. Radioactivity retained on the filters was determined by liquid scintillation counting.

Studies on inositol phosphate turnover

In these studies 1321 N1 cells were cultured in 12 well Costar plates. When the cells became confluent,

[^{3}H]-myo-inositol (1 μCi per well) was added to the medium and the cells were incubated for 18 h. After removing the medium the cells were washed once with 1 ml of oxygenated modified Krebs-Bicarbonate buffer of the following composition (mM): NaCl 118, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$: $(H_2O)_6$ 1.25, $MgCl_2$ 1.1, glucose 11. The cells were then incubated at 37°C for 30 min in 0.90 ml of the modified Krebs-bicarbonate supplemented with LiCl (5 mM) and the antagonist drugs when studied. After this pre-equilibration period, carbachol (100 μl) was added to the cells and allowed to act for a further 30 min at 37°C before terminating the reaction by aspirating the buffer and replacing with 1 ml of chloroform/methanol (1:2, vol:vol). After several minutes, the chloroform/methanol was transferred to a scintillation vial. To effect extraction of the inositol phosphates (IP) the wells were sequentially washed with 1 ml of water, 2 ml of chloroform/methanol (1:2, vol:vol), 1 ml of chloroform and 1 ml of water. The combined washings were shaken for 20 min before determination of total IP by a procedure based upon that of Minneman & Johnson (1984). Briefly, 2 ml of the aqueous layer of the cell washings were applied to Dowex columns (200–400 mesh in the formate form). Following two 10 ml washes with 5 mM myo-inositol, the inositol phosphates were eluted with 2 ml of 1 M ammonium formate in 0.1 N formic acid; 1.8 ml of this eluate was transferred to a scintillation vial containing 10 ml aquasol and counted.

Data analysis

All data were analysed by iterative curve fitting techniques. Saturation binding data were analysed with LIGAND (Munson & Rodbard, 1980) while competition binding data were analysed by a curve fitting programme (Michel & Whiting, 1984) based on the method of Parker & Waud (1971). In the latter case IC_{50} values were converted to K_i values using the Cheng-Prusoff approximation (1973). In the functional studies carbachol concentration-response curves (CRC) were analysed by iterative curve fitting techniques (based on the method of Parker & Waud, 1971) in order to determine agonist ED_{50} values and to determine the slope and maxima of the carbachol CRC. pA_2 values were calculated according to the method of Arunlakshana & Schild (1959). The pA_2 value and the slope of the Arunlakshana & Schild (AS) plot were calculated by linear regression analysis.

Materials

[^{3}H]-NMS (specific activity 72 Ci mmol^{-1}) and [^{3}H]-Pir (specific activity 87 Ci mmol^{-1}) were obtained from Amersham and New England Nuclear, respectively. [^{3}H]-myo-inositol (specific activity 15 Ci mmol^{-1}) was obtained from American Radiolabeled Chemicals Inc. Pirenzepine hydrochloride was obtained from Boehringer Ingelheim. Atropine sulphate was purchased from Sigma Chemical Company as were all chemicals and reagents used. 4-DAMP (4-diphenylacetoxy-N-methylpiperidine methiodide) was obtained from Research Biochemical Inc. AF-DX 116 (11-[[2-(diethylamino)-methyl]-1-piperidinyl]-acetyl-5,11-dihydro-6H-pyrido[2,3-b]-[1,4]benzodiazepine-6-one), CPPS (cyclohexylphenyl(2-piperidinoethyl)silanol), and methoctramine (N,N′-bis[6-[(2-methoxybenzyl)amino]-hexyl]-1,8-octanediamine tetrahydrochloride) were synthesized by J. Berger, Dr R. Clark and D. Repke (IOC, Syntex, Palo Alto). Himbacine was obtained from Dr Taylor, University of Sydney, Australia.

Results

Intact cell binding assays

[^{3}H]-NMS bound to intact 1321 N1 cells in a saturable manner and with high affinity (see below). Measurable levels of binding could be achieved using as little as 0.5×10^6 cells per assay tube, although in routine studies $1–2 \times 10^6$ cells were added to each assay tube.

In contrast to studies with [^{3}H]-NMS, it was not possible to detect any binding of [^{3}H]-pirenzepine to 1321 N1 cells using our filtration assay even when concentrations of 10 nM [^{3}H]-pirenzepine were used.

Binding of [^{3}H]-NMS to 1321 N1 cells was rapid and fully reversible at 37°C (data not shown). In two experiments equilibrium was attained within 30 min and was maintained for up to 4 h when using a concentration of 0.2 nM [^{3}H]-NMS. The half life for dissociation of [^{3}H]-NMS was 10 min in two experiments.

Saturation studies

Equilibrium saturation binding of [^{3}H]-NMS indicated that this ligand bound to an apparently homogeneous population of sites in the 1321 N1 cells. The K_d value was 0.21 ± 0.04 nM while the B_{max} was 23 fmol mg^{-1} protein (Table 1). The K_d for [^{3}H]-NMS in the 1321 N1 cells was marginally lower than in the submaxillary gland and cardiac membranes. In Table 1 saturation data obtained using rat membrane preparations are shown. As can be seen, [^{3}H]-NMS identified apparently homogeneous populations of sites in cardiac and submaxillary gland membranes while [^{3}H]-pirenzepine appeared to

Table 1 Binding parameters for muscarinic radioligands in the intact 1321 N1 cells and in rat membrane preparations

Preparation	K_d (nM)	B_{max} (fmol mg^{-1} protein)
1321 N1 cells	0.14 ± 0.014	23 ± 2
Cardiac membranes	0.47 ± 0.031	495 ± 21
Submaxillary gland membranes	0.26 ± 0.029	340 ± 48

In this table the radioligand used was [^{3}H]-N-methylscopolamine ([^{3}H]-NMS). In rat cerebral cortex membranes [^{3}H]-pirenzepine displayed a K_d of 14.8 ± 1.4 nM and a B_{max} of 1690 ± 230 fmol mg^{-1} protein.

Values shown are the best fit parameters obtained after fitting specific and total binding data either to models describing the binding of the radioligand to one or more specific binding sites or to a model which assumed binding in a cooperative manner. In addition, specific binding data were fitted to a model which assumed the presence of single or multiple populations of radioligand binding sites together with a non-specific component of binding. In all cases the data could only be described in terms of an interaction of the radioligand with homogeneous populations of sites in the respective preparations. Data are those obtained from fitting specific binding data. In each preparation the data are from 4–7 experiments. Values are the mean ± standard error of the mean.

identify a single population of muscarinic receptor binding sites in cerebrocortical membranes.

Competition studies

The affinity estimates for a series of compounds in inhibiting [^{3}H]-NMS binding in the intact 1321 N1 cells are shown in Table 2. In this Table, affinity estimates of the compounds for the three subtypes of muscarinic receptor identified in rat membranes are also presented for comparative purposes. In the membrane preparations the M_1 receptor of cerebral cortex was labelled with [^{3}H]-pirenzepine while the M_2 receptors present in cardiac and submaxillary gland membranes were labelled with [^{3}H]-NMS.

The pharmacology of the three subtypes of muscarinic receptor identified in the membrane preparations can be seen in Table 2. Methoctramine and himbacine were 100 and 10 fold, respectively, selective for the M_2-cardiac over the M_2-gland muscarinic receptor. Pirenzepine was nearly 100 fold more potent at the M_1 than at the M_2-cardiac receptor. The pharmacological differences between the M_1 and M_2-gland receptors were not as dramatic. Pirenzepine and methoctramine were both approximately 10 fold more potent at M_1 than at M_2-gland muscarinic receptors.

In the 1321 N1 cells, all of the compounds studied produced mass action displacement isotherms with Hill coefficients close to unity (Table 2). Given that these compounds displayed up to a 100 fold selectivity between muscarinic receptor subtypes in direct binding studies (Table 2) this would indicate that only one population of muscarinic receptors was being labelled by [^{3}H]-NMS in the 1321 N1 cells.

The pharmacological profile of the muscarinic receptors in the 1321 N1 astrocytoma cells was clearly dissimilar to that of the M_1 and M_2-cardiac muscarinic receptors depicted in Table 2 but showed striking similarities to the M_2-gland receptor. Thus, both methoctramine and pirenzepine, which could differentiate M_1 and M_2-cardiac muscarinic receptors, displayed approximately 10 fold lower potency for muscarinic receptors of 1321 N1 cells than for M_1 muscarinic receptors in rat cerebral cortex. These data indicated that the muscarinic receptors in the 1321 N1 cells were unlikely to represent M_1 muscarinic receptors. Furthermore, the 1321 N1 muscarinic receptors were also dissimilar to the M_2-muscarinic receptor of cardiac membranes since both AF-DX 116 and himbacine were some 5 fold, and methoctramine some 100 fold less potent at the 1321 N1 muscarinic receptor than at the M_2-cardiac muscarinic receptor. In marked contrast, affinity estimates of the ligands at the M_2-gland muscarinic receptor and at the 1321 N1 muscarinic receptor were almost identical. In membrane preparations of the 1321 N1 cells the pKi values obtained for AF-DX 116 (6.22 ± 0.08; $n = 3$), atropine (8.92 ± 0.06; $n = 3$), himbacine (6.72 ± 0.09; $n = 3$), methoctramine (5.82 ± 0.11; $n = 3$) and pirenzepine (6.81 ± 0.05; $n = 3$) were not significantly different from those obtained in the intact cells.

Functional studies of carbachol-stimulated IP accumulation

In the 1321 N1 cells, carbachol stimulated the accumulation of IP 3–4 fold above basal levels. The ED_{50} for this effect was 8 μM. At the concentrations studied, the muscarinic antagonists indicated in Table 3 were able to inhibit this response in a competitive manner as adjudged by their failure to alter either the slope or maximum of the carbachol CRC and by the demonstration of linear Arunlakshana-Schild (AS) plots for these compounds. The pA_2 values for pirenzepine, methoctramine, AF-DX 116 and himbacine were in close agreement with the pKi values obtained in the radioligand binding studies in the intact cells (Table 2). The functional affinity data are clearly not consistent with the presence of an M_2-cardiac muscarinic receptor in the 1321 N1 cells. For AF-DX 116 and himbacine the pA_2 values were almost identical to their M_1 muscarinic receptor

Table 2 Affinity of competing compounds for radioligand binding sites in 1321 N1 intact cells and in rat membrane preparations

	Membrane preparations			
Ligand	*Cerebral cortex* pKi nH	*Cadiac* pKi nH	*Submaxillary gland* pKi nH	*1321 N1 cells* pKi nH
AF-DX 116	6.55 (0.11) 1.05 (0.06)	7.10 (0.08) 0.92 (0.07)	6.10 (0.11) 1.04 (0.08)	6.29 (0.03) 1.09 (0.08)
Atropine	9.02 (0.09) 0.93 (0.05)	8.67 (0.05) 1.03 (0.10)	8.97 (0.07) 0.93 (0.07)	9.01 (0.05) 0.97 (0.04)
CPPS	8.44 (0.07) 1.02 (0.04)	7.37 (0.08) 1.08 (0.06)	7.73 (0.06) 0.94 (0.07)	7.69 (0.06) 1.04 (0.02)
4-DAMP	8.49 (0.03) 1.06 (0.03)	7.96 (0.03) 0.94 (0.04)	8.68 (0.08) 0.95 (0.04)	8.59 (0.06) 1.00 (0.03)
Himbacine	7.12 (0.08) 0.89 (0.08)	7.72 (0.11) 0.99 (0.03)	6.91 (0.09) 0.92 (0.06)	6.83 (0.11) 1.11 (0.07)
Methoctramine	6.91 (0.07) 1.07 (0.07)	7.86 (0.09) 0.94 (0.08)	6.10 (0.09) 1.07 (0.07)	5.82 (0.06) 0.94 (0.08)
Pirenzepine	7.81 (0.09) 0.98 (0.06)	6.35 (0.02) 0.97 (0.04)	6.81 (0.06) 1.05 (0.07)	6.83 (0.08) 1.04 (0.05)

In all studies the radioligand was [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) except for those using cerebrocortical membranes where the radioligand was [^{3}H]-pirenzepine.

All affinity values are expressed as the negative logarithm of the inhibitory affinity constant (K_i) and are in mol litre^{-1}. The Hill coefficient (nH) values shown were not significantly different ($P > 0.05$) from unity. Parameters shown represent the mean (±s.e.mean) from 4–7 experiments.

AF-DX 116 = 11-[[2-(diethylamino)-methyl]-1-piperidinyl]-acetyl-5,11-dihydro-6H-pyrido[2,3-6][1,4] benzodiazepine-6-one; CPPS = cyclohexylphenyl(2-piperidinoethyl)silanol; 4-DAMP = 4-diphenylacetoxy-N- methylpiperidine methiodide.

affinity and marginally (0.2 to 0.4 log units) different from their M_2-gland muscarinic receptor affinity. For pirenzepine, the pA_2 value was 0.5 log unit different from both the M_1 and M_2-gland muscarinic receptor affinity estimates obtained in binding studies. For methoctramine the pA_2 value was identical to the M_2-gland affinity.

Discussion

Previous studies in membrane preparations have not defined the muscarinic receptor subtype present in the 1321 N1 cells. It has been shown that pirenzepine displays similar affinity for both the 1321 N1 receptors and the high affinity pirenzepine binding site (presumably M_1 muscarinic receptor) found in cerebrocortical preparations (Evans *et al.*, 1984). However, in the intact cell the potency of pirenzepine appears to decrease and in both binding and functional studies the muscarinic receptors of 1321 N1 cells display low affinity for pirenzepine (Evans *et al.*, 1984; Brown *et al.*, 1985).

In the present study we used the cardiac M_2 muscarinic receptor subtype selective ligands AF-DX 116 (Hammer *et al.*, 1986), methoctramine (Melchiorre *et al.*, 1987; Michel & Whiting, 1988)

Table 3 pA_2 values for muscarinic antagonists in 1321 N1 cells

Compound	pA_2	*As slope*	*Concentrations tested* (μM)
AF-DX 116	6.52 ± 0.04	1.02 ± 0.04	1, 3, 10, 30
Himbacine	7.03 ± 0.07	1.15 ± 0.08	0.1, 0.3, 1, 3
Methoctramine	6.10 ± 0.06	0.95 ± 0.06	1, 3, 10, 30
Pirenzepine	7.31 ± 0.06	0.93 ± 0.05	0.1, 0.3, 1, 3

The parameters shown represent the mean (±s.e.mean) and were obtained by linear regression analysis of between 8 and 14 data points obtained using the concentrations of antagonist indicated. The AS slopes shown are not significantly different from unity ($P > 0.05$).

and himbacine (Gilani *et al.*, 1986) to characterize the muscarinic receptor of the 1321 N1 cells. The binding results with these ligands confirmed previous findings (Brown *et al.*, 1985) that the receptor does not appear to represent an M_1 receptor and indicated that the receptor was probably of the M_2-gland subtype. Thus, the receptor displayed a low affinity for the M_1 selective ligand pirenzepine (Hammer *et al.*, 1980) and also displayed very low affinity for methoctramine which can also differentiate between M_1 and M_2-cardiac receptors. These findings excluded the possibility that the receptors were of the M_1 subtype.

Methoctramine and himbacine are M_2-cardiac selective ligands that display a 10 and 100 fold, higher affinity at the M_2-cardiac than at the M_2-gland receptor (Delmendo *et al.*, 1989). The data obtained with these ligands indicated that the receptors were not of the M_2-cardiac subtype and therefore were probably of the M_2-gland subtype. This was also suggested by the close similarity of affinity estimates at the M_2-gland receptor present in submaxillary gland membranes and the muscarinic receptor of the intact 1321 N1 cells.

The data obtained in the functional studies provided further evidence that the muscarinic receptors present in the 1321 N1 cells were of the M_2-gland subtype. These studies provided definitive evidence that the receptor was not of the M_2-cardiac subtype since pA_2 values for all four ligands differed 10 fold from that expected of such a receptor.

It was, however, not easy to differentiate between the possibility that the receptor was an M_1 or an M_2-gland receptor. Thus, both himbacine and AF-DX 116 produced pA_2 values consistent with an M_1 pharmacology although the values differed by 0.2 and 0.4 from that expected of an M_2-gland receptor. For pirenzepine the pA_2 value was intermediate between the M_1 and M_2-gland values obtained in membranes although it was at least 10 fold lower than the affinity of pirenzepine for the M_1-receptor in cerebral cortex that is coupled to IP accumulation (Kunysz *et al.*, 1988). The most convincing evidence for the receptor representing an M_2-gland receptor was obtained with methoctramine which displayed an identical pA_2 at the 1321 N1 cells and at the M_2-gland receptor.

With regard to the difficulty in differentiating between M_1 and M_2-gland receptors, several factors should be borne in mind. Firstly, we have previously noted (Delmendo *et al.*, 1989) that there are no ligands which provide definitive separation of the M_1 and M_2-gland receptors. At best, methoctramine and pirenzepine display a 5–8 fold lower affinity for the M_2-gland than for the M_1 muscarinic receptors while AF-DX 116 and himbacine display low selectivity (2–3 fold) between M_1 and M_2-gland receptors. Secondly for receptor subtypes to be differentiated Furchott (1972) has suggested that at least a 3 fold difference in affinity should be apparent. With this caveat in mind, then it is clear that AF-DX 116 and himbacine would not be expected to differentiate M_1 and M_2-gland receptors. Since the data obtained using the more selective agents methoctramine and pirenzepine were more consistent with the presence of an M_2-gland receptor we feel that it is more likely that the muscarinic receptors of 1321 N1 cells linked to IP accumulation belong to the M_2-gland subtype.

Such a finding, that the muscarinic receptors of the 1321 N1 cells are of the M_2-gland subtype, may explain the difficulty in characterizing this receptor in previous studies since pirenzepine, which has been used exclusively for characterizing this receptor, displays intermediate affinity for the M_2-gland subtype of the muscarinic receptor.

Additional more indirect evidence in favour of the M_2-gland receptor nature of the astrocytoma 1321 N1 muscarinic receptor comes in the recent study of Liang *et al.* (1987). In that study the muscarinic receptor of the 1321 N1 cell line was shown to possess an apparent size of 92,000 Da which differed substantially from that obtained for the M_1 and M_2-cardiac muscarinic receptors. The M_r of the 1321 N1 muscarinic receptor was however similar to that reported for muscarinic receptors in exocrine glands (Hootman *et al.*, 1984) which appear to possess predominantly M_2-gland muscarinic receptors (De Jonge *et al.*, 1986; Delmendo *et al.*, 1989). It is also worth noting that gene sequence studies have identified 4 muscarinic receptor genes one of which has been predicted to exhibit a M_r of 90,000 daltons when glycosylated (Kerlavage *et al.*, 1987). It would be interesting to determine the pharmacology of this receptor, termed the m_3 receptor, when it has been expressed and characterized in order to compare it with the M_2-gland receptor identified in the 1321 N1 cells and in exocrine glands.

Returning to the issue of muscarinic receptor subtypes and their interaction with second messenger systems, the results of the present study would indicate that the M_2-gland receptor present in the 1321 N1 cells can couple to IP turnover. Incidently, if this is correct then the present study represents the first quantitative *in vitro* functional characterization of the M_2-gland receptor. Given the similarities between the M_2-gland muscarinic receptor identified in direct binding studies and the ideal muscarinic receptor identified in functional studies, a comparison of the present functional data on the M_2-gland muscarinic receptor with that obtained in the guinea-pig ileum could enable a direct comparison between these receptors in order to determine if they are separate entities or both belong to the same subtype. Such studies are now in progress.

The demonstration that the M_2-gland receptor subtype couples to PI turnover may indicate that the suggestion by Harden *et al.* (1986) that muscarinic receptors can be characterized by their second messenger system is valid. Thus, there is now ample evidence that M_1 muscarinic receptors couple to PI turnover (Gill & Wolfe, 1985; Lazereno *et al.*, 1985; Fisher & Bartus, 1986; Kunysz *et al.*, 1988). The interaction of the M_2-cardiac muscarinic receptor with the adenylate cyclase system is well established (Flemming *et al.*, 1987) while the present study has indicated that M_2-gland muscarinic receptors can stimulate IP production. It should of course be stressed that a more indirect inference concerning the ability of M_2-gland muscarinic receptors to stimulate PI turnover can be made from the binding classification of exocrine glands as containing M_2-gland muscarinic receptors (De Jonge *et al.*, 1986) and the well-established finding that muscarinic receptor agonists stimulate IP accumulation in exocrine glands (Berrige *et al.*, 1983).

It is still clear that there are receptors with low pirenzepine affinity (presumed to be M_2) which stimulate IP accumulation (Lazereno *et al.*, 1985; Fisher & Bartus, 1986) as well as receptors with high affinity for pirenzepine (presumed to be M_1 receptors) which attenuate adenylate cyclase activity in chick atria (Brown *et al.*, 1985). Perhaps, given the dual problems of classifying receptors using pirenzepine and the demonstration that two muscarinic receptor subtypes can stimulate IP production, further studies are required to reevaluate the pharmacology of the atypical receptors indicated above, in order to clarify the nature of the interaction of muscarinic receptors with effector systems.

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Neurotransmission in pig renal artery: the actions of angiotensin II and dopamine

D.R. Ferguson & [1]M.D. Randall

Department of Pharmacology, University of Cambridge, Hills Road, Cambridge CB2 2QD

1 Electrical stimulation of pig renal arteries causes contractions which are potentiated in the presence of angiotensin II (5×10^{-8} M).

2 The potentiation is followed by a long-lasting inhibitory phase which is mimicked by dopamine and 2-amino-6,7,dihydroxy-1,2,3,4,-tetrahydronaphthalene (ADTN), but blocked by sulpiride (5×10^{-8} M).

3 Release of noradrenaline from renal artery rings by 25 mM K^+ was measured by h.p.l.c. and found to be increased by 5×10^{-8} M angiotensin II. As this was done in the presence of 1×10^{-6} M desmethylimipramine it was concluded that the increase was due to increased neuronal release of noradrenaline, not inhibition of neuronal uptake.

4 The experiments indicate that dopamine is present in the artery wall and produces its effects through DA_2-receptors.

Introduction

It has been known since 1968 (Zimmerman & Gisslen) that angiotensin II potentiates the vasoconstrictor effects of peripheral sympathetic nerve stimulation. As angiotensin II is known to have a vasoconstrictor action in its own right, and to interact with the sympathetic nervous system there has been debate about the relative importance of its pre- and post-synaptic actions (Antonaccio & Kerwin, 1981). It has not been clear either whether the angiotensin II (AII) which acts on sympathetic nerve endings in large vessels diffuses in from the circulation, or is converted within the wall from angiotensinogen or angiotensin I (Clough *et al.*, 1982).

Presynaptic receptors for both noradrenaline and dopamine have long been recognised (Starke, 1977), but whereas a physiological role for such noradrenaline receptors is widely accepted, a similar role for dopamine on sympathetic nerves has been disputed (Langer, 1981). The present paper describes the results of experiments in which the potentiation of electrical stimulation of pig renal arteries by AII at concentrations below those required to cause contraction directly was demonstrated. This potentiation was followed by an inhibitory phase in which the release of endogenous dopamine seems likely to play a part. Finally high potassium Krebs solution was used to release noradrenaline from renal arteries in the presence or absence of AII and the released catecholamines measured chemically.

[1] Author for correspondence.

Methods

Transmural electrical stimulation of pig renal arteries was carried out essentially as described previously (Ferguson *et al.*, 1985). Briefly arteries were tied over a platinum wire electrode and surrounded by a platinum wire spiral, and stimulated with a Harvard Research Stimulator (50-7459). Stimulation parameters were rectangular pulses of 1 ms, 150 V for 30 s at from 5 to 40 Hz. The arteries were suspended in Krebs solution of the following composition: (mM) NaCl 124, KCl 15, $NaHCO_3$ 26, $CaCl_2$ 0.8, $MgCl_2$ 1.3, KH_2PO_4 1.4, glucose 10, pH 7.4, bubbled with 95% O_2/5% CO_2 (Normal Krebs). All drugs were administered into the organ bath and experiments performed at 37°C.

Measurement of noradrenaline release

Renal arteries were cut into small rings and threaded onto cotton so that arteries from different animals were randomized to control and experimental conditions. The rings were suspended in a 5 ml jacketed organ bath with a tap at the base to allow the

bathing fluid to be rapidly released into ice-cold siliconed tubes.

Stimulation of release of noradrenaline was achieved by addition to the bath of high K^+ Krebs solution of the following composition (mM): NaCl 115.4, KCl 23.6, other components as above. When noradrenaline was being released into either normal Krebs solution or high K^+ Krebs solution, the following were also included: bovine serum albumin 3×10^{-5} M, pargyline 1×10^{-4} M, desmethylimipramine 1×10^{-6} M, cystamine dihydrochloride 1.3×10^{-4} M, Na_2EDTA 2.7×10^{-5} M.

Separation of noradrenaline from the bathing fluid was effected by using Supelco LC-WCX SPE ion exchange columns. Dihydroxybenzylamine (DHBA) standards were added to the bathing solution to estimate recovery. Volumes (5 ml) of the bathing medium were added directly to the columns which were then washed with 1 ml H_2O, 250 μl 1 M NH_4OH, 1 ml H_2O, then eluted with 2×0.5 ml 0.2 M perchloric acid.

Noradrenaline and DHBA were assayed by high performance liquid chromatography (h.p.l.c.) on an octadecylsilane column in 0.025 M citric acid, 0.025 M Na_2HPO_4, 0.005 mM Na_2EDTA and 0.15 mM octane sulphonic acid pH 3.4, using electrochemical detection.

Drugs

2 - Amino - 6,7,dihydroxy - 1,2,3,4 - tetrahydronaphthalene HBr (ADTN) was obtained from Burroughs Wellcome Co, Research Triangle Park, N.C, U.S.A. Noradrenaline, dopamine, (−)-sulpiride, desmethylimipramine, cystamine, angiotensin II and pargyline were obtained from Sigma Chemical Co, Poole. Remaining chemicals of Analar grade came from Fisons plc, Loughborough.

Statistics

Results are expressed as means ± s.e.mean. Student's *t* test was used to test for differences between means. The results in Figure 2 were analysed by 2-way analysis of variance.

Results

Transmural stimulation of pig renal arteries between 5 and 20 Hz showed that the size of contractions increased with the frequency, but were still submaximal. When 5×10^{-8} M AII was present in the organ bath it increased the size of the contractions. At 40 Hz maximal contractions were produced and they were not potentiated by AII. It was possible to produce potentiation of noradrenaline release at AII concentrations which failed to cause contraction of the arterial muscle directly (Ferguson *et al.*, 1985).

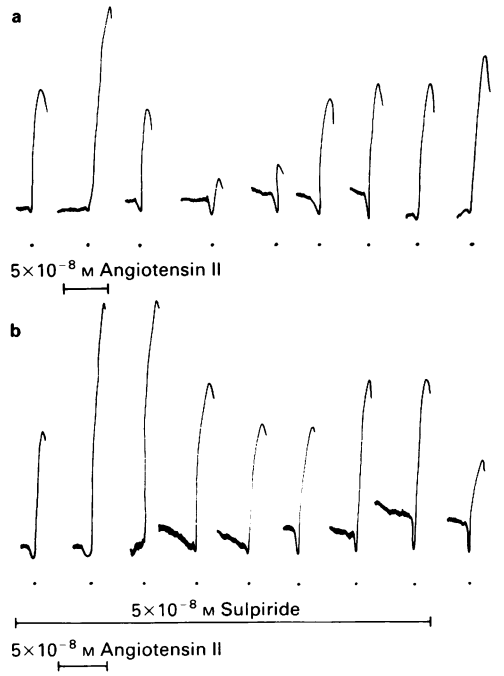

Figure 1 Contractions of the isolated renal artery of the pig in the presence (b) and absence (a) of sulpiride (5×10^{-8} M). Stimulus frequency 5 Hz, 30 s, with 10 min between each 30 s period. Angiotensin II (AII) was present in the bathing fluid as indicated by the shorter line. Note that the potentiation caused by AII is followed by inhibition which slowly recovers and that this effect is blocked by sulpiride.

If AII was washed from the organ bath and electrical stimulation continued at 10 min intervals using the same stimulus parameters, the responses became smaller than the initial values, but recovered after 40–50 min (Figure 1). This reduction in response following administration of AII was found to be blocked by (−)-sulpiride (5×10^{-8} M), and was mimicked by addition to the organ bath of dopamine (IC_{50} $1.31 \pm 0.14 \times 10^{-7}$ M). If the responses to electrical stimulation before addition of AII were taken as 100%, it was possible to compare control responses with those in the presence of sulpiride (Figure 2), there being significant differences 10 and 20 min after angiotensin II addition. The inhibitory phase could not be blocked by propranolol (1×10^{-7} M) or α-flupenthixol at concentrations between 1 and 4×10^{-7} M. The rigid dopamine analogue 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (ADTN) gave the same responses as dopamine. In experiments to compare the potency of

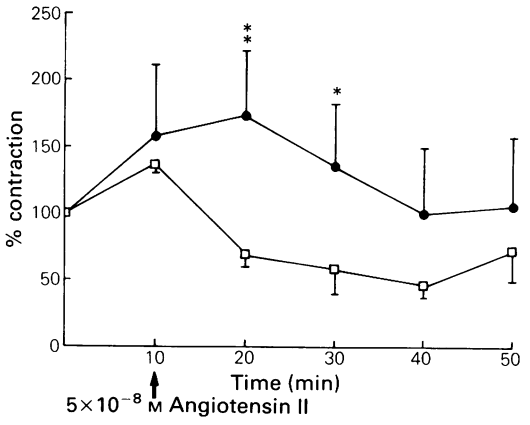

Figure 2 Summary of results obtained in the isolated renal artery of the pig using 10 Hz stimulation frequency for 30 s, in the absence and presence of sulpiride (5×10^{-8} M), with angiotensin II (5×10^{-8} M) present where indicated by the arrow. Control (□); plus sulpiride (●). The results analysed by 2-way analysis of variance show significant inhibition by sulpiride at times indicated by $*P < 0.05$ and $**P < 0.01$ ($n = 4$).

ADTN with dopamine in the same preparations the IC_{50} of dopamine was $1.3 \pm 0.14 \times 10^{-7}$ M, that of ADTN $4.7 \pm 0.01 \times 10^{-8}$ M ($n = 3$). It was concluded that dopamine was present in the arterial wall and inhibited the responses to electrical stimulation through DA_2-receptors (Woodruff, 1986).

Measurement of noradrenaline release from artery rings and the effects of angiotensin II

Initial experiments using known amounts of noradrenaline added to the bath showed that electrical stimulation for 30 s at 37°C resulted in its rapid degradation. However, the use of Krebs solution containing 25 mM potassium produced a submaximal sustained release of noradrenaline which could readily be measured. Artery rings were preincubated for 30 min in oxygenated Krebs solution containing pargyline 1×10^{-4} M at 37°C. They were then bathed for 5 min in the incubation medium containing normal potassium, bovine serum albumin, pargyline, cystamine and desmethylimipramine, followed by 3 min in the same solution containing 25 mM potassium. Both these media were collected and the noradrenaline extracted and assayed. The results are summarised in Table 1.

Discussion

AII has been demonstrated to increase release of noradrenaline both in the central nervous system (Huang *et al.*, 1987) and in the peripheral autonomic system (Ziogas *et al.*, 1985). The present work differs from that described previously because, released noradrenaline has been measured chemically rather than by measuring release of tritiated noradrenaline and its metabolites.

Recently, authors have doubted the relevance of the interaction between AII and sympathetic nervous activity in human subjects under physiological conditions (Nicholls *et al.*, 1981). Their experiments were based on infusion of AII or captopril and measurements of plasma catecholamine levels. Our work has uncovered some important mechanisms which indicate that such methods may not be successful. The first is the likelihood that AII is present within the arterial wall and may be released by electrical stimulation (Ferguson *et al.*, 1985); therefore uptake of AII from the circulation is not necessary. In fact it may not be possible for AII to diffuse from the circulation as far as the autonomic nerve plexuses in large arteries. The present work demonstrates that AII is effective in low concentrations. Experiments on human subjects on normal sodium intake demonstrate that the role of AII in cardiovascular regulation following postural changes is very localised (Lewis & Ferguson, 1986). If the interactions between AII and noradrenaline release in arteries are similarly localised, sampling mixed venous plasma from the forearm will not allow such interactions to be detected (Barrand & Callingham, 1981).

The results presented here confirm that AII potentiates release of noradrenaline from arterial sympathetic nerve terminals; as maximal concentrations of desmethylimipramine were present, this pre-

Table 1 Noradrenaline (NA) release from pig renal artery with or without angiotensin II

Bathing medium	*No angiotensin II*	*Angiotensin II* (5×10^{-8} M)	n	
Normal Krebs solution	(a) 5.6 ± 2.0	(b) 8.12 ± 3.5	4	(a) v (b) NS
High K^+ Krebs solution	(c) 11.8 ± 3.5 (a) v (c) $P < 0.05$	(d) 23.5 ± 5.2 (b) v (d) $P < 0.05$	4	(c) v (d) $P < 0.05$

NA released ng min^{-1} g^{-1} wet weight of artery, means ± s.e.mean, sig. of differences by *t* test. NS = not significant.

cluded an inhibition of neuronal uptake. Potentiation by AII is followed by an inhibitory phase of small responses which seem likely to be due to a presynaptic action of dopamine acting through DA_2-receptors: the evidence being that ADTN is more potent than dopamine, and the effect is blocked by sulpiride though not by doses of α-flupenthixol sufficient to block DA_1-receptors. As it is possible to stimulate arteries at 10 min intervals for long periods and to produce stable consistent contractions, it is possible that the dopamine actions are seen only after potentiation of noradrenaline release by AII.

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5-HT_1 agonists reduce 5-hydroxytryptamine release in rat hippocampus *in vivo* as determined by brain microdialysis

Trevor Sharp, Steven R. Bramwell & David G. Grahame-Smith

MRC Clinical Pharmacology Unit, University Department of Clinical Pharmacology, Radcliffe Infirmary, Woodstock Road, Oxford OX2 6HE

1 An intracerebral perfusion method, brain microdialysis, was used to assess changes of 5-hydroxytryptamine (5-HT) release in the ventral hippocampus of the chloral hydrate-anaesthetized rat in response to systemic administration of a variety of 5-HT_1 receptor agonists.

2 A stable output of reliably detectable endogenous 5-HT was measured in dialysates collected from ventral hippocampus with the 5-HT reuptake inhibitor, citalopram, present in the perfusion medium.

3 Under these conditions the putative 5-HT_{1A} agonist 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) caused a dose-dependent (5–250 $\mu g\,kg^{-1}$, s.c.) reduction of 5-HT in hippocampal dialysates.

4 Similarly, the putative 5-HT_{1A} agonists gepirone (5 $mg\,kg^{-1}$, s.c.), ipsapirone (5 $mg\,kg^{-1}$, s.c.) and buspirone (5 $mg\,kg^{-1}$, s.c.) markedly reduced levels of 5-HT in hippocampal perfusates whereas their common metabolite 1-(2-pyrimidinyl) piperazine (5 $mg\,kg^{-1}$, s.c.), which does not bind to central 5-HT_{1A} recognition sites, had no effect.

5 5-Methoxy-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H-indole (RU 24969), a drug with reported high affinity for brain 5-HT_{1B} binding sites, also produced a dose-dependent (0.25–5 $mg\,kg^{-1}$, s.c.) decrease of hippocampal 5-HT output.

6 These data are direct biochemical evidence that systemically administered putative 5-HT_{1A} and 5-HT_{1B} agonists markedly inhibit 5-HT release in rat ventral hippocampus *in vivo*.

Introduction

Radioligand binding studies have detected three pharmacologically distinct populations of 5-hydroxytryptamine (5-HT) recognition sites in rat brain tissue; 5-HT_1 sites bind [^{3}H]-5-HT with high affinity (Peroutka & Snyder, 1979), 5-HT_2 sites are preferentially labelled by [^{3}H]-spiperone (Leysen *et al.*, 1978; Peroutka & Snyder, 1979) and 5-HT_3 sites are recognised with high affinity by the ligand [^{3}H]-GR65630 (Kilpatrick *et al.*, 1987). The 5-HT_1 binding site has been further subdivided on the basis of evidence that [^{3}H]-5-HT binding sites display high and low affinity for spiperone, these being designated 5-HT_{1A} and 5-HT_{1B} recognition sites, respectively (Pedigo *et al.*, 1981). Additional 5-HT_1 subtypes, 5-HT_{1C} and 5-HT_{1D}, are also proposed (Pazos *et al.*, 1984; Heuring & Peroutka, 1987).

Currently, there are a number of ligands which show high selectivity for the 5-HT_{1A} binding site in rat brain including 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT), buspirone, and its structurally related compounds, gepirone and ipsapirone (Middlemiss & Fozard, 1983; Hall *et al.*, 1985; Hamon *et al.*, 1986; McMillen *et al.*, 1987; Traber & Glaser, 1987). Further, these drugs behave as central 5-HT_{1A} agonists or partial agonists in both biochemical (De Vivo & Maayani, 1986; Bockaert *et al.*, 1987) and electrophysiological (Andrade & Nicoll, 1987; Rowen & Anwyl, 1987) experiments. On the other hand there are drugs such as 5-methoxy-3-(1,2,3,6-tetrahydro-4-pyrindinyl)-1H-indole (RU 24969) which have high affinity for the 5-HT_{1B} binding site (Sills *et al.*, 1984; Hoyer *et al.*, 1985; Peroutka, 1986).

There is increasing evidence that 5-HT_{1A} and 5-HT_{1B} binding sites function as autoreceptors on rat 5-HTergic neurones ascending from the midbrain raphé nuclei into regions such as hippocampus

and cortex. For instance, putative 5-HT_{1A} agonists, both when systemically administered and microiontophoretically applied into the dorsal raphé nucleus, cause a marked reduction of the rate of firing of 5-HTergic neurones in the dorsal raphé nucleus (De Montigny *et al.*, 1984; Vander Maelen *et al.*, 1986; Blier & De Montigny, 1987; Sprouse & Aghajanian, 1987). These findings together with the observation that 5-HT_{1A} binding sites in the dorsal raphé nucleus are diminished by the neurotoxic lesion of brain 5-HT-containing neurones (Verge *et al.*, 1985; Weissmann-Nanopoulos *et al.*, 1985) indicate that the somatodendritic 5-HT autoreceptor is of the 5-HT_{1A} subtype. Conversely, data showing that the potency of 5-HT agonists in inhibiting the release of preloaded [^{3}H]-5-HT from cortical slices *in vitro* correlates better with their affinity for the 5-HT_{1B} than 5-HT_{1A} recognition sites, suggest that the nerve terminal 5-HT autoreceptor is of the 5-HT_{1B} subtype (Middlemiss, 1984a,b; Engel *et al.*, 1986).

Consistent with the idea of a 5-HT_1 autoreceptor are data showing that putative 5-HT_{1A} and 5-HT_{1B} agonists reduce regional rat brain 5-HT turnover and metabolism measured *ex vivo* (Hjorth & Carlsson, 1982; Hjörth *et al.*, 1982; Anderson *et al.*, 1987; McMillen *et al.*, 1987) and *in vivo* (Brazell *et al.*, 1985; Marsden & Martin, 1986). Additionally, Brazell *et al.* (1985) reported that RU 24969 reduced cortical 5-HT release measured in the intact rat using brain microdialysis (Ungerstedt *et al.*, 1982; Zetterström *et al.*, 1983), but studies on the effect of 5-HT_1 agonists on this more direct *in vivo* measure of brain 5-HT release are currently lacking. The aim of the present study was to determine whether putative 5-HT_{1A} agonists inhibit brain 5-HT release as assessed by the microdialysis technique, with the view that this method may prove to be a useful tool to probe the pharmacology of the central 5-HT autoreceptor *in vivo*. This work has been presented in a preliminary form to the British Pharmacological Society (Sharp *et al.*, 1988).

Methods

Dialysis probe preparation and implantation

Male Sprague Dawley rats (280–320 g) were anaesthetized with a bolus injection of chloral hydrate (360 mg kg^{-1}) and supplementary doses were given as the experiment progressed (approximately 60 mg kg^{-1} h^{-1}). A dialysis probe of the loop type (3 mm length, 270 μm diameter membrane, Cordis Dow Medical Inc., Brussels, Belgium) was prepared as previously described (Sharp *et al.*, 1986) and stereotaxically implanted into either ventral hippocampus (co-ordinates for probe tip; rostal-caudal −4.8 mm, lateral −4.6 mm, ventral −8.5 mm, from bregma and dura surface according to Paxinos & Watson, 1982) or medial frontal cortex (rostral-caudal +2.5 mm, lateral −0.6 mm, ventral −2.8 mm) and secured in place with dental cement and skull screws. The probe was continuously perfused (1 μl min^{-1}) with artificial CSF composition, mM: (NaCl 140, KCl 3, $CaCl_2$ 2.5, MgCl 1.0, Na_2HPO_4 1.2, NaH_2PO_4 0.27, glucose 7.2, pH 7.4) with or without the selective 5-HT reuptake inhibitor citalopram (1 μM). At the end of most experiments, brains were removed and frozen for examination of the cannula track in razor blade-cut fine sections. In all cases the track was correctly localized, terminating within the confines of either ventral hippocampus or medial frontal cortex.

Perfusate analysis

Perfusates were collected every 20 min and analysed directly for 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) using h.p.l.c. with electrochemical detection as described by Sharp *et al.* (1987). Separation was achieved by reversed-phase ion-pair chromatography conditions comprising a mobile phase of 0.15 M NaH_2PO_4, 0.1 mM EDTA, 12% (v/v) methanol, 0.01 mM sodium octane sulphonate, pH 4.0 with an Ultrasphere ODS column (150 × 4.6 mm). Detection was by a carbon paste or glassy carbon working electrode coupled to a BAS-4A electrochemical detector.

Experimental design

It was found that perfusion of the ventral hippocampus with artificial CSF containing citalopram 10^{-6} M provided dialysates with readily detectable and constant amounts of 5-HT (see Results) which enabled reliable determination of drug-induced decreases in 5-HT output. Under these conditions, 20 min dialysates were taken until similar amounts of 5-HT were detected in 2–3 consecutive samples (typically 120–160 min post probe implantation). Drug or saline vehicle was then administered subcutaneously, and dialysates were collected for a further 2 h.

Data analysis

5-HT in dialysates from rats treated with drug or saline is expressed as a percentage of the absolute amount of 5-HT contained in the dialysates collected immediately before drug or saline injection. Areas under the curve for the 2 h post injection period were calculated and statistical comparisons are made between the drug-treated groups and the saline-treated control group using Student's unpaired *t* test (two tailed) following analysis of variance where

appropriate. The approximate dose producing half the maximal observed effect was determined by extrapolation from plots of drug dose versus area under the curve data.

Drugs

Drugs with the following sources were used: 8-hydroxy-2-(di-n-propylamino)tetralin. HBr. (8-OH-DPAT, Research Biochemicals Inc., SEMAT, St. Albans), gepirone.HCL, buspirone.HCl and 1-(2-pyrimidinyl)-piperazine.HCl (1-PP; all from Bristol-Myers Co., Evansville, IN, U.S.A.), ipsapirone.HCl (TVX Q 7821, Troponwerke, Cologne, W. Germany) and 5-methoxy-3-(1,2,3,6-tetrahydro-4-pyridinyl)-IH-indole (RU 24969, ICI). All drugs were soluble in normal or slightly acidified (ipsapirone) saline and injected in a volume of 1 ml kg^{-1} (except RU 24969; 2 ml kg^{-1}).

Results

Basal levels of 5-HT and 5-HIAA in rat hippocampal and cortical dialysates

Endogenous 5-HT in dialysates collected from frontal cortex of the chloral hydrate-anaesthetized rat, in which artificial CSF was used as the perfusion medium, declined from high amounts detected 20–40 min following probe implantation (0.94 ± 0.1 (7) pmol 20 μl^{-1}, mean value ± s.e.mean (n)) to considerably lower amounts 5 h later (0.1 ± 0.04 (7) pmol 20 μl^{-1}) without reaching a constant level of output. In comparison, 5-HT in hippocampal dialysates declined from initially high amounts to a more constant output during the 2–4 h post implantation period (Figure 1) although levels of 5-HT during this time interval were considered to be too close to the limit of detection to assess accurately drug-induced reductions. Addition of citalopram (1 μM) to the perfusion medium raised 5-HT levels in hippocampal dialysates by about 3 fold with output being relatively constant 100–120 min after implantation (Figure 1). All experiments were subsequently carried out with citalopram present and in the hippocampus.

In contrast to 5-HT, basal levels of 5-HIAA in both cortical (12.07 ± 1.63 (7) pmol 20 μl^{-1} perfusate, 3 h post implantation) and hippocampal (17.7 ± 5.1 (7) pmol 20 μl^{-1} perfusate, 3 h post implantation) dialysates were constant over the initial 4 h of perfusion. Addition of citalopram (1 μM) to the perfusion medium tended to increase 5-HIAA in dialysates from hippocampus although this effect was not statistically significant (Figure 1). Introduction of citalopram to the perfusion medium following a 2 h control period also produced about a 4 fold rise of 5-HT with no consistent change in 5-HIAA in the dialysates (data not shown).

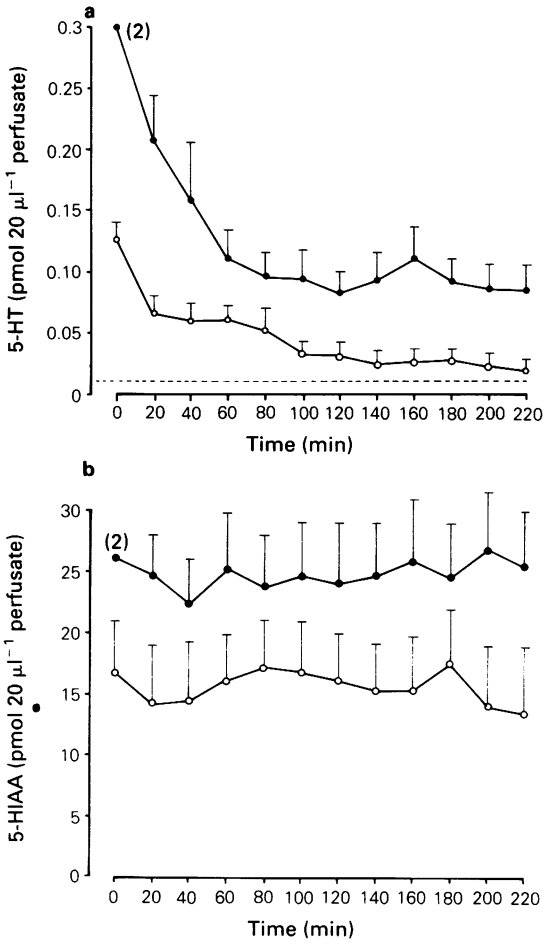

Figure 1 Basal amounts of 5-hydroxytryptamine (5-HT) (a) and 5-hydroxyindoleacetic acid (5-HIAA) (b) in 20 min dialysates collected from ventral hippocampus of the chloral hydrate-anaesthetized rat. Perfusion medium was artificial CSF with (●, 6 rats) or without (○, 5 rats) 1 μM citalopram. Dotted line represents limit of detection for 5-HT. Mean values are shown with s.e.mean indicated by vertical bars.

Effect of 8-OH-DPAT on 5-HT in hippocampal dialysates

In comparison to saline-treated controls, the 5HT$_{1A}$ agonist 8-OH-DPAT (5–250 μg kg^{-1}, s.c.) caused an immediate and dose-dependent decrease in 5-HT in dialysates of the ventral hippocampus (Figure 2). The doses 100 and 250 μg kg^{-1} reduced 5-HT output to about 75% of preinjection values within 40 min of administration and 5-HT output remained at this level for the following 80 min. The approximate dose

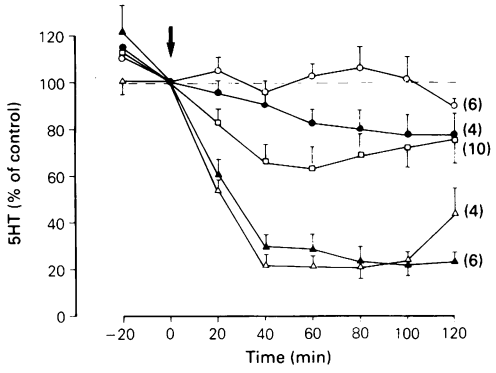

Figure 2 Effect of the 5-HT_{1A} agonist 8-hydroxy-2-(di-n-propylamino)tetralin HBr (8-OH-DPAT) on 5-hydroxytryptamine (5-HT) in rat ventral hippocampus dialysates. Saline (○) or 8-OH-DPAT, at doses 5 (●), 10 (□), 100 (△) and 250 (▲) $\mu g\,kg^{-1}$, were injected subcutaneously at time zero. 5-HT in dialysates is expressed as a percentage of the absolute amount of 5-HT in the dialysate collected immediately before injection. Each curve is constructed from mean time point values obtained from the number of rats shown in parentheses; s.e.mean shown by vertical bars.

of 8-OH-DPAT producing half the maximal observed effect was $10\,\mu g\,kg^{-1}$.

Effect of gepirone, buspirone, ipsapirone and 1-PP on 5-HT in hippocampal dialysates

Each of the putative 5-HT_{1A} agonists gepirone, buspirone and ipsapirone, at a dose of $5\,mg\,kg^{-1}$ s.c., markedly reduced 5-HT output in ventral hippocampus (Figure 3). A major metabolite of these compounds, 1-PP, which does not have significant affinity for 5-HT_{1A} binding sites (see Discussion), had no effect on 5-HT in hippocampal dialysates at the dose ($5\,mg\,kg^{-1}$, s.c.) and over the time (120 min) tested when compared to saline-injected controls (Figure 3, Table 1).

Effect of RU 24969 on 5-HT in hippocampal dialysates

RU 24969, a drug with high affinity for the 5-HT_{1B} binding site, dose-dependently ($0.25–5\,mg\,kg^{-1}$) decreased the amount of 5-HT in hippocampal dialysates (Figure 4). Note the slower onset of RU 24969 effect compared to the 5-HT_{1A} agonists. The highest dose tested ($5\,mg\,kg^{-1}$) reduced 5-HT levels to 78% of preinjection values 120 min postdrug. The approximate dose of RU 24969 producing half the maximal observed effect was $0.5\,mg\,kg^{-1}$.

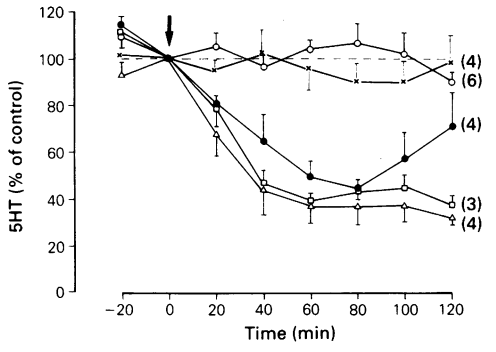

Figure 3 Effect of putative 5-HT_{1A} agonists on 5-hydroxytryptamine (5-HT) in rat ventral hippocampus dialysates. Saline (○) or gepirone (●), ipsapirone (□) and buspirone (△) and their common metabolite 1-(2-pyrimidinyl-piperazine HCl (1-PP, x), all at $5\,mg\,kg^{-1}$, were injected subcutaneously at time zero. See legend to Figure 1 for further details.

Effect of 5-HT agonists on 5-HIAA in hippocampal dialysates

Both 8-OH-DPAT and RU 24969 caused a decrease of 5-HIAA in hippocampal dialysates although these responses were considerably smaller (Table 1) and slower in onset (time course data not shown) than the change in 5-HT. In contrast to their effects on 5-HT output, neither gepirone, nor buspirone, nor ipsapirone reduced 5-HIAA output at the doses and over the time tested (Table 1). Similarly 1-PP had no effect on 5-HIAA in hippocampal dialysates.

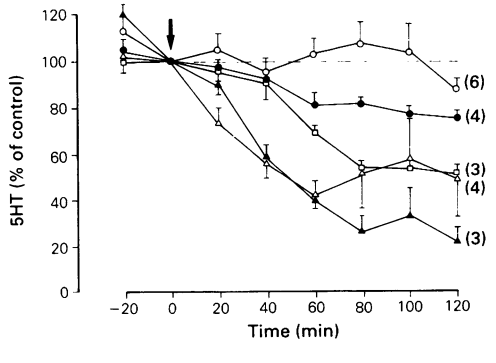

Figure 4 Effect of the putative 5-HT_{1B} agonist RU 24969 on 5-hydroxytryptamine (5-HT) in rat ventral hippocampus dialysates. Saline (○) or RU 24969, at doses 0.25 (●), 1.0 (□), 2.5 (△) and 5.0 (▲) $mg\,kg^{-1}$, were injected subcutaneously at time zero. See legend to Figure 1 for further details.

Table 1 Summary of effect of 5-hydroxytryptamine (5-HT) agonists on 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) in ventral hippocampus dialysates of the rat

Treatment	*5-HT* (AUC)	*5-HIAA* (AUC)
Saline	100.0 ± 4.8 (6)	100.0 ± 3.3 (6)
8-OH-DPAT (μg kg^{-1})		
5	83.0 ± 7.6 (4)	87.7 ± 1.2 (4)*
10	71.6 ± 9.2 (10)*	88.9 ± 3.4 (10)*
100	32.1 ± 5.6 (4)**	77.4 ± 2.2 (4)**
250	34.2 ± 10.6 (5)**	82.9 ± 3.1 (5)**
RU 24969 (mg kg^{-1})		
0.25	82.3 ± 3.6 (4)*	87.7 ± 3.1 (4)*
1	69.1 ± 2.1 (3)**	79.7 ± 5.3 (3)*
2.5	55.5 ± 17.3 (4)*	77.9 ± 1.4 (4)*
5	44.8 ± 8.9 (3)**	42.7 ± 1.8 (3)**
Gepirone 5 mg kg^{-1}	59.8 ± 9.7 (4)**	93.9 ± 4.2 (4)
Ipsapirone 5 mg kg^{-1}	48.8 ± 4.7 (3)**	89.5 ± 8.9 (3)
Buspirone 5 mg kg^{-1}	43.5 ± 16.3 (4)**	90.6 + 9.6 (4)
1-PP 5 mg kg^{-1}	94.4 ± 7.7 (4)	95.6 ± 5.9 (4)

Areas under the curves (AUC) are the accumulated amounts of 5-HT or 5-HIAA in dialysates collected over the 2 h post injection period and are expressed as a percentage of the saline-injected control group. Mean values ± s.e.mean (n = number of rats) are shown. *$P < 0.05$, **$P < 0.01$ versus saline treated group (unpaired t test on raw data following 1-way ANOVA).
1-PP = 1-(2-pyrimidinyl)-piperazine HCl.

Discussion

The present study demonstrates that systemic administration of the putative 5-HT$_{1A}$ agonists 8-OH-DPAT, gepirone, buspirone and ipsapirone markedly reduces 5-HT in dialysates of the ventral hippocampus of the chloral hydrate-anaesthetized rat. This finding is direct biochemical evidence that 5-HT$_{1A}$ agonists decrease hippocampal 5-HT release *in vivo*, and it is probable that this is due to activation of somatodendritic 5-HT$_{1A}$ autoreceptors causing a reduction of 5-HTergic impulse flow. Thus, electrophysiological studies have shown that microionotophoretic application of 8-OH-DPAT, gepirone, buspirone or ipsapirone into the rat dorsal raphé nucleus, which is rich in 5-HT$_{1A}$ binding sites localized on 5-HTergic neurones (Verge *et al.*, 1985; Weismann-Nanopoulos *et al.*, 1985), causes a reduction in the rate of 5-HTergic cell firing similar to that seen when these drugs are administered systemically (Sprouse & Aghajanian, 1986; Vander-Maelen *et al.*, 1986; Blier & De Montigny, 1987). It seems less likely that nerve terminal 5-HT autoreceptors mediate the effects of 5-HT$_{1A}$ agonists on 5-HT release since 8-OH-DPAT, gepirone and ipsapirone are reported to have weak or little effect on the release of [^{3}H]-5-HT from rat hippocampus and cortex *in vitro* (Middlemiss, 1984a; Engel *et al.*, 1986; this laboratory, unpublished observations).

Although our data show that four drugs, which have high selectivity for the 5-HT$_{1A}$ versus 5-HT$_{1B}$, 5-HT$_2$ and other neurotransmitter binding sites (see Introduction), reduce 5-HT in ventral hippocampal dialysates, the pharmacological identity of the receptor mediating these effects awaits further characterization. A recent study reported that the α_2-adrenoceptor antagonist, idazoxan, prevents the decrease of 5-HT metabolism in the rat suprachiasmatic nucleus induced by 8-OH-DPAT (Marsden & Martin, 1986). Also, buspirone, gepirone and ipsapirone, as well as their common metabolite 1-PP, have some affinity for the α_2-adrenoceptor binding site in rat cortex (Rimele *et al.*, 1987). Our finding that 1-PP, which has little affinity for the 5-HT$_{1A}$ binding site (Traber & Glaser, 1987), had no effect on 5-HT output in ventral hippocampus, strengthens the idea that the decrease in 5-HT output produced by its parent compounds and 8-OH-DPAT is mediated through 5-HT$_{1A}$ receptors and not α_2-adrenoceptors.

Various effects of 8-OH-DPAT and the buspirone-related compounds in rats are thought to relate to reduced central 5-HTergic neurotransmission following activation of somatodendritic 5-HT$_{1A}$ autoreceptors; increased feeding (Dourish *et al.*, 1986), hypothermia (Goodwin *et al.*, 1987 but see Hutson *et al.*, 1987), anxiolysis (Traber & Glaser, 1987) and possibly increased sexual activity (Ahlenius *et al.*, 1981). Interestingly, the functional effects of 8-OH-DPAT which are believed to correlate with decreased brain 5-HT function, as well as the 8-OH-DPAT-induced decrease of hippocampal 5-HT output (present study), occur at doses of 8-OH-DPAT well below those that induce the postsynaptically-mediated 5-HT behavioural syndrome.

The putative 5-HT$_{1B}$ selective agonist, RU 24969, also dose-dependently reduced 5-HT levels in hippocampal dialysates. This finding is consistent with a previous report that RU 24969 decreases 5-HT in rat cortical dialysates (Brazell *et al.*, 1985). On the basis of biochemical evidence that the nerve terminal 5-HT autoreceptor is of the 5-HT$_{1B}$ subtype

(Middlemiss, 1984a,b; Engel *et al.*, 1986) and electrophysiological data showing that systemic and intraraphé injection of the putative 5-HT_{1B} agonists mCPP and TFMPP has little effect on the firing of 5-HTergic neurones in the rat dorsal raphé nucleus (Sprouse & Aghajanian, 1987), it would seem reasonable to assume that the effect of RU 24969 on hippocampal 5-HT output relates to activation of the nerve terminal 5-HT_{1B} autoreceptor in hippocampus. Indeed, local administration of RU 24969 (10^{-5} M) into hippocampus via the perfusion medium markedly reduces 5-HT in the dialysates (unpublished data). However, while there is little evidence from functional studies that RU 24969 activates central 5-HT_{1A} receptors (e.g. Goodwin & Green, 1985; Dourish *et al.*, 1986; Tricklebank *et al.*, 1987) this drug has significant affinity for the 5-HT_{1A} binding site (Peroutka, 1986; Hamon *et al.*, 1986). Therefore, whether RU 24969 reduces 5-HT released from the hippocampus *in vivo* through stimulation of 5-HT autoreceptors on the 5-HT nerve terminals without involving those on the 5-HT cell body, remains an open question.

In the present study it was found necessary to add the selective 5-HT uptake blocker, citalopram, to the perfusion medium to provide readily measurable, constant amounts of 5-HT in the hippocampal dialysates. It has recently been reported that 5-HT in rat striatal dialysates collected on the day of probe implantation, but without a 5-HT reuptake inhibitor in the perfusion medium, predominantly originates from the blood (Kalèn *et al.*, 1988). However, in view of our finding of a marked decrease of 5-HT in hippocampal dialysates in response to the 5-HT_{1A} agonists, and the known potent inhibitory actions of these drugs on 5-HTergic cell firing, it seems highly likely that a major component of extracellular 5-HT measured under our conditions arises from central 5-HT neurones. Furthermore, we recently observed that local infusion of tetrodotoxin, a drug that inhibits action potentials, causes a marked decrease of 5-HT in the hippocampal dialysates (Sharp *et al.*, unpublished).

Both 8-OH-DPAT and RU 24969 reduced extracellular 5-HIAA in rat hippocampus, which is consistent with previous experiments on the effect of these drugs on brain 5-HT metabolism (Hjorth *et al.*, 1982; Brazell *et al.*, 1985; Marsden & Martin, 1986). It is notable that the responses of 5-HIAA to 8-OH-DPAT and RU 24969 were small compared to those of 5-HT. Furthermore, while gepirone, ipsapirone and buspirone markedly reduced 5-HT output, they had no significant effect on 5-HIAA output at the doses and over the time-course tested. Given that the basal amounts of 5-HIAA in the hippocampal dialysates are much higher than 5-HT, the proportionate reductions in 5-HIAA in response to the 5-HT agonists are likely to be much smaller. Also, extracellular 5-HIAA need not necessarily change in close accordance with extracellular 5-HT if, as previously proposed (Grahame-Smith, 1974; Kuhn *et al.*, 1985), a significant part of 5-HIAA is derived from the catabolism of unreleased 5-HT. Our finding that the presence of a 5-HT reuptake inhibitor, while increasing 5-HT overflow, did not reduce 5-HIAA, is consistent with the latter hypothesis.

In conclusion, brain microdialysis was used to provide direct biochemical evidence that putative 5-HT_{1A} and 5-HT_{1B} agonists reduce 5-HT release in the rat ventral hippocampus *in vivo*. Whilst further characterization of the drug responses is necessary, it seems likely that the microdialysis method will be a useful approach to probe 5-HT autoreceptor function *in vivo*.

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The classification of prostaglandin DP-receptors in platelets and vasculature using BW A868C, a novel, selective and potent competitive antagonist

Heather Giles, [1]P. Leff, Mary L. Bolofo, *M.G. Kelly & *A.D. Robertson

Analytical Pharmacology Group, Department of Pharmacology and *Department of Medicinal Chemistry, Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS

1 BW A868C, a novel compound, behaved as a simple competitive antagonist in a human washed platelet aggregation assay. Anti-aggregatory concentration-effect curves to BW 245C were displaced in a parallel manner. The shifts accorded with a Schild plot slope of unity and a pK_B of 9.26.

2 Inhibition of platelet aggregation by prostaglandin D_2 (PGD_2) was antagonized with a similar potency, as were the relaxation effects of BW 245C and PGD_2 in the rabbit jugular vein. BW A868C can, therefore, be classified as a DP-receptor antagonist.

3 Actions of BW A868C at other prostaglandin receptors (IP, EP_1, EP_2, TP and FP) were excluded at concentrations up to 1,000 times higher than the DP-receptor affinity.

4 Analyses of BW 245C- and PGD_2-mediated effects were complicated by additional agonist receptor interactions which were revealed by BW A868C. In rabbit jugular vein a resistant phase of agonism was detectable, indicating that both agonists exerted effects through another receptor (possibly EP_2). Also, PGD_2, in addition to its anti-aggregatory effect on platelets, demonstrated a pro-aggregatory action in the presence of BW A868C.

5 The contractile effects of PGD_2 in guinea-pig tracheal strips were resistant to 10 μM BW A868C indicating that they were not mediated through DP-receptors.

6 To our knowledge this is the first account of a well-classified competitive antagonist at the DP-receptor. Its potency and selectivity make it an important new tool in prostanoid receptor classification and identification.

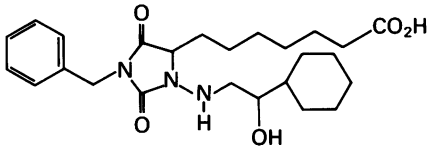
Introduction

The classification of prostaglandin receptors has in general been hampered by the lack of selective agonists and antagonists. It has been proposed (Kennedy *et al.*, 1982; Coleman *et al.*, 1984) that distinct receptors types exist for each of the naturally occurring prostaglandins: a thromboxane, TP-receptor; a prostacyclin, IP-receptor; a prostaglandin D_2, DP-receptor; a prostaglandin $F_{2\alpha}$, FP-receptor and at least two prostaglandin E_2 receptors, EP_1 and EP_2; more recently the same workers have invoked the presence of a third E type receptor, EP_3 (Coleman *et al.*, 1987b,c). This scheme was based originally on the discontinuity of potency orders of the naturally occurring agonists in different tissues. However, as its proponents explain (Coleman *et al.*, 1984), identification of a particular receptor type on this basis is compromised by the multiplicity of actions that these prostaglandins exhibit. Although this classification scheme was based in part on antagonist information, SC-19220 and AH19437 were claimed to antagonize selectively EP_1- and thromboxane-receptors respectively, selective antagonists for the majority of the putative receptor types remained to be discovered.

In the case of the claimed DP-receptor, several antagonists have been reported, among them NO164 (MacIntyre & Gordon, 1977), diphloretin phosphate (Westwick & Webb, 1978), desacetyl-1-nantradol (Horne, 1984), and AH6809 (Keery & Lumley, 1985), but none of them possess the selectivity or potency necessary for use in the quantitative classification of receptors.

In this paper, we describe the pharmacological actions of BW A868C, (3-benzyl-5-(6-carboxyhexyl)-

[1] Present address: Dept. of Pharmacology, Fisons Research and Development Labs., Bakewell Road, Loughborough, Leicestershire LE11 0RH.



BW A868C

Figure 1 Chemical structure of BW A868C: The synthesis and chemical properties of BW A868C will be described in a separate publication.

1-(2-cyclohexyl-2-hydroxyethylamino)-hydantoin) a novel, 3-benzyl substituted hydantoin (Figure 1), in human washed platelets and rabbit vasculature. We demonstrate that this compound behaves as a highly potent, simple competitive antagonist at the DP-receptor in platelets and vasculature with negligible actions at the other claimed prostaglandin receptors.

Methods

Washed human platelets

Washed platelet suspensions were used rather than platelet-rich plasma in order to avoid possible errors in the analysis of drug action caused by plasma-protein binding. Suspensions were prepared according to a modified version of the method of Vargas *et al.* (1982).

Blood was drawn from healthy male and female volunteers who had not taken any drugs known to affect platelet function for at least two weeeks. Blood was collected from the antecubital vein into (3.15% w/v) sodium citrate in proportion 9:1 v/v. The blood was centrifuged for 20 min at 225 *g* and the resulting supernatant platelet-rich plasma was collected. PGI_2 (0.3 $\mu g\,ml^{-1}$) was added before further centrigugation at 760 *g* for 10 min. The platelet-poor plasma supernatant was then discarded and the platelet pellet gently resuspended in calcium-free Tyrode buffer pH 7.4 of the following composition (mM): NaCl 136.89, $NaHCO_3$ 11.90, KCl 2.68, NaH_2PO_4 0.42, $MgCl_2$ 1.05, glucose 5.55, warmed to 37°C. PGI_2 (0.3 $\mu g\,ml^{-1}$) was added again and a final centrifugation, 400 *g* for 10 min, was performed. The Tyrode buffer was then removed and the platelets resuspended in a volume of fresh buffer sufficient to give a platelet concentration of $2 \times 10^8\,ml^{-1}$.

The suspension was stored at 4°C for 2 h. Before use the following additions were made: Ca^{2+} 1 mM, indomethacin 5 $\mu g\,ml^{-1}$, fibrinogen 400 $\mu g\,ml^{-1}$. Indomethacin was used in all assays to prevent production of cyclo-oxygenase products which could compromise analysis of exogenous drug effects. Aggregation responses were measured in 0.5 ml aliquots of the suspension maintained at 37°C and stirred with a metal bar at 900 r.p.m. in 300 BD-S Payton dual channel aggregometers and recorded on Gould BS 272 pen recorders.

Rabbit jugular veins

External jugular veins were removed from male New Zealand White rabbits (2.4–3.0 kg) killed by injection of phenobarbitone sodium (Sagatal, 60 $mg\,kg^{-1}$) into a marginal ear vein. The vessels were cannulated, which caused the removal of the endothelium, and then cleared of adhering tissue.

Three ring segments, approximately 5 mm wide, were obtained from each vein. Each ring was suspended between two wire hooks, one attached to a Grass FT03C force displacement transducer and the other to a stationary support in a 20 ml organ bath containing Krebs solution pH 7.4 of the following composition (mM): NaCl 118.41, $NaHCO_3$ 25.00, KCl 4.75, KH_2PO_4 1.19, $MgSO_4$ 1.19, glucose 11.10, $CaCl_2$ 2.50 and indomethacin 1 $\mu g\,ml^{-1}$. This was maintained at 37°C and continuously gassed with 95% O_2:5% CO_2. Tissue responses were measured as changes in isometric force and recorded on Gould BS 272 pen recorders.

Guinea-pig trachea

Male Dunkin Hartley guinea-pigs (400 g) were killed by a blow to the head and the trachea was removed and cleared of fat and connective tissue. The trachea was cut open longitudinally to expose the trachealis muscle. Transverse strips containing 3 cartilage rings were cut and suspended by cotton thread in the organ bath, as described above. Responses were measured as changes in isometric force.

Experimental protocols

Human washed platelets: In most experiments prostaglandin receptor agonists were incubated in the platelet suspension for 6 min before addition of ADP (50 μM). The reproducibility of the ADP-induced aggregation response was checked at intervals throughout the experiment. When an antagonist was used it was added 2 min before the agonist. Responses to agonists were expressed as percentage inhibitions of the standard ADP-induced aggregation. The total volume of solutions added to the suspension was always less than 5% of the platelet suspension volume.

In experiments with PGD_2 the order of addition of agents was reversed. A steady level of aggregation was induced by ADP (50 μM) before addition of PGD_2. Antagonist was incubated in the suspension for 5 min before ADP was added.

Rabbit jugular vein: These experiments followed a paired curve design. This was necessary due to substantial inter-tissue variation in the position of prostaglandin receptor agonist concentration-effect curves. Also the thromboxane antagonist BM 13.177 (Patscheke *et al.*, 1984) was routinely used in order to inhibit thromboxane receptor-mediated effects which occurred at higher concentrations of the agonists. Data illustrating the effect of BM 13.177 on responses to PGD_2 are shown in Figure 2, together with the results of an experiment indicating that BM 13.177 acts as a competitive antagonist of U46619, the stable thromboxane-mimetic (Coleman *et al.*, 1980a), in the jugular vein assay, demonstrating the same affinity as described previously for its effects in rabbit aorta and human platelets (Patscheke *et al.*, 1984).

Initially, jugular vein ring segments were subjected to a force of 0.75 g then allowed to stabilize for a period of 60 min during which time preparations were washed three times. BM 13.177 (30 μM) was incubated for 60 min, then histamine (1 μM) was added in order to produce tone. After establishing a stable contraction, relaxation concentration-effect curves were obtained using prostaglandin receptor agonists. Responses were expressed as percentage relaxations of the histamine-induced force. Preparations were then washed thoroughly and allowed to re-stabilise. After re-introduction of BM 13.177, antagonists, when used, were incubated for 60 min. The preparations were contracted again with histamine (1 μM) and a second prostaglandin agonist concentration-effect curve was obtained.

A separate experiment established that repeated histamine concentration-effect curves were superimposable (data not shown).

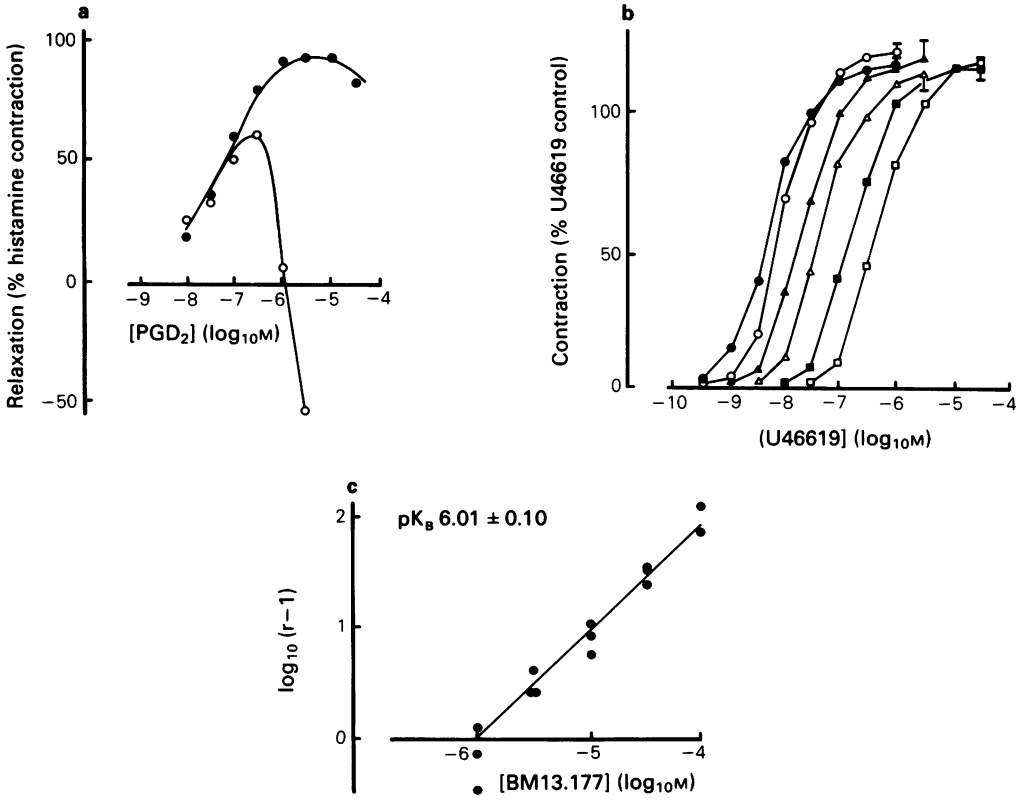


Figure 2 (a) Representative prostaglandin D_2 (PGD_2) concentration-effect curves in rabbit jugular vein in the absence (○) and presence (●) of 30 μM, BM 13.177. (b) Antagonism of U46619 effects in rabbit jugular vein by BM 13.177 at the following concentrations (number of replicate curves in parentheses): zero (●), (3); 1 μM (○), (3); 3 μM (▲), (3); 10 μM (△), (3); 30 μM (■), (3); 100 μM (□), (2). Error bars denote standard errors. (c) Schild plot for the antagonism of U46619 by BM 13.177. Analysis of [A_{50}] values by Equation (2) showed that n, the slope parameter was not significantly different from unity, and the line drawn through the data set was obtained by fitting the data to Equation 2 with n constrained to unity.

The experiment which demonstrated the effects of BM 13.177 on U46619 contractions was conducted by a one curve per preparation design. U46619 was used to provide a reference contraction then, following thorough washing BM 13.177 or vehicle was added to the organ baths for 60 min before obtaining cumulative U46619 concentration-effect curves.

Guinea-pig trachea: An initial force of 1 g was applied to the tissues. After stabilization the tissues were challenged with 10 μM histamaine to establish viability and to provide a reference contraction that was used to normalise subsequent agonist responses. After thorough washout of histamine, BW A868C or vehicle was added to the organ bath 60 min before cumulative addition of PGD_2. A single concentration-effect curve was obtained from each preparation. Contractile responses were expressed as a percentage of the histamine control response.

Rat fundic strip and guinea-pig longitudinal ileum muscle These tissues were used for exclusion assays and were set up in a similar manner to that already described for rabbit jugular vein. In both cases atropine sulphate 0.4 μM was added to the bathing medium. Single cumulative concentration-effect curves were performed on each tissue.

Analysis of data

Curve fitting Each concentration-effect curve data set was fitted to a logistic function of the form:

$$E = \frac{\alpha[A]^m}{[A_{50}]^m + [A]^m} \quad (1)$$

in which α, $[A_{50}]$ and m are the asymptote, location and slope parameters respectively. Location parameters were actually estimated as $-\log_{10}[A_{50}]$.

Analysis of antagonism: single curves designs: A one-way analysis of variance tested for treatment effects on the computed estimates of α and m. In the platelet study inhibition of aggregation greater than 100% of the ADP aggregation cannot be obtained therefore analysis of variance on the asymptote (α) was inappropriate. Tests for parallelism relied on slope analysis in this case. If the antagonist did not significantly modify these parameters then computed $\log_{10}[A_{50}]$ values were fitted to the linear form of the Schild equation used previously (Black *et al.*, 1985a,b):

$$\log_{10}[A_{50}] = \log_{10}[A_{50}^c] + \log_{10}(1 + [B]^n/K_B) \quad (2)$$

where $[A_{50}^c]$ is a control A_{50} value, [B] is the concentration of antagonist, K_B is its equilibrium dissociation constant and n is its order of reaction with the receptor. If n was not significantly different from unity, indicating simple competition, it was constrained to this value in order to obtain an estimate of K_B. The dissociation constant was actually estimated as pK_B ($-\log_{10} K_B$). In this experimental design a complete study of antagonism was conducted using each suspension or tissue and so equation (2) was used to provide an estimate of pK_B in each case. The average of these values is quoted in the text together with the standard error.

Paired curve design: One-way analyses of variance were performed on the differences between computed α and m estimates for each pair of concentration-effect curves. Further analysis of curve displacements was inappropriate in this case as will be seen, although a concentration-ratio was calculated for each pair and used for display in Schild plot form.

Computer-simulation of two-receptor systems: In order to interpret the data from one of the experiments it was necessary to consider the action of a selective antagonist on a one agonist: two-receptor system. The model of Furchgott (1981) was used to simulate the expectations of such an interaction. This model is essentially an extension of the traditional model for a one-receptor system (Stephenson, 1956; Furchgott, 1966) in which pharmacological effect is assumed to be a function of stimulus. In the two-receptor case the combined stimulus is imparted by the interaction of the agonist at each receptor, thus:

$$S = S_1 + S_2 = e_1 p_1 + e_2 p_2 \quad (3)$$

where e_1 and e_2 are the efficacies of the agonist at each receptor and p_1 and p_2 are the respective fractional occupancies.

Furchgott assumed that the relation between S and pharmacological effect can be described by:

$$\frac{E}{E_m} = \frac{S^n}{1 + S^n} = \frac{(e_1 p_1 + e_2 p_2)^n}{1 + (e_1 p_1 + e_2 p_2)^n} \quad (4)$$

When a competitive antagonist able to interact with each receptor is present the occupancies p_1 and p_2 are defined by:

$$p_1 = \frac{[A]}{K_{A1}(1 + [B]/K_{B1}) + [A]} \quad (5)$$

$$p_2 = \frac{[A]}{K_{A2}(1 + [B]/K_{B2}) + [A]} \quad (6)$$

Equations (4), (5) and (6) were used to generate theoretical agonist concentration-effect curves in the presence and absence of competitive antagonists. Concentration-ratios were measured between the curves in order to produce theoretical Schild plot profiles.

Drugs

PGD_2, PGE_1, PGE_2 and U46619 (9,11-dideoxy-9α,11α-methanoepoxy-$PGF_{2\alpha}$) were purchased from Cayman Chemical, Denver, Colorado, U.S.A. Carbacyclin ((5E)-6a-carbaprostaglandin I_2), epoprostenol sodium, Grade 1 (PGI_2), BW245C (5-(6-carboxyhexyl)-1-(3-cyclohexyl-3-hydroxypropyl-hydantoin), BW A868C ((±)-3-benzyl-5-(6-carboxyhexyl)-1-(2-cyclohexyl-2-hydroxyethylamino)-hydantoin) were all obtained from Wellcome Research Laboratories. Fluprostenol sodium was a gift from Coopers Animals Health Ltd., Berkhamsted, Herts. AH6809 (6-isopropoxy-9-oxaxanthene-2-carboxylic acid) was a gift from Glaxo Group Research Ltd., Ware, Herts. BM13.177 (4-[2-(benzenesulphonamido)-ethyl]-phenoxyacetic acid, Boehringer Mannheim GmbH) was prepared at the Medicinal Chemical Laboratories, Wellcome Research Laboratories. Indomethacin, adenosine-5-diphosphate, histamine-HCl and atropine sulphate were all purchased from Sigma Chemical Co. Ltd. Phenoxybenzamine hydrochloride was obtained from Smith, Kline and French, Welwyn Garden City, Herts, and fibrinogen was obtained from Kabi Vitrum, Stockholm, Sweden.

Results

Antagonism of PGD_2 by BW A868C in human washed platelets

Figure 3 shows the effects of BW A868C on PGD_2-mediated responses in human washed platelets. This compound had no intrinsic effect on the aggregations. The results illustrated are from one platelet suspension; they typify those of two other replicate experiments. Although BW A868C evidently antagonized the deaggregatory effects of PGD_2 in a concentration-dependent way, the 'bell-shaped' nature of the PGD_2 concentration-effects curve precluded quantitative analysis of the antagonism.

Antagonism of BW245C by BW A868C in human washed platelets

Unlike PGD_2, BW245C was shown in preliminary experiments to be devoid of pro-aggregatory effects. Therefore this putative DP-receptor agonist (Town *et al.*, 1983; Whittle *et al.*, 1983) was investigated in the presence of BW A868C. Figure 4 shows the results of this study. BW A868C displaced BW245C concentration-effect curves in a manner consistent with simple competitive antagonism. No significant differences between curve gradients were detected by analyses of variance. Analysis of com-

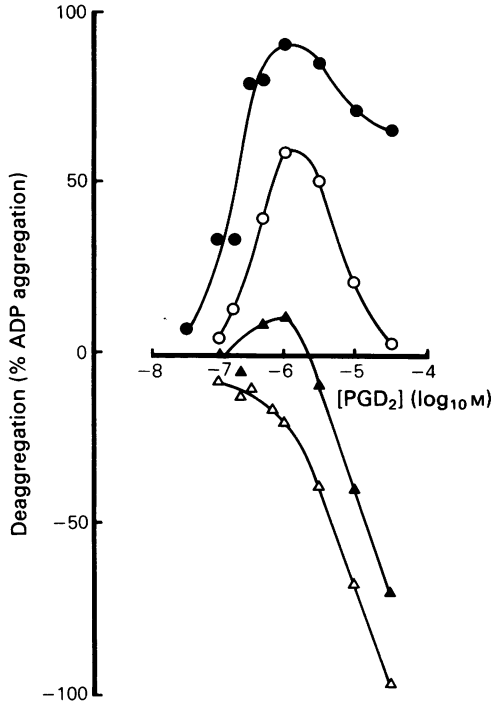


Figure 3 Representative effects of prostaglandin D_2 (PGD_2) in the presence of increasing concentrations of BW A868C; control (●); 10 nM (○); 30 nM (▲); 100 nM (△) PGD_2 was added to human washed platelet suspensions just after maximum aggregation to ADP was obtained. On the ordinate scale, positive values indicate deaggregation, negative values indicate further aggregation.

puted [A_{50}] values by equation (2) indicated that n, the Schild slope parameter, was 1.25 ± 0.04 (s.e. 3 d.f.) which was significantly greater than unity. The broken line in Figure 4b corresponds to this fit.

We considered the possibility that this departure from simple competitive behaviour was the consequence of inadequate antagonist equilibration (Kenakin, 1980). In order to examine this in more detail the inhibition of aggregation achieved after incubating BW A868C and BW245C for differing periods of time (1–10 min) before the addition of ADP was measured. Time-course profiles were constructed in the absence of antagonist or in the presence of 10 nM or 300 nM BW A868C. A steady state response to BW245C in the absence of antagonist, or in the presence of 300 nM BW A868C was achieved within 6 min. However, in the presence of 10 nM BW A868C there was, with increasing incubation time, a gradual decrease in the degree of agonism. A steady state response appeared to have been achieved after 10 min incubation.

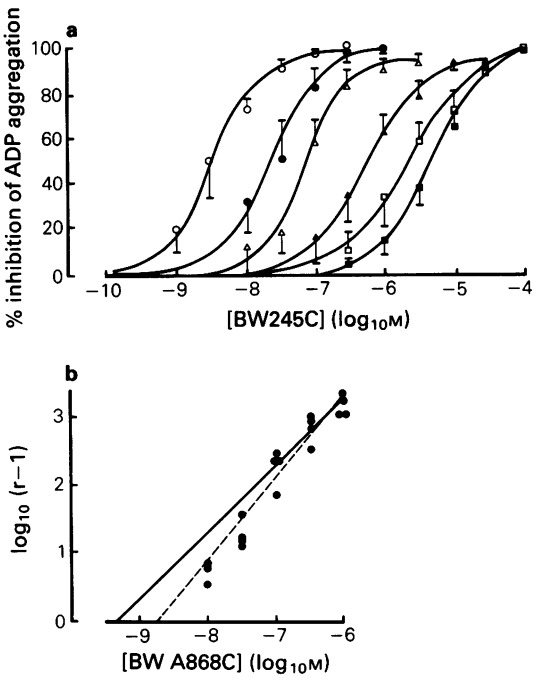


Figure 4 (a) Antagonistic effects of BW A868C on BW245C concentration-effect curves in human washed platelet suspension at the following concentrations (replicate number of curves in parentheses): zero (○), (4); 10 nM (●), (3); 30 nM (△), (4); 100 nM (▲), (4); 300 nM (□), (4); 1 μM (■), (4). Error bars represent standard errors. The lines drawn through the data are the results of logistic curve fitting using Equation (1). (b) Schild plot for the antagonism of BW245C by BW A868C. [A_{50}] values were analysed using Equation (2). The broken line drawn through the data was calculated using all the data, as described in the Methods section, and has a slope of 1.25 ± 0.04 (s.e. 3 d.f.). The solid line has a slope of unity and was calculated, omitting data points at 10 nM and 30 nM BW A868C, as explained in the results section.

The antagonist study was, therefore, repeated (in triplicate) using an extended period of agonist and antagonist co-incubation. This period was limited (in total) to 10 min for reasons of experimental feasibility. Under these conditions the estimate of n was still greater than unity, 1.14 ± 0.05, although the departure was less than previously, a result which would be expected if an equilibration problem existed.

However, under both conditions analysis of the higher concentration-ratio data (obtained at 0.1, 0.3 and 1 μM BW A868C, see Figure 4b) provided estimates of n of 0.89 ± 0.10 (6 min incubation) and 1.09 ± 0.08 (10 min incubation), neither of which is significantly different from unity. Again, these results are consistent with the above explanation which, if accepted, means that the pK_B estimates obtained from the higher concentration-ratios with n constrained to unity would be the most reliable (Kenakin, 1980). When analysed in this way, the data shown in Figure 4 provided a pK_B estimate of 9.26 ± 0.10 (s.e., 3 d.f.), corresponding to the continuous line in Figure 4b.

An equivalent experiment was carried out with platelet-rich plasma (obtained as described in the Methods section). Although broadly parallel rightward shift was obtained, the curve displacements accorded with a much lower affinity for BW A868C, and the resulting Schild plot was curvilinear. It is likely that these results are a result of antagonist loss due to binding to plasma proteins.

Antagonism of BW245C by BW A868C in rabbit jugular vein

The effects of BW A868C on BW245C concentration-effect curves are shown in Figure 5a. The antagonist had no intrinsic effects on the tone of preparations. The control curve shown is the average of all the first curves obtained and the antagonist treatment curves are average second curves. Paired control curves were also obtained during the course of the experiment (8 replicates) and found to be superimposable. Although low concentrations (10 nM and 30 nM) of the antagonist caused displacement of the curves, no further rightward shift could be achieved at higher concentrations. As the curves shown in Figure 5a consist of average data they do not accurately represent paired information. The average paired concentration-ratios, which reflect the antagonistic effects of BW A868C more accurately, are displayed in Figure 5b.

The profile of the Schild plot accords with expectations of a one-agonist: two-receptor system (Furchgott, 1981) in which (i) the agonist can elicit full effect through both receptors, (ii) the two agonist-receptor processes are separated in potency terms by approximately an order of magnitude, (iii) the antagonist acts exclusively on the more potent process. Equations (4), (5) and (6) (after Furchgott, 1981) were used to simulate the present data assuming that BW245C acts non-selectively and BW A868C acts exclusively at the DP-receptor. Figure 5b shows that the data were fitted adequately assessing a pK_B for BW A868C of 8.7 (the other parameters used are given in the figure legend).

The presence of a second phase of agonism, resistant to antagonism by BW A868C was also detected in the case of PGD_2 in the jugular vein. The nature of this secondary action was investigated further by examining BW245C and PGD_2 concentration-effect curves in the presence of AH6809 (Coleman *et al.*,

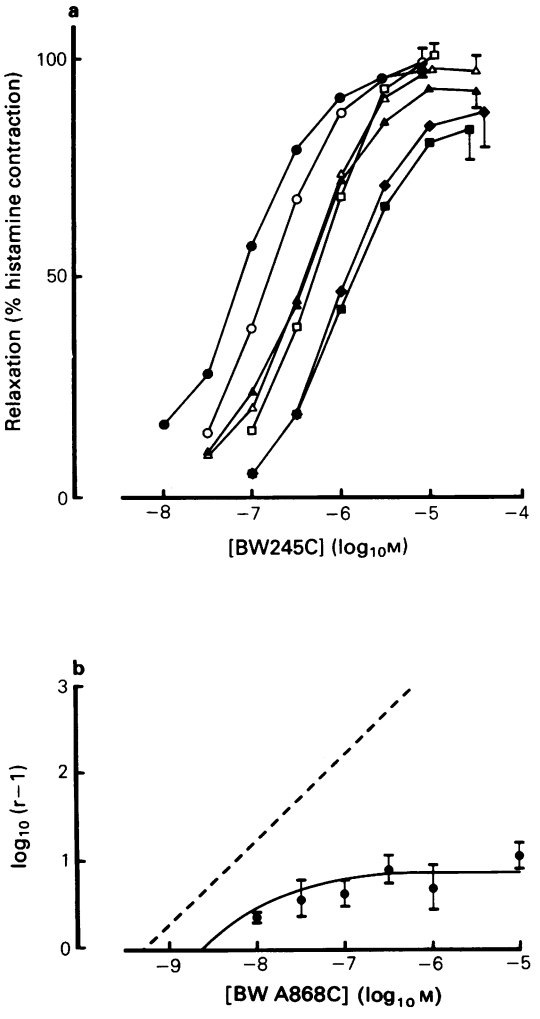


Figure 5 (a) Antagonism by BW A868C of BW245C effects in rabbit isolated jugular vein rings preconstricted with 1 μM histamine. Concentration-effect curves were obtained in a paired design using the following concentrations of BW A868C: zero (●), 10 nM (○); 30 nM (▲); 100 nM (△); 300 nM (■); 1 μM (□); 10 μM (◆). The diagram shows the mean of the 24 replicate control curves obtained by this design and the average of four curves obtained at each BW A868C concentration. (b) Schild plot for the antagonism by BW A868C of BW245C in rabbit jugular vein. The paired curve experimental design allowed a concentration ratio (r) to be calculated for each pair of curves. The data points are the mean for four experiments; s.e. shown by vertical bars. The line through the data was generated from a computer-simulation of a one agonist: two-receptor system, as described in the Methods section. The dashed line indicates the position of the Schild plot obtained from the equivalent platelet experiment (Figure 4b).

1985b). This putative EP_1-receptor antagonist, at 10^{-5} M, had no effect on the BW A868C resistant phase, in each case, the latter being applied at 1 μM in order to expose the secondary action. This experiment was performed in triplicate using BW245C, and on one occasion using PGD_2 (data not shown).

The effect of BW A868C on responses to PGD_2 in guinea-pig trachea

PGD_2 caused a dose-dependent contraction of isolated segments of guinea-pig trachea, although at high concentrations (10 μM or greater) a dose-dependent relaxation occurred, which resulted in a bell-shaped concentration-effect curve. This curve was unaffected by the presence of 10 μM BW A868C (Figure 6) and BW A868C alone had no effect on basal tone.

Selectivity of BW A868C

Concentration-effect curves for PGI_2, carbacyclin (a stable PGI_2 analogue), PGE_1 and U46619 were unaffected by BW A868C up to 1 μM (in platelets) and 10 μM (in jugular veins). Also, basal tone and PGE_2 concentration-effect curves in guinea-pig

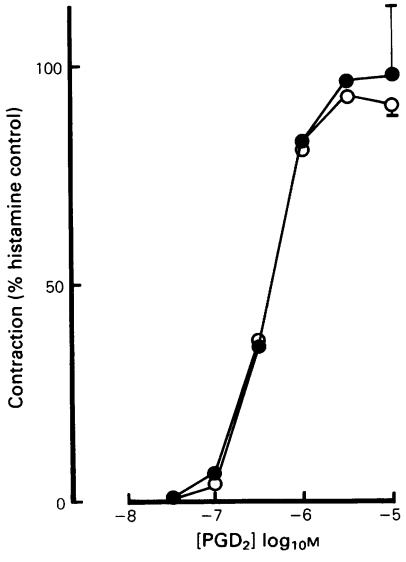


Figure 6 Prostaglandin D_2 (PGD_2) concentration-effect curves in isolated segments of guinea-pig trachea in the absence (○) and presence of BW A868C 10 μM (●). Each point is the mean of three experiments and bars represent standard errors.

ileum, and fluprostenol concentration-effect curves in rat stomach fundic strips were unaffected by 10 μM BW A868C, as were PGE_2-mediated relaxations of cat trachea pre-contracted with 3 μM bethanechol. These experiments were performed in triplicate.

Discussion

In this paper we have shown that BW A868C acts as a highly potent antagonist at the DP-receptor in human washed platelets. The DP-designation relies partly on the prior designation of BW245C as an agonist at this receptor. Binding studies (Town *et al.*, 1983) as well as agonist potency order information (Whittle *et al.*, 1983) indicate that BW245C and PGD_2 interact with the same receptor on platelets. Also, in the present experiments, BW A868C was shown to antagonize PGD_2-induced deaggregatory responses, albeit in a complex way. Together these results support the classification of BW A868C.

Evidently, this antagonist is extremely selective for DP-receptors. In the platelet assay neither IP-receptor-mediated inhibition of aggregation (by PGI_2, PGE_1 and carbacyclin) nor TP-receptor-mediated aggregation (by U46619) was affected by concentrations of BW A868C 10^3 times higher than those at which DP-receptor antagonism is demonstrable. Vehicle (ethanol) effects prevented use of BW A868C at higher concentrations. In the jugular vein, IP-receptor-mediated relaxations and TP-receptor-mediated constrictions were unaffected by yet higher concentrations, in this case 10^4 times greater than the DP-receptor K_B. The same concentration of BW A868C failed to alter the contractile effects of PGE_2 in the guniea-pig ileum, or relaxant effects in the cat trachea, which are claimed to be mediated by the EP_1- and EP_2-receptors respectively (Kennedy *et al.*, 1982; Coleman *et al.*, 1984; 1987a). The exposure of a BW A868C resistant relaxation response in the rabbit jugular vein assay, which we presume to be mediated by the putative EP_2-receptor (this is discussed below), in itself provides evidence for selectivity of the compound over this receptor type. As shown by Figure 5, concentrations of BW A868C 10^4 times higher than the apparent DP-receptor K_B again failed to displace this relaxation effect. The contractile effects of the putative selective FP-receptor agonist fluprostenol (Dukes *et al.*, 1974; Coleman *et al.*, 1984) in the rat fundus preparation (Dong *et al.*, 1986) were similarly unchanged by this concentration of BW A868C.

Although BW A868C is evidently highly potent and selective for DP-receptors it did not satisfy all the criteria for simple competitive antagonism under the conditions employed in the platelet assay. While the antagonist produced apparently parallel displacement of BW245C concentration-effect curves, the Schild plot slope parameter, n, exceeded unity. This deviation was significant according to the method of data analysis used here, that is, on the basis of the average of four independent estimates of n made in different platelet suspensions. In fact, the same estimate of n, namely 1.25, was obtained when all the curve $[A_{50}]$ data from the four experiments were pooled and analysed (analysis not shown), although, in this case, the estimated standard error (0.16) made the deviation appear insignificant. However, in the interests of rigour it seemed appropriate to accept the implications of the more stringent statistical evaluation. As it was found that increasing the equilibrium period for the antagonist lowered the value of n, albeit not to unity, we conclude that the estimate of 1.25 was meaningful as well as significant. On this basis, as explained in the results section, a reliable estimate of antagonist affinity could only be derived from the concentration-ratio data obtained at higher concentrations of BW A868C. This estimate 9.26 is, in fact, very similar to that obtained in a human adenylate cyclase assay as described in the following paper (Trist *et al.*, 1989).

It was not possible to confirm this estimate of affinity with the natural agonist, PGD_2, in the platelet assay due to expression of pro-aggregatory effects by PGD_2. In the jugular vein assay also, PGD_2 demonstrated additional properties producing contractile effects, which due to their BM 13.177 susceptibility, appeared to be TP-receptor-mediated. These results are consistant with similar reports of PGD_2 actions in other smooth muscle preparations (Jones *et al.*, 1982; Toda *et al.*, 1986). Although the pro-aggregatory effects of PGD_2 in the human platelet assay were not examined in the presence of BM 13.177 it is likely that they are TP-receptor-mediated in view of previous evidence of PGD_2 action at this receptor in guinea-pig platelet (Hamid-Bloomfield & Whittle, 1986).

Unfortunately, the BW A868C-resistant relaxation responses of BW245C meant that a reliable affinity estimate for the antagonist could not be made in the jugular vein assay with this agonist either. However, assuming that a one agonist: two receptor system was operating here, it was possible to show that antagonistic effects were consistent with the antagonism of BW245C-mediated effects by BW A868C with a pK_B at the DP-receptor of 8.7. This value is clearly lower than that obtained in the platelet assay(s) but under the circumstances we feel it is unwise to invoke receptor heterogeneity.

Further evidence, using other agonist and antagonist probes, is necessary for the similarity or otherwise of the platelet and vascular DP-receptors to be established. We are currently undertaking such studies.

Regarding the BW A868C-resistant relaxation responses of BW245C and also PGD_2, the receptor mediating these effects is unlikely to be of the EP_1-type as AH6809 produced no antagonism at concentrations approaching two orders of magnitude higher than the reported EP_1-receptor affinity of this compound (Coleman *et al.*, 1985). It is also unlikely that these responses are IP-receptor-mediated because the potency of PGI_2 is higher in the platelet than in the jugular vein assay (data not shown) implying that any BW245C-mediated IP-receptor effect should have been more marked in the platelet assay rather than absent as was actually the case. Also, binding studies have shown the BW245C has negligible affinity for PGI_2 binding sites in platelets (Town *et al.*, 1983). In the absence of evidence to the contrary and for the sake of parsimony it seems reasonable to conclude that these effects are mediated by the EP_2-receptor class. Previous reports indicate that this receptor subserves relaxation responses in dog vasculature (Lumley *et al.*, 1982; Dong *et al.*, 1986) although firm classification of this receptor as a distinct type awaits the discovery of further selective agonist and antagonist probes.

In addition to its effects in platelets and vasculature, PGD_2 has also been shown to mediate bronchoconstriction in the dog *in vivo* (Wasserman *et al.*, 1977) and contraction of guinea-pig isolated tracheal preparations (Coleman & Kennedy, 1980). Here we have shown in the guinea-pig trachea that the receptor(s) mediating contraction to PGD_2 is not of the DP-class; 10 μM BW A868C had no effect on these responses. Indeed it has previously been suggested that PGD_2 produces these effects through TP- and EP_1-receptors (Jones *et al.*, 1982; Coleman & Kennedy, 1985).

In summary, BW A868C appears to be a very potent competitive antagonist of DP-receptors in human washed platelets. It demonstrates negligible affinity for TP- IP-, EP_1-, EP_2- and FP-receptors at concentrations three to four orders of magnitude higher than its expressed DP-receptor dissociation constant. Antagonists of this potency and selectivity have not been previously reported for DP-receptors or for most of the other putative prostaglandin receptor classes. The exception is the TP-class for which antagonists of similar profile exist: EP045 (Jones *et al.*, 1982), BM 13,177 (Patscheke *et al.*, 1984), AH23848 (Brittain *et al.*, 1984). As in the case of the available TP-receptor antagonists, BW A868C should prove to be an important tool for the reliable identification and quantitative classification of prostaglandin receptors.

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The antagonism by BW A868C of PGD_2 and BW245C activation of human platelet adenylate cyclase

[1]D.G. Trist, B.A. Collins, *J. Wood, †M.G. Kelly & †A.D. Robertson

Department of Biochemistry, *Department of Scientific Computing and Statistics and †Department of Medicinal Chemistry, Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS

1 In glycerol-lysed human platelets, prostaglandin D_2 (PGD_2) and the hydantoin BW245C both activate adenylate cyclase in a biphasic manner. These activations are qualitatively different from those of carbacyclin, iloprost and prostaglandin E_2 (PGE_2) whose E/[A] curves can be adequately described by rectangular hyperbolae.

2 Prostaglandin E_1 (PGE_1) had E/[A] curves of slope significantly lower than that expected for a rectangular hyperbola.

3 The selective PGD_2 antagonist BW A868C shifts the first phase of the PGD_2 and BW245C E/[A] curves but has no effect on the second phase.

4 Applying a two-receptor model enables a pK_B to be derived for BW A868C of 9.11.

5 BW A868C has no effect on carbacyclin, iloprost, prostacyclin, PGE_1 and PGE_2 at a concentration 1,000 fold that of its K_B against PGD_2 and BW245C.

6 These results indicate that PGD_2 and BW245C are capable of activating adenylate cyclase in human platelets through the DP-receptor and by another mechanism as yet uncharacterized.

Introduction

Prostacyclin (PGI_2), stable analogues of prostacyclin such as carbacyclin and iloprost, prostaglandin E_1 (PGE_1) and prostaglandin D_2 (PGD_2) inhibit human platelet aggregation (Moncada *et al.*, 1976; Whittle *et al.*, 1978). Binding studies with radioligands suggest that whereas PGI_2, carbacyclin, iloprost and PGE_1 appear to interact at the same site, PGD_2 binds to a separate receptor (Siegl *et al.*, 1979). Similar binding studies have classified the hydantoin, prostaglandin-mimetic BW245C as acting on the PGD_2 receptor rather than the PGI_2 receptor (Town *et al.*, 1983; Hall & Strange, 1984).

The preceding paper (Giles *et al.*, 1989) describes a novel, competitive, high affinity, selective antagonist, BW A868C (3-benzyl-5-[6-carboxylhexyl]-1-[2-cyclohexyl-2-hydroxyethylamino]hydantoin), against PGD_2- and BW245C-inhibited platelet aggregation. As BW A868C was ineffective against PGI_2, PGE_1 and carbacyclin, these results support the classification of at least two separate prostaglandin receptors in human platelets.

Concomitant with the inhibition of platelet aggregation, all the above mentioned prostaglandin-mimetics elevate platelet intracellular cyclic AMP (Miller & Gorman, 1976; 1979; Tateson *et al.*, 1977) and have been shown to activate adenylate cyclase in human platelet membranes (Gorman *et al.*, 1977).

The activation of adenylate cyclase by PGI_2 and its stable analogues is quantitatively different from that of PGD_2. PGI_2 maximally stimulates human platelet cyclase to a much greater degree than PGD_2. Furthermore, we have extended the range of PGD_2 concentrations tested and observed a second phase of stimulation of adenylate cyclase by PGD_2, which is not apparent with carbacyclin, PGE_1, iloprost or prostacyclin.

This paper describes these observations and their partial characterization by use of the new selective PGD_2 antagonist BW A868C.

Methods

Platelet lysis

Human platelets were separated from peripheral blood and subsequently lysed by the method of Steer & Wood (1979). Human blood was collected by venepuncture into a bottle containing EDTA (Sigma, $1.2\,g\,l^{-1}$ of blood) as anticoagulant. Platelet-rich

[1] Present address: Glaxo S.p.A., Via Fleming 2, 37100 Verona, Italy.

plasma (PRP) was obtained by centrifugation (1,000 g for 20 min at 4°C).

Platelets were isolated from the PRP by further centrifugation (4300 g for 10 min at 4°C) and resuspended in a buffer containing 1 mM EDTA, 150 mM NaCl, 10 mM Tris HCl, pH 7.6, 12 ml buffer being used per 34 ml of original blood. The platelets were recentrifuged under the same conditions and resuspended in 1–2 ml of the same buffer per 34 ml of original blood. This was layered onto a glycerol gradient in 150 mM NaCl (0%–40% v/v in 5% steps). The platelets were then centrifuged through the gradient (2000 g for 35 min at 4°C). The pellet was resuspended in a buffer containing 0.32 M sucrose, 5 mM Tris HCl, 1 mM EGTA and 1 mM 1,4-dithiothreitol (DTT, Sigma), pH 7.6; this buffer is hypotonic to the glycerol-loaded platelets and causes lysis. The lysed platelets were pelleted at 8700 g for 10 min at 4°C, washed 4 times with the same buffer and finally resuspended in that buffer at a protein concentration of 0.2 to 1.0 mg ml^{-1}.

Adenylate cyclase assay

Each experiment was done with a fresh platelet preparation. The standard reaction mixture contained 75 mM Tris/HCl (pH 7.6) (Sigma), 0.25 mM ATP (Boehringer), 1.25 mM $MgCl_2$, 1 mM cyclic AMP (Boehringer), 5 mM creatine phospate (Boehringer), 7 units creatine kinase (E.C. 2.7.3.2) (Boehringer), 0.5 mM 3-isobutyl-1-methyl xanthine (IBMX, Sigma), 10 μM GTP (Boehringer), 100 mM sucrose (B.D.H.), 0.3 mM EGTA (Sigma), 0.3 mM DTT (Sigma), enzyme protein (20 to 100 μg), [α-^{32}P]-ATP 1–2 μCi (NEN, 800 Ci mmol^{-1}), plus test substances in a final volume of 500 μl. All assays were performed in triplicate or quadruplicate. All additions were made with a randomised allocation of treatment to the assay tubes on ice. They were then transferred to a water bath and preincubated at 30°C. The time allowed for the tubes to equilibrate depended on the substance(s) being tested, but was usually 2 min. After preincubation [α-^{32}P]-ATP was added and the reaction carried out for 5 min before being stopped by the addition of 250 μl of 1% sodium dodecyl sulphate. After addition of 1.3 ml [^{3}H]-cyclic AMP (18–20,000 c.p.m., Amersham, 26 μCi mmol^{-1}) to each tube to monitor recovery, the labelled cyclic AMP was isolated by passage through Dowex AG-50 W (Bio-Rad) and alumina columns (Sigma, type WN-S) (Salomon *et al.*, 1974). The reaction was linear with platelet protein over the range used and for up to 2 h after the addition of [α-^{32}P]-ATP. The test substances had no effect on the recovery of cyclic AMP from the columns, nor on the linearity of the reaction and had reached a steady state in respect of cyclase activation within the perincubation period.

Compounds

BW245C 5-(6-carboxyhexyl)-1-(3-cyclohexyl-3-hydroxypropyl hydration) and BW A868C were synthesized by Dr C. Caldwell and Dr M. Kelly respectively (Department of Medicinal Chemistry, Wellcome Research Laboratories). Iloprost was obtained from Amersham International, PGE_1 from Sigma and 5-E-carbacyclin from Upjohn Limited, U.S.A.

Carbacyclin, PGD_2, PGE_1, BW A868C and BW245C were all made up as stock solutions in 95% ethanol at 10 mg ml^{-1}.

These stocks were diluted, as appropriate, with adenylate cyclase buffer. All control buffers were adjusted to the same ethanol concentration.

Analysis of data

Where the data for a particular agonist were observed to produce monophasic E/[A] curves, simple logistics of the following general form were fitted:

$$\text{cyclic AMP produced} = \beta + \frac{\alpha}{1 + 10^{-n(\log [A] - \log [A_{50}])}} \quad (1)$$

where β is basal activity of adenylate cyclase, α the maximal response to agonist [A], $\log [A_{50}]$ is the location parameter and n the slope of the logistic.

The rectangular hyperbola (with n = 1) is a special case of the logistic and this model was preferred when the fit to the data was not significantly improved (as judged by an F-test) by the estimation of n.

Where the E/[A] curves of the agonist were clearly not monophasic then a more complex model was fitted. As BW A868C only seemed to move the first phase of the E/[A] curve the model assumed 2 receptors for the same agonist both of which activate adenylate cyclase but only the more sensitive one able to bind BW A868C. The model is essentially the sum of two logistic curves with separate location parameters and upper asymptotes, and common slopes. The location parameter of the first logistic is modified by BW A868C in line with simple competition. A further parameter, equivalent to the Schild-plot slope was included to allow for departures from simple competition.

The full model is:

$$\text{cyclic AMP produced} = \beta + \frac{\alpha'}{1 + 10^{-n(\log [A] - \log [A'_{50}] - \log(1 + 10r(pK)B + \log B))}} + \frac{\alpha''}{1 + 10^{-n(\log [A] - \log [A''_{50}])}} \quad (2)$$

where [B] is the concentration of antagonist (BW A868C), pK_B the negative logarithm of the antagonist dissociation constant, log $[A'_{50}]$ and log $[A''_{50}]$ the locations of the two phases of the E/[A] curve with upper asymptotes of α' and α'' respectively, and r is effectively the Schild-plot slope.

In practice the second logistic is incompletely defined as the second phase did not reach an asymptote over the range of agonist that it was practical to use. In consequence there was insufficient information to estimate slope, location and upper asymptote of the second logistic independently. Therefore the slope of the second logistic was set equal to the first.

Simple competitivity at the first site was assessed by checking for deviation of the Schild-plot slope (r) from unity that were consistent across data sets.

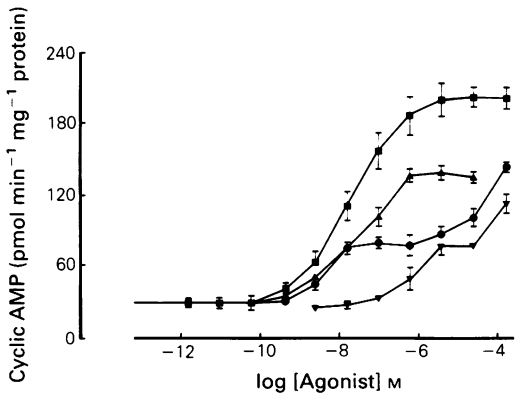

Figure 1 Representative E/[A] curves for the activation of adenylate cyclase in lysed human platelets from a single donor by carbacyclin (■), prostaglandin E_1 (PGE_1) (▲), BW245C (●) and PGD_2 (▼). The points are the means with s.e. (vertical bars) from triplicate determinations.

Results

Activation of platelet adenylate cyclase by prostaglandins and prostaglandin-mimetics

Prostaglandin D_2 (PGD_2) (Figures 1 and 2) and BW245C (Figures 1 and 3) both exhibit biphasic E/[A] curves for the activation of human platelet adenylate cyclase. This is a property not exhibited in the E/[A] curves of PGE_1 (Figure 1), carbacyclin (Figures 1 and 2), iloprost (Figure 2), prostacyclin and PGE_2 (not shown).

Fitting the E/[A] curves either by simple logistics, equation (1), (in the case of carbacyclin, PGE_2 and iloprost and prostacyclin) or by a more complex model (in the case of PGD_2 and BW245C), equation (2) as described in the methods, gave slopes not significantly different from that expected for rectangular hyperbolae. PGE_1 consistently was fitted better by a logistic of low slope (0.82 ± 0.03, mean ± s.e., $n = 7$ donors) than by a rectangular hyperbola. This is a finding which has been reported previously (Lombroso *et al.*, 1984). Table 1 gives maximal activations and concentrations giving half maximal stimulation (log $[A_{50}]$) for the agonists examined in platelets from a number of different donors. For PGD_2 and BW245C only the first phase of activation has been considered. PGD_2 and BW245C produced stimulations significantly lower than that of carbacyclin, iloprost, prostacyclin, PGE_2 and PGE_1, with iloprost and BW245C being the most potent (log $[A_{50}] = -8.3$ M) and PGE_2 being the least potent (log $[A_{50}] = -4.8$ M).

Table 1 The location parameters (log $[A_{50}]$s) and upper asymptote values (expressed as stimulation above basal activity) for the activation of human platelet adenylate cyclase by the prostaglandin-mimetics studied

Agonist	log $[A'_{50}]$ (M)	log $[A''_{50}]$ (M)	*Fold stimulation (relative to basal)*
PGE_2	-4.8 ± 0.04 (4)	N.A.	7.2 ± 0.40
PGE_1	-7.2 ± 0.06 (7)	N.A.	8.0 ± 0.80
Carbacyclin	-7.4 ± 0.10 (12)	N.A.	9.3 ± 1.00
Prostacyclin	-7.9 ± 0.10 (5)	N.A.	10.7 ± 0.40
Iloprost	-8.3 ± 0.09 (6)	N.A.	9.2 ± 0.90
PGD_2	-6.4 ± 0.10 (5)	-4.3 ± 0.07 (5)	2.9 ± 0.16^E
BW245C	-8.3 ± 0.08 (6)	-4.4 ± 0.05 (6)	2.7 ± 0.14^E

[E] Derived from first phase only.

log $[A_{50}]$s and maximal stimulations were obtained by fitting the E/[A] curves to the equations described in the methods. The estimates are the means for platelets from a number of different donors (in parentheses). Where there was no second phase of activation log $[A''_{50}]$ is not applicable (N.A.).

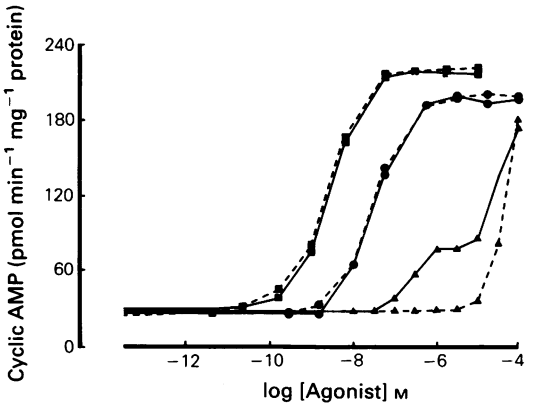

Figure 2 E/[A] curves for the activation of adenylate cyclase in lysed human platelets from a single donor by iloprost (■), carbacyclin (●) and prostaglandin D_2 (▲) in the absence (———) or presence (– – – –) of BW A868C (1.35 μM). The points are the means of triplicates, the s.e. bars have been omitted for clarity but never exceeded ±5% of the mean value.

Antagonism by BW A868C

BW A868C at 1.35 μM, a concentration more than 1,000 fold greater than its K_B in the platelet aggregation assay (Giles *et al.*, 1989), shifted the first phase of the PGD_2 E/[A] curve to the right but had no effect on the E/[A] curves for iloprost and carbacyclin (Figure 2). In similar experiments, but not shown, BW A868C at the same concentration shifted the E/[A] curve of BW245C but failed to shift E/[A] curves for prostacyclin, PGE_1 and PGE_2.

In the case of PGD_2, the two phases of activation were located too close together to allow a detailed analysis of BW A868C. Therefore, a pK_B for BW A868C was estimated using BW245C as the agonist because of the wider separation of the two phases (Figure 1).

Figure 3 shows a representative experiment in which BW A868C was used over the range of 5 nM to 1.22 μM. Only the first phase is affected, the right shift of the E/[A] curve approaching a limit at the highest concentrations of BW A868C. Fitting a two-receptor model where BW A868C competitively antagonized the higher affinity receptor for BW245C gave a pK_B of 9.11 ± 0.16 (s.e.) as estimated on 8 platelet preparations each from a different donor. Values for r other than unity gave better fits to two of the individual experiments but there was little sign of a consistent difference which was taken to indicate that the assumption of simple competitivity was reasonable. Deviations of n from unity were assessed in a similar way, and again no consistent differences were noted. The model was therefore fitted with n and r constrained to unity, and estimates of the pK_B and the remaining parameters for each experiment taken from this fit.

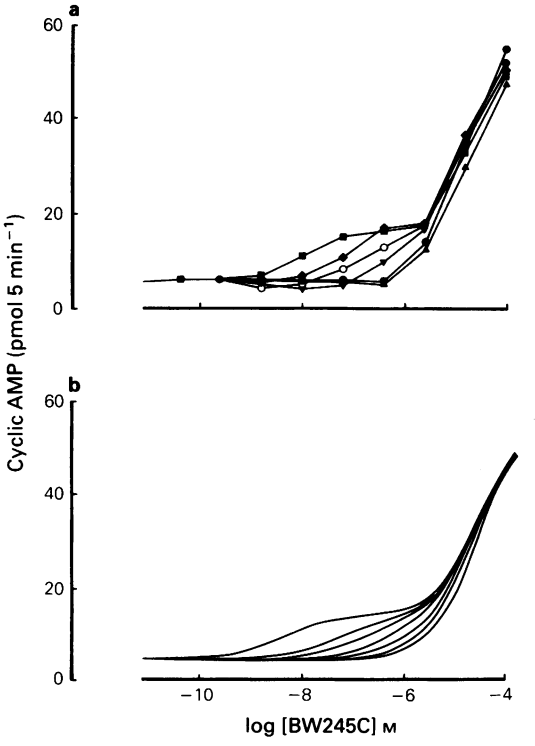

Figure 3 (a) E/[A] curves for the activation by BW245C of adenylate cyclase in lysed human platelets from a single donor in the presence of BW A868C at the following concentrations: 0 nM (■), 5 nM (◆), 15 nM (○), 45 nM (▼), 135 nM (●), 405 nM (▲) and 1215 nM (▲). The points are the means of triplicates. The s.e. bars have been omitted for clarity, but never exceeded ±5% of the mean value. (b) Fitted curves to the data shown in (a) using the two-receptor model described in Methods. The parameters estimated in this experiment were: $\beta = 4.9$, $\alpha' = 9.1$, $\alpha'' = 44.0$, $\log [A'_{50}] = -8.4$, $\log [A''_{50}] = -4.7$ and $pK_B = 9.4$.

Discussion

The finding in the preceding paper that BW A868C is a highly selective competitive DP-receptor antagonist offers a high affinity tool to help elucidate complex E/[A] curves of the type described in this paper for PGD_2 and BW245C.

The results show that BW A868C antagonizes only one of the two phases of activation of human platelet adenylate cyclase exhibited by both PGD_2 and BW245C. The degree of shift of this first phase translates into a pK_B value for BW A868C of 9.11, which is not significantly different from the 9.26

found in the whole platelet aggregation assay (Giles *et al.*, 1989). Like the aggregation assay, BW A868C did not give Schild-plot slope parameters of unity in two of the eight experiments. Of these two, one was significantly above and one significantly below unity. As there were no consistent deviations from unity and as the pooled value of r was 1.03 ± 0.05 (s.e.) it was taken that the criteria for BW A868C as a competitive antagonist was met in the cyclase assay. Neither did there appear to be an equilibrium problem as found in the aggregation assay. This was supported by experiments (not shown) where the progress of the adenylate cyclase activity was followed. These suggested that BW A868C at concentrations as low as that producing 20% inhibition were in a steady state with BW245C within the period of preincubation (2 min).

The second phase of activation by PGD_2 and BW245C is resistant to BW A868C and therefore appears not to be mediated through the same receptor and is not yet identified. It is possible that this phase is a reflection of some physical property of high concentrations of the prostaglandin-mimetic acting on adenylate cyclase itself or indirectly on the membrane environment of the enzyme. In estimating the pK_B for BW A868C we have ignored these possibilities and assumed that the second phase is receptor-mediated. This was done because a two-receptor model seemed a simple starting point with a pharmacological basis to which the data fitted tolerably well and because binding studies with [^{3}H]-iloprost and human platelets have shown that BW245C displaces this ligand with an IC_{50} of 78.3 μM (Hall & Strange, 1984). Assuming simple competition at the same site an estimated K_D of approximately 40 μM can be calculated. This value is very close to the ED_{50} computed for the second phase of adenylate cyclase activation by BW245C (Table 1), suggesting a specific receptor. Furthermore, using human platelet membranes we have found a similar close correspondence between the log [A_{50}] derived from adenylate cyclase assays and the log K_D derived from [^{3}H]-iloprost displacement studies for a large number of prostaglandin analogues.

In the rabbit jugular vein, BW245C produces a second relaxation phase thought to be through the EP_2-receptor (using the nomenclature of Kennedy *et al.*, 1982) rather than any other (Giles *et al.*, 1989). Displacement of [^{3}H]-iloprost by BW245C could be interpreted as the BW245C second phase in the platelet being mediated through the IP-receptor. We favour the IP-receptor as the second receptor in the human platelet because of agonist potencies in stimulating adenylate cyclase. Iloprost, PGI_2, carbacyclin and PGE_2 all show simple monophasic E/[A] curves with iloprost > PGI_2 > carbacyclin > PGE_2 which is the rank order expected for action on the IP-receptor (Dong *et al.*, 1986). The same authors show that for EP_2-receptors the rank order would be expected to be PGE_2 > carbacyclin > iloprost = PGI_2.

In conclusion this paper supports the classification of BW A868C as a highly selective, competitive antagonist at the DP-receptor with a pK_B of 9.11. This compound discriminates between the two mechanisms of activation of human platelet adenylate cyclase by PGD_2 and BW245C, one being characterized as through the DP-receptor and the other, as yet undefined, possibly through the IP-receptor.

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Antagonism of PGD_2 vasodepressor responses in the rat *in vivo* by the novel, selective antagonist, BW A868C

S. Hamid-Bloomfield & B.J.R. Whittle

Department of Pharmacology, Wellcome Research Laboratories, Langley Court, Beckenham, Kent, BR3 3BS

1 Bolus intravenous injection of prostaglandin D_2 (PGD_2, 1–160 $\mu g\,kg^{-1}$), the hydantoin prostanoid BW245C (0.25–160 $\mu g\,kg^{-1}$) or prostacyclin (PGI_2, 0.05–0.5 $\mu g\,kg^{-1}$) caused a dose-dependent fall in systemic arterial blood pressure (BP) in the anaesthetized rat, lasting 2–4 min.

2 Intravenous infusion of the novel 3-benzyl substituted hydantoin, BW A868C (1–10 $\mu g\,kg^{-1}\,min^{-1}$), in doses that had no direct effect on BP, dose-dependently reduced the vasodepressor action of PGD_2.

3 Bolus injection of BW A868C (30 and 100 $\mu g\,kg^{-1}$, i.v.) likewise dose-dependently antagonized the vasodepressor responses to PGD_2, causing a 3.4 and 13.2 fold rightward shift of the dose-response curve.

4 The thromboxane-receptor antagonist, BM 13.177 (2.5 $mg\,kg^{-1}$ i.v.) had little effect on the PGD_2 vasodepressor responses, suggesting minimal contribution of a PGD_2 interaction at thromboxane receptor-sites in the systemic vasculature of this species.

5 BW A868C (10 $\mu g\,kg^{-1}\,min^{-1}$ i.v.) caused a rightward shift (59 fold) of the dose-response relationship for BW245C, the putative PGD_2-receptor agonist. This antagonism lasted for at least 1 h after termination of the BW A868C infusion. Higher doses of BW A868C (20–100 $\mu g\,kg^{-1}\,min^{-1}$) caused no further antagonism of the vasodepressor responses to BW245C, suggesting that this prostanoid also acts at vascular receptors other than of the DP-type.

6 BW A868C (10 $\mu g\,kg^{-1}\,min^{-1}$, i.v.) failed to alter the vasodepressor actions of prostacyclin.

7 These findings in the rat *in vivo* support the characterization of BW A868C as a potent and selective antagonist of the cardiovascular actions of PGD_2 at the DP-receptor.

Introduction

Prostaglandin D_2 (PGD_2), a naturally occurring prostanoid, exhibits a wide variety of pharmacological activities. It has potent anti-aggregatory activity on platelets of several species (Whittle *et al.*, 1978), produces vasodilatation or constriction depending on the vascular bed and species studied (Wasserman *et al.*, 1977; Chapnick *et al.*, 1978) and produces bronchoconstriction in animals and in asthmatic subjects (Wasserman *et al.*, 1977; Patterson *et al.*, 1980; Hardy *et al.*, 1984).

Pharmacological and binding studies suggest that there are at least two types of receptors on the platelet which mediate the anti-aggregatory actions of the prostanoids, one through which prostacyclin (PGI_2) and PGE_1 act, and the second through which PGD_2 exerts its actions (Whittle *et al.*, 1978; Siegl *et al.*, 1979; Schafer *et al.*, 1979; Miller & Gorman, 1979). It has also been suggested that BW245C interacts with similar binding sites to PGD_2 on platelets of various species (Whittle *et al.*, 1983; Town *et al.*, 1983). Another study classifies the receptors for prostacyclin and prostaglandin D_2 as IP- and DP-receptors respectively (Kennedy *et al.*, 1982). This classification, however, remains qualitative in the absence of highly selective receptor-antagonists. Furthermore, there is minimal information available to classify the nature of prostanoid receptors mediating vasodepressor effects *in vivo*.

The present study *in vivo* extends the findings *in vitro* described in the accompanying two papers (Giles *et al.*, 1989; Trist *et al.*, 1989) of the selective antagonism of PGD_2 at the DP-receptor by a novel, 3-benzyl substituted hydantoin, BW A868C ((±)-3-benzyl-5-(6-carboxyhexyl)-1-(2-cyclohexyl-2-hydro-

xyethylamino)hydantoin]. Thus, the actions of BW A868C on the vasodepressor effects of PGD_2, BW245C and prostacyclin following intravenous administration in the anaesthetized rat have been investigated.

Methods

Anaesthesia was induced in male Wistar rats (250–300 g body weight) with the long-acting barbiturate, inactin (100 mg kg^{-1}, i.p.). The trachea was cannulated to facilitate respiration. Rectal temperature was maintained at 37°C by thermistor-controlled radiant heat. Systemic arterial blood pressure (BP) was recorded from a cannulated carotid artery. In these animals, resting BP was 100–140 mmHg and heart rate derived from the arterial pulse was 350–450 beats min^{-1}. Both femoral veins were cannulated for the administration of drugs. For bolus i.v. administration, compounds were injected into the femoral vein in incremental doses, in volumes of 1 ml kg^{-1} and flushed in with 0.25 ml of isotonic saline (0.9% w/v). In some experiments, compounds were infused (0.1–0.25 ml min^{-1}) into the femoral vein with a Spritze Perfuser pump.

Incremental doses of PGD_2 (1–160 μg kg^{-1}), BW245C (0.25–160 μg kg^{-1}) or prostacyclin (0.05–0.5 μg kg^{-1}) were administered by bolus i.v. injection, and their effect on the BP recorded. Intravenous infusion of BW A868C (1–100 μg kg^{-1} min^{-1}) was started 5 min before the study and maintained throughout the period of administration of the bolus intravenous injections of PGD_2, BW245C or prostacyclin. In further experiments, BW A868C (30 or 100 μg kg^{-1} i.v.) or BM 13.177 (2.5 mg kg^{-1} i.v.), a thromboxane antagonist (Patscheke & Stegmeier, 1984), was administered 10 min before injection of PGD_2.

Drugs

Prostacyclin as the sodium salt (Wellcome Foundation Ltd) was freshly dissolved in 1 M Tris buffer (pH 9.6 at 4°C) immediately before use and diluted in ice-cold 1.25% w/v $NaHCO_3$ solution. Prostaglandin D_2 (Upjohn Company, Kalamazoo, U.S.A.) and the hydantoin prostaglandin BW245C, 5-(6-carboxyhexyl)-1-(3-cyclohexyl-3-hydroxypropyl hydantoin (synthesized in the Department of Medicinal Chemistry, Wellcome Research Laboratories) were stored in ethanol (10 mg ml^{-1}; 4°C) and diluted with 1.25% $NaHCO_3$ solution when required.

BW A868C [(±)-3-benzyl-5-(6-carboxyhexyl)-1-(2-cyclohexyl-2-hydroxyethyl-amino) hydantoin] and the thromboxane antagonist, BW 13.177 (4-[2-(benzyenesulphon-amide)-ethyl]-phenoxyacetic acid) were synthesized in the Department of Medicinal Chemistry, Wellcome Research Laboratories. These compounds were stored in ethanol (10 mg ml^{-1}; 4°C) which was evaporated off under N_2 before being diluted with 1.25% w/v $NaHCO_3$ solution when required. 11α,9α-epoxy-methano PGH_2, U-46619 was from the Upjohn Company, Kalamazoo, U.S.A.

Statistical analysis

Results are expressed as mean ± s.e.mean, where (n) is the number of values. The difference between groups was evaluated by Student's t test for unpaired data with $P < 0.05$ being taken as significant. The slopes and shifts of the log dose-response curves were evaluated by regression analysis and $P < 0.05$ was taken as significant.

Results

Effect of BW A868C on PGD_2 responses

Bolus intravenous injection of PGD_2 caused a dose-dependent fall in the BP lasting 2–4 min (Figure 1) with the time to complete the incremental dose-

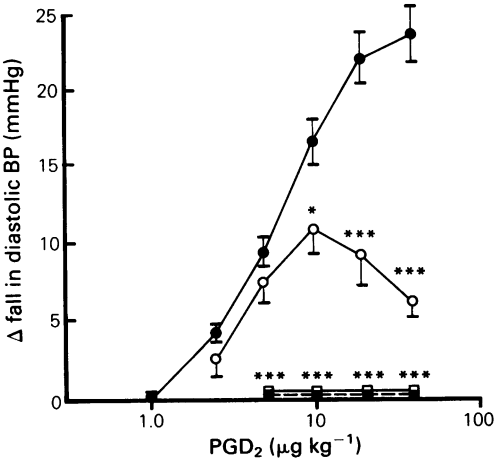

Figure 1 Effect of intravenous infusion of BW A868C on the vasodepressor responses to prostaglandin D_2 (PGD_2) in the rat. Control bolus injection of PGD_2 alone (●, $n = 24$), and with BW A868C (1 μg kg^{-1} min^{-1}, ○, $n = 8$), with 5 μg kg^{-1} min^{-1} (□, $n = 4$) and with 10 μg kg^{-1} min^{-1} (■, $n = 4$). Results, expressed as Δ fall in diastolic blood-pressure (BP, mmHg), are shown as mean with s.e.mean indicated by vertical bars; statistical differences from control PGD_2 responses are shown as $^*P < 0.05$; $^{***}P < 0.001$.

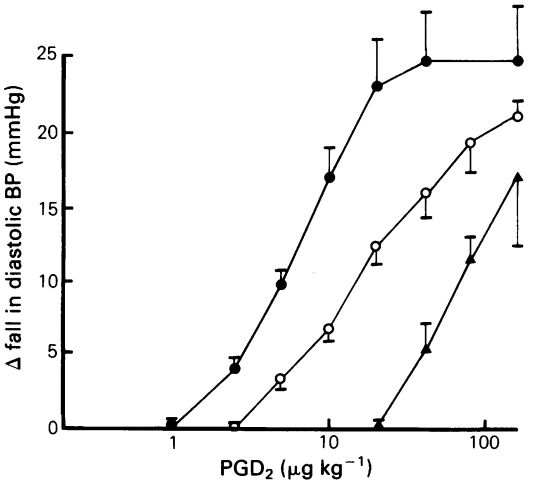

Figure 2 Effect of bolus i.v. administration of BW A868C (30 and 100 $\mu g\,kg^{-1}$) on the vasodepressor responses to prostaglandin D_2 (PGD_2) in the rat. Control i.v. bolus injection of PGD_2 alone (●, $n = 24$), with BW A868C (30 $\mu g\,kg^{-1}$, ○, $n = 4$) and BW A868C (100 $\mu g\,kg^{-1}$; ▲, $n = 4$). Results, expressed as Δ fall in diastolic blood-pressure (BP, mmHg), are shown as mean, with s.e.mean shown by vertical bars; statistical difference from the control PGD_2 dose-response curve by regression analysis was $P < 0.05$, and $P < 0.001$ in the presence of the lower and higher doses of BW A868C respectively.

response study being 40 min. The vasodepressor actions of PGD_2 (10–80 $\mu g\,kg^{-1}$, i.v.) were significantly ($P < 0.001$) reduced during an i.v. infusion of BW A868C (1 $\mu g\,kg^{-1}\,min^{-1}$), started 5 min before and maintained during the administration of PGD_2 (10–80 $\mu g\,kg$, i.v.). Furthermore, higher doses of BW A868C (5–10 $\mu g\,kg^{-1}\,min^{-1}$, i.v.) abolished the fall in BP induced by PGD_2 (Figure 1). These doses of BW A868C (1–10 $\mu g\,kg^{-1}\,min^{-1}$, i.v.) alone had no significant effect on resting BP ($n = 4$).

In further studies, bolus intravenous injection of BW A868C (30 and 100 $\mu g\,kg^{-1}$), administered 10 min before PGD_2, caused a significant rightward shift (3.4 and 13.2 fold respectively; $P < 0.05$ and $P < 0.001$) of the PGD_2 dose-response curve, with no significant reduction in the maximal vasodepressor response of PGD_2 with either dose (Figure 2).

Effect of BM 13.177 on PGD_2 responses

In preliminary experiments, in which the potency of BM 13.177 as a thromboxane antagonist *in vivo* was determined, the increase in BP (Δ 15 ± 5 mmHg, $n = 4$) following intravenous bolus injection of the thromboxane mimetic, U-46619 (1.0 $\mu g\,kg^{-1}$ i.v.) was abolished by pretreatment (5–10 min) with BM 13.177 (1–2.5 $mg\,kg^{-1}$, i.v.)

Pretreatment with BM 13.177 (2.5 $mg\,kg^{-1}$ i.v.), 5 min prior to PGD_2, caused a small rightward shift (2.1 fold, $P < 0.025$) of the PGD_2 dose-response curve. However, the maximal vasodepressor response (24 ± 4 mmHg, $n = 4$) achieved with PGD_2 (40 $\mu g\,kg^{-1}$) was not significantly reduced by this dose of BM. 13.177 (18 ± 2 mmHg, $n = 4$; $P > 0.05$).

Effect of BW A868C on BW245C responses

Intravenous injection of BW245C (0.25–160 $\mu g\,kg^{-1}$) produced dose-related falls in BP, as shown in Figure 3. BW A868C (10 $\mu g\,kg^{-1}\,min^{-1}$ i.v.), at a dose that abolished the PGD_2-induced fall in BP, caused a significant rightward shift (59 fold, $P < 0.001$) of the dose-response curve to BW245C (Figure 3). However, higher doses of BW A868C (20–

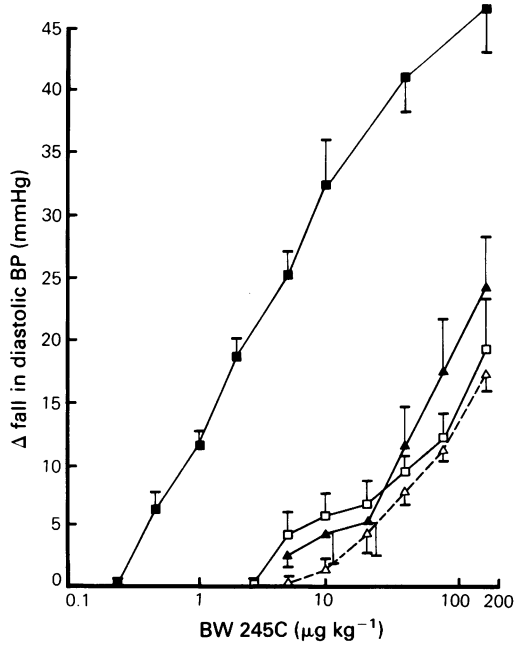

Figure 3 Effect of intravenous infusion of BW A868C on the vasodepressor responses to bolus i.v. injection of BW245C in the rat. Control BW245C responses alone (■, $n = 20$), with BW A868C 10 $\mu g\,kg^{-1}\,min^{-1}$ (□, $n = 5$), with 20 $\mu g\,kg^{-1}\,min^{-1}$ (▲, $n = 4$) and with 100 $\mu g\,kg^{-1}\,min^{-1}$ (△, $n = 8$). Results, expressed as Δ fall in diastolic blood pressure (BP, mmHg) are shown as mean with s.e.mean shown by vertical bars. Statistical differences from control BW245C dose-response curve by regression analysis were $P < 0.001$ for each group in the presence of BW A868C.

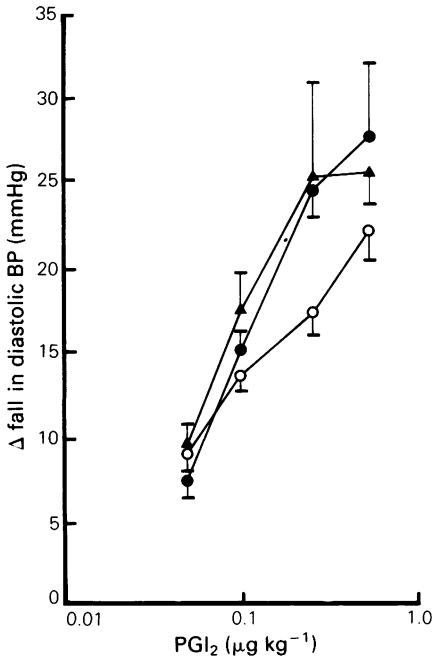

Figure 4 Effect of intravenous infusion of BW A868C on the vasodepressor responses to bolus i.v. injection of prostacyclin (PGI_2) in the rat. Control prostacyclin responses alone (●, $n = 10$), with BW A868C $10\,\mu g\,kg^{-1}\,min^{-1}$ (▲, $n = 6$) and with $100\,\mu g\,kg^{-1}\,min^{-1}$ (○, $n = 4$). Results, expressed as Δ fall in diastolic blood-pressure (BP, mmHg), are shown as mean, with s.e.mean shown by vertical bars. Statistical differences for parallelism by regression analysis from the control prostacyclin dose-response curve was $P < 0.01$ in the presence of the high dose of BW A868C ($100\,\mu g\,kg^{-1}\,min^{-1}$). However, there was no significant antagonism at this dose of BW A868C of the BP fall induced by any of the prostacyclin doses.

$100\,\mu g\,kg^{-1}\,min^{-1}$) caused no further shift of the dose-response curve. There was still significant inhibition of this response to BW245C, 75 min after termination of the infusion of BW A868C ($20\,\mu g\,kg^{-1}\,min^{-1}$), with the dose-response curve to BW245C being still shifted by 12.4 fold ($P < 0.05$) to the right of the control.

Effect of BW A868C on prostacyclin responses

Prostacyclin (0.05–0.5 $\mu g\,kg^{-1}$ i.v.) caused a dose-dependent fall in BP (Figure 4). However, infusion of BW A868C ($10\,\mu g\,kg^{-1}\,min^{-1}$, i.v.) failed to alter the vasodepressor actions of prostacyclin. A higher dose of BW A868C ($100\,\mu g\,kg^{-1}\,min^{-1}$, i.v.) induced a significant change in the slope of the prostacyclin dose-response curve (from 9.3 to 5.4, $P < 0.01$) although there was no significant inhibition of the hypotensive action of any of the prostacyclin doses studied.

Discussion

The full characterization of both *in vitro* and *in vivo* responses to PGD_2 in various tissues requires the use of specific DP-receptor antagonists. N-0164 has been shown to be an antagonist of the anti-aggregatory actions of PGD_2 on human platelets *in vitro* (MacIntyre & Gordon, 1977; Whittle *et al.*, 1978). However, our previous studies showed that N-0164 not only acted as a DP-receptor antagonist, but also as a thromboxane-receptor (TP) antagonist in guinea-pig platelets *in vitro* (Hamid-Bloomfield & Whittle, 1986). Similarly, diphloretin phosphate (Westwick & Webb, 1978), deacetyl-1-nantradol (Horne, 1984) and AH6809 (Keery & Lumley, 1988) have been reported as DP-receptor antagonists, but none exhibit the selectivity or potency required for stringent quantitative receptor classification.

The findings from the present *in vivo* study in the rat have confirmed BW A868C as a potent, selective antagonist for the vasodepressor actions of PGD_2, supporting the *in vitro* studies of its selective DP-receptor antagonism reported in the accompanying two papers (Giles *et al.*, 1989; Trist *et al.*, 1989). BW A868C potently and selectively reduced the vasodepressor action of PGD_2. A profile characteristic of competitive antagonism could not be obtained following intravenous infusion of BW A868C in these studies, since there was a reduction in the vasodepressor response to the maximal dose of PGD_2 that could be used under these conditions. This may reflect the increasing plasma accumulation of BW A868C during the period of infusion. Following bolus intravenous injection of BW A868C however, a dose-related parallel rightward shift of the PGD_2 response was observed, suggesting competitive antagonism at the DP-receptor sites *in vivo* under these conditions.

In our previous studies on guinea-pig platelets, PGD_2 exhibited a bell-shaped dose-inhibition relationship, the second phase of this curve reflecting its interaction at TP-receptor sites (Hamid & Whittle, 1985; Hamid-Bloomfield & Whittle, 1986). In the current study, pretreatment with the selective thromboxane receptor antagonist BM 13.177 (Patscheke & Stegmeier, 1984; Stegmeier *et al.*, 1984) had little effect on the vasodepressor responses to PGD_2. Thus, the vasodepressor actions of PGD_2 at these doses in the rat appear to be predominantly brought about by interaction at DP-receptor sites, with minimal actions at TP-receptor sites. Furthermore, it would be unlikely that the antagonism by

BW A868C *in vivo* of the vasodepressor responses to PGD_2 could be brought about by effects at vascular TP-receptors, since this compound is devoid of such effects *in vitro* (Giles *et al.*, 1989). The mechanism by which PGD_2 brings about the fall in systemic arterial blood pressure is not known, but it presumably reflects vasodilatation in peripheral vascular beds (Chapnick *et al.*, 1978; Wendling & DuCharme, 1981; Giles & Leff, 1988).

BW A868C, at doses that abolished the PGD_2-induced vasodepression, produced a rightward shift of the vasodepressor responses to the hydantoin prostanoid, BW245C. These findings are consistent with those of previous studies that BW245C interacts with DP-receptor sites (Whittle *et al.*, 1983; Town *et al.*, 1983; Keery & Lumley, 1988). However, the BW245C vasodepressor responses were not further shifted to the right or abolished with higher doses of BW A868C. This failure of BW A868C to suppress completely these vasodepressor responses gives support to the concept that BW245C also acts on vascular prostanoid receptors that are not of the DP-type (Whittle *et al.*, 1983, Giles *et al.*, 1988).

In doses sufficient to antagonize the responses to PGD_2 and BW245C, BW A868C failed to antagonize the vasodepressor action of prostacyclin, indicating that the effect of this compound was not simply a non-specific inhibition of vasodilatation and supporting a selective action of this compound on DP-vascular receptors *in vivo*. The current findings indicate that this compound is not rapidly cleared from the circulation, as demonstrated by the antagonism of BW245C responses observed 1 hour after administration. Thus, the profile of antagonism with BW A868C *in vivo* gives further support for its use in classifying DP-prostanoid receptors in the vasculature or other tissues and for potential therapeutic intervention in diseases that involve inappropriate release of PGD_2.

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Binding of [^{3}H]-muscimol to $GABA_A$ sites in the guinea-pig urinary bladder: biochemical assay and autoradiography

[1]*†S.L. Erdö, **M.C. Mione, **F. Amenta & *J.R. Wolff

*Department of Anatomy, Georg August University, 36 Kreuzbergring, D-3400 Göttingen, F.R.G.,
**Department of Neurological Sciences, 'La Sapienza' University, 50 Via A. Borelli, I-00161 Rome, Italy and
†Pharmacological Research Centre, Chemical Works of Gedeon Richter Ltd., H-1475 Budapest, Hungary

1 The specific binding of [^{3}H]-muscimol, a γ-aminobutyric $acid_A$ receptor ($GABA_A$) agonist, to whole membranes of the guinea-pig urinary bladder was examined. In addition, the distribution of specific muscimol binding sites within the bladder was visualized by autoradiography.

2 It was demonstrated that in a frozen-thawed whole membrane preparation of the organ the specific binding of [^{3}H]-muscimol is reproducible, reversible and saturable.

3 Saturable binding was of a single component with an equilibrium dissociation constant (K_d) of 12 nM and a maximal density (B_{max}) of about 80 fmol mg^{-1} protein.

4 In displacement experiments with several model compounds, [^{3}H]-muscimol binding sites showed the characteristics of a $GABA_A$ receptor site.

5 Autoradiographic experiments revealed uneven distribution of specifically bound [^{3}H]-muscimol in the bladder. The density of binding sites was high in clusters within the smooth muscle layers of the bladder fundus and of the urethra, while the apex and the neck were not specifically labelled.

6 The present findings show that $GABA_A$ type receptor sites in the guinea-pig urinary bladder may be labelled by [^{3}H]-muscimol in a specific and reproducible manner. Moreover, the localization of these binding sites is consistent with the presence of $GABA_A$ receptors in only a subpopulation of vesical ganglia.

Introduction

Pharmacological (Hill & Bowery, 1986), biochemical (Erdö, 1986) and autoradiographic (Amenta, 1986) lines of evidence indicate that specific receptor sites for γ-aminobutyric acid (GABA) are not restricted to the central nervous system, but are present also in various mammalian organs other than the brain.

Ligand binding studies with homogenates revealed the presence of specific, bicuculline-sensitive ($GABA_A$) receptor sites in several organs, such as female sex organs (Erdö & Lapis, 1982a,b; Erdö *et al.*, 1983; Erdö, 1984; Erdö & Laszlo, 1984), placenta (Erdö *et al.*, 1985), several types of blood vessels (Krause *et al.*, 1980a,b; Napoleone *et al.*, 1987), hepatocytes (Minuk *et al.*, 1987), and adrenal medulla (Kataoka *et al.*, 1984, Martinez *et al.*, 1987).

On the other hand, binding studies with membrane preparations of the gastro-intestinal tract, and urinary bladder, where GABA is regarded as a neurotransmitter (Jessen *et al.*, 1986, 1987; Taniyama & Tanaka, 1986) have not been published as yet, even though the presence of $GABA_A$ and $GABA_B$ receptors in these organs has been convincingly documented in pharmacological experiments with isolated organs (see Taniyama & Tanaka, 1986; Jessen *et al.*, 1987).

Therefore, we decided to develop a reproducible technique for identification of $GABA_A$ receptor sites in homogenates of the above mentioned organs. Here we show the presence of high-affinity binding

[1] Author for correspondence at Department of Anatomy, Georg August University, 36 Kreuzbergring, D-3400 Göttingen, F.R.G.

sites for the $GABA_A$ receptor agonist, muscimol in membranes of the guinea-pig urinary bladder. Moreover, the distribution of binding sites within the bladder has been analysed by autoradiography.

Methods

Animals and membrane preparation

White guinea-pigs, weighing 200–300 g, were decapitated and the urinary bladder as a whole, was dissected and cleaned of external connective tissue and fat. The bladder was opened by a razor blade and repeatedly rinsed by immersion in cold distilled water. The tissue was then placed on dry-ice and stored frozen overnight. On the next day, pooled tissue was homogenized in 10 volumes of ice-cold distilled water by an Ultra-Turrax (Janke & Kunkel, Ika Werk). The crude homogenate was sedimented at 48,000 *g* for 15 min (Hi-Spin, MSE) and the resulting pellet was suspended in water by an ultrasonic disintegrator (Soniprep 150, MSE) for 30 s. This rinsing procedure was repeated once more to reduce endogenous GABA content in the membranes. The resulting pellet was stored at −20°C for at least 16 h, but less than one week. This storage failed to influence the characteristics of [^{3}H]-muscimol binding to the membranes, as controlled in a separate experiment (data not shown). Prior to the assay, membranes were resuspenced in ice-cold Tris-citrate buffer (0.05 M, pH 7.1) and the rough tissue fragments, which had been present throughout, were removed by filtration through a fine mesh nylon screen (Eterlon 62 GG).

Binding assay

The binding assay was performed by a minor modification of a previously described centrifugation method (Erdö & Laszlo, 1984), with the following modifications. [^{3}H]-muscimol (specific activity 30.8 Ci mmol^{-1}, New England Nuclear Co.) was used as the ligand instead of labelled GABA and the incubation time was extended to 20 min. The saturability of the specific binding was examined at 8 different [^{3}H]-muscimol concentrations ranging from 2 to 120 nM. Binding parameters were determined by log-probit analysis of the Scatchard data. Non-specific binding was estimated in the presence of 0.1 mM unlabelled muscimol or 0.5 mM GABA. Specific binding was defined as the difference between total binding (in the absence of unlabelled displacers) and non-specific binding.

Protein measurement

Estimates of protein content were made according to the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Autoradiography

Dissected bladders were divided into the following portions: apex plus corpus (upper and lower bodies), fundus (base), neck, urethra. Terms in parentheses refer to the terminology used by Kusunoki *et al.* (1984). Separated tissue blocks were embedded in a cryoprotectant medium (OCT, Ames, U.S.A.), then rapidly frozen in a mixture of dry-ice and acetone. Serial sections of 7–8 μm thickness were then cut with a −20°C microtome cryostat. Sections were mounted on gelatine-coated microscope slides, air dried, preincubated (20 min) in cold buffer and subsequently incubated (30 min) in Tris-citrate buffer (50 mM, pH 7.1) with 5 nM [^{3}H]-muscimol as described earlier in detail (Napoleone *et al.*, 1987). Control sections were incubated in the same way, but with the addition of 0.1 mM unlabelled muscimol or 0.5 mM GABA. The same non-specific labelling was found with these two compounds.

Slides were postfixed in formaldehyde vapours (60 min, 80°C) and coverslips coated with nuclear emulsion (Ilford L4, 1:1 dilution with distilled water) were attached to the slides (see also Napoleone *et al.*, 1987). This fixation procedure preserved the morphology of the sections, failed to reduce the specifically bound radioactivity, and did not cause any translocation of the ligand, as compared in a pilot experiment with unfixed sections. After an exposure time of 8 to 10 weeks, they were developed in D19 Kodak.

Results

Membranes of the urinary bladder showed the capacity to bind [^{3}H]-muscimol in a specific manner. Non-specific binding was relatively low, representing 20 to 30% of the total binding (data not shown).

The specific binding of 5 nM [^{3}H]-muscimol increased with time up to about 15 min, at 4°C, then equilibrium was achieved (Figure 1). Binding proved to be reversible, as unlabelled GABA could displace equilibrium binding in a few minutes (Figure 1).

Incubations with increasing ligand concentrations revealed the presence of a single class of high-affinity, and saturable binding sites in membrane homogenates of the bladder (Figure 2). The equilibrium

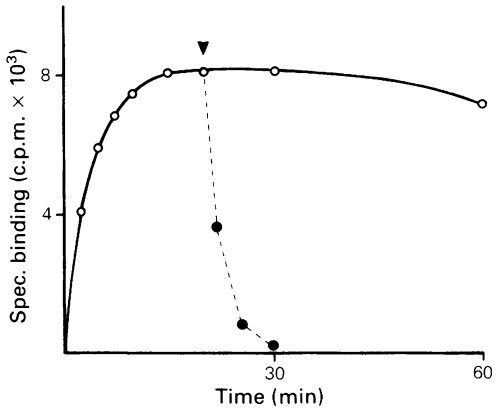

Figure 1 Time-course and reversibility of specific [^{3}H]-muscimol binding to membranes of the guinea-pig urinary bladder. Membranes were incubated at 4°C in 5 nM radioligand. Note the rapid displacement of specifically bound muscimol (broken line) by the addition of 0.1 mM GABA (arrowhead). Each point is the mean of two triplicate determinations, with less than 7% s.e.mean.

dissociation constant (K_D) and the maximal density of specific binding sites (B_{max}) were calculated from Scatchard plots (Figure 2), and they were found to be 12 ± 3 nM and 78 ± 39 fmol mg^{-1} protein, respectively. Values are mean ± s.d. of 5 determinations with separate membrane preparations.

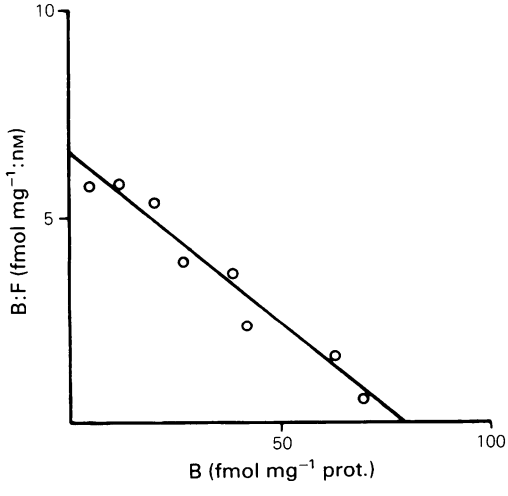

Figure 2 Representative Scatchard plot showing a single population of high-affinity binding sties for [^{3}H]-muscimol on membranes of the guinea-pig urinary bladder. Membranes were incubated at 4°C, for 20 min, in the presence of increasing (2 to 120 nM) muscimol concentrations. Points represent means of triplicate determinations. For binding parameters, see text.

Table 1 Displacement of specific [^{3}H]-muscimol binding from membranes of the guinea-pig urinary bladder

Compound	*IC_{50} value (μM)*
Muscimol	0.04
GABA	0.09
THIP	1.2
Bicuculline	44
Baclofen	>500
L-DABA	>500

Membranes were incubated for 20 min in the presence of 5 nM radioligand and of at least 5 different concentrations of each model-compound tested. IC_{50} values are given as the mean of 3 independent determinations with less than 10% s.d.
THIP = 1,2,3,4,-tetrahydro-isoxasolo-pyridinol;
L-DABA = L-diaminobutyric acid

In displacement experiments high concentrations of various compounds (see Table 1) known to act competitively at $GABA_A$ sites produced more than 97% reduction of the specific binding, while the uptake-blocker L-diamino-butyric acid (L-DABA) and the $GABA_B$ receptor agonist baclofen were ineffective. The concentrations of the compounds producing 50% displacement of the specific binding, i.e. the IC_{50} values, are presented in Table 1.

In autoradiographic experiments, silver grains representing specific [^{3}H]-muscimol binding sites were unevenly distributed. No specific binding occurred in the apex and the neck of the bladder. In these parts, only some non-specific accumulation of silver grains was seen in the intercellular spaces or over bundles of the connective tissue dividing bladder smooth muscle (not shown).

Specific binding sites were visualized in the fundus of the bladder, where silver grains were arranged in clusters within the smooth muscle layers (Figure 3). No specific binding was seen in the ureters and in their transitional zone in the bladder wall (not shown).

The highest density of specific binding sites was present in the urethra. Silver grains representing specific binding sites were concentrated in clusters within the smooth muscle (Figure 4). The density of binding sites was lower in the circular than in the longitudinal muscle layer (not shown).

Discussion

It has been demonstrated that GABA and its major biosynthetic enzyme, i.e. glutamate decarboxylase, are present in the guinea-pig urinary bladder, and that their distribution closely correlates with the dis-

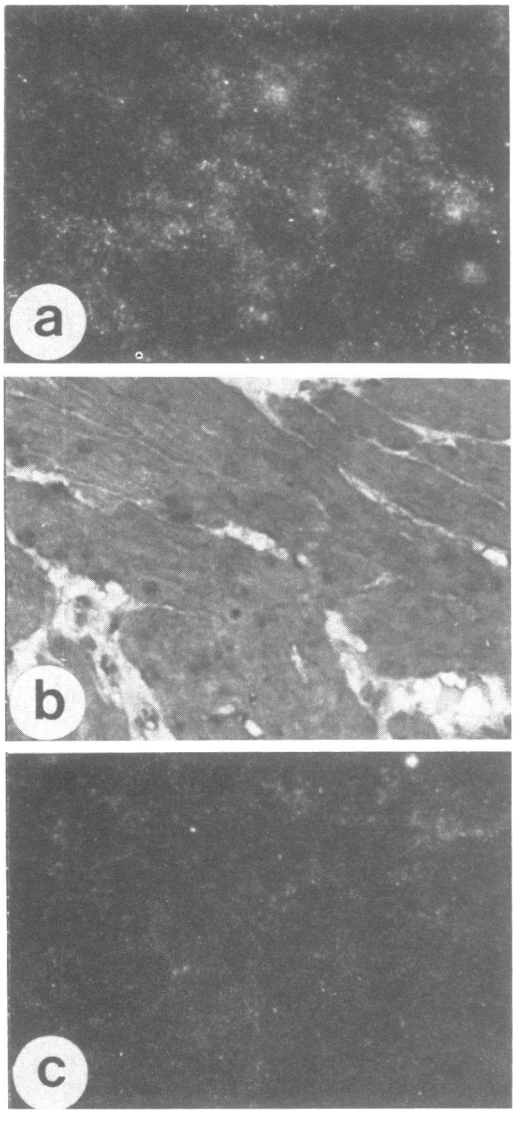

Figure 3 Autoradiogram of a section of the guinea-pig urinary bladder (fundus) incubated with 5 nM [^{3}H]-muscimol. (a) Dark field illumination; (b) bright field picture stained with toluidine blue to verify microanatomical details; (c) dark field picture of an adjacent control section incubated additionally with 0.1 mM unlabelled muscimol. Note the occurrence of silver grains representing specific binding primarily within the smooth muscle, where they appear to be accumulated in clusters (× 180).

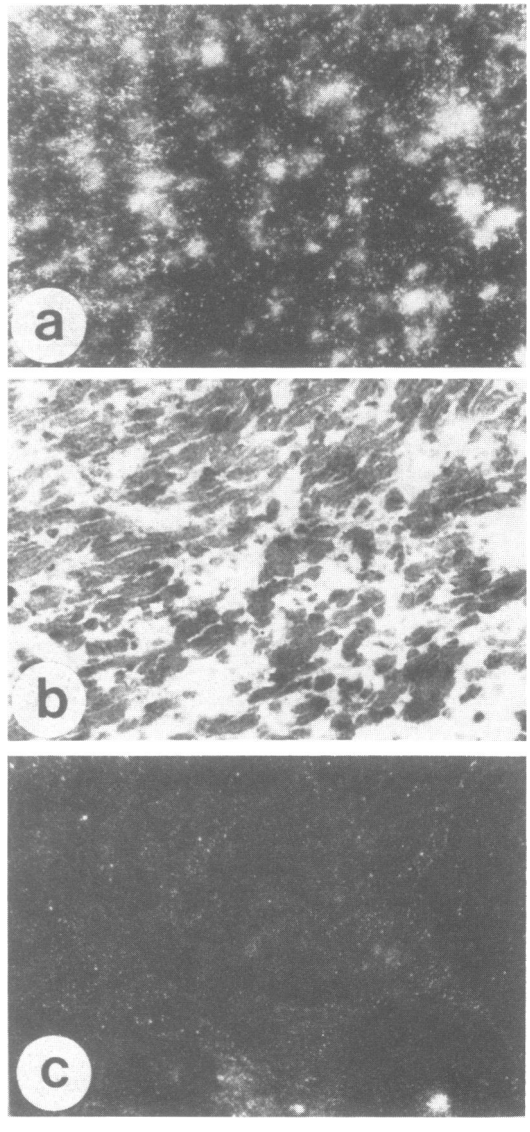

Figure 4 Autoradiogram of a section of the guinea-pig urethra incubated with [^{3}H]-muscimol as above. (a) Picture in dark field illumination; (b) bright field picture showing microanatomical details; (c) dark field picture of an adjacent control section incubated additionally with 0.5 mM unlabelled GABA. Note the high density of specific silver grains in clusters near smooth muscle cells (× 160).

tribution of ganglion cells within this organ (Kusunoki *et al.*, 1984). These authors also reported that electrical stimulation evokes the release of GABA from the bladder and that GABA inhibits the efflux of acetylcholine from strips of the organ in a bicuculline-sensitive manner (Kusunoki *et al.*, 1984). Moreover, through specific $GABA_A$ and $GABA_B$ receptors, GABA induces contractile responses of the isolated bladder (Taniyama *et al.*, 1983; Maggi *et al.*, 1985a,b). These findings, along with electrophysiological observations, have led to the hypothesis of a GABAergic innervation of the urinary bladder (Taniyama & Tanaka, 1986).

In the guinea-pig urinary bladder, $GABA_A$ receptors inhibit the cholinergic component of the contraction to electrical stimulation as well as the nicotine-induced contraction, in a manner consistent with the presence of the receptor sites on postganglionic cholinergic neurones (see Taniyama & Tanaka, 1986).

The occurrence of bicuculline-sensitive GABA receptors in homogenates of several organs other than the urinary bladder has also been demonstrated by ligand binding techniques (see Erdö, 1986). Moreover, autoradiographic evidence has been provided for the occurrence of specific $GABA_A$ binding sites in the guinea-pig ovary (Amenta *et al.*, 1986), in rat cerebral blood vessels (Napoleone *et al.*, 1987), in rat atria (Bowery & Hudson, 1983) and in the pancreas of neonatal rats (Reusens-Billen *et al.*, 1984).

The occurrence of a single population of high-affinity, saturable binding sites for [^{3}H]-muscimol, as well as the capacity of various displacers to reduce specific binding in bladder homogenates are consistent with former findings for peripheral $GABA_A$ sites (see Erdö, 1986). Thus, these binding sites correspond most probably with $GABA_A$ receptors.

It has been proposed that in the guinea-pig urinary bladder, $GABA_A$ receptors are located in vesical ganglia, on postganglionic cholinergic nerves (Kusunoki *et al.*, 1984; Maggi *et al.*, 1985a; Taniyama & Tanaka, 1986) and it has been demonstrated that the number of vesical ganglion cells, and the levels of GABA and glutamate decarboxylase show similar distribution patterns, with the highest densities in the upper body and decreasing levels towards the neck (Kusunoki *et al.*, 1984). By contrast, the present experiments failed to demonstrate any specific binding in the upper portion (apex plus corpus) of the bladder where both ganglion cell density and GABA concentration are high. This lack of correspondence might suggest that the GABA content of the ganglia in this upper region of the bladder may not act as transmitter through $GABA_A$ receptors, but rather through the baclofen-sensitive ($GABA_B$) receptor subclass which cannot be labelled by muscimol (Bowery *et al.*, 1983). In the fundus and the urethra, however, specific muscimol binding sites are located in clusters corresponding most probably to vesical ganglia within the musculature. Thus, these specifically labelled sites probably represent ganglionic $GABA_A$ receptors. Consequently, in the fundus of the bladder and in the urethra, GABA may act as transmitter via $GABA_A$ receptors.

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Structure-activity relationships of new analogues of arecaidine propargyl ester at muscarinic M_1 and M_2 receptor subtypes

[1]U. Moser, G. Lambrecht, M. Wagner, J. Wess, & E. Mutschler

Department of Pharmacology, University of Frankfurt/M, Theodor-Stern-Kai 7, Geb. 75 A, D-6000 Frankfurt/M, F.R.G.

1 The potency of arecaidine propargyl ester (APE) and of several analogues containing a modified ester side chain has been assessed at M_1 and M_2 muscarinic receptor subtypes. APE was shown to act as a potent agonist at ganglionic M_1 receptors in the pithed rat, at M_2 receptors in guinea-pig isolated atria ($-\log EC_{50} = 8.22$) and ileum ($-\log EC_{50} = 7.77$).

2 The arecaidine 2-butynyl and 2-pentynyl esters were approximately equipotent with APE at M_1 and M_2 receptors, whereas the 2-hexynyl derivative was found to be less potent than APE in atria ($-\log EC_{50} = 6.80$) and ileum ($-\log EC_{50} = 6.70$) by about one order of magnitude. The 2-heptynyl and 3-phenyl propargyl esters exhibited no agonist actions in atria and ileum.

3 Shifting the triple bond from the 2 to the 3 position and introducing a bulky group at position 1 of the ester side chain of APE and analogues resulted in competitive antagonists (pA_2 ranging from 4.9 to 7.3).

4 APE and its 2-butynyl analogue showed some agonistic selectivity for cardiac M_2 receptors (potency ratio, ileum/atria = 2.8 and 4.6 respectively). All antagonists in this series of compounds were not selective in terms of affinity since their pA_2 values at cardiac and ileal M_2 receptors were similar (potency ratios, ileum/atria = 0.4 to 1.2).

Introduction

Muscarinic receptors are composed of two major subpopulations which have been designated M_1 and M_2 (Hammer & Giachetti, 1982; Birdsall *et al.*, 1987; Lambrecht *et al.*, 1987). In addition, it is now well established that M_2 receptors can be further subdivided into an M_2 'cardiac' and an M_2 'smooth muscle/glandular type' (Mutschler & Lambrecht, 1984; Eglen & Whiting, 1986; Birdsall *et al.*, 1987; Doods *et al.*, 1987; Lambrecht *et al.*, 1987). This concept is mainly based on the availability of selective antagonists, such as pirenzepine, AF-DX 116, methoctramine, 4-DAMP or hexahydro-sila-difenidol (Birdsall *et al.*, 1987; Mutschler *et al.*, 1987). Although various agonists have also been reported to discriminate between the proposed muscarinic receptor subtypes (Mutschler *et al.*, 1987), the development of more selective potent muscarinic agonists still remains a highly important goal in muscarinic receptor research.

[1] Author for correspondence.

It has previously been reported that the muscarinic agonist arecaidine propargyl ester (APE, 1a; Figure 1) shows some selectivity for cardiac M_2 receptors as compared to those in the ileum (Mutschler & Hultzsch, 1973; Mutschler & Lambrecht, 1984). This selectivity profile of APE was confirmed by Barlow & Weston-Smith (1985). Additionally, APE also displayed considerable activity at ganglionic M_1 receptors in the pithed rat (Wess *et al.*, 1987).

In order to assess the role of the propargyl moiety for the observed activity/selectivity profile of APE, we have synthesized and tested several analogues of APE containing a modified ester side chain.

The following structural modifications were made (Figure 2): elongation of the ester side chain at C3; shift of the triple bond from C2/C3 to C3/C4; introduction of a methyl group at C1 or a phenyl group at C1 or C3.

All compounds (Table 2) were tested *in vitro* at ileal and atrial M_2 receptors of the guinea-pig. Based on the results of the *in vitro* experiments, several

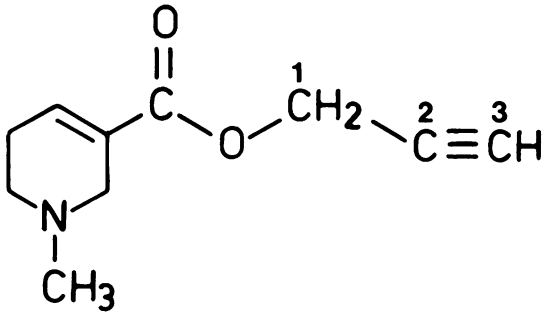


Figure 1 Chemical structure of arecaidine propargyl ester (APE, 1a)

agents were additionally tested on ganglionic M_1 receptors in the pithed rat by recording heart rate responses (Wess *et al.*, 1987).

A preliminary account of this investigation was presented at the 6th Camerino-Noordwijkerhout Symposium, Camerino, Italy, 1987.

Methods

Guinea-pig isolated ileum

Guinea-pigs (250–350 g) of either sex were killed by cervical dislocation. Strips of ileal longitudinal muscle (1.5–2 cm) were prepared according to the technique described by Paton & Zar (1968), transferred to 6 ml organ baths and loaded with a tension of 500 mg. Tyrode solution (pH = 7.4; temperature 32°C), bubbled with carbogen (95% O_2; 5% CO_2), was the bathing fluid. It had the following composition (mM): NaCl 137.0, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaHCO_3$ 11.9, NaH_2PO_4 0.42, glucose 5.6. The preparation was allowed to equilibrate for 30 min, during which time the bath fluid was changed every 10 min. Contractions were recorded isotonically with an electromechanical transducer connected to a Hellige amplifier and a Rikadenki recorder. Dose-response curves to APE were obtained by cumulative addition of the agonist (van Rossum, 1963). The concentration of agonist in the organ bath was increased approximately 3 fold at each step, with each addition being made only after the response to the previous addition had attained a maximal level and remained steady. Dose-response curves were repeatedly established until constant responses were obtained, allowing 30 min between each curve. Putative agonists were tested in the same manner as described for APE. Effects were expressed as percentages of the maximum effect induced by APE. $-\log EC_{50}$ values and intrinsic activities were determined graphically (van Rossum, 1963; Ariens & Simonis, 1964).

Antagonist affinities were determined by constructing concentration-response curves to APE at 3 different concentrations of antagonist (log conc. interval = 0.5), allowing 20–30 min equilibration time. EC_{50} values of APE were determined graphically for the control and the shifted concentration-response curves. Dose-ratios were calculated and Schild plots (Arunlakshana & Schild, 1959) were constructed from which pA_2 values were assessed according to Tallarida *et al.* (1979).

Guinea-pig isolated electrically paced left atria

Isolated left atria of guinea-pigs were suspended in Tyrode solution (composition and temperature as above) and oxygenated continuously with carbogen. The organ was electrically paced by supramaximal rectangular impulses of 3 ms duration (2 Hz, 4–10 V) using platinum electrodes. Preparations were preloaded with 500 mg and left to stabilize for 60 min. The effects of agonists were expressed as the percentage inhibition of the force of isometric contractions. Agonist and antagonist potencies were determined in essentially the same manner as described above for the ileum.

Pithed rat

Male normotensive White Wistar rats (200–300 g) were anaesthetized with pentobarbitone sodium (60 mg kg^{-1}, i.p.). The left jugular vein was cannulated for the administration of drugs. The heart rate was monitored continuously by means of a rate-meter (Hellige) which was triggered by the blood pressure pulse in the carotid artery. After catheterization of the trachea, heparin (300 iu kg^{-1}) was given i.v. to prevent blood coagulation. The rats were then pithed by introducing a steel rod into the spinal canal and artificial respiration with room air was provided (10 ml kg^{-1} body weight; 60 strokes min^{-1}). The body temperature was kept at 37 ± 1°C throughout the experiment by means of an overhead heating lamp.

All drugs were dissolved in saline (0.9% w/v) and injected i.v. in a volume of 1 ml kg^{-1}. To characterize agonist responses pharmacologically, pretreatment with pirenzepine (300 $\mu g\,kg^{-1}$ i.v.) was carried out 20 min before the administration of agonists. ED_{50} values were calculated graphically from log dose-response curves. The putative M_1 agonist McN-A-343 (Hammer & Giachetti, 1982; Wess *et al.*, 1984) served as a reference drug.

Statistics

The data are presented as means ± s.e.mean of n experiments. Differences between mean values were

tested for statistical significance ($P < 0.05$) by means of Student's t test.

Compounds

The following were used: atropine sulphate (Merck), heparin sodium (Promonta), hexamethonium iodide (Fluka), McN-A-343 (4-[N-(3-chlorophenyl) carbamoyloxy]-2-butynyltrimethylammonium chloride, kindly provided by Dr R. Hammer (Boehringer Ingelheim Zentrale, F.R.G.), pentobarbitone sodium (Abott), pirenzepine dihydrochloride (Thomae).

Chemistry

The esters 1a–1h and 2a–2c (Table 2) were prepared by azeotropic esterification of 1-methyl-1,2,5,6-tetrahydropyridine -3-carboxylic acid (arecaidine) with the corresponding alcohols under acid conditions by the procedure of Mutschler & Hultzsch (1973). The alcohols were commercially available and used directly for synthesis. All compounds were obtained as tosylates (Table 1).

Melting points were determined in open glass capillaries with a Büchi-Tottoli melting point apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer 1420 spectrophotometer. ^1H n.m.r. spectra were obtained on a Perkin Elmer R 23 spectrometer with Me_4Si as an internal standard. All spectral and analytical data for compounds 1a–1h and 2a–2c were consistent with the assigned structures. Microanalyses (C, H, N) were performed by the Department of Organic Chemistry, University of Frankfurt, F.R.G., and were correct within ± 0, 4% of the theoretical values. Thin layer chromatography was performed on Merck silica gel plates 60 F 245 in toluene/chloroform/acetone (9:4:14). Spots were visualized under 245 nm illumination or with Dragendorff spraying reagent.

Results

In vitro *studies*

The results obtained at atrial and ileal M_2 receptors are summarized in Table 2.

Lengthening of the ester side chain in APE (1a) from propargyl to 2-hexynyl (1b–1d) resulted in full agonists in both preparations. The effects of 1a–1d could be competitively blocked by atropine (30–300 nM), but were not affected by hexamethonium (30 μM) (data not shown). The arecaidine 2-butynyl (1b) and 2-pentynyl ester (1c) were approximately equipotent with APE in the atria ($P > 0.05$), whereas 1c was slightly (≈ 2 fold) more potent than 1a and 1b in the ileum ($P < 0.05$). In contrast, the arecaidine 2-hexynyl ester (1d) showed an about 10–30 fold lower potency than 1a–1c in both tissues (Table 2). While 1c and 1d displayed similar $-\log EC_{50}$ values at atrial and ileal M_2 receptors, compounds 1a and 1b were about 3–5 fold more potent in the atria than in the ileum (Table 2). Further elongation of the ester side chain led to the 2-heptynyl derivative 1e which behaved as a weak competitive muscarinic antagonist in both preparations.

Shift of the triple bond from 2- to 3-position in 1b–1d resulted in compounds 2a–2c which were devoid of agonist activity in both ileum and, except 2a (i.a. ≈ 0.2), in atria. In both preparations, 2a–2c proved to be competitive antagonists (Table 2).

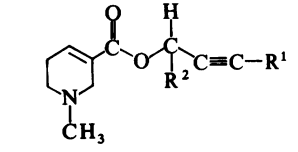
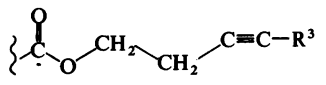
Introduction of a methyl group at C1 (Figure 1) in the ester side chain of APE led to a complete loss of efficacy in the ileum whereas weak partial agonist activity (i.a. = 0.2) was retained in the atria (1g). Introduction of a phenyl group at C1 or C3 in the ester side chain of APE resulted in competitive antagonists (1f, 1h). However, the 1-phenyl derivative (1h) showed an approximately 100 fold higher affinity than the 3-phenyl derivative (1f) in both preparations.

Table 1 Physical constants of arecaidine esters related to arecaidine propargyl ester (APE, 1a)

*Compound**	MP (°C)	Yield (%)	M_r	Formula
1b	128–129	23	365.45	$C_{18}H_{23}NO_5S$
1c	131–132	67	379.47	$C_{19}H_{25}NO_5S$
1d	122–123	26	393.50	$C_{20}H_{27}NO_5S$
1e	114–115	43	407.53	$C_{21}H_{29}NO_5S$
1f	135–137	44	427.52	$C_{23}H_{25}NO_5S$
1g	130–131	45	365.45	$C_8H_{23}NO_5S$
1h	177–178	10	427.52	$C_{23}H_{25}NO_5S$
2a	112–113	50	365.45	$C_{18}H_{23}NO_5S$
2b	104–105	39	379.48	$C_{19}H_{25}NO_5S$
2c	107–108	60	393.50	$C_{21}H_{29}NO_5S$

* Tosylates; colourless crystals; recrystn. solvent: acetone/ether.

Table 2 Chemical structures of arecaidine propargyl ester (APE, 1a) and analogues (1b–1h, 2a–2c) and their muscarinic/antimuscarinic activity at atrial and ileal M_2 receptors: potency ratios (ileum/atria) are given as a measure of receptor selectivity

General formula	*Compound*	R^1	R^2	R^3	*Atria (force)* $-\log EC_{50}$	*Atria (force)* pA_2	*Ileum* $-\log EC_{50}$	*Ileum* pA_2	*Potency ratio*[a] (ileum/atria)
	1a	H	H		8.22 ± 0.04		7.77 ± 0.02		2.8
	1b	CH_3	H		8.33 ± 0.04		7.67 ± 0.04		4.6
	1c	C_2H_5	H		8.27 ± 0.08		8.04 ± 0.07		1.7
	1d	$n-C_3H_7$	H		6.80 ± 0.03		6.70 ± 0.04		1.3
	1e	$n-C_4H_9$	H			5.77 ± 0.06[b]		5.89 ± 0.04	0.8
	1f	C_6H_5	H			4.86 ± 0.02[b]		5.25 ± 0.03	0.4
	1g	H	CH_3			6.38 ± 0.04[c]		6.45 ± 0.03	0.8
	1h	H	C_6H_5			6.96 ± 0.04		7.26 ± 0.03	0.5
	2a			H		5.97 ± 0.04[c]		6.01 ± 0.03	0.9
	2b			CH_3		6.76 ± 0.06		6.69 ± 0.03	1.2
	2c			C_2H_5		6.74 ± 0.05		6.67 ± 0.04	1.2

Values are mean ± s.e.mean, n = 4–7 for agonists and 4–16 for antagonists.

[a] EC_{50} ileum/EC_{50} atria in case of agonists; K_D ileum/K_D atria for antagonists ($pA_2 = -\log K_D$).

[b] Only two concentrations were investigated. The pA_2 values were therefore determined from the individual dose-ratios according to Tallarida *et al.*, 1979.

[c] Partial agonists: intrinsic activity relative to APE ≈ 0.2.

In vivo *studies*

Compounds 1a–1c and 2a–2b were also tested for M_1 receptor activity in the pithed rat. The resting heart rate of the pithed rats before drug treatment was 275 ± 7 beats min^{-1} (n = 27).

1a–1c (0.2–6 μmol kg^{-1}, i.v.) caused a brief transient decrease in heart rate (max. decrease about 40 beats min^{-1}), followed by a dose-dependent tachycardia (ED_{50} values approx. 1 μmol kg^{-1}; Figure 2). These increases in heart rate proved to be highly sensitive to blockade by a low dose of pirenzepine (300 μg kg^{-1}, i.v.), indicating the involvement of ganglionic M_1 receptors. Compounds 1a–1c produced similar maximum responses as McN-A-343, but were about 2.5–5 times less potent than the reference drug (Figure 2). On the other hand, 2a and 2b (0.1–30 μmol kg^{-1}, i.v.) showed no agonist activity in the pithed rat.

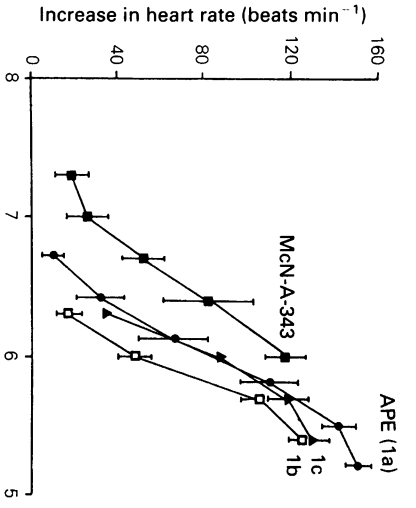


Figure 2 Dose-response curves for the increase in heart rate caused by McN-A-343 (■), 1a (●), 1b (□) and 1c (▲) in the pithed rat. Mean values are given (n = 3–10); s.e.means shown by vertical bars.

Discussion

The data show that muscarinic potency of APE and its analogues at both M_1 and M_2 receptors is highly dependent on the position of the triple bond in the ester side chain. In general, agonist activity could only be observed with compounds where the triple bond is located between C2 and C3 (Figure 1); in these compounds, elongation of the ester side chain up to C_6 was tolerated without loss of full agonist activity.

The arecaidine 2-butynyl and 2-pentynyl ester (1b, 1c) were approximately equipotent with APE at atrial and ileal M_2 receptors in the *in vitro* experiments as well as at ganglionic M_1 receptors in the pithed rat. In contrast, the corresponding analogues 2a and 2b (triple bond shifted to C3/C4) displayed virtually no agonist activity at either M_1 and M_2 receptors. Thus, the activities of 1a–1c and 2a, 2b at M_2 receptors closely paralleled those observed at M_1 receptors and no selectivity for either M_1 or M_2 receptors was found.

Introduction of a methyl group at C1 of the propargyl chain in APE led to compound 1g which showed an almost complete loss of efficacy and proved to be a competitive antagonist of APE-induced responses ($pA_2 = 6.4$). This finding indicates that bulk tolerance with respect to agonist activity is low at the C1 position. Similar results were obtained in a series of methyl-substituted oxotremorine derivatives (Ringdahl & Jenden, 1983a,b; Amstutz *et al.*, 1985). These findings suggest that among APE analogues, agonists and antagonists bind to a common site on the receptor molecule, in contrast to other antimuscarinic agents (e.g. atropine type, benzilic acid ester type) which are believed to interact predominantly with accessory sites adjacent to the agonist binding site (Ariens & Simonis, 1967).

Whereas 1h (phenyl-substitution at C1) showed a pharmacological profile similar to that of 1g (methyl-substitution at C1), 1f (phenyl-substitution at C3) displayed an approximately 100 fold lower affinity for both atrial and ileal M_2 receptors, indicating that introduction of bulky groups at C3 is associated with a dramatic loss in affinity. Moreover, these data suggest that highly potent muscarinic antagonists may be obtained by introduction of even larger substituents at C1 in APE-related compounds. Although none of the compounds could clearly discriminate between M_1 and both M_2 receptor subtypes, APE and the arecaidine 2-butynyl ester (1b) showed some degree of cardio-selectivity (about 3–5 fold). Interestingly, structure-activity studies of APE analogues in which the N-methyl group had been replaced by larger N-alkyl substituents revealed that the N-ethyl derivative of APE (N-ethyl guvacine propargyl ester) shows an even more pronounced cardioselectivity. This compound proved to be a rather potent partial agonist at rat cardiac M_2 receptors ($-\log EC_{50} = 6.56$; i.a. = 0.8 relative to APE), but a competitive antagonist at rat ileal M_2 receptors ($pA_2 = 6.06$) (Mutschler *et al.*, 1987).

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Neuroanatomical sites of action of 5-HT_3 receptor agonist and antagonists for alteration of aversive behaviour in the mouse

B. Costall, M.E. Kelly, [1]R.J. Naylor, E.S. Onaivi & *M.B. Tyers

Postgraduate School of Studies in Pharmacology, University of Bradford, Bradford, BD7 1DP and *Neuropharmacology Department, Glaxo Group Research Ltd, Ware, SG12 0DJ

1 The cerebral topography of the action of diazepam and the action of the 5-hydroxytryptamine 5-HT_3 receptor antagonists GR38032F and ICS 205–930 in attenuating an aversive response was studied in the mouse.

2 Mice which had been cannulated to allow drug injection into the dorsal and median raphe nuclei, the amygdala, nucleus accumbens or caudate-putamen were placed in a two compartment black (dimly illuminated) and white (brightly illuminated) test box. Measurements were made of the time spent, rearing and line crossings in the two sections and the latency of initial movement from the white to the black area.

3 The injection of diazepam (0.1–10 ng), GR38032F (0.01–1.0 ng) and ICS 205–930 (1.0–10 ng) into the dorsal raphe nucleus and amygdala, and the injection of diazepam (0.1–10 ng) into the median raphe nucleus, reduced an aversive response to the brightly illuminated white area, delaying the initial movement into the black section and increasing the time spent, rearings and line crossings in the white area. Concomitantly such activities were decreased in the black section.

4 The injection of the 5-HT_3 agonist 2-methyl-5-hydroxytryptamine (0.1–10 ng) into the dorsal raphe nucleus and amygdala caused the opposite response, decreasing the time taken to move into the black section and increasing the time spent, rearings and line crossings in the black section, decreasing such activities in the white area.

5 The 5-HT_3 agonist and antagonists showed little or no effect following injection into the median raphe nucleus and there were no changes in exploratory behaviour following their injection, or injection of diazepam, into the nucleus accumbens or caudate-putamen.

6 It is concluded that in the mouse the cerebral topography of action of GR38032F and ICS 205–930 in attenuating an aversive response follows that of diazepam in the dorsal raphe nucleus and amygdala but that diazepam may have additional effects mediated via the median raphe nucleus.

Introduction

Based on findings that open fields appear to have aversive properties which inhibit rodent exploratory behaviour (Christmas & Maxwell, 1970; Oliverio *et al.*, 1973), Crawley & Goodwin, (1980) described a mouse model where benzodiazepines produced a facilitation of exploratory behaviour between a lighted open field and a dark enclosure. The model was shown subsequently to detect anxiolytic agents specifically (Crawley, 1981; Blumstein & Crawley, 1983; Smith & Crawley, 1986) and, using a similar mouse model, and models developed in the rat and primate, the profile of action of 5-HT_3 receptor antagonists has been shown to be similar to that of the benzodiazepines (Costall *et al.*, 1987; 1988a,b; Jones *et al.*, 1987; 1988; Tyers *et al.*, 1987). This may indicate that 5-HT_3 receptor antagonists such as GR38032F (Brittain *et al.*, 1987; Butler *et al.*, 1988) and ICS 205–930 (Richardson *et al.*, 1985) have anxiolytic potential and in the present studies, using the mouse black and white test box system we investigate whether the topography of action of GR38032F and ICS 205–930 for modification of aversive responding follows that of diazepam.

[1] Author for correspondence.

Methods

The studies used male BKW mice (25–30 g) housed in groups of 10 in conditions of constant temperature (22 ± 1.0°C) and controlled lighting (dark

period 07 h 00 min–19 h 00 min) and fed *ad libitum* on a standard laboratory chow.

Behavioural experiments

Mice were taken from the dark holding room at 08 h 30 min in a dark container to a dimly illuminated room (red illumination) where, after 1 h period of adaptation to the new environment they were placed in the centre of the white area of the test box. The box (45 × 27 × 27 cm high) was open-topped and the base lined into 9 cm squares, two fifths painted black and illuminated under a dim red light (1 × 60 W, zero Lux) and partitioned from the remainder of the box which was painted white and brightly illuminated (1 × 60 W, 400 Lux), the red and white light sources being located 17 cm above the box. The compartments were connected by an opening 7.5 × 7.5 cm located at floor level in the centre of the partition. The mice were observed for 5 min by remote videorecording and four measurements were made (a) the time spent in the white and black section, (b) the number of rearings (a lifting of the head with or without lifting of the forelimb(s) from the floor) in the white and black areas, (c) the number of line crossings (movements of both the front and hind limbs across a line) in the white and black sections and (d) the latency of the initial movement from the white to the black area.

Stereotaxic implantation of cannulae and intracerebral drug injection

Fourteen days before the test, mice were anaesthetized with chloral hydrate (450 mg kg^{-1}, i.p.) and placed in the Kopf stereotaxic frame. Using standard stereotaxic techniques, holes were made at the point of guide cannulae penetration. Dental acrylic cement mixed with cyanoacrylate glue was used to bond the cannula unit to the skull. The wound was sealed and a stylet placed in the cannula which extended 0.5 mm below the bottom of the guide. Prior to intracerebral injection, the mice were manually restrained in a soft cloth and after removing the stylet(s) an injection unit(s), connected by pp25 polythene tubing to 5 μl Hamilton syringes, was inserted into the guide and the drug or vehicle solution administered over a 5 s period, the unit(s) remaining in position for a further 55 s. A volume of 0.25 μl was injected bilaterally into the nucleus accumbens (ACB), the central nucleus of the amygdala (ACE) or unilaterally into the dorsal or median raphe nuclei (DRN, MRN); a 0.5 μl volume was injected bilaterally into the caudate-putamen (CP). The injection unit(s) were then withdrawn, the stylet(s) replaced, and the animal placed immediately in the test box for behavioural measurements.

The guide cannulae were constructed from 6 mm lengths of stainless steel tubing (0.65 mm external diameter inserted and bonded (with Araldite Epoxy Resin) into predrilled (No. 73 drill) perspex sheets of 3 mm depth. The perspex was also drilled and tapped to allow attachment to the tool carrier of the stereotaxic instrument. The stainless steel tubing extended 3 mm below the base of the perspex and some 1.5 mm into the brain tissue. The small size of the perspex blocks (approximately 5.0 mm deep and 5.0 to 8.0 mm wide) ensured a non-intrusive presence on implantation.

The coordinates and lengths of the injection unit made from stainless steel tubing (0.3 mm external diameter) were selected such that the drug or vehicle would be injected into the 'centre' of the area under investigation. Cannulae were implanted vertically for injections into the ACB (Ant. 4.5, Vert. 4.2, Lat. ±1.0), the CP (Ant. 3.0, Vert. 3.8, Lat. ±2.3) and ACE (Ant. 2.5, Vert. 4.8, Lat. ±2.8) and angled at 30° posterior for injection into the MRN (Ant. −0.5, Vert. −5.4, Lat. 0.0) and DRN (Ant. −0.5, Vert. −3.1, Lat. 0.0; Atlas of Slotnick & Leonard, 1975).

Experimental design

Behavioural testing was carried out immediately following intracerebral injection. Control data were obtained with non-cannulated untreated mice and cannulated mice receiving intracerebral injection of vehicle (saline). In each test period, non-treated mice and cannulated mice receiving vehicle or drug treatments were used in groups of 5. Drug treatment regimes were randomised between test periods and each treatment assessed in 3 groups of mice, the mean values being determined from 15 determinations. No significant differences were recorded between the control values obtained in different test periods or days (see for example the control data reported on Figure 1) and to simplify the presentation of data obtained from non-treated and vehicle-injected animals, data are presented from only 15 animals in Figures 1 to 4. It is emphasised that animals were used on a single occasion only and all cannulated mice were subsequently killed and the site(s) of drug/vehicle deposition identified from the site of the injection cannula track in frozen sections.

Drugs

GR38032F (1,2,3,9-tetrahydro-9-methyl-3[(2-methyl-1H-imidazol-1-yl)]-methyl-4H-carbazol-4-one, HCl.$2H_2O$), (Glaxo Group Research Ltd), ICS 205–930 ([3α-tropanyl]-1H-indole-3-carboxylic acid ester.HCl), (Sandoz) and 2-methyl-5-hydroxytryptamine.HCl (2-methyl-5-HT) (Glaxo Group) diazepam (Roche Products Ltd) was dissolved in the

minimum quantity of polyethylene glycol and prepared to volume with distilled water. Doses quoted in the text refer to the amount of parent drug used rather than the salt.

Results

Behavioural experiments

Non-drug treated mice placed in the centre of the white section explored the area for some 10 to 12 s before moving into the black arena. This latency of the initial movement from the white to the black section was reproducible between experiments and was not significantly ($P > 0.05$) modified by the cannulation procedure or by the injection of vehicle into the DRN, MRN or ACE (Figure 1). However, the latency of movement from the white to the dark section was delayed 2 to 3 fold following the injection of diazepam (0.1–10 ng) into the DRN and ACE; GR38032F (0.01–1.0 ng) and ICS 205–930 (0.1–10 ng) caused a similar profile of behavioural change. The injection of diazepam into the MRN had a similar effect although GR38032F and ICS 205–930 caused no change in the latency excepting at the large dose of 10 ng GR38032F. The injection of 2-methyl-5-HT into the DRN and ACE reduced the latency of the initial movement into the dark area; the injection of 2-methyl-5-HT into the MRN was ineffective (Figure 1).

The relative size of the two compartments, two-fifths black and three-fifths white, and the lighting intensity used, ensured that mice spent an approximately equal time in each area. This was not modified by either the cannulation procedure or the intracerebral injection of vehicle (Figure 2). Following the injection of diazepam into the DRN, MRN and ACE, mice spent more time in the white area, achieving a 1.5–2.0 fold increase. A similar profile of behavioural change followed the injection of GR38032F and ICS 205–930 into the DRN and ACE; only GR38032F (10 ng) caused an increase in time spent in the white area following injection into the MRN. An injection of 2-methyl-5-HT into either the DRN or ACE caused changes opposite to those

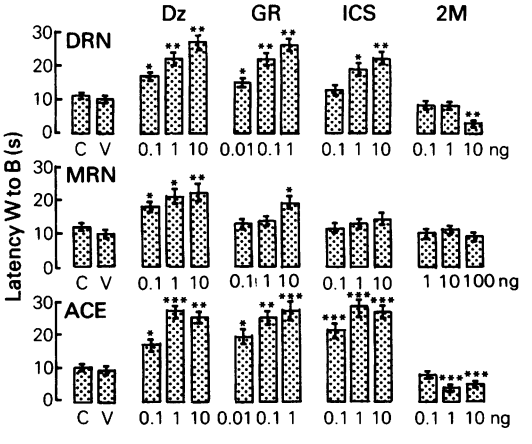

Figure 1 Effects of diazepam (Dz), GR38032F (GR), ICS 205–930 (ICS) and 2-methyl-5-hydroxytryptamine (2M) in modifying the latency of the movement of mice from the white (W) to the black (B) section of the test box (after first placement in the white area) following injection into the dorsal raphe nucleus (DRN), the median raphe nucleus (MRN) and central nucleus of the amygdala (ACE). Each column is the mean of 15 determinations with s.e.mean shown by vertical bar. Significant increases in responding compared with vehicle (V)-treated controls (C is the response of non-treated non-cannulated mice) are indicated $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (one-way ANOVA followed by Dunnett's t test).

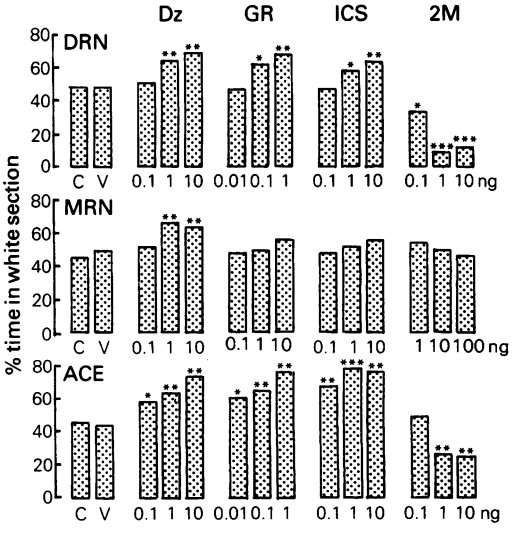

Figure 2 Effects of diazepam (Dz), GR38032F (GR), ICS 205–930 (ICS) and 2-methyl-5-hydroxytryptamine (2M) injected into the dorsal raphe nucleus (DRN), the median raphe nucleus (MRN) and central nucleus of the amygdala (ACE) in modifying the time spent by mice in the black and white compartments of the test box. Data are expressed as the % time spent in the white section during the 5 min test period. Each value is the mean of 15 determinations; s.e.means calculated on the original data were in the range 5.8–9.3%. Significant increases or decreases in responding compared to vehicle (V) control (C indicates the response of non-cannulated non-treated mice) are indicated $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ (one-way ANOVA followed by Dunnett's t test).

of diazepam and the 5-HT_3 antagonists, i.e. an increase in the time spent in the black area. The injection of 2-methyl-5HT into the MRN failed to modify the proportion of time spent in either section of the test box (Figure 2).

The increases in time spent in the white area caused by the injection of diazepam, GR38032F and ICS 205–930 into the DRN, ACE or MRN were associated with an increased number of rearings and line crossings. Whereas the drug treatments increased the time spent in the white area by 1.5–2.0 fold, the increases in rearings were in the range of 2–5 fold. Whilst the increase in rearing may reflect an increased level of locomotor activity this is unlikely to be a major factor since line crossings were only increased by 1.5–2 fold. The latter may simply mirror the increased time spent in the white area. Also, it is emphasised that the increases in rearings and line crossings in the white area were associated with a marked decrease in these activities in the black area. This again indicates that the increased rearing and line crossing in the white area does not reflect a drug-induced general increase in activity throughout the test box (Figures 3 and 4).

Following its injection into the DRN and ACE, the ability of 2-methyl-5-HT to increase the amount of time spent in the black section was associated with an increase in rearing and line crossings. Such changes were associated with a decrease in rearing and line crossings in the white area. Whilst the injection of 2-methyl-5-HT (0.1 and 10 ng) into the MRN failed to modify the time spent in rearing or line crossing in either the white or black sections of the test box, a higher dose of 2-methyl-5-HT (100 ng) increased rearing and line crossing in the black section but failed to modify significantly such activities in the white (Figures 3 and 4).

The injection of diazepam, GR38032F, ICS 205–930 and 2-methyl-5-HT (0.01, 0.1, 1.0, 10 and 100 ng) into the nucleus accumbens and caudate putamen failed to modify the latency of the first movement from the white to the black section, the time spent or the rearing and line crossings in either the black or white areas of the test box ($n = 5$ for each treatment); the values obtained were indistinguishable from the control values shown in Figures 1 to 4.

The modification of mouse behaviour in the white and black test box following drug injection into the DRN, MRN or ACE was not associated with 'non-specific' changes in activity such as tremors, stereotyped movements or seizure activity. In approximately 10% of the mice cannulated to allow drug injection into the MRN, the injection of vehicle or drug caused a circling response. The circling is probably the consequence of asymmetric damage to the raphe complex (revealed on histological examination) and such animals were removed from the study.

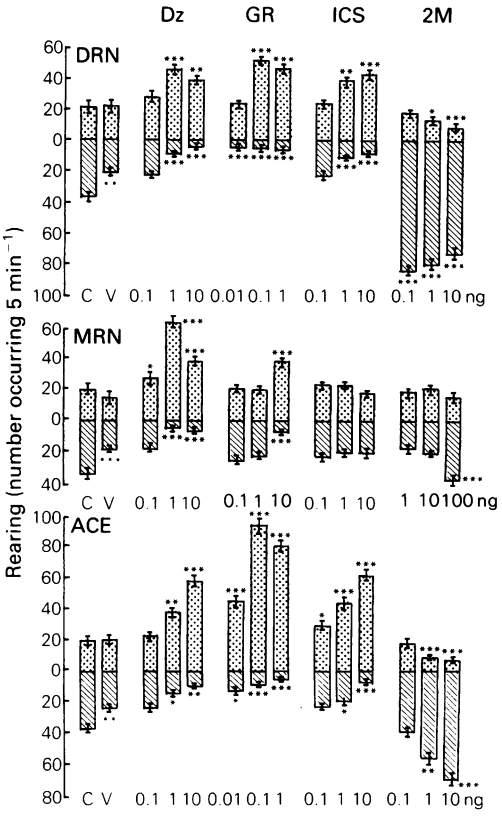

Figure 3 Effects of diazepam (Dz), GR38032F (GR), ICS 205–930 (ICS) and 2-methyl-5-hydroxytryptamine (2M) injected into the dorsal raphe nucleus (DRN), the median raphe nucleus (MRN) and central nucleus of the amygdala (ACE) in modifying mouse rearing behaviour in the black and white test box. Rearing is expressed as the number occurring in the black (hatched histogram) and white (stippled histograms) sections. Each value is the mean of 15 determinations; s.e.means shown by vertical bars. Significant increases or decreases in responding compared with the vehicle (V) controls (C is the response of non-cannulated non-treated mice) are indicated $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$; significant reductions in responding in vehicle treated animals as compared to C are indicated $^{\bullet\bullet}P < 0.01$ and $^{\bullet\bullet\bullet}P < 0.001$ (one-way ANOVA followed by Dunnett's t test).

Histology

The brains of all cannulated animals were examined to determine the site of drug or vehicle deposition.

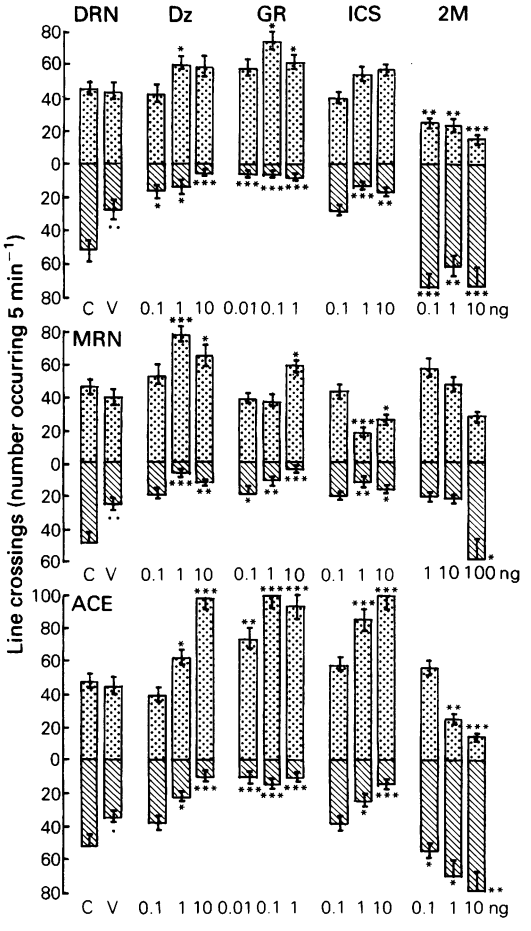

Figure 4 Effects of diazepam (Dz), GR38032F (GR), ICS 205–930 (ICS) and 2-methyl-5-hydroxytryptamine (2M) injected into the dorsal raphe nucleus (DRN), the median raphe nucleus (MRN) and central nucleus of the amygdala (ACE) of the mouse in modifying line crossing activity in the black and white test box. Line crossings are expressed as the number occurring in the black (hatched histogram) and white (stippled histograms) sections. Each column is the mean of 15 determinations; s.e.means shown by vertical bars. Significant increases or decreases in responding compared with the vehicle (V) controls (C is the response of non-cannulated non-treated mice) are indicated $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$; significant reductions in responding in vehicle treated animals as compared to C are indicated $\bullet P < 0.05$ and $\bullet\bullet P < 0.01$ (one-way ANOVA followed by Dunnett's t test).

Since over 300 animals were prepared for each of the 5 brain areas to be investigated, only the data for every 30th animal examined are presented. The routine preparation of large numbers of animals facilitated an accurate placement of cannulae and subsequent injection. The cannulae locations were accurate to within 0.4–0.6 mm of the desired injection point, and, given the small size of the structures involved, it was considered that drug or vehicle diffusion would be adequate to ensure drug action within the area of interest. For example, the central nucleus of the amygdala and nucleus accumbens extend over 1.3 mm only, and the raphe nuclei are considerably smaller. Thus injections aimed at the central nucleus of the amygdala almost certainly influence other nuclei of the amygdala complex, e.g. the basal and lateral nuclei. It remains possible that the injections aimed at the median and dorsal raphe nuclei may influence the nucleus reticularis tegmenti pontis and substantia grisea periventricularis respectively, and that injections into the nucleus accumbens may diffuse to influence the head of the caudate putamen (Figure 5).

Discussion

Mice taken from a dark environment and placed in a two compartment black and white box exhibited an aversion to the brightly illuminated white area. It should be noted that given the relative size of the two compartments, two-fifths black and three-fifths white, a preference for the black section was shown by the approximately equal time spent and number of line crossings in the two areas and a greater number of rearings in the black section. The peripheral administration of a benzodiazepine reverses the above preference by increasing the time spent, rearings and line crossings in the white area and decreasing such activities in the black; mice placed in the white area are also slower in moving to the black section (Costall *et al.*, 1986; 1988a).

In the present studies a similar profile of action was observed following the injection of diazepam into the amygdala and dorsal and median raphe nucleus of the mouse brain: injections into the nucleus accumbens and striatum were ineffective. The injection of vehicle into the amygdala and raphe nuclei also reduced rearing and line crossings in the dark section, although the time spent in the two compartments and rearing and line crossings in the white section were not modified. The vehicle effects may reflect non-specific damage since lesion or disruption of the 5-hydroxytryptamine system may release behaviours suppressed by punishment (Geller & Blum, 1970; Tye *et al.*, 1977; 1979); for example, lesions of the median or dorsal raphe nuclei may increase social interaction in the rat (File *et al.*, 1979). In any event, the effect of the injection of diazepam greatly exceeded that of the vehicle and

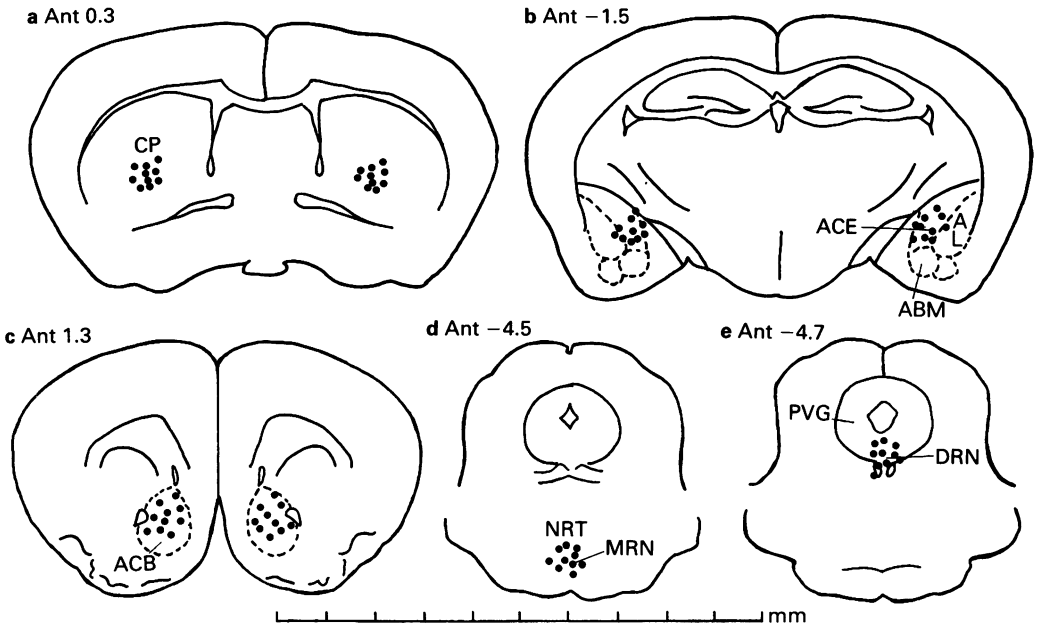

Figure 5 Diagrammatic representation of the location of injection sites (●) in the mouse brain prepared by reference to the Atlas of Slotnick & Leonard (1975), anterior coordinates indicated. Injections were directed at (a) the caudate putamen (CP), (b) the central nucleus of the amygdala (ACE), (c) the nucleus accumbens (ACB), (d) the median raphe nucleus (MRN) and (e) the dorsal raphe nucleus (DRN). Data are presented from every thirtieth brain examined; AL = nucleus amygdaloideus lateralis, ABM = nucleus amygdaloideus basalis, PVG = substantia grisea periventricularis, NRT = nucleus reticularis tegmenti pontis.

reduced the aversive response to the white environment.

The ability of diazepam to attenuate an aversive response in the mouse following injection into the raphe nuclei may be analogous to the effects of chlordiazepoxide in releasing behaviour suppressed by punishment following its injection into the dorsal raphe nucleus of the rat (Thiebot *et al.*, 1982). Since the inhibition of the firing of 5-hydroxytrytamine cells in the raphe nuclei by γ-aminobutyric acid (GABA) is enhanced by diazepam (Gallagher, 1978), it is possible that the benzodiazepines may at least in part, exert their anti-aversive actions via the raphe nuclei, attenuating 5-hydroxytryptamine function in the forebrain. Certainly, there is considerable evidence that 5-hydroxytryptamine pathways are involved in the control of anxiety, with a reduction in 5-HT function leading to an anxiolytic effect (see reviews by Stein *et al.*, 1975; Gardner, 1985; Chopin & Briley, 1987). Within the forebrain the amygdala may be a further site of benzodiazepine action since the injection of diazepam into the amygdala also attenuated the aversive response of mice in the black and white test box. Indeed, injections into the amygdala were as effective as injections into the raphe nuclei which supports observations that injections of benzodiazepines into the amygdala of the rat reduces conflict behaviour (Shibata *et al.*, 1982).

The profile of behavioural change of mice in the black and white test box following the injection of GR38032F and ICS 205–930 into the amygdala and dorsal raphe nucleus was very similar to that caused by diazepam. However, the abilities of the 5-HT_3 receptor antagonists to attenuate an aversive response in the mouse was not consistently observed following their injection into the median raphe nucleus. In this respect the site(s) of action of GR38032F and ICS 205–930 would appear to differ from diazepam. Similar differences are also apparent in the site of action of the 5-HT_3 receptor agonist 2-methyl-5-HT (Richardson *et al.*, 1985). 2-Methyl-5-HT injected into the dorsal raphe nuclei and amygdala exacerbated the aversive behaviour of mice, i.e. it increased the time spent, rearing and line crossings in the black section, but the median raphe nuclei was much less responsive. Indeed, the injection of a high dose of 2-methyl-5-HT into the median raphe nucleus was required to increase the aversive response, and this may have influenced areas outside the median raphe nucleus itself. Therefore a 5-HT_3

receptor involvement in the dorsal raphe nucleus and amygdala would appear important for the control of aversive responding in the mouse and provide possible sites of action for peripherally administered 5-HT$_3$ receptor antagonists; the data offer little support for a 5-HT$_3$ receptor involvement in the median raphe nucleus.

In summary, there is a similarity in the topography of action of GR38032F, ICS 205–930 and diazepam for attenuation of an aversive response in the mouse, such effects being obtained from injection into the dorsal raphe nucleus and amygdala whilst injections into the nucleus accumbens and caudate putamen were ineffective. However, differences were apparent in the actions of diazepam and the 5-HT$_3$ receptor antagonists since the median raphe nucleus was sensitive to the actions of diazepam but not GR38032F and ICS 205–930. If an ability to attenuate an aversive response can be translated into changes in anxiety responding, then the conclusion may be drawn that the raphe projection to the amygdala may be an important locus of anxiolytic action for the benzodiazepines and a site of action for the 5-HT$_3$ receptor antagonists.

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Interactions of calcium antagonists and the calcium channel agonist Bay K 8644 on neurotransmission of the mouse isolated vas deferens

Giles A. Rae & João B. Calixto

Department of Pharmacology, CCB, Universidade Federal de Santa Catarina, 88000 Florianópolis, SC-Brazil

1 The present study compares the effects of verapamil and Bay K 8644 on twitches of the mouse vas deferens induced by field stimulation at 0.1 Hz. The influence of interactions between these drugs and nifedipine on neurotransmission was also investigated.

2 Bay K 8644 (0.1 nM–3 μM) and verapamil (1–100 μM) potentiated twitches maximally by about 1000% (EC_{50} 17.3 nM) and 300% (EC_{50} 17.5 μM), respectively. Nifedipine (0.1 nM–1 μM) only reduced twitch magnitude (IC_{50} 7.7 nM). All effects were reversed following washout.

3 Yohimbine (1–100 μM) reversed twitch potentiation caused by verapamil but not by Bay K 8644. Prazosin (1 μM) did not reduce basal twitch tension nor antagonize twitch potentiation by verapamil.

4 Twitch inhibition by nifedipine was unaltered by previous incubation with verapamil (30 μM), but Bay K 8644 (1 μM) shifted the curve to nifedipine 120 fold to the right. Previous incubation with nifedipine (1 μM) blocked potentiation induced by verapamil but did not modify responsiveness to Bay K 8644.

5 Previous addition of verapamil (30 μM) markedly enhanced twitch potentiation caused by Bay K 8644 in a supra-additive fashion. In experiments conducted in the reversed condition, Bay K 8644 (1 nM but not 10 nM) potentiated the effect of verapamil in a similar manner but to a lesser extent.

6 It is concluded that verapamil, in contrast to nifedipine, markedly enhances neurally-evoked twitches of the mouse vas deferens. Bay K 8644 produces essentially the same effect as verapamil, but its potency is 1000 fold and its maximal effect about 3 fold greater than that observed for verapamil. It is suggested that the mechanism of twitch potentiation by verapamil is different from that of Bay K 8644 and may involve an increased release of non-adrenergic co-transmitter(s).

Introduction

A vast number of pharmacological, electrophysiological and binding studies have characterized the inhibitory effects of different subclasses of organic calcium antagonists at voltage- and receptor-operated calcium channels on cardiac and smooth muscles (for recent reviews see Spedding, 1985; Cauvin & Van Breemen, 1987; Kamp & Miller, 1987). However, much less is known about the influence of such compounds on neuronal tissues, which posess a high density of calcium channels (Murphy & Snyder, 1982). The number of studies is limited and these have provided controversial results. Although some studies have shown that calcium antagonists are inactive or only weakly active on neurones (Daniell *et al.*, 1983; Freedman & Miller, 1984; Rampe *et al.*, 1984; Taube & Schwarzkroin, 1986), others demonstrated that such compounds can effectively inhibit calcium influx and/or transmitter release under certain circumstances (Middlemiss & Spedding, 1985; Turner & Goldin, 1985; Nowycky *et al.*, 1985; Woodward & Leslie, 1986; Kingsbury & Balazs, 1987).

It is becoming increasingly evident that the population of calcium channels in neuronal and muscular cells are not identical (for review see Hoffman *et al.*, 1987). Such differences may account for the differential sensitivity of autonomically innervated tissues to inhibition, by calcium antagonists, of responses induced by agonists and those evoked by nerve stimulation. Studies have shown that verapamil increases electrically-induced release of [^{3}H]-noradrenaline from sympathetic nerves innervating rat

caudal artery, guinea-pig vas deferens (Zsotér *et al.*, 1984) and rabbit aorta (Karaki *et al.*, 1984) and urethra (Larsson *et al.*, 1984). Moreover, verapamil enhances the magnitude of field stimulation-evoked twitches of the prostatic portion of the rat vas deferens (French & Scott, 1981; 1983; Hay & Wadsworth, 1983). Recently, Moritoki *et al.* (1987) reported that verapamil potentiates non-adrenergic nerve-mediated contractions of the rat vas deferens, as does diltiazem and the calcium-channel activator dihydropyridine Bay K 8644. However, verapamil was found only to inhibit twitches of the field-stimulated mouse vas deferens (Zetler & Kaschube, 1985). This discrepant finding, which is surprising in view of the very dense and apparently exclusive motor sympathetic innervation of the mouse vas deferens (Stjärne & Lundberg, 1986), prompted us to reinvestigate the effects of verapamil on neurotransmission in this preparation. Also, we have investigated the influence of interactions between verapamil, nifedipine and Bay K 8644 on nerve-induced twitches.

Methods

Animals

Male Swiss albino mice (25–35 g), raised in temperature-controlled (22 ± 1°C) ambience with 12 h light/dark cycle and allowed free access to water and Purina lab chow, were used throughout the study.

Mouse isolated vas deferens

The general set up and procedure employed has been described by Rae & De Moraes (1983). Mice were killed by cervical dislocation and both vasa deferentia were carefully excised, united by a small portion of the prostate gland, and placed in a Petri dish containing physiological salt solution (see composition below). After removing the excess of adherent connective and adipose tissues, each preparation (consisting of two vasa deferentia) was transfered to a double-jacketed organ bath containing 5 ml of a modified Krebs-Henseleit solution at 30°C bubbled with 5% CO_2 in O_2. The composition of the salt solution was (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, KH_2PO_4 0.9 and glucose 11 (pH = 7.3). Preparations were submitted to a basal tension of 0.15 g and allowed a 60 min equilibration period, during which the bathing solution was renewed every 15 min, before initiating electrical field stimulation. Rectangular wave electrical pulses of 1 ms duration, delivered at 0.1 Hz and of supramaximal strength, were applied via a pair of platinum electrodes consisting of a hook below and a ring above each preparation. Isometric contractions were registered by means of strain gauge transducers coupled to a pen recorder (Narco Biosystems).

Once the field stimulation-evoked twitches became stable, preparations were exposed to increasing and cumulative concentrations of the calcium-channel activator Bay K 8644 (0.1 nM to 3 μM), or of the calcium-channel antagonists verapamil (1 to 100 μM) or nifedipine (0.1 nM to 1 μM). In some experiments, a second cumulative concentration-response curve to one of these drugs was obtained in the presence of a maximally- or submaximally-effective concentration of another.

Other sets of experiments examined the influence of prazosin (1 μM) or yohimbine (0.3 to 300 μM) on the magnitude of field stimulation-evoked twitches and on the responsiveness to verapamil. In the latter experiments prazosin was added to the bathing solution at least 10 min before exposure to verapamil (1 to 100 μM), whereas yohimbine (0.3 to 300 μM) was given in the presence of a maximally effective concentration of verapamil (30 μM).

In some experiments, single vas deferens were challenged repeatedly with noradrenaline (100 μM) or acetylcholine (1 mM) at 15 min intervals. The mean value of the first three control exposures to the agonist was taken as the 100% response. Subsequent challenges with the agonist were conducted in the presence of increasing concentrations of verapamil (1 to 30 μM), added 10 min prior to each challenge.

Drugs

Drugs used were acetylcholine iodide, nifedipine, noradrenaline bitartrate, tetrodotoxin, verapamil hydrochloride, yohimbine hydrochloride (all from Sigma Chemical Company), Bay K 8644 (methyl 1,4-dihydro - 2,6 - dimethyl - 3 - nitro - 4 - (2 - tri - fluoromethylphenyl)-pyridine-5-carboxylate, Bayer A.G.) and prazosin hydrochloride (Pfizer, São Paulo, Brazil). All drugs were stored as 10 mM stock solutions for up to a week at −4°C and diluted to the desired concentrations in distilled and deionized water just before use. Stock solutions of Bay K 8644, nifedipine, prazosin and yohimbine were made up in 100% ethanol before dilution with distilled water. Other stock solutions were made up in water and that of noradrenaline also contained 0.1 N HCl. At lower concentrations ethanol (0.01%) failed to modify twitch tension, so no corrections were necessary. In highest concentration (0.1 and 0.3%), ethanol caused a discrete inhibition in field stimulation-evoked twitches. Control experiments were performed in order to correct for the inhibition caused by ethanol alone. Solutions containing the dihydropyridines Bay K 8644 and nifedipine were protected from light and, when experiments were

conducted with these compounds, the organ baths were covered with aluminium foil and the laboratory lights were switched off, leaving only enough illumination to enable the experimenter to make drug additions.

Statistics

The EC_{50}'s and IC_{50}'s are presented with 95% confidence limits. All other values are presented as the means ± s.e.mean. The significance of drug-induced effects was assessed by Student's t test for unpaired samples. Differences between groups were considered to be significant at $P < 0.05$.

Results

Characterization of field stimulation-evoked twitches

Responses to field stimulation were entirely of neurogenic origin as they were completely blocked by tetrodotoxin (0.1 μM, $n = 3$, not shown). Basal field stimulation-evoked twitches were unaffected by yohimbine (up to 300 μM, twitch tension 104.3 ± 8.7% of basal value at 300 μM, $n = 3$) or prazosin (1 μM, twitch tension 99.2 ± 6.1% of basal value, $n = 7$) indicating that they derived mainly from the release of a non-adrenergic transmitter.

Effects of verapamil, Bay K 8644 and nifedipine on twitch tension

As shown in Figure 1, verapamil (1 to 100 μM) enhanced twitches evoked by supramaximal field stimulation. These effects were concentration-dependent and reversed upon washout. Twitch potentiation induced by verapamil usually peaked at concentrations between 30 and 100 μM. In half of the experiments, potentiation followed by depression of twitches was observed upon exposure to 100 μM verapamil (Figure 1). The depressant effect of verapamil possibly derives from a local anaesthetic action (Hay & Wadsworth, 1982; Beattie *et al.*, 1986). The

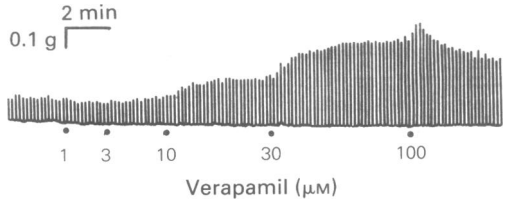

Figure 1 Representative isometric recording showing the changes of twitch tension of the isolated field-stimulated vas deferens of the mouse caused by cumulative additions of verapamil (1 to 100 μM).

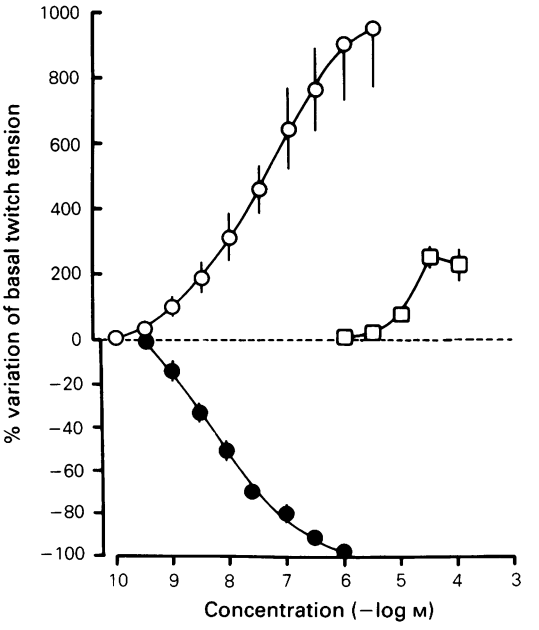

Figure 2 Mean concentration-response curves for Bay K 8644 (○) and for the calcium antagonists verapamil (□) and nifedipine (●) in altering basal twitch tension in the isolated field-stimulated vas deferens of the mouse. Each point represents the mean of 8 to 12 experiments and the vertical lines indicate s.e.mean. Note that scales used to illustrate potentiation and inhibition are different.

α_1-adrenoceptor antagonist prazosin (1 μM) failed to modify basal twitch tension or the twitch enhancing effect of verapamil (EC_{50} 13.0 μM, 8.8–19.2; maximal potentiation 299.6 ± 56.0%, $P > 0.05$, $n = 5$). Bay K 8644 (0.1 nM to 3 μM) also promoted concentration-dependent and reversible twitch potentiation. Comparison of the mean results illustrated in Figure 2 indicates that Bay K 8644 was 1000 fold more potent than verapamil; EC_{50} values were 17.3 nM (9.0–33.3) and 17.5 μM (13.7–22.5), respectively. Figure 2 also shows that maximal twitch potentiation afforded by Bay K 8644 (952.3 ± 208.8%) was 3 fold greater than that caused by verapamil (314.7 ± 35.3%). In contrast, nifedipine (0.1 nM to 1 μM)-induced inhibition of field stimulation evoked twitches, yielding an IC_{50} of 7.7 nM (5.0–11.8) and complete inhibition at 1 μM (Figure 2). This effect of nifedipine was concentration-dependent and reversed by washout.

Interactions between nifedipine and Bay K 8644 or verapamil

Figure 3 shows the inhibitory concentration-response curves obtained for nifedipine in the absence or presence of a maximally potentiating con-

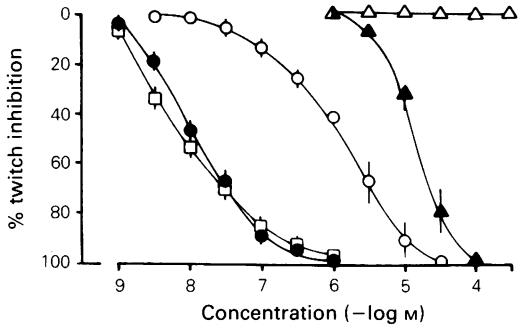

Figure 3 Mean concentration-response curves to nifedipine: control curve (□); in presence of verapamil 30 μM (●); or Bay K 8644, 1000 nM (○); to yohimbine: alone (△); in presence of Bay K 8644, 1000 nM (△) or in presence of verapamil 30 μM (▲) in inhibiting twitch tension in the field-stimulated mouse vas deferens. Note that yohimbine did not modify basal twitch tension up to 300 μM in the absence or presence of Bay K 8644. Each point represents the mean of 5 to 8 experiments and the vertical lines indicate s.e.mean.

centration of Bay K 8644 (1 μM) or verapamil (30 μM). The presence of Bay K 8644 caused a marked parallel rightward shift of the concentration-response curve to nifedipine. The IC_{50} determined for nifedipine in the presence of Bay K 8644 (0.9 μM, 0.3–2.2) was 120 fold greater than that determined in the absence of dihydropyridine agonist (7.7 nM, 5.0–11.8). In sharp contrast, simultaneous exposure to verapamil failed to affect significantly the inhibitory concentration-response curve to nifedipine (IC_{50} 12.6 nM, 8.0–19.7).

When twitches were abolished by nifedipine (1 μM), cumulative additions of Bay K 8644 initially at 0.1 or 0.3 nM restored the twitch responses to their original magnitude and at higher concentrations (1 to 1000 nM) caused concentration-dependent potentiations of twitch tension (EC_{50} 5.2 nM, 1.8–15.1; maximal response 1137.3 ± 381.8%; $n = 6$), similar to those observed in the absence of nifedipine (EC_{50} 17.3 nM, 9.0–33.3; maximal response 952.3 ± 208.8%; $n = 8$; $P > 0.05$). Interestingly, the presence of nifedipine (1 μM) rendered the preparation completely insensitive to the potentiating effects of verapamil (1 to 100 μM, $n = 4$). In addition, concentrations of verapamil in excess of 100 μM caused concentration-dependent sustained increases in tone of preparations bathed in nifedipine 1 μM (results not shown, $n = 4$).

Inhibition of verapamil-induced potentiation by yohimbine

The selective α_2-adrenoceptor antagonist yohimbine (up to 300 μM) did not affect basal twitch tension ($n = 3$, results not shown). However, Figure 3 shows that yohimbine (1 to 100 μM) caused a concentration-dependent inhibition of twitches potentiated by a maximally effective concentration of verapamil (30 μM) (IC_{50} 13.6 μM, 7.0–24.9; $n = 5$). In contrast, yohimbine failed to inhibit twitches potentiated by a maximally effective concentration of Bay K 8644 (1 μM, $n = 4$) (Figure 3).

Interactions between Bay K 8644 and verapamil

Previous incubation with verapamil (30 μM) enhanced the responsiveness of the preparation to Bay K 8644 (0.1 to 10 nM). As shown in Figure 4, the twitch potentiations observed in the presence of both compounds were significantly greater than those expected from the mere summation of their respective effects when given alone. Therefore, in the presence of verapamil, the potency of Bay K 8644 was enhanced nearly 60 fold at the EC_{50} level ($P < 0.05$) from 17.3 nM (9.0–33.3) to 0.3 nM (0.2–0.6). The maximal response to Bay K 8644 in the presence of verapamil (1442.0 ± 348.4%) was not significantly different ($P > 0.05$) from that detected in the absence of this drug (952.3 ± 208.8%).

When experiments were performed in the reverse conditions, i.e., the preparations were exposed to Bay K 8644 before cumulative additions of verapamil (1 to 30 μM), the results depended on the dose of Bay K 8644 employed. At 1 nM, a concentration that

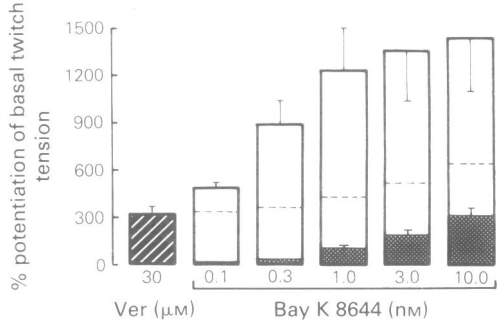

Figure 4 Influence of preincubation with verapamil (30 μM, hatched column) on the potentiation by Bay K 8644 (0.1 to 10 nM) of field stimulation-evoked twitches of the mouse isolated vas deferens. Cross-hatched columns represent the effect of Bay K 8644 alone and open columns the effect of Bay K 8644 in the presence of verapamil 30 μM. The broken lines indicate the values to be expected from simple addition of the individual effects of each agent alone. Each column represents the mean of 4 to 8 experiments and vertical bars the s.e.mean. Note that verapamil markedly potentiated the action of Bay K 8644 when compared to the control responses obtained in the absence of the calcium antagonist.

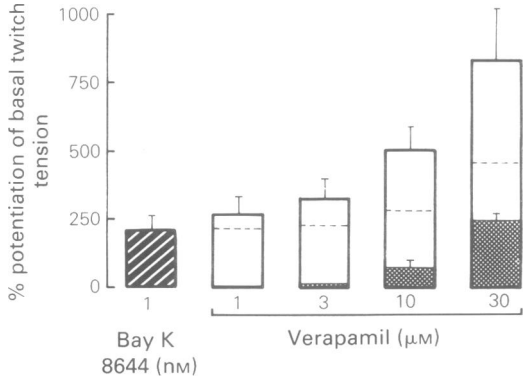

Figure 5 Influence of preincubation with Bay K 8644 (1 nM, hatched column) on the potentiation by verapamil (1 to 30 μM) of field stimulation-evoked twitches of the isolated mouse vas deferens. Cross-hatched columns represents the effect of verapamil alone and open columns the effect of verapamil in the presence of Bay K 8644 1 nM. The broken lines indicate the values to be expected from simple addition of the individual effects of each agent alone. Each column represents the mean of 6 to 12 experiments and vertical bars the s.e.mean. Note that in presence of Bay K 8644, the effect caused by verapamil (3, 10 and 30 μM) was potentiated when compared to the control responses obtained in the absence of Bay K 8644.

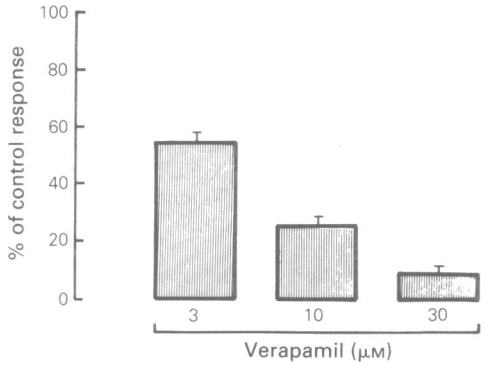

Figure 6 Inhibition by verapamil (3 to 30 μM) of noradrenaline-induced contractions of the mouse isolated vas deferens. Each column represents the mean of 11 experiments and the vertical bars indicate s.e.mean.

potentiated twitches to a lesser extent than maximally effective concentrations of verapamil, Bay K 8644 produced a supra-additive potentiation of the effect of verapamil (Figure 5), displacing its concentration-response curve to the left about 3 fold ($P < 0.05$), reducing the EC_{50} from 17.5 μM (13.7–22.5) to 5.3 μM (3.8–7.3) and increasing the maximal response from 314.7 $\pm$ 35.3% to 778.8 $\pm$ 186.2% ($P < 0.05$). However, at 10 nM, a concentration producing about half maximal twitch potentiation, Bay K 8644 potentiated the response to verapamil in a merely additive fashion ($n = 6$, results not shown). Thus, true potentiation by Bay K 8644 of the responses to verapamil was observed only when the former compound was incubated at a low concentration.

Effects of verapamil on noradrenaline- and acetylcholine-induced contractions

In the absence of field stimulation contractions of the mouse vas deferens to maximally effective concentrations of noradrenaline (100 μM) were depressed in a concentration-dependent manner by exposure to verapamil (3 to 30 μM) (Figure 6), yielding a mean pD'_2 value of 5.50 $\pm$ 0.13. Similar results were obtained with verapamil (1 to 10 μM) against responses to maximally effective concentrations of acetylcholine (1 mM), giving a mean pD'_2 value of 5.68 $\pm$ 0.09 ($n = 6$, results not shown).

Discussion

It is clear from the present results that verapamil induces a concentration-dependent enhancement of twitches evoked by electrical stimulation of the mouse isolated vas deferens at 0.1 Hz. This effect was essentially similar to that produced by Bay K 8644, but the potency of and maximal response to verapamil were 1000 and 3 fold smaller, respectively, than those seen with the dihydropyridine agonist. Almost identical findings have been obtained with verapamil in the rat vas deferens (French & Scott, 1983; Hay & Wadsworth, 1983; Moritoki *et al.*, 1987). In contrast, Zetler & Kaschube (1985) reported that nerve-mediated contractions of the mouse vas deferens elicited by high frequency field stimulation (trains of 0.03–0.8 ms pulses at 8–15 Hz for 1 s every 30 s) are depressed by verapamil. Indeed, when we applied trains of 0.5 ms pulses at 10 Hz for 0.5 s every 10 s, addition of verapamil only caused twitch inhibition (results not shown). Therefore, it appears that the twitch enhancing effect of verapamil only occurs at low frequencies of field stimulation.

Moritoki *et al.* (1987) suggested that, in the rat vas deferens, twitch potentiation by verapamil is mediated through α_2-adrenoceptors and binding of verapamil to α_2-adrenoceptors has been demonstrated (Van Meel *et al.*, 1981; Baranathan *et al.*, 1982). However it is difficult to envisage that the calcium antagonist elicits twitch potentiation by interaction with these receptors. In the mouse vas deferens, which appears to contain only prejunctional α_2-adrenoceptors (Baker & Marshall, 1983), field stimulation-evoked contractions are inhibited by the

α_2-adrenoceptor agonist clonidine and this effect is antagonized by yohimbine (Rae & De Moraes, 1984). In the present study, yohimbine failed to modify basal twitch tension but antagonized the potentiating effect of verapamil. Since clonidine and verapamil promote opposite yohimbine-sensitive effects on twitch tension, it seems likely that yohimbine antagonized verapamil-induced twitch potentiation through mechanisms independent of α_2-adrenoceptors.

Motor innervation of the mouse vas deferens appears to consist mainly, if not exclusively (Jones & Spriggs, 1975), of sympathetic neurones which release noradrenaline, ATP and neuropeptide Y (Stjärne & Lundberg, 1986; Forsyth & Pollock, 1988). In this preparation, exogenous administration of these co-transmitters each produces a distinct pattern of contraction (Stjärne *et al.*, 1986). Verapamil enhances the release of [^{3}H]-noradrenaline from sympathetic neurones in rat and guinea-pig vasa deferentia and in the aorta and tail artery of the rat (Karaki *et al.*, 1984; Zsóter *et al.*, 1984; Beattie *et al.*, 1986; Moritoki *et al.*, 1987). In the present study, however, the selective α_1-adrenoceptor antagonist prazosin (1 μM) failed to modify basal twitch tension or the twitch enhancing effect of verapamil. Moreover, verapamil markedly inhibited contractions induced by exogenous maximally effective concentrations of noradrenaline and acetylcholine. Taken together, these findings would indicate that twitches evoked by field stimulation at 0.1 Hz are due mainly to the action of a non-adrenergic transmitter, as suggested by Jenkins *et al.* (1975) and Marshall *et al.* (1978), and that verapamil acts to enhance the release of this transmitter. It is also tempting to speculate that ATP is the neurotransmitter preferentially released by field stimulation at low frequency, since this substance induces twitch-like phasic contractions of the mouse vas deferens, contrasting with the slowly developing sustained contractions caused by neuropeptide Y (Stjärne *et al.*, 1986).

Although verapamil and Bay K 8644 both increased twitch tension, there is evidence to suggest that different mechanisms underlie the effects of these drugs. Yohimbine, which antagonized twitch potentiation induced by verapamil, did not affect the response to Bay K 8644. Pre-incubation with a maximal inhibitory concentration of nifedipine (1 μM) abolished the twitch-enhancing effect of verapamil but did not modify the potency of or maximal potentiation induced by Bay K 8644. Conversely, nifedipine was equally effective in inhibiting basal or verapamil (30 μM)-potentiated twitches, but was 120 fold less potent against twitches maximally potentiated by Bay K 8644 (1 μM).

The observation that pre-incubation with Bay K 8644 antagonized nifedipine in a competitive manner whereas no antagonism occurred in the reverse condition deserved some consideration. In depolarized smooth muscle preparations contracted with calcium, competitive interaction between these dihydropyridines has been reported to occur both ways (Spedding & Berg, 1984; Spedding, 1985). Perhaps such differences are related to the lack of a persistent depolarized state in the present experiments (Sanguinetti & Kass, 1984; Middlemiss & Spedding, 1985).

One of the more interesting findings of the present study was that preincubation with verapamil (30 μM) markedly potentiated the twitch-enhancing effect of Bay K 8644, i.e. the concentration-response curve to Bay K 8644 was shifted to the left and its EC_{50} reduced 60 fold. Therefore, the effects produced by the combination of both compounds was clearly greater than that expected by summation of the effects produced by each drug alone. This result suggests that verapamil and Bay K 8644 enhance twitches through different mechanisms. When the reverse experiments were performed i.e. the preparation was pre-incubated with a low concentration of Bay K 8644 (1 nM, but not 10 nM) and then exposed to verapamil, a significant but less marked synergism was observed (verapamil EC_{50} reduced 3 fold). However, the maximal response observed when Bay K 8644 (1 nM) and verapamil (30 μM) were combined was the same, irrespective of the sequence of drug administration, and did not differ significantly from that caused by Bay K 8644 alone at a concentration of 1 μM.

Together, these results indicate that verapamil potentiates twitches evoked by low frequency field stimulation of the mouse vas deferens through a mechanism(s) distinct from that of Bay K 8644. Moreover, it is suggested that, in this preparation, twitch potentiation by verapamil may reflect a prejunctional action leading mainly to enhanced release of a non-adrenergic co-transmitter.

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Release-regulating autoreceptors of the $GABA_B$-type in human cerebral cortex

Giambattista Bonanno, *Paolo Cavazzani, *Gian Carlo Andrioli, Daniela Asaro, Graziella Pellegrini & [1]Maurizio Raiteri

Istituto di Farmacologia e Farmacognosia, Università degli Studi di Genova, Viale Cembrano 4, 16148 Genova, Italy & *Divisione di Neurochirurgia, Ospedali Galliera, Via A. Volta 8, 16128 Genova, Italy

1 The depolarization-evoked release of γ-aminobutyric acid (GABA) and its modulation mediated by autoreceptors were investigated in superfused synaptosomes prepared from fresh human cerebral cortex.

2 The release of [^{3}H]-GABA provoked by 15 mM K^+ from human cortex nerve endings was almost totally (85%) calcium-dependent.

3 In the presence of the GABA uptake inhibitor SK&F 89976A (N-(4,4-diphenyl-3-butenyl)-nipecotic acid), added to prevent carrier-mediated homoexchange, GABA (1–10 μM) decreased in a concentration-dependent manner the K^+-evoked release of [^{3}H]-GABA. The effect of GABA was mimicked by the $GABA_B$ receptor agonist (−)-baclofen (1–100 μM) but not by the $GABA_A$ receptor agonist muscimol (1–100 μM). Moreover, the GABA-induced inhibition of [^{3}H]-GABA release was not affected by two $GABA_A$ receptor antagonists, bicuculline or SR 95531 (2-(3′-carbethoxy-2′-propenyl)-3-amino-6-paramethoxy-phenyl-pyridazinium bromide).

4 (−)-Baclofen also inhibited the depolarization-evoked release of endogenous GABA from human cortical synaptosomes.

5 It is concluded that GABA autoreceptors regulating the release of both newly taken up and endogenous GABA are present in human brain and appear to belong to the $GABA_B$ subtype.

Introduction

Studies on the laboratory animal have shown that autoregulation of transmitter release mediated by receptors sited on the releasing terminals (autoreceptors) appears to be a common feature of the major transmitter systems (Langer, 1981; Starke, 1981; de Belleroche, 1982; Raiteri *et al.*, 1984). Autoreceptors for noradrenaline (for reviews see: Langer, 1981; Starke, 1981), 5-hydroxytryptamine (Engel *et al.*, 1986; Maura *et al.*, 1986; Bonanno *et al.*, 1986) and acetylcholine (Marchi & Raiteri, 1985; Meyer & Otero, 1985; Mash & Potter, 1986) have been widely studied and characterized in terms of types and subtypes. In the case of γ-aminobutyric acid (GABA) the mechanisms of autoregulation have been less investigated. GABA autoreceptors have been reported to be muscimol-sensitive (Mitchell & Martin, 1978; Brennan *et al.*, 1981) and therefore of the $GABA_A$ type. However a number of recent papers suggest the presence of autoreceptors regulating the release of GABA in the rat brain of the $GABA_B$ receptor subtype (Anderson & Mitchell, 1985; Pittaluga *et al.*, 1987; Waldmeier *et al.*, 1988).

It has been proposed that abnormalities in GABA transmission are associated with various human disease states. The GABA system appears to be altered in certain types of epilepsy (Meldrum, 1975), in Huntington's and Parkinson's disease (Hornykiewicz *et al.*, 1976; Schwarcz *et al.*, 1977) and in a number of neuropsychiatric disorders including depression (Lloyd *et al.*, 1985).

Due to this postulated involvement of GABA in neurological and psychiatric diseases and in view of the existence of different types of GABA receptors (Bowery, 1983) it was of relevance to determine: (a) whether GABA autoreceptors are present in human brain; (b) whether or not their pharmacological properties are similar to those of the autoreceptors found in the rodent brain. We here show that functional autoreceptors belonging to the $GABA_B$ subtype are present in the cerebral cortex of man.

[1] Author for correspondence.

Methods

Characteristics of human specimens

Samples of human cerebral cortex were obtained from patients undergoing neurosurgery. The tissues used were removed by the surgeon to reach deeply located tumours. The samples used represented parts of frontal (2), temporal (3), parietal (2) and occipital (2) lobes and were obtained from 5 female and 4 male patients (aged 45–69 years). The tissues were obtained and processed separately on different days. After premedication with atropine and meperidine, anaesthesia was induced with Pentothal and maintained with 70% nitrous oxide in 30% oxygen and 0.5–1% isoflurane. Pancuronium was employed to obtain muscular relaxation.

Preparation of synaptosomes

Immediately after removal, the tissue was placed in a physiological salt solution kept at 2–4°C and a synaptosomal fraction was obtained within 60 min.

Crude synaptosomes were prepared essentially as previously described (Gray & Whittaker, 1962) with minor modifications. Briefly, the brain cortex was homogenized in 40 vol of 0.32 M sucrose buffered at pH 7.4 with phosphate using a glass-teflon tissue grinder (clearance 0.25 mm). The homogenate was centrifuged (5 min, 1000 *g* at 0–4°C) to remove nuclei and debris and synaptosomes were isolated from the supernatant by centrifugation at 12,000 *g* for 20 min. The synaptosomal pellet was then resuspended in a physiological medium having the following composition (mM): NaCl 125, KCl 3, $CaCl_2$ 1.2, $MgSO_4$ 1.2, NaH_2PO_4 1, $NaHCO_3$ 22, glucose 10 (aerated with 95% O_2 and 5% CO_2 at 37°C) pH 7.2–7.4. Protein was measured by the method of Petersen (1977).

Release experiments

Synaptosomes were incubated in a rotatory water bath at 37°C for 15 min in the presence of 0.04 μM [^{3}H]-GABA or without label (experiments on endogenous GABA release). After incubation, identical aliquots of the synaptosomal suspension (ranging between 0.4–0.8 mg protein in different experiments) were distributed on 0.65 μm Millipore filters placed at the bottom of a set of parallel superfusion chambers maintained at 37°C (Raiteri *et al.*, 1974; Raiteri & Levi, 1978) and layered under moderate vacuum filtration. Superfusion was then started with standard medium aerated with 95% O_2 and 5% CO_2 at a rate of 0.6 ml min^{-1} and continued for 48 min. After 36 min to equilibrate the system, fractions were collected according to the following scheme: two 3 min samples (basal release) before and after one 6 min sample (evoked release). A 90 s period of depolarization with 15 mM KCl was applied at the end of the first fraction collected. GABA, (−)-baclofen or muscimol was added to the superfusion medium concomitantly with the depolarizing stimulus. Bicuculline, or the novel $GABA_A$ receptor antagonist SR 95531 [2-(3′-carbethoxy-2′-propenyl)-3-amino-6-paramethoxy-phenyl-pyridazinium bromide] (Wermuth & Bizière, 1986) was added 8 min before GABA. SK&F 89976A [4,4-diphenyl-3-butenyl)-nipecotic acid], a novel and potent inhibitor of GABA uptake (Yunger *et al.*, 1984; Bonanno & Raiteri, 1987; Larsson *et al.*, 1988) was present in the superfusion medium at a final concentration of 30 μM to minimize carrier-mediated exchange between intraterminal [^{3}H]-GABA or endogenous GABA and extrasynaptosomal GABA or muscimol. Aminooxyacetic acid (AOAA; final concentration 50 μM) was present throughout the experiment to prevent [^{3}H]-GABA metabolism. AOAA was not used in experiments on endogenous GABA release.

Endogenous GABA in the superfusates was measured with a radioreceptor assay, essentially as described by Enna & Snyder (1976). High performance liquid chromatography with fluorescent detection after pre-column derivatization by *o*-phathalaldehyde according to Tonnaer *et al.* (1983) was applied in some cases. The results obtained did not differ significantly from those obtained with the radioreceptor assay. At the concentration used in our experiments (−)-baclofen did not interfere in the radioreceptor assay (Hill & Bowery, 1981).

The amount of radioactivity released into each fraction was expressed as the percentage of the tritium content of synaptosomes at the start of the respective collection period. The depolarization-evoked overflow was estimated by subtracting the percent of tritium content of the basal release from the release evoked in the 6 min fraction collected during and after the depolarization pulse. The endogenous GABA released was expressed as pmol of amino acid per mg of synaptosomal protein. The drug effects were evaluated as the ratio of the depolarization-evoked overflow calculated in the presence of the drugs vs. that calculated under control conditions.

Drugs

[^{3}H]-GABA (105 Ci mmol^{-1}) was obtained from Amersham Radiochemical Centre; aminooxyacetic acid and (+)-bicuculline were purchased from Sigma Chemical Co (St. Louis, MO, U.S.A.); GABA from Serva (Heidelberg, F.R.G.). The following drugs were generous gifts by the companies indicated: (−)-baclofen (Ciba Geigy, Basel, Switzerland); muscimol (Zambon Farmaceutici, Milan, Italy); SK&F 89976A

Table 1 Calcium-dependence of the K^+-evoked release of [^{3}H]-GABA from human cortical brain synaptosomes

	Standard medium[a]	Ca^{2+}-*free medium*
Basal release min^{-1}	0.62 ± 0.08	0.56 ± 0.06
K^+ (15 mM)-evoked overflow	2.96 ± 0.38	0.32 ± 0.05*

Values are the mean ± s.e. mean of four experiments in triplicate.
[a] Measured as percentage of total [^{3}H]-GABA content.
* Statistical significance of changes versus the K^+ (15 mM)-evoked overflow in standard medium were determined by Student's t test: $P < 0.001$.

(Smith Kline & French, Welwyn, England) and SR 95531 (Sanofi, Bruxelles, Belgium).

Results

As shown in Table 1, when human cerebral cortex synaptosomes were exposed during superfusion to 15 mM KCl, the depolarization-evoked release of tritium was almost totally calcium-dependent.

The Ca^{2+}-dependency of the release, the presence of 50 μM of the GABA transaminase inhibitor AOAA throughout the experiment, the characteristics of the superfusion technique used (see Raiteri & Levi, 1978), together with the data obtained previously in various laboratories (for review see Levi, 1984) make it likely that the radioactivity released by 15 mM KCl consisted largely of unmetabolized [^{3}H]-GABA. Therefore, in the remainder of the text, we refer to the K^+-evoked release of tritium as K^+-evoked [^{3}H]-GABA release.

Figure 1 shows that exogenous GABA (1–10 μM), added to the superfusion fluid, decreased in a concentration-dependent manner the K^+-evoked release of [^{3}H]-GABA. The (−)-enantiomer of baclofen produced a concentration-inhibition curve almost superimposable on that of GABA. In contrast, muscimol (1–100 μM) was totally ineffective.

Table 2 shows that neither (+)-bicuculline (10 μM) nor SR 95531 (10 μM) was able to antagonize significantly the inhibition of [^{3}H]-GABA release caused by an equimolar concentration of exogenous GABA.

The results obtained when (−)-baclofen was tested on both the basal and the K^+-evoked release of endogeneous GABA from human synaptosomes are shown in Table 3. While the basal release was not significantly affected, the depolarization-evoked overflow was inhibited by about 50% when the agonist was present at 10 μM.

Table 2 Effects of bicuculline and SR 95531 on the GABA-induced inhibition of the K^+ (15 mM)-evoked release of [^{3}H]-GABA

	% *inhibition*
GABA 10 μM	40.4 ± 2.9
GABA 10 μM + bicuculline 10 μM	41.2 ± 2.8
GABA 10 μM + SR 95531 10 μM	37.8 ± 3.2

Values are the mean ± s.e.mean of 4–6 experiments in triplicate.

Discussion

The aims of this work were to ascertain the presence of GABA autoreceptors in the human brain and to classify them in terms of GABA receptor subtype.

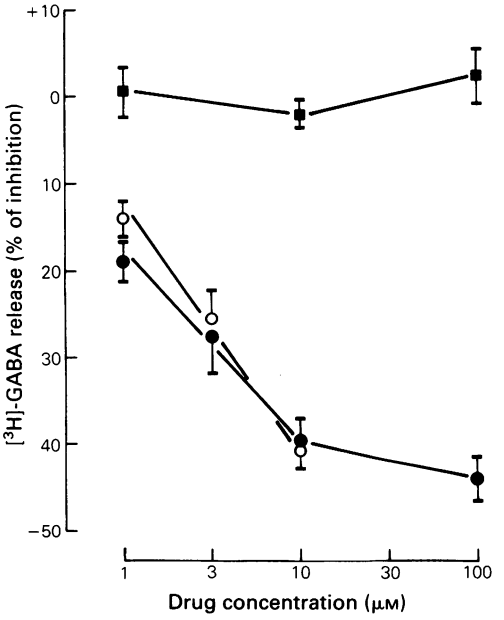

Figure 1 Effects of γ-aminobutyric acid (GABA, (○), (−)-baclofen (●) or muscimol (■) on the release of [^{3}H]-GABA evoked by depolarization of human brain cortical synaptosomes. When GABA was used as an agonist, SK&F 89976A 30 μM, an inhibitor of GABA uptake was added to the superfusion medium. Aminooxyacetic acid (50 μM) was present throughout the experiment to prevent [^{3}H]-GABA metabolism. Points represent the mean of 3–4 separate experiments with s.e.mean shown by vertical lines.

Table 3 Effect of (−)-baclofen (10 μM) on the K^+-evoked release of endogenous GABA from human cortical brain synaptosomes

	Controls[a]	*(−)-Baclofen*
Basal release min^{-1}	28.3 ± 3.2	25.2 ± 2.7
K^+ (15 mM)-evoked overflow	149.6 ± 21.7	76.7 ± 11.5*

Values are the mean ± s.e.mean of five experiments in triplicate.
[a] Measured as pmol of endogenous GABA released mg^{-1} protein.
* Statistical significance of changes versus controls were determined by Student's t test: $P < 0.05$.

When studying release-regulating autoreceptors, a critical point is to show that the natural transmitter is able to depress its own release from nerve terminals. In the case of GABA this has always been a problem due to the fact that exogenous GABA, used as an autoreceptor agonist, stimulates very actively the release of the radioactive GABA used to label the nerve terminals through an homoexchange process (Levi & Raiteri, 1974). This exchange obviously obscures the expected inhibitory effect of the natural transmitter unless a very efficient blocker of the GABA carrier is present. The novel compound used in this study (SK&F 89976A) is a potent and selective inhibitor of the uptake of GABA (Yunger *et al.*, 1984; Bonanno & Raiteri, 1987). Moreover, SK&F 89976A is a pure GABA uptake inhibitor devoid of intrinsic releasing activity (data not shown). The compound is therefore different from other uptake inhibitors such as nipecotic acid which is known also to be a substrate for the GABA uptake system and to release [^{3}H]-GABA through a heteroexchange process (Szerb, 1983). Thus, SK&F 89976A appears to be particularly suitable for studying GABA autoreceptors when the natural transmitter is used as an agonist. Our data show that, in the presence of SK&F 89976A, GABA inhibited the K^+-evoked release of [^{3}H]-GABA, indicating that autoreceptors involved in the regulation of [^{3}H]-GABA release exist on the GABA nerve terminals of human cerebral cortex.

It is now well accepted that GABA receptors exist in two different types, termed $GABA_A$ and $GABA_B$ (Bowery, 1983). It has been proposed that the latter type inhibits the stimulus-evoked release of several transmitters (Bowery *et al.*, 1980). The receptor involved in the autoinhibition of GABA release was characterized by using various pharmacological tools. The findings that: (a) the effect of GABA was insensitive to the $GABA_A$ receptor antagonists (+)-bicuculline or SR 95531 (Wermuth & Bizière, 1986); (b) muscimol, a $GABA_A$ receptor agonist, did not mimic the effect of GABA and (c) the $GABA_B$ receptor agonist (−)-baclofen (Bowery *et al.*, 1980) produced a dose-dependent inhibition of [^{3}H]-GABA release, allow us to suggest that the GABA autoreceptors in human cerebral cortex belong to the $GABA_B$ subtype. A note of caution is necessary, however, because potent and selective $GABA_B$ receptor antagonists have not been available. Only recently, it has been proposed that the phosphonic acid derivative of baclofen (phaclofen) is an antagonist at $GABA_B$ receptor on the basis of electrophysiological studies (Kerr *et al.*, 1987; Dutar & Nicoll, 1988). In preliminary experiments, phaclofen partly antagonized the action of GABA on [^{3}H]-GABA release (data not shown).

As to the anatomical localization of the $GABA_B$ receptor regulating GABA release, the experimental set up used (a thin layer of synaptosomes in superfusion) should exclude the possibility that we are dealing with somato-dendritic autoreceptors. It should be added that indirect effects involving transmitters other than GABA are minimized by use of the above technique (see Raiteri & Levi, 1978 for more details). Moreover, it has been reported that neuronal but not glial release of [^{3}H]-GABA was stimulated by K^+ depolarization (Neal & Bowery, 1979). These considerations, together with the calcium-dependency of the release of GABA (which is generally taken as an indication of a neuronal origin of the transmitter released) make it legitimate to conclude that the human GABA autoreceptors characterized in this study are sited on GABA-releasing nerve terminals.

Considering that newly taken up radioactive GABA may in some cases behave differently from the endogenously synthesized amino acid (see Szerb, 1983), we tested the effect of baclofen on the release of endogenous GABA. The finding that (−)-baclofen inhibited not only the release of previously taken up [^{3}H]-GABA but also that of endogenous GABA strengthens the idea that $GABA_B$ autoreceptors are present on GABAergic nerve endings in human cerbral cortex.

Finally, when the present results in human brain cortex are compared with those obtained in the cerebral cortex of the rat (Pittaluga *et al.*, 1987), no clear differences appear to exist between the release-regulating GABA autoreceptors present in the two species. Actually, the effects of GABA and (−)-baclo-

fen are strikingly similar in man and rat. As an important consequence, the laboratory animal appears to be a useful model for studying $GABA_B$ autoreceptors and for testing new drugs of potential therapeutic use.

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Cardiovascular interactions between vasopressin, angiotensin and noradrenaline in the Brattleboro rat

[1]J.F. Laycock & *S.L. Lightman

Departments of Physiology and *Medicine, Charing Cross & Westminster Medical School, Fulham Palace Road, London W6 8RF

1 The cardiovascular effects of bolus intravenous injections of vasopressin, angiotensin II and noradrenaline were studied in 6-hydroxydopamine pretreated, anaesthetized Brattleboro rats with hereditary diabetes insipidus and normal rats of the parent Long Evans strain.

2 Pretreatment with 6-hydroxydopamine did not significantly affect control values for mean arterial blood pressure, cardiac output or total peripheral resistance in either Brattleboro or Long Evans rats but the pressor response to haemorrhage was reduced in both strains compared to the control animals.

3 The pressor responses of the untreated Brattleboro rats to 250 mu kg^{-1} vasopressin were significantly greater and more prolonged than in control rats of the Long Evans strain.

4 Pretreatment with 6-hydroxydopamine significantly enhanced the peak pressor response to vasopressin, but not to angiotensin II (1 $\mu g\,kg^{-1}$), in Brattleboro and Long Evans rats.

5 Pretreatment with 6-hydroxydopamine resulted in an enhanced pressor response to 1 $\mu g\,kg^{-1}$ noradrenaline in both Brattleboro and Long Evans rats, but the effect was significantly greater in the vasopressin-deficient animals.

6 These results indicate differences in the pressor responsiveness of Brattleboro rats to vasopressin and noradrenaline, but not to angiotensin II, compared with control Long Evans rats and provide evidence for important interactions between the sympathetic nervous system and these pressor hormones.

Introduction

There is increasing evidence to support the view that the cardiovascular system is continuously regulated not only by the autonomic nervous system but also by a variety of hormones. The principal hormones of current interest in the control of arterial blood pressure are the catecholamines (mainly noradrenaline and adrenaline), angiotensin II and arginine vasopressin.

In addition to their possible direct effects, interactions between these hormones have been described. Thus vasopressin infusions into anaesthetized vagotomized dogs undergoing major vessel occlusion, or into pithed rats, appear to potentiate the pressor effect of intra-aortic injections of noradrenaline (Bartelstone & Nasmyth, 1965). The converse interaction was observed by Burnier & Brunner (1983). There is also evidence that the renin-angiotensin system interacts with circulating vasopressin, particularly regarding control of release (e.g. see review by Share, 1979), but also regarding peripheral pressor activity. Infusions of angiotensin II into conscious rats depressed the pressor effect of vasopressin (Burnier & Brunner, 1983), while blockade of angiotensin II converting enzyme with captopril resulted in an enhanced pressor response to vasopressin (Spertini *et al.*, 1981).

Other possible interactions are between individual pressor hormones and the sympathetic nervous system. Again there is some evidence for such interdependent actions. Recently, a central interaction between vasopressin and the sympathetic nervous system was suggested by Feuerstein *et al.* (1984). Patients with high cervical cord transections have a greater than normal pressor sensitivity to vasopressin (Poole *et al.*, 1987), suggesting the possibility of some relationship between the peptide hormone and the sympathetic nervous system. The mechanism of action of angiotensin II has also been related to

[1] Author for correspondence at Department of Physiology.

interactions with sympathetic nerve catecholamine synthesis or release, and with stimulation of adrenal medullary catecholamine release (for review see Peach, 1977).

The present study was devised to re-examine some of the interactions between the pressor hormones themselves and their relationships with the sympathetic nervous system. One animal model was used to characterize in particular the involvement of vasopressin in the interactions between the different systems. This model consisted of the Brattleboro rat with hereditary diabetes insipidus (BDI) lacking circulating vasopressin. Normal rats of the parent Long Evans (LE) strain were used as controls. Some rats of these two strains were pretreated with 6-hydroxydopamine, a drug which destroys peripheral sympathetic postganglionic nerve terminals (Thoenen & Tranzer, 1968). Preliminary reports on aspects of this study have been published (Laycock & Lightman, 1986; 1987a,b).

Methods

All studies were carried out on adult male Brattleboro rats with hereditary diabetes insipidus (BDI) and Long Evans (LE) rats of comparable age (60 ± 5 days) and within the same weight range, from the Charing Cross and Westminster Medical School Animal Unit. Twelve animals of each strain were pretreated with 6-hydroxydopamine (6-OH-DA). The drug was administered intravenously via the tail vein in two doses, given 24 h apart, each of $100\,mg\,kg^{-1}$. Groups of these animals were used in the cardiovascular study 5 to 12 days after treatment with the drug. Eight untreated animals of each strain were also used as controls for the cardiovascular study.

On the day of study the pre-surgical procedure described by Walter *et al.* (1979) was followed. Briefly, this consisted of Brattleboro rats first being given 3 ml water by gavage in order to maintain fluid balance during the first part of the subsequent surgical procedure. Then these rats were given hydrocortisone sodium succinate (Organon; 5 mg, i.m.) because of lower plasma glucocorticoid levels in these animals compared with control rats (Mohring *et al.*, 1974). Then all rats were anaesthetized with Inactin ($120\,mg\,kg^{-1}$, i.p.) and surgically prepared as described elsewhere (Chapman *et al.*, 1986). Briefly, cannulae were inserted into the bladder, a femoral artery for measurement of heart rate and arterial blood pressure and a femoral vein for infusion of 150 $mmol\,l^{-1}$ NaCl at a rate of $0.5\,ml\ 100\,g^{-1}\,h^{-1}$ (and, via an additional cannula in the BDI rats only, 2% glucose solution at a rate determined from the urinary excretion rate to maintain fluid balance). A cannula was also inserted into the right atrium via the right jugular vein for the rapid injection of 0.1 ml of $150\,mmol\,l^{-1}$ NaCl with a Hamilton syringe for the determination of cardiac output by a standard thermodilution technique (Hosie, 1962). A thermocouple was inserted down the left carotid artery so that the tip just entered the aortic arch. Finally a tracheostomy was performed. Rectal temperature was maintained at 37.4°C throughout the experiment by means of a thermostatically controlled heating blanket. At the end of the surgical procedure 1 ml of $150\,mmol\,l^{-1}$ NaCl was given intravenously over a period of approximately 3 min to replace fluid loss during surgery.

Cardiac output was calculated from the changes in aortic temperature recorded by the thermocouple following the right atrial injection of NaCl at room temperature. Stroke volume and total peripheral resistance were calculated from the cardiac output, heart rate and mean arterial blood pressure measurements.

After an initial stabilization period of at least 20 min, control values for cardiac output, arterial blood pressure and heart rate were determined.

After at least two control cardiac output determinations, either vasopressin ($250\,mu\,kg^{-1}$, Sigma), noradrenaline ($1\,\mu g\,kg^{-1}$, Sigma) or angiotensin II ($1\,\mu g\,kg^{-1}$, Sigma) in $150\,mmol\,l^{-1}$ NaCl was injected intravenously in a volume of $0.5\,ml\,kg^{-1}$ body weight. Cardiac output, heart rate and arterial blood pressure determinations were made between 20 and 30 s after injection of either noradrenaline or angiotensin II, and then repeated 5 min after injection once the pressor response was over. The same cardiovascular determinations were made 1 and 15 min after the vasopressin injections, and also after 30 min in the Brattleboro rats only. Additional determinations of heart rate and arterial blood pressure were made 5 and 10 min after vasopressin injection in all animals, and after 20 and 25 min in the Brattleboro rats only. Subsequently, the two hormones not administered initially were injected intravenously in sequence, so that each animal received all three pressor hormones in randomized order. There was no evidence of any significant carry-over of pressor effects associated with any particular sequence of hormone administration in any of the four groups of animals. A second run of the three hormone injections was then carried out in each rat and then finally, in the majority of animals, a haemorrhage of $1\,ml\ 100\,g^{-1}$ body weight was induced. Cardiac output (usually), heart rate and arterial blood pressure were determined 1, 5, 10 and 20 min after the removal of blood via the femoral arterial catheter.

Because of the number of injections of saline for cardiac output determinations as well as the drug injections, the infusion of NaCl was stopped in all

animals after the initial stabilization period, and infusion of the glucose solution in the BDI rats was altered as appropriate in order to minimize changes in fluid balance.

Total peripheral resistance and stroke volume were calculated from the cardiovascular measurements made on each occasion that the cardiac output was determined.

Samples of tissue from heart, spleen and adrenal gland were obtained from some animals at the end of the experiments. These samples were immediately frozen in liquid nitrogen, ground to a fine powder and weighed quantities added to 1 ml volumes of 1 N HCl. After approximately 10 min, the samples were centrifuged at 4°C and the supernatent solutions stored at −70°C. Tissue catecholamine contents were later assayed by h.p.l.c. with electrochemical detection using an adaptation of the method of Martin *et al.* (1983).

All between-group results were analysed by a one way analysis of variance (ANOVA). Within-group differences were analysed by Student's paired *t*-test.

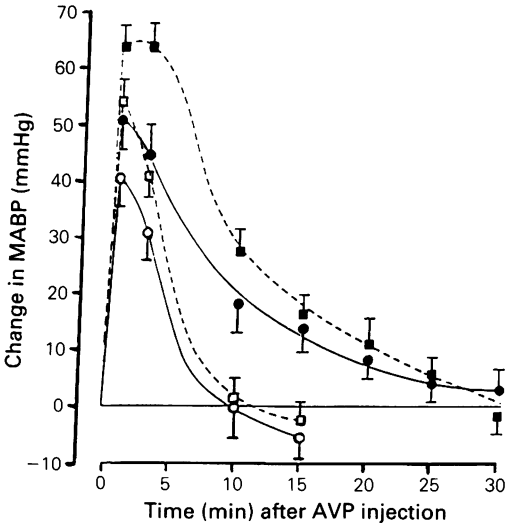

Figure 1 Changes in mean arterial blood pressure (MABP, means with s.e.mean shown by vertical bars) following the intravenous injection of 250 mu kg^{-1} vasopressin in the BDI control (●) and 6-hydroxydopamine pretreated (■) rats and the LE control (○) and pretreated (□) rats. For further details see text and legend to Table 1.

Results

There was no evidence of any significant effect of pretreatment with 6-OH-DA on mean arterial blood pressure, cardiac output, total peripheral resistance and stroke volume in either BDI or LE rats (Table 1). However, the mean heart rate of the control BDI rats was significantly greater than that of the control LE rats and those of the 6-OH-DA pretreated animals of both strains.

The bolus injection of vasopressin (250 mu kg^{-1}) produced very different pressor responses in the BDI and LE rats (Figure 1). Not only was the peak effect of the hormone greater in the BDI rats than in the LE rats, but it was also more prolonged. Fifteen minutes after the injection the blood pressure had returned to pre-injection values in the two groups of LE rats but was still significantly ($P < 0.0001$) raised in the two BDI groups. Only after 30 min was the pressor response over in the latter two groups of animals. The effect of pretreatment with 6-OH-DA in both BDI and LE rats was to increase significantly the peak pressor effect of vasopressin in both BDI and LE rats but not to alter the durations of response.

For all animals the increase in mean arterial blood pressure was associated with a significant increase in total peripheral resistance but a decrease in cardiac output (Figure 2). The prolonged effect of vasopressin in the BDI rats was also associated with a maintained raised peripheral resistance 15 min after

Table 1 Control values for the mean arterial blood pressure (MABP), heart rate (HR), cardiac output (CO), total peripheral resistance (TPR) and stroke volume (SV) in 6-hydroxydopamine (6-OH-DA) pretreated and untreated Brattleboro rats with diabetes insipidus (BDI) and normal rats of the parent Long Evans (LE) strain

	BDI				*LE*			
	Control		*6-OH-DA*		*Control*		*6-OH-DA*	
MABP (mmHg)	90 ± 6	(8)	92 ± 5	(12)	97 ± 6	(8)	93 ± 5	(12)
HR (beats min^{-1})	370 ± 12	(8)*	321 ± 10	(12)	335 ± 12	(8)	333 ± 10	(12)
CO (ml kg^{-1} min^{-1})	371 ± 30	(8)	367 ± 30	(8)	429 ± 32	(7)	338 ± 25	(12)
TPR ($\times 10^9$ N s kg m^{-5})	2.1 ± 0.2	(8)	2.0 ± 0.2	(8)	2.0 ± 0.2	(7)	2.2 ± 0.2	(12)
SV (ml kg^{-1})	1.0 ± 0.1	(8)	1.2 ± 0.1	(8)	1.3 ± 0.1	(7)	1.0 ± 0.1	(12)

Values are mean ± s.e.mean.

* $P < 0.05$ between BDI control rats and all other groups.

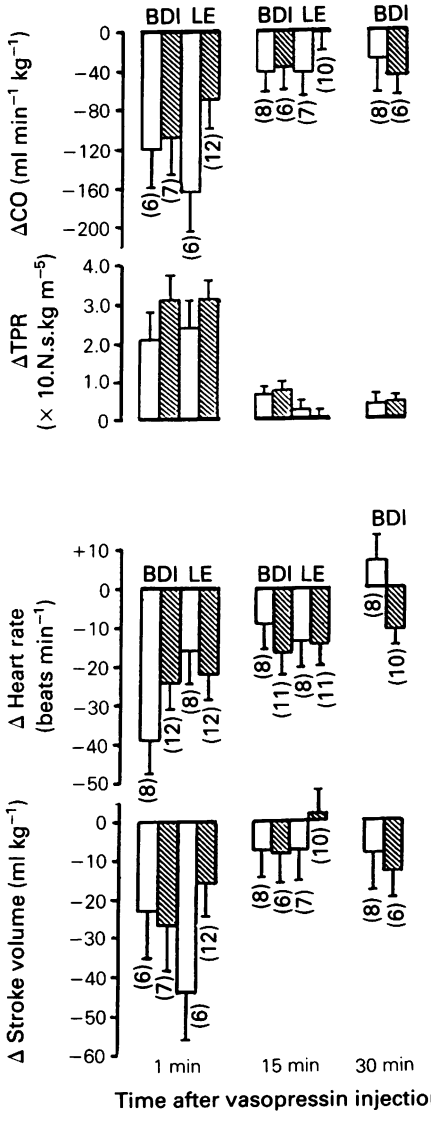

Figure 2 Mean changes (s.e.mean shown by vertical bars) in cardiac output (CO), total peripheral resistance (TPR), heart rate (HR) and stroke volume (SV) following the injection of 250mu kg^{-1} vasopressin in 6-hydroxydopamine pretreated (hatched columns) and control (untreated, open columns) BDI and LE rats.

injection in these animals compared with the LE rats. The decreases in cardiac output were associated with the significantly reduced heart rate and stroke volume observed, at least during the peak response, in each of the four groups of animals (see Figure 2).

The intravenous injection of 1 μg kg^{-1} noradrenaline produced a marked increase in arterial blood pressure within seconds. By 20 to 30s, when the pressor response was already beginning to diminish, the increase in blood pressure was significant in all four groups of animals and was clearly more pronounced in those rats which had been pretreated with 6-OH-DA. Furthermore, the pressor effect was significantly greater in the 6-OH-DA pretreated BDI rats than in the comparable LE animals (Figure 3).

The pressor response was associated with a significant increase in cardiac output and no change in total peripheral resistance in the two control groups of animals, but with an increase in peripheral resistance and a smaller change in cardiac output in the 6-OH-DA-treated groups. The increases in cardiac output in the control BDI and LE rats were associated with increases in heart rate and stroke volume. On the other hand, in the 6-OH-DA pretreated rats the increases in heart rate were significantly greater than in the respective control animals while there were no significant increases in stroke volume.

The single intravenous injection of 1 μg kg^{-1} angiotensin II had a similar pressor effect in the 6-OH-DA pretreated and control rats of the two strains, the response being slightly greater in the pretreated groups (Figure 4).

However, the changes in cardiac output and total peripheral resistance were different in the pretreated and control rats. In the control animals the increase in mean arterial blood pressure was associated with large increases in cardiac output which were significantly greater than those of the 6-OH-DA pretreated rats. The control rats of both strains also showed small, insignificant increases in total peripheral resistance unlike the significantly larger increases in peripheral resistance observed in the pretreated animals.

The second, later, sequence of injections of each of the three pressor hormones had almost identical cardiovascular effects to those described above.

Pretreatment with 6-OH-DA was associated with a depressed blood pressure recovery following the removal of 1 ml blood 100 g^{-1} body weight in both strains of rat, but this was only significant over the whole 20 min study period in the BDI animals (Figure 5). In the pretreated LE rats there was a significant ($P < 0.02$) depression in blood pressure only 5 min after haemorrhage compared with untreated control animals of this strain. Furthermore, even 1 min after the end of the haemorrhage the blood pressure was significantly lower ($P < 0.02$) in the BDI rats than in the LE animals.

The pre-haemorrhage mean cardiac output for the 6-OH-DA pretreated BDI rats was significantly lower than the corresponding values for the untreated BDI rats ($P < 0.005$) and the two groups of LE rats ($P < 0.001$). However, there were no further significant differences between the mean

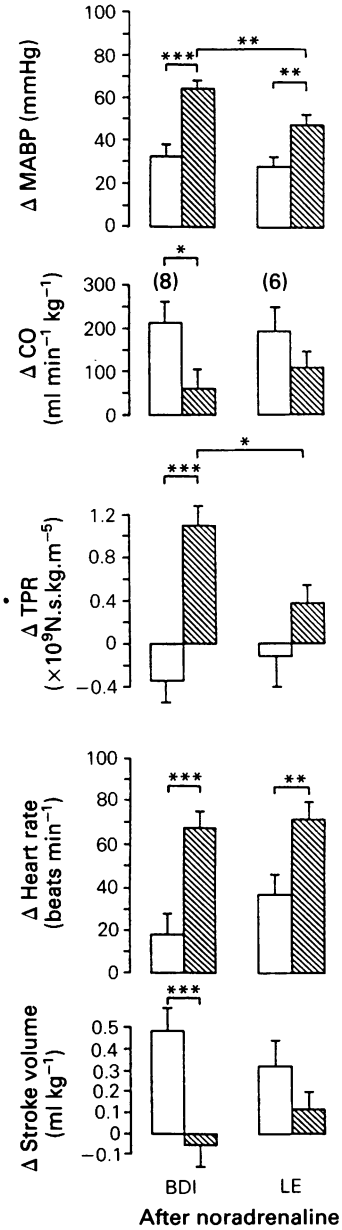

Figure 3 Mean changes (with s.e.mean shown by vertical bars) in mean arterial blood pressure (MABP), cardiac output (CO), total peripheral resistance (TPR), heart rate (HR) and stroke volume (SV) after injection of $1\,\mu g\,kg^{-1}$ noradrenaline in 6-hydroxydopamine pretreated (hatched columns) and control (untreated, open columns) BDI and LE rats. $^{*}P < 0.025$; $^{**}P < 0.01$; $^{***}P < 0.001$.

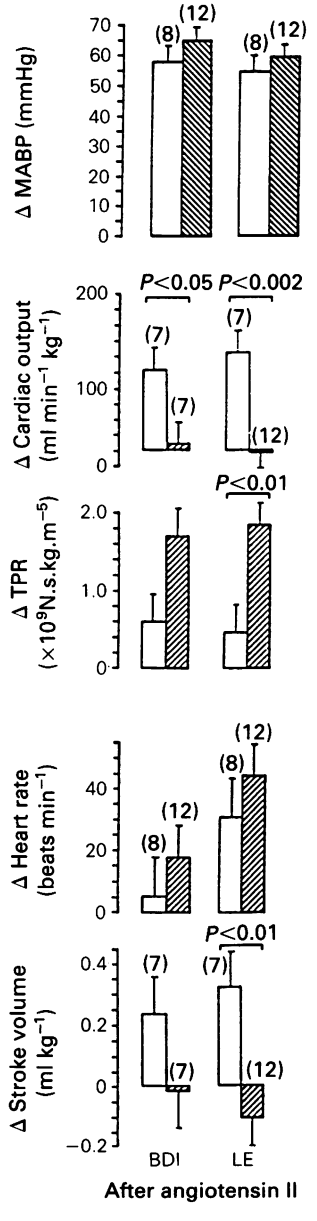

Figure 4 Mean changes (s.e.mean shown by vertical bars) in mean arterial blood pressure (MABP), cardiac output (CO), total peripheral resistance (TPR), heart rate (HR) and stroke volume (SV) after injection of $1\,\mu g\,kg^{-1}$ angiotensin II in 6-hydroxydopamine pretreated (hatched columns) and control (untreated, open columns) BDI and LE rats.

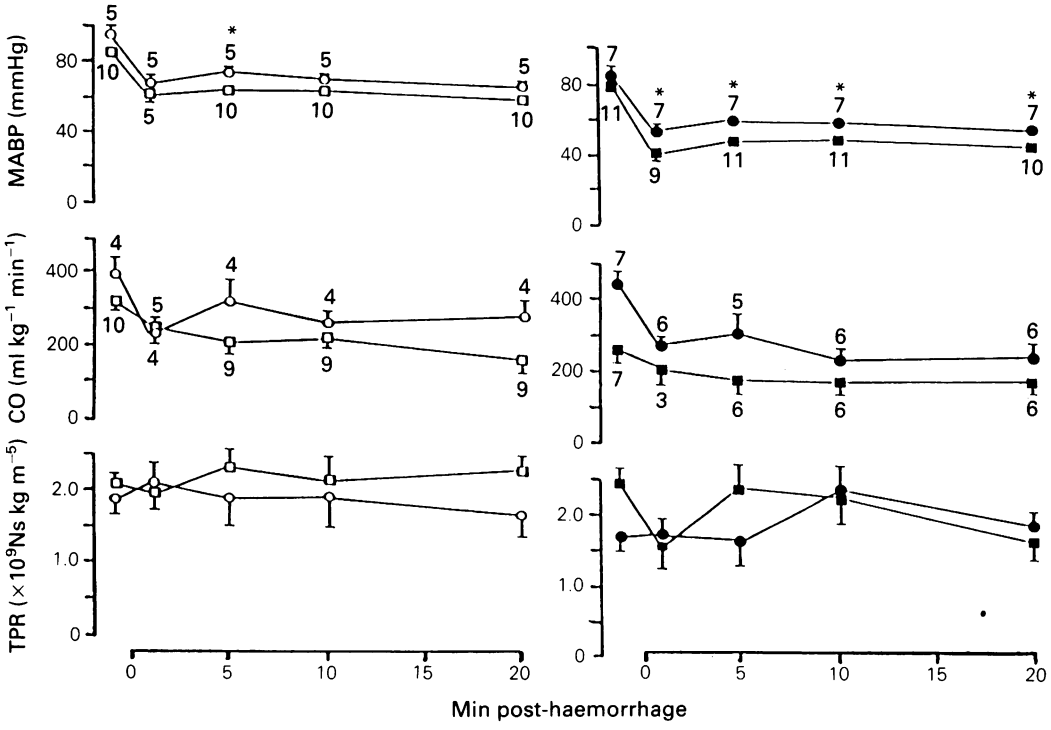

Figure 5 Mean changes (s.e.mean shown by vertical bars) in mean arterial blood pressure (MABP), cardiac output (CO) and total peripheral resistance (TPR) following haemorrhage (1 ml $100\,g^{-1}$ body weight) in 6-hydroxydopamine (6-OH-DA) pretreated BDI (■) and LE (□) rats and in control (untreated) animals of these two strains (●, ○ respectively). * = $P < 0.05$ for comparisons between 6-OH-DA pretreated and control (untreated) animals of either strain. For further details see text.

cardiac output values for any of the groups of rats after the haemorrhage. Nevertheless, it was noticeable that in the two control untreated groups the decreases in cardiac output were greater than in the animals of both strains pretreated with 6-OH-DA. There were no significant differences between the mean total peripheral resistance values at any stage, either before or during the haemorrhage.

While the pre-haemorrhage heart rate was markedly higher in the BDI control (untreated) rats than in the other groups, there were no changes within groups following the haemorrhage so that even 20 min after the blood loss the heart rate for the BDI rats was still higher than the corresponding values for the other groups. Mean stroke volume for the 6-OH-DA-treated BDI and LE rats were lower (significantly, $P < 0.01$, for BDI rats compared with the untreated controls) than the corresponding values for the untreated animals of the two strains. While stroke volume decreased after haemorrhage in all groups, there were no further significant differences between groups because the decreases in the untreated control animals of the two strains were greater than in the 6-OH-DA-treated animals.

Tissue noradrenaline contents (mean ± s.e.mean) in heart and spleen samples from 6-OH-DA pretreated BDI animals were 87 ± 27 and $86 \pm 10\,ng\,g^{-1}$ respectively ($n = 2$), compared with 542 ± 49 and $626 \pm 227\,ng\,g^{-1}$ for control BDI animals ($n = 3$), representing 83 and 86% reductions for these two tissues respectively. Similar findings were made for the LE rats (e.g. heart noradrenaline contents for pretreated ($n = 2$) and control ($n = 2$) LE rats respectively: 66 ± 12 and $446 \pm 20\,ng\,g^{-1}$). Adrenaline and dopamine contents for heart were also markedly reduced in the 6-OH-DA pretreated animals of both strains.

Discussion

Peripheral postganglionic sympathetic nerve terminals were chemically destroyed by the intravenous administration of 6-OH-DA to normal Long Evans

and Brattleboro DI rats in order to investigate subsequently interactions between the sympathetic nervous system and various pressor hormones in animals with and without endogenous vasopressin.

That the 6-OH-DA-treatment was effective is indicated by the reduction in tissue (heart, spleen) catecholamine concentrations. While not total, the degree of depletion produced by the drug was comparable to that observed by other workers (e.g. Baumgarten *et al.*, 1972) and was clearly associated with an abnormal responsiveness to the various pressor substances administered, and the haemorrhage induced in the treated animals compared with their controls. It is appreciated that while nerve terminals are destroyed by this drug in addition to the tissue depletion of catecholamines, receptors will still be present and adrenomedullary hormone production is unaffected.

In the present study the large intravenous dose of vasopressin produced pressor responses which were quite different in the four groups of rats. There were two main differences: (a) Brattleboro DI rats had more prolonged pressor responses than the normal Long Evans animals and (b) pretreatment with 6-OH-DA significantly enhanced the initial maximal pressor effect of vasopressin in both strains of rats without influencing the duration of response.

Two experimental factors that might have some bearing on the prolonged pressor response to vasopressin in the Brattleboro DI rats are possible differences in fluid balance between these animals and the rats of the parent Long Evans strain, and the initial injections of cortisol to the Brattleboro DI animals. Regarding the first of these possible factors, fluid balance was maintained as far as possible within the confines of the experimental protocol but differences cannot be ruled out since measures of haematocrit and plasma osmolality were not obtained before each injection. With respect to the replacement of glucocorticoid in the Brattleboro DI rats, recent experiments in our laboratory show that the prolonged pressor response to vasopressin is still present in animals not given hydrocortisone initially (Obika & Laycock, 1988). Excluding these possibilities, the prolonged pressor response to vasopressin in the Brattleboro DI rats could be due to either (a) the differential clearance of vasopressin in the two strains or (b) changes in the number or distribution of receptors for vasopressin or (c) different post receptor regulation. Since there are various other differences (probably related to the absence of vasopressin) between the Brattleboro DI rat and rats of the parent Long Evans strain, such as lower plasma levels of aldosterone and corticosterone (Milne *et al.*, 1982), and angiotensin II (Mohring *et al.*, 1974), there could be other factors involved in the differential pressor response to vasopressin. The present study was not designed to identify the mechanism by which the pressor effect of this peptide is prolonged, and this remains to be investigated.

The pretreatment with 6-OH-DA resulted in an enhanced pressor response to vasopressin in both Brattleboro DI and Long Evans rats which was not related to the duration of response. This suggests that sympathetic activity normally depresses the pressor sensitivity of vascular smooth muscle to vasopressin. Thus when this tonic inhibition is removed by sympathectomy there would be an increased response to vasopressin. Such a possible reduction in sensitivity to vasopressin by noradrenaline has been shown in isolated vascular strips by Abraham & Nasmyth (1976). Alternatively, since there is some evidence for the co-existence of noradrenaline with other peptides, including a vasopressin-like peptide (Hanley *et al.*, 1984) it is possible that destruction of postganglionic nerve terminals could result in local up-regulation of vasopressin- and noradrenaline-receptors.

The pressor effect of noradrenaline was associated with an increased cardiac output but no effect on total peripheral resistance, in agreement with other workers (Imms *et al.*, 1974). These workers also showed that following β-adrenoceptor blockade with propranolol the pressor effect of noradrenaline was now associated with a marked increase in total peripheral resistance. This finding is remarkably similar to that found in the present study in the rats of both strains pretreatedwith 6-OH-DA.

The intravenous injection of noradrenaline into the 6-OH-DA pretreated rats produced significantly greater increases in arterial blood pressure than in the control rats of the two strains indicating receptor up-regulation. The significantly greater pressor response in the pretreated Brattleboro DI rats compared with the similarly pretreated Long Evans animals indicates the existence of an important interaction between the sympathetic nervous system and vasopressin. However, the interpretation of this finding is complicated by the existence of other differences between Brattleboro DI and Long Evans rats (see earlier), and in view of the various findings by workers using other experimental models. For example, Bartelstone & Nasmyth (1965) showed that infusions of vasopressin potentiate the pressor response to noradrenaline in pithed rats. On the other hand subpressor infusions of vasopressin in conscious nephrectomized rats reduced the pressor response to high ($200\,ng\,kg^{-1}$ and greater) doses of noradrenaline (Elijovich *et al.*, 1984), an effect abolished by ganglion blockade with pentolinium. One possible explanation for our results is that the circulating vasopressin in the LE rats exerts a tonic vasoconstrictor effect, which in the Brattleboro DI rats is

compensated for by enhanced sympathetic and/or renin-angiotensin system activity. When the sympathetic activity is abolished by 6-OH-DA pretreatment there would be a greater degree of catecholamine receptor up-regulation in the Brattleboro rats, hence an enhanced sensitivity to exogenous noradrenaline.

The increased pressor sensitivity to noradrenaline and to vasopressin in the Brattleboro rat is not simply due to a general increase in vascular reactivity. This is clearly demonstrated by the observation that angiotensin II produced the same pressor response in both Brattleboro and Long Evans animals. Furthermore, pretreatment with 6-OH-DA was only associated with a small but insignificant further increase in arterial blood pressure in both strains of animals. Despite the higher basal plasma levels of angiotensin II in Brattleboro rats compared with Long Evans rats (Mohring *et al.*, 1974) isolated vascular (aortic) smooth muscle of the vasopressin-deficient animals is less sensitive to angiotensin II than that of normal Long Evans animals (Altura, 1982). Any decreased sensitivity of Brattleboro DI rats to this peptide was not apparent in the present study. However, it did provide further evidence for an important interaction between angiotensin II and the sympathetic nervous system. There are reports that angiotensin II stimulates the synthesis and/or the release of noradrenaline from sympathetic neurones, and that it also stimulates the release of catecholamines from the adrenal medulla (see Peach, 1977). The present study indicated that in both Brattleboro DI and Long Evans rats with intact sympathetic nervous systems, the angiotensin II-induced increase in arterial blood pressure was associated with an increase in cardiac output rather than the total peripheral resistance, possibly by stimulating noradrenaline release. In the rats pretreated with the 6-OH-DA the pressor effect of angiotensin II was associated entirely with an increased total peripheral resistance, presumably as a direct consequence of its vasoconstrictor action.

The effect of haemorrhage on blood pressure and its subsequent recovery confirmed that Brattleboro rats are more sensitive to blood volume depletion (Laycock *et al.*, 1979; Johnston *et al.*, 1981; Chapman *et al.*, 1986) and that destruction of peripheral sympathetic nerve endings has a more profound effect on the pressor response to haemorrhage in these animals than in the control Long Evans rats. Pretreatment with 6-hydroxydopamine did appear to be associated with a greater susceptibility to the hypotensive effect of haemorrhage in both strains but the depressor effect was significantly more marked in the Brattleboro DI rats. This reduced pressor recovery in the pretreated Brattleboro DI rats was associated with a lower cardiac output than that measured in the untreated rats of this strain, even 20 min after the event. This may be related to the generally lower pre-haemorrhage control cardiac output values for those animals pretreated with 6-OH-DA by this stage of the study. This in turn indicates, perhaps not surprisingly, a greater collapse of cardiovascular recovery mechanisms when peripheral sympathetic and vasopressin-induced responses are both absent compared with the lack of either one of these responses alone.

In conclusion, this study demonstrates important interactions between the sympathetic nervous system and pressor hormones in the anaesthetized rat. Furthermore the absence of endogenous circulating vasopressin in the Brattleboro DI rat appears to influence the interaction between noradrenaline and vasopressin since this animal is more sensitive to the pressor effects of both these hormones than normal rats of the parent strain.

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The effect of propranolol on the hyperaemic response of the hepatic artery to portal venous occlusion in the dog

B. Alexander, [1]L.H. Blumgart & [2]R.T. Mathie

Department of Surgery, Royal Postgraduate Medical School, London

1 It has been reported that activation of β-adrenoceptors may be responsible for the hyperaemic response of the hepatic artery to portal venous blood flow reduction.

2 The effect of β-adrenoceptor blockade on the hepatic arterial response to portal vein occlusion was investigated in 6 anaesthetized dogs. A side-to-side portacaval shunt was established to prevent loss of venous return and arterial blood pressure during periods of portal occlusion. Measurements of hepatic arterial and portal venous blood flows were made by use of electromagnetic flow probes.

3 Intravenous propranolol injection, at a dose sufficient to block the vasodilator effect of low doses of exogenous adrenaline, did not alter the magnitude of the hyperaemic response of the hepatic artery. Propranolol also produced no change in baseline portal venous pressure.

4 It is concluded that hepatic β-adrenoceptors are unlikely to be involved in the arterial response to portal occlusion. The absence of any reduction in basal portal venous pressure by propranolol is of interest in view of the current application of the drug in the treatment of patients with portal hypertension.

Introduction

A compensatory hyperaemic response of hepatic arterial (HA) blood flow occurs during periods of reduced portal venous (PV) flow (Greenway & Stark, 1971; Kock *et al.*, 1972; Mathie *et al.*, 1980). The mechanisms that control this hyperaemic response of the HA (the hepatic arterial 'buffer response': Lautt, 1981) remain poorly understood, though recent studies have implicated vasodilator metabolite involvement (Lautt, 1983; Mathie & Blumgart, 1983). Nevertheless, other mechanisms also postulated to control arterial blood flow have not been excluded. One such possible mechanism was investigated in a previous paper (Mathie *et al.*, 1980), which demonstrated that an extrinsic neurogenic phenomenon neither initiated nor modified the response. Another group has proposed the hypothesis that the mechanism involves activation of β-adrenoceptors through an intrinsic neurogenic vasodilator system or through circulating catecholamines (Fischer *et al.*, 1970).

The current study was carried out to determine if hepatic β-adrenoceptors are involved in the HA 'buffer response' to PV occlusion. The investigation provided an opportunity also to study the effect of propranolol on PV pressure in the normal dog, information relevant to discussions regarding the efficacy of the drug in the treatment of patients with portal hypertension (Conn, 1984).

Methods

Experiments were carried out in a total of 11 mongrel dogs of either sex, weighing 22.2–31.5 kg (mean 27.5 kg). The animals were deprived of food but not water for 24 h before the operation. Anaesthesia was induced with thiopentone (25 mg kg^{-1}, i.v.) and maintained with pentobarbitone (30 mg kg^{-1}, i.v.). After endotracheal intubation, the dogs were ventilated with a 3:1 mixture of nitrous oxide and oxygen using a Starling pump. The minute volume and the inspired oxygen concentration were adjusted to maintain the Po_2/Pco_2 at normal levels (approximately 100 mmHg [13.3 kPa] and 40 mmHg [5.3 kPa] respectively). The base deficit was maintained at 4 mmol l^{-1} by use of sodium bicarbonate i.v. as required. Fluid balance was achieved by infusion of 150 mM sodium chloride i.v.; haematocrit remained above 40% throughout each experiment. Body temperature remained at 36–38°C, maintained when necessary by means of radiant heat lamps.

[1] Present address: Klinik für Viszerale Chirurgie, Inselspital, Berne, Switzerland.

[2] Author for correspondence.

Operative procedures

The experimental model has been used extensively and is described in detail elsewhere (Mathie *et al.*, 1980; Mathie & Blumgart, 1983). After right femoral artery cannulation (for blood pressure measurement), a mid-line laparotomy was performed. A pre-calibrated electromagnetic flow probe (Statham) was applied to both the HA and the PV (3 mm and 6 mm diameter respectively). The HA probe was positioned about 2 cm from the coeliac axis while the PV probe was placed mid-way between the gastroduodenal and splenic veins (see Figure 1).

The gastroduodenal artery and vein were then ligated and the latter vessel cannulated to allow PV pressure measurement. Hepatic vein (HV) cannulation was achieved via the right external jugular vein, the location of the catheter tip being confirmed by direct palpation; the catheter was withdrawn about 5 mm from a 'wedged' position for 'free' HV pressure measurement.

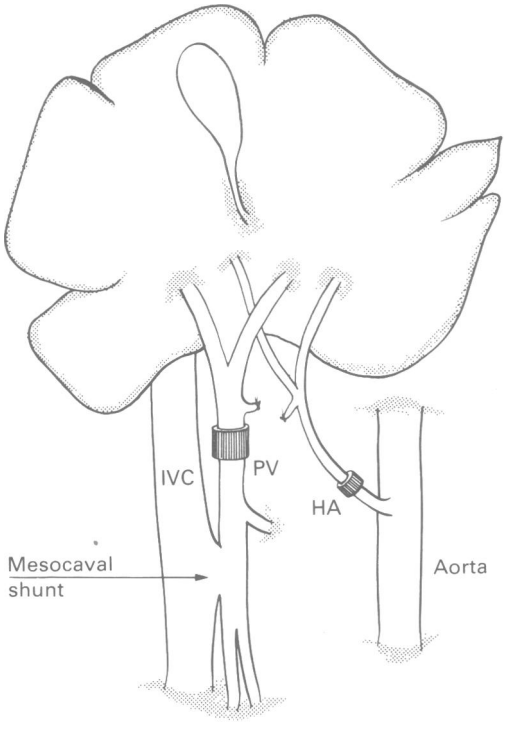

Figure 1 Diagrammatic illustration of experimental preparation, showing electromagnetic flow probes on hepatic artery (HA) and portal vein (PV), and position of the mesocaval shunt which allows diversion of PV blood into the inferior vena cava (IVC) during periods of PV occlusion. (Reproduced from Mathie *et al.*, 1980, by permission of Springer-Verlag).

A side-to-side mesocaval shunt was formed, using 5/0 silk suture, by the construction of an anastomosis between the inferior vena cava and the superior mesenteric vein, just below the entry of the splenic vein (Figure 1). After haemostasis had been obtained, the shunt was closed by means of a small bulldog clip placed along the suture line, thus restoring normal PV flow to the liver until the start of the experimental measurements.

Experimental protocol

The effect of PV flow interruption was investigated approximately 1 h following the operation. Measurements were made of the basal HA and PV blood flows and the pressures in the femoral artery, PV and HV, prior to any alterations in blood flow. The PV was then cross-clamped just proximal to the flow probe, and the shunt immediately opened. The PV occlusion was maintained for approximately 10 min, at which time blood flow and pressure measurements were repeated. PV flow was then restored by reclosing the shunt with the bulldog clip after release of the cross-clamp on the PV. These manoeuvres were repeated in order to obtain duplicate information on the magnitude of the 'buffer response'.

In 6 dogs the effect of intravenous adrenaline (Phoenix Pharmaceuticals) was then investigated to establish the normal response of the HA circulation to adrenergic stimulation. Doses in the vasodilator range (0.02–2.56 $\mu g\,kg^{-1}$, i.v.) were chosen. The peak of the hyperaemic response to adrenaline injection was plotted against the dose employed in each case.

Propranolol (ICI) was then administered at a loading dose of 0.3 $mg\,kg^{-1}$, i.v., followed by an infusion at 0.3 $mg\,kg^{-1}\,min^{-1}$ for the remainder of the experiment. After 30 min, two further 'buffer responses' were elicited for comparison with the control response. Potency of the β-blockade was confirmed immediately after the second 'buffer response' by the construction of a further adrenaline dose-response curve.

Calculations

Blood flows were recorded on the flowmeters in $ml\,min^{-1}$ and subsequently recalculated in ml $100\,g^{-1}\,min^{-1}$ by relating the readings to the wet weight of the liver, determined at the end of each experiment. Total liver blood flow was calculated by addition of the individual HA and PV flows.

The 'buffer capacity' (or 'buffering efficiency') of the HA was calculated as the increase in HA flow/decrease in PV flow and expressed as a percentage.

Vascular resistance for the HA, PV and mesenteric vasculature were calculated in the normal manner (Hughes *et al.*, 1979).

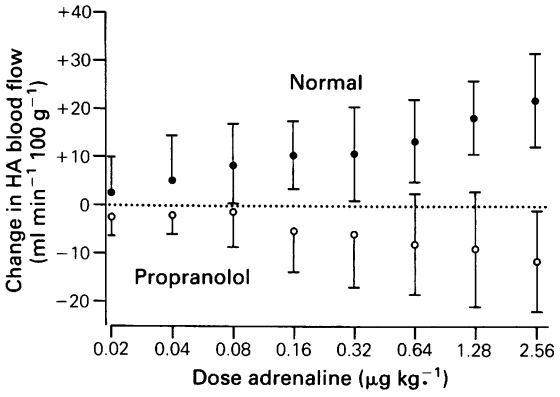

Figure 2 Hepatic arterial (HA) blood flow response to increasing doses of adrenaline i.v. before (●) and after (○) propranolol in 6 dogs.

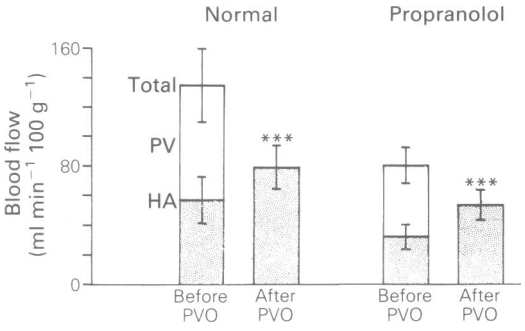

Figure 3 Effect of portal venous (PV) occlusion on liver blood flow before and after propranolol in 6 dogs. Before PV occlusion (PVO), total liver blood flow comprises both PV and hepatic arterial (HA) components; during PVO there remains only HA blood flow (shaded areas). *** Significant difference from baseline HA flow ($P < 0.001$).

Statistics and presentation of data

Student's paired t test was used to test the significance of (a) the differences in measured parameters before and after PV occlusion, and (b) the differences in the magnitude of responses before and after propranolol administration. All results are quoted as mean ± s.e.mean.

Results

Dose-response to adrenaline

The changes in HA blood flow produced by adrenaline in 6 dogs before and after propranolol are illustrated in Figure 2. It is evident that increasing doses of adrenaline caused increasing dilatation of the HA, and that propranolol inhibited the flow increase, thus indicating effective β-adrenoceptor blockade. This is confirmed by the systemic arterial blood pressure changes due to adrenaline. Before propranolol these showed a 10–20 mmHg decrease at each adrenaline dose up to 0.32 $\mu g\,kg^{-1}$ followed by a 10–30 mmHg increase at the three highest doses, whereas after propranolol there was an increase in pressure at every dose of adrenaline used, implying absence of β-mediated vasodilatation.

Hepatic artery 'buffer response'

In the normal situation, PV occlusion caused HA blood flow to increase by 22.0 ± 1.9 ml $100\,g^{-1}\,min^{-1}$; after propranolol, the increase remained essentially unchanged at 21.0 ± 1.8 ml $100\,g^{-1}\,min^{-1}$ (Figure 3). In both instances, the increase was statistically significant ($P < 0.001$). However, the basal HA flow was significantly different in the two groups (57.5 ± 15.4 and 31.7 ± 7.5 ml $100\,g^{-1}\,min^{-1}$ respectively). Baseline PV blood flow was significantly reduced by propranolol from 77.4 ± 9.9 to 47.5 ± 6.4 ml $100\,g^{-1}\,min^{-1}$ ($P < 0.05$). Total liver blood flow was also significantly reduced by propranolol (134.9 ± 25.0 cf. 79.2 ± 13.1 ml $100\,g^{-1}\,min^{-1}$; $P < 0.05$); the magnitude of decrease caused by PV occlusion was smaller after propranolol (55.4 ± 10.5 cf. 26.5 ± 5.6 ml $100\,g^{-1}\,min^{-1}$; $P < 0.05$) due to the smaller baseline PV flow in the latter situation. Interestingly, the 'buffer capacity' of the HA increased from 31.1 ± 5.0% in the normal situation to 50.1 ± 8.6% after propranolol, but this increase was not statistically significant; the difference from normal was primarily due to the smaller baseline PV flow following propranolol infusion.

Mean arterial blood pressure did not change significantly from baseline levels during PV occlusion with or without propranolol infusion. However, there was a small, non-significant decrease in baseline arterial pressure (Table 1), attributable to the systemic actions of propranolol. HV pressure remained unaltered by any procedure (Table 1). Similarly, basal PV pressure was not affected by propranolol administration (Table 1). The effects of PV occlusion on prehepatic venous pressure could not be assessed due to the absence of a recording catheter below the cross-clamp.

The changes in HA vascular resistance were reciprocal to those of HA blood flow (Table 1). There was a significant increase in basal HA resistance due to propranolol ($P < 0.05$). Propranolol also caused an increase in both portal and mesenteric baseline vascular resistances, though only the latter was statistically significant (Table 1); mesenteric vascular resistance was not calculated during the

Table 1 Pressure and vascular resistance measurements during hepatic artery (HA) 'buffer response' before and after propranolol in 6 dogs

	Normal		*Propranolol*	
	Before PV occlusion	*During PV occlusion*	*Before PV occlusion*	*During PV occlusion*
Blood pressure (mmHg)	123.2 ± 8.4	123.8 ± 8.7	109.8 ± 5.4	109.7 ± 5.9
PV pressure† (mmHg)	6.3 ± 0.5	(4.1) ± (0.6)	6.9 ± 0.7	(5.2) ± (0.7)
HV pressure (mmHg)	4.0 ± 0.4	3.7 ± 0.3	4.7 ± 0.4	4.8 ± 0.4
HA resistance ($mmHg\,ml^{-1}\,100\,g^{-1}\,min^{-1}$)	3.4 ± 1.2	1.8 ± 0.3	5.2 ± 1.5*	2.2 ± 0.3
PV resistance ($mmHg\,ml^{-1}\,100\,g^{-1}\,min^{-1}$) × 10^{-2}	3.1 ± 0.9	—	4.7 ± 1.8	—
Mesenteric vascular resistance§ ($mmHg\,ml^{-1}\,min^{-1}$)	0.31 ± 0.03	—	0.45 ± 0.03*	—

PV: portal vein; HV: hepatic vein.
* Significant difference from normal baseline value ($P < 0.05$).
† PV pressure measurements after PV occlusion do not represent prehepatic pressure readings, due to the position of the recording catheter (see text).
§ Mesenteric resistance calculations after PV occlusion were not possible because flow through the anastomosis was not measured (see also text).
All values are mean ± s.e.mean.

'buffer response' since PV flow through the anastomosis was not measured.

Effect of controlled hypotension on hepatic artery 'buffer response'

In another series of experiments the effect of haemorrhagic hypotension on the magnitude of the HA 'buffer response' was examined in 5 mongrel dogs. The animals were bled from the femoral artery until arterial blood pressure remained steady at 75% of control values. Results showed that the 'buffer response' remained at a normal magnitude ($17.5\,ml\,100\,g^{-1}\,min^{-1}$) despite the reduction in blood pressure; hypotension *per se* did not diminish HA blood flow (see Table 2).

Discussion

This study has demonstrated the absence of any significant change in the magnitude of the HA hyperaemic response to PV occlusion following β-adrenoceptor blockade. We can therefore conclude it is unlikely that activation of hepatic β-receptors is of any significance in the normal 'buffer response' of the HA, either through an intrinsic neural vasodilator mechanism or by circulating catecholamines. This conclusion is in contrast to that proposed by Fischer *et al.* (1970), who suggested that β-receptor stimulation played an important, though not exclusive role. However, these authors observed a reduction in the response after propranolol in only 5 out of 11 dogs studied; the remaining animals did not show any change in response from controls

Table 2 Effect of portal vein (PV) occlusion in 5 dogs subjected to haemorrhagic hypotension

	Control	*Hypotension alone*	*Hypotension + PV occlusion*
Blood pressure (mmHg)	121.0 ± 6.9	91.6 ± 5.1*	87.4 ± 3.7*
HA blood flow ($ml\,100\,g^{-1}\,min^{-1}$)	29.6 ± 7.2	28.3 ± 5.1	45.8 ± 5.7†*
PV blood flow ($ml\,100\,g^{-1}\,min^{-1}$)	62.0 ± 10.7	32.0 ± 6.6*	—
HA resistance ($mmHg\,ml^{-1}\,100\,g^{-1}\,min^{-1}$)	3.8 ± 0.9	2.8 ± 0.5	1.5 ± 0.2†*
PV resistance ($mmHg\,ml^{-1}\,100\,g^{-1}\,min^{-1}$) × 10^{-2}	8.4 ± 3.0	19.6 ± 8.5	—

For abbreviations see Table 1, except: * significant difference from control ($P < 0.05$); † significant difference from hypotension alone ($P < 0.05$).

although greater doses of propranolol were administered. The standard dose administered in Fischer's investigations was identical to that used in the present study, but the experimental model differed: diversion of PV blood was achieved via a portofemoral shunt and it is not clear from their published description if the degree of diversion was controlled in a reproducible way. We suggest, therefore, that the report did not conclusively demonstrate the partial involvement of β-adrenoceptors as claimed by the authors. The relatively high dose of propranolol used in the present study conformed with that employed by Fischer and demonstrated that β-adrenoceptors play little or no part in the response. Indeed, this dose of propranolol transformed the HA response to exogenous adrenaline from vasodilatation to vasoconstriction.

The experimental model used in the present work has been employed in several previous studies by our group: it achieves complete PV occlusion in a repeatable manner without loss of systemic venous return or development of arterial hypotension (Mathie *et al.*, 1980; Mathie & Blumgart, 1983; 1987). The magnitude of the HA 'buffer response' is also highly reproducible with repeat observations in individual animals: in a series of 17 normal dogs, a repeat 'buffer response' was only 1.1% smaller than the first measurement (18.6 ± 1.8 ml $100\,g^{-1}\,min^{-1}$ and 18.8 ± 1.8 ml $100\,g^{-1}\,min^{-1}$ respectively; R.T. Mathie and B. Alexander, unpublished observations).

Propranolol infusion resulted in a small decrease in arterial blood pressure, an increase in HA, PV and mesenteric vascular resistance and a decrease in HA and PV blood flow. If it is assumed that the drug produced a generalised peripheral vasoconstriction, it may be surmised that the slight drop in mean blood pressure was the result of a significant fall in cardiac output, a noted action of propranolol (Lebrec *et al.*, 1982). The reduced values of basal HA and PV blood flow after propranolol administration did not influence the absolute magnitude of the HA flow increase during PV occlusion. We believe that such basal haemodynamic conditions do not compromise our conclusions regarding the absence of β-receptor involvement, since the results of our other experiments demonstrated a normal 'buffer response' of 17.5 ml $100\,g^{-1}\,min^{-1}$ even after 25% reduction of mean arterial blood pressure by controlled haemorrhage.

Propranolol administration in our experiments caused a substantial increase in the 'buffer capacity' of the HA from 31% to 50%. This was felt to be a feature solely of the reduced basal PV blood flow and was not taken to imply an increased hyperaemic response of the HA *per se*. The figures may, nevertheless, be advanced as supportive evidence for the conclusion that the response is not diminished by propranolol.

It is known that both α- and β-adrenoceptors exist in the HA (Richardson & Withrington, 1981). The effect of adrenaline is complicated by the dose-dependency of its action on α- and β-receptors: at low blood concentrations, vasodilatation predominates, whereas at higher concentrations vasoconstriction occurs. Only dilatation was observed at the concentrations used in the present study (below $10\,\mu g\,kg^{-1}$, i.v.), which ensured a valid basis for comparison of the haemodynamic response before and after β-blockade and for assessment of its potency. By contrast, Richardson & Withrington (1977) found predominantly vasoconstriction of the HA, but at a higher effective dose than in the present experiment since the adrenaline was injected intra-arterially. However, their observation that propranolol potentiated the basal vasoconstrictor response supports the present findings. We used adrenaline in preference to the more selective β-agonist isoprenaline in our study because of the more profound effects of the latter on systemic haemodynamics: it produces generalised vasodilatation and causes a fall in blood pressure coupled with a marked increase in heart rate (Bowman *et al.*, 1975). In addition, the dilator action of adrenaline on the HA has been more extensively documented than isoprenaline (Richardson & Withrington, 1981). The selection of adrenaline was vindicated by the modest changes in blood pressure observed at the doses used and by the unequivocal effect of propranolol on the dose-response curve.

The current study has also provided further data relating to the effect of propranolol on PV pressure and blood flow. Propranolol has recently been used as a means of decreasing PV pressure in order to reduce the risk of variceal bleeding in patients with portal hypertension (Lebrec *et al.*, 1980; Bercoff *et al.*, 1984; Ohnishi *et al.*, 1985), though not with universal success (Burroughs *et al.*, 1983; Anderberg *et al.*, 1984). One mode of action is thought to be related to its vasoconstrictor action on the mesenteric vascular bed, caused by β_2-adrenoceptor blockade, which results in a fall in PV blood flow and pressure (Kroeger & Groszmann, 1985; Jenkins *et al.*, 1985). However, in the current series of investigations PV (and HV) pressure remained unaltered despite a decrease in PV flow. This effect may be a result of the increase in intrahepatic PV resistance; it is widely believed that the PV vascular bed contains α- but not β-adrenoceptors (Richardson & Withrington, 1981), and therefore it is not evident why PV resistance should have increased during β-blockade in this preparation.

Propranolol can also produce a decrease in PV pressure by a reduction in PV flow as a result of a fall in cardiac output (Lebrec *et al.*, 1982), due to

β_1-adrenoceptor blockade (Ohnishi *et al.*, 1985). Most authors now conclude that propranolol achieves its reduction in PV flow and pressure through a combination of β_1-adrenoceptor blockade (causing a reduction in cardiac output) and β_2-adrenoceptor blockade (causing a reduction in mesenteric blood flow) (Hillon *et al.*, 1982; Kroeger & Groszmann, 1985; Jenkins *et al.*, 1985).

It is probable that blood flow in the normal canine liver behaves in a quite different manner from the human cirrhotic hepatic circulation, but our results do indicate some caution in the administration of propranolol during treatment of portal hypertension in man. Recent haemodynamic studies in dogs with chronic bile duct ligation and portal hypertension have demonstrated minimal hepatic circulatory or pressure effects of propranolol despite significant systemic responses (Willems *et al.*, 1986). As these authors stated, 'the factors responsible for the discrepancy between man and dog are not known.' It is of interest that other authors have demonstrated a significant reduction in PV pressure with propranolol in cirrhotic rats (Jenkins *et al.*, 1985), and it is therefore possible that differences between the dog and other species may be explained by the relative absence of potential portal-systemic collateral vessels in the canine circulation.

This investigation has demonstrated that hepatic β-adrenoceptors have little or no role in the vasodilator response of the HA to PV occlusion. Further studies are currently under way in an attempt to discover the possible mechanisms. The present study has indicated an absence of PV pressure reduction in response to intravenous propranolol injection in the normal dog.

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Pharmacological modulation of Paf-induced rat pleurisy and its role in inflammation by zymosan

[1]Marco A. Martins, Patricia M.R. e Silva, Hugo C. Castro Faria Neto, Patricia T. Bozza, Paulo M.F.L. Dias, Marcia C.R. Lima, Renato S.B. Cordeiro & *B. Boris Vargaftig

Dept. Fisiologia e Farmacodinâmica, Fundação Oswaldo Cruz, Av. Brasil, 4365, Caixa Postal 926, CEP 20010, Rio de Janeiro, Brazil and *Unité de Pharmacologie Cellulaire, Unité Associée Institut Pasteur, INSERM 285, 25, Rue du Dr Roux, 75015 Paris, France

1 The intrapleural injection of Paf-acether into rats caused, at 30 min, a marked exudation accompanied by a reduction in the pleural leucocyte count. At 6 h, the exudate volume had decreased and a significant increase in the total leucocyte count, particularly eosinophils was noted.

2 Two Paf-acether antagonists, WEB 2086 and 48740 RP abrogated the pleural leucopenia observed 30 min after Paf-acether administration, whereas the exudation was inhibited only by the former. Pleurisy was also reduced by about 60% with dexamethasone, by about 45% with BW 755C or LY 171883, a mixed cyclo-oxygenase/lipoxygenase inhibitor and a peptido-leukotriene antagonist respectively, and by about 30% with indomethacin, flurbiprofen or piroxicam.

3 Repeated daily intrapleural injections of Paf-acether led to a state of progressive desensitization to Paf-acether itself, whereas responsiveness to 5-hydroxytryptamine was maintained. In addition, the Paf-induced auto-desensitization was largely inhibited by WEB 2086.

4 Pleurisy induced by zymosan, but not by carrageenin, was significantly reduced in Paf-acether-desensitized animals. These results were consistent with those obtained with WEB 2086 which supressed zymosan-induced but not carrageenin-induced pleurisy.

5 This study suggests that Paf-acether-induced pleurisy in the rat may be mediated by lipoxygenase arachidonic acid metabolites and that pleurisy induced by zymosan, but not by carrageenin, is largely dependent upon Paf-acether.

Introduction

Since the initial studies with Paf-acether (1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine), in the early seventies, several lines of evidence have accumulated indicating that this phospholipid may play an important role in acute inflammation. Paf-acether: (1) increases vascular permeability in several animal species (Wedmore & Williams, 1981; Humphrey *et al.*, 1982; Bjork & Smedegard, 1983; Pirotzky *et al.*, 1984; Hwang *et al.*, 1985), (2) is produced by or activates different cell types such as mast cells, platelets, macrophages, monocytes, endothelial cells, neutrophils, lymphocytes and eosinophils which are important participants in inflammation (for reviews see Braquet *et al.*, 1987 and Pinckard *et al.*, 1988), (3) is a potent inducer of oedema, hyperalgesia (Vargaftig & Ferreira, 1981) and pleurisy (Silva *et al.*, 1986; Tarayre *et al.*, 1986) and (4) is chemotactic for mononuclear and polymorphonuclear leucocytes (Shaw *et al.*, 1981; Archer *et al.*, 1985; Henocq & Vargaftig, 1988).

There is evidence for and against the participation of Paf-acether in the inflammatory reaction induced by carrageenin. Hwang *et al.* (1986) demonstrated that Paf-acether antagonists such as kadsurenone and L-652,731 reduce the first phase of the carrageenin-induced rat paw oedema. In addition, they detected a significant release of Paf-acether-like materials in the carrageenin-injected paw. In contrast, Cordeiro *et al.* (1986), reported that neither the antagonist BN 52021 nor selective desensitization to Paf-acether modify rat paw oedema induced by carrageenin.

Repeated daily intraplantar injections of Paf-acether or 2-methyl-carbamate-Paf lead to topical

[1] Author for correspondence.

cross-desensitization between these structurally related lipids under conditions where the responsiveness to 5-hydroxytryptamine (5-HT) was not modified (Cordeiro *et al.*, 1986). Therefore, the selective desensitization to Paf-acether, together with the development of specific Paf-acether antagonists, are effective tools for further elucidation of the physiopathological significance of this lipid.

In view of the potential importance of Paf-acether in inflammation, we have now studied the mechanism of pleurisy induced by this lipid in rats and, also, the potential involvement of Paf-acether in pleurisy induced by carrageenin or zymosan. Our results show that the pleurisy induced by zymosan is markedly reduced after the Paf-acether-induced desensitization, a finding which is not duplicated with carrageenin. In addition, pretreatment of animals with the Paf-acether antagonist WEB 2086 inhibited pleurisy induced by zymosan, but failed to modify that induced by carrageenin, suggesting an important role of Paf-acether in the inflammatory reaction due to the former, but not to the latter.

Methods

Animals and treatments

Male Wistar rats weighing 150–200 g were used. Indomethacin (2 mg kg^{-1}), pirozicam (1.8 mg kg^{-1}), flurbiprofen (1.0, 2.5, and 5.0 mg kg^{-1}) and BW 755C (25 mg kg^{-1}) were given intraperitoneally 1 h before the agonist. All drugs were diluted in sterile NaCl 0.9% solution (saline) except piroxicam which was dissolved in Tween 80 and further diluted with saline. Dexamethasone (Decadron, 0.5 mg kg^{-1}) was diluted in saline and given i.p. 12 and 1 h before the agonist. LY 171883 (Fleish *et al.*, 1985), a leukotriene D_4 antagonist, was dissolved in 1 M sodium hydroxide and subsequently neutralized with 2 M HCl to be administered (3 mg kg^{-1}, orally) 2 h before Paf-acether.

Paf-acether antagonists 48740 RP (Sédivy *et al.*, 1985) and WEB 2086 (Casals-Stenzel *et al.*, 1986; 1987) were given intrathoracically (i.t.) into the pleural cavity 5 min before Paf-acether at 2, 6 and 10 μg and 2, 6, 10 and 15 μg, respectively, in a volume of 0.1 ml. In some experiments, WEB 2086 was administered intraperitoneally (15 mg kg^{-1}) 1 h before Paf-acether, carrageenan or zymozan. The Paf antagonists were diluted in sterile saline, immediately before use, with the aid of 0.1 N HCl in the case of WEB 2086.

Induction of pleurisy

Pleurisy was induced by the i.t. injection of Paf-acether (0.5 to 16 μg/cavity), 5-hydroxytryptamine (100 μg/cavity), zymosan (1000 μg/cavity) or carrageenin (800 μg/cavity) in a final volume of 0.2 ml. Each experiment included an equivalent number of control animals receiving the same volume of sterile saline. The animals were killed 30 min or 6 h after Paf-acether, 1 h after 5-hydroxytryptamine and 4 h after carrageenin and zymosan. The pleural cavity was opened and rinsed with 3 ml of saline containing heparin (20 iu ml^{-1}). The fluid was collected and the volume measured with a graduated syringe.

Desensitization procedure

Auto-desensitization to Paf-acether was produced by successive daily i.t. administrations of Paf-acether (1 μg/cavity) for 5 successive days. In control groups saline was used as a substitute for Paf-acether. It is important to note that the exudative response induced by Paf-acether disappeared 24 h after i.t. injection of the lipid (data not shown). To verify the effect of Paf antagonists on Paf-induced desensitization, 6 μg doses of WEB 2086 were injected i.t. 5 min before each dose of Paf-acether administered daily for 3 days. On the 4th day, only Paf-acether (1 μg/cavity) was injected and the pleurisy determined. To test the potential existence of cross-desensitization, 5-hydroxytryptamine (100 μg/cavity), carrageenin (800 μg/cavity) or zymosan (1000 μg/cavity) were injected i.t. in Paf-desensitized animals.

Total and differential leukocyte counts

Total leucocytes from the pleural cavity were counted (in acetic acid 2%) in Neubauer chambers by means of an optical microscope. Differential counts were made with May-Grünwald-Giemsa dye in smears prepared in a cytocentrifuge (Incibras) and examined under an oil immersion objective. The fluid collected from the cavity was centrifuged for 5 min at 1500 r.p.m. (1000 *g*), and the total proteins were quantified in the supernatant by the Biuret technique in a spectrophotometer (Incibras MF 190).

Drugs

Paf-acether (1-O-hexadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine) was from Bachem (Switzerland), 48740 RP (3-(3-pyridyl)-1H, 3H-pyrrolo-(1,2-c)-thiazole-7-carboxamide) from Rhône-Poulenc Santé (France), WEB 2086 (3-4-(2-chlorphenyl)-9-methyl-6H-thieno 3,2-f 1,2,4 triazolo-4,3-a 1,4-diazepin-2-yl-1-(4-morpholinyl)-1-propanone) was a gift from Dr H. Heuer (Boehringer Ingelheim, Federal Republic of Germany), LY 171883 (1- < 2-hydroxy-3-propyl-4- < 4 - (1H - tetrazol - 5 - yl) butoxy > phenyl > ethanone) was from Eli Lilly and Company, Indianapolis, U.S.A. The other drugs were obtained from the

following sources: 5-hydroxytryptamine (5-HT), zymosan, carrageenin (lambda) and indomethacin (Sigma); piroxicam (Pfizer); flurbiprofen was a gift from Dr Marcio Falci (Boehringer Ingelheim, S. Paulo, Brazil); BW 755C (3-amino-1-[*m*-(trifluoromethyl)-phenyl]-2pyrazoline (Wellcome Laboratories, Beckenham) and dexamethasone (Decadron (R), Merck, Sharp, and Dohme).

Statistical analysis

The data were analysed statistically by means of Student's two tailed t test for unpaired samples. P values of 0.05 or less were considered significant.

Results

Exudation and cell migration induced by intrapleural injections of Paf-acether

Table 1 shows that intrapleural injection of Paf-acether (1 μg/cavity) caused a significant exudation after 30 min, which was accompanied by protein extravasation and by a marked reduction in the total pleural leucocyte count. At 6h, the exudate volume and its protein content decreased from 867 ± 34 μl to 395 ± 20 μl and from 36.6 ± 2.4 μg to 12.2 ± 0.8 μg, respectively, under conditions where a significant increase in the total leucocyte count was noted (Table 1). Differential cell counts performed 30 min and 6 h, after intrapleural saline showed the predominance of mononuclear leucocytes in the cavity. When the differential counts were performed on samples collected 30 min after Paf-acether, a decrease in the number of neutrophils, eosinophils and mononuclear cells was noted. In contrast, at 6 h, the increase in the number of eosinophils was markedly above that of neutrophils and mononuclear cells (Table 1). As indicated in Figure 1, intrapleural injections of increasing amounts of Paf-acether (0.25–16 μg/cavity) yielded, at 30 min, bell-shaped

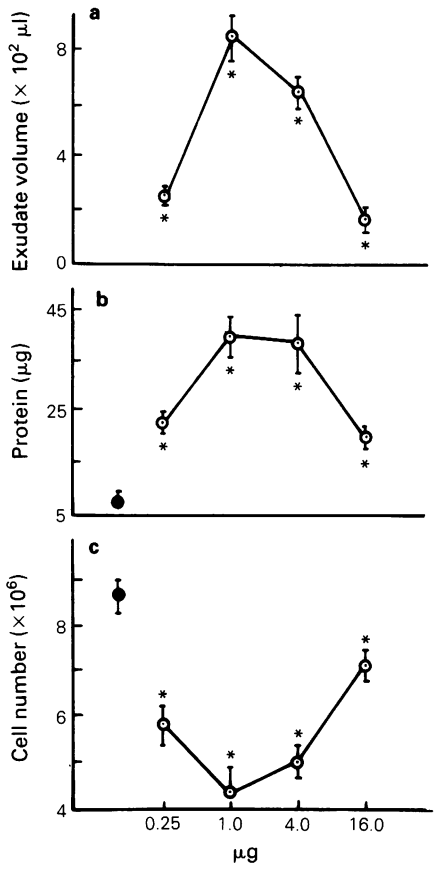

Figure 1 The biphasic dose-effect curves of exudate volume (a), extravasated protein (b) and reduction of pleural leucocyte counts (c) induced by i.t. injection of Paf-acether (0.25–16 μg/cavity). The analyses were made 30 min after injection of the lipid; (●) values obtained from animals injected with saline. Each point represents the mean from at least 6 animals with vertical bars showing s.e.mean and statistically significant ($P < 0.05$) differences are indicated by an asterisk.

Table 1 Exudative and cellular alterations induced by Paf-acether in rats

Time (h)	*Exudate* (μl)	*Total protein* (μg) Sal	Paf	*Total leucocytes* (× 10⁶) Sal	Paf	*Mononuclear cells* (× 10⁶) Sal	Paf	*Neutrophils* (× 10⁶) Sal	Paf	*Eosinophils* (× 10⁶) Sal	Paf
0.5	866.6* ±33.3	6.3 ±0.6	36.6* ±2.4	10.3 ±0.5	4.9* ±0.4	8.6 ±0.6	3.9* ±0.3	0.4 ±0.1	0.2 ±0.5	1.2 ±0.2	0.6* ±0.1
6.0	395.0* ±20.4	5.5 ±0.8	12.2* ±0.8	8.3 ±0.9	17.2* ±2.1	6.5 ±0.2	10.8* ±0.5	0.4 ±0.1	0.8 ±0.2	1.0 ±0.1	4.7* ±0.3

Exudate volume, protein extravasation and total and differential leucocyte counts were measured, in the pleural fluid, at 30 min and at 6 h after i.t. injection of Paf-acether (1 μg/cavity). Values in the table represent the mean ± s.e.mean from at least 6 animals and statistically significant differences are indicated by an asterisk.

dose-response curves for pleural exudation (Figure 1a), for protein (Figure 1b) and for the reduction in the number of leucocytes (Figure 1c),

Interference of potential inhibitors with Paf-acether-induced pleurisy

Figure 2 shows the effects of the *in situ* administration of WEB 2086 or 48740 RP in pleurisy triggered by Paf-acether (1 μg/cavity). WEB 2086 (2–15 μg/cavity) inhibited dose-dependently the exudate volume, the amount of extravasated protein and the reduction in the leucocyte count caused by Paf-acether, whereas 48740 RP (2–15 μg/cavity) inhibited only the latter. Table 2 summarizes the effect of several drugs on exudation induced by 1 μg of Paf-acether. Dexamethasone was the most effective among the tested compounds. The cyclo-oxygenase antagonists piroxicam, flurbiprofen and indomethacin, the dual cyclo- and lipoxygenase inhibitor BW 755C, and the leukotriene D_4 antagonist LY 171883 were also effective against Paf-induced pleurisy. It is noteworthy that in no instance was inhibition greater than 60%.

Auto-desensitization to Paf-acether-induced rat pleurisy

As shown in Figure 3a, repeated daily intrapleural injections of Paf-acether (1 μg) led to a progressive state of auto-desensitization, down to about 20% of initial response after 4 daily re-stimulations. The refractoriness to Paf-acether was selective since the responses to 5-hydroxytryptamine were maintained (Figure 3b). In addition, as indicated in Figure 4, the Paf-acether receptor antagonist WEB 2086 (10 μg/cavity) inhibited significantly the auto-desensitization caused by repeated administration of Paf-acether.

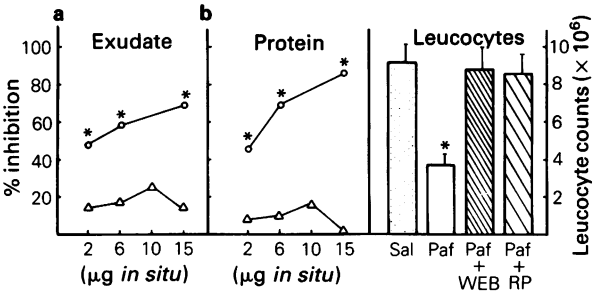

Figure 2 The effect of *in situ* administration of WEB 2086 (2–15 μg/cavity) (○) or 48740 RP (2–15 μg/cavity) (△) on exudate volume (a) and extravasated protein (b) induced by Paf-acether (1 μg/cavity). Section (c) shows the inhibitory effect of WEB 2086 (WEB, 6 μg/cavity) or 48740 RP (RP, 1 μg/cavity) on the reduction in numbers of pleural leucocyte induced by Paf-acether (1 μg/cavity). Each column represents the mean (with s.e.mean shown by vertical bars) from at least 6 animals and statistically significant ($P < 0.05$) differences are indicated by an asterisk.

Interference of Paf-induced desensitization and of WEB 2086 with carrageenin- or zymosan-induced rat pleurisy

Figure 5 shows that zymosan-induced pleurisy was markedly reduced in animals desensitized to Paf-acether under conditions where the response to carrageenin was preserved. The protective role against zymosan, assessed by cross desensitization with Paf-acether (about 50% inhibition, $P < 0.001$), was seen for both exudate volume (left panel) and for extravasated proteins (right panel).

Table 2 Effect of anti-inflammatory drugs on the pleural exudate induced by Paf-acether

Drug	*No. of rats*	*Dose* (mg kg^{-1})	*% inhibition*	*Degree of significance (P)*
Dexamethasone	5	0.5	62.2	<0.001
Indomethacin	5	2.0	26.9	<0.03
Flurbiprofen	4	1.0	30.9	<0.02
	5	2.5	28.4	<0.02
	6	5.0	37.0	<0.006
Piroxicam	6	1.8	38.1	<0.002
BW 755C	6	25.0	44.0	<0.001
LY 171883	6	3.0	47.9	<0.004

The anti-inflammatory drugs were administered 1 h before and the exudate volume was measured 30 min after the i.t. injection of Paf-acether (1 μg/cavity).

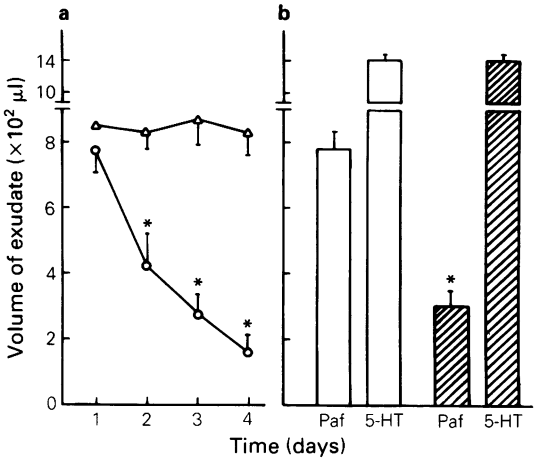

Figure 3 (a) The kinetics of the auto-desensitization induced by 1 to 4 repeated stimulations with 1 µg of Paf-acether (○), under conditions where the responsiveness was maintained after 1 up to 4 repeated injections of saline (△). (b) The effect of an i.t. injection of Paf (1 µg) or 5-hydroxytryptamine (5-HT, 100 µg) to animals which received one daily injection of Paf-acether (hatched columns) or saline (open columns) for 4 days. Each column represents the mean (with s.e.mean shown by vertical bars) from at least 6 animals. Statistically significant ($P < 0.05$) differences are indicated by an asterisk.

A close relationship between the kinetics of the progressive auto-desensitization induced by Paf-acether and the refractoriness to zymosan was also noted (Figure 6). The involvement of Paf-acether in zymosan-induced inflammation was reinforced by pretreating the animals with the Paf-acether antagonist WEB 2086 (15 mg kg^{-1}), which supressed the pleurisy induced by Paf-acether or zymosan, but not by carrageenin (Figure 7). In addition, a significant reduction in the zymosan-induced leucocyte migration was noted in animals either desensitized to Paf-acether or pretreated with WEB 2086 (Table 3).

Discussion

Intrathoracic injections of Paf-acether induced, at 30 min, a marked exudative response accompanied by a reduction in pleural leucocyte count. After 6 h, the volume of exudate was reduced and a significant increase in total number of leucocytes present was noted, confirming studies by Tarayre *et al.* (1986). Exudation is indeed induced by Paf-acether (Wedmore & Williams, 1981; Humphrey *et al.*, 1982; Handley *et al.*, 1984; Pirotzky *et al.*, 1984; Tarayre *et al.*, 1986), and probably results from an initial short-

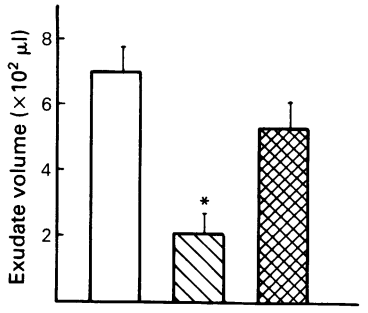

Figure 4 The effect of the i.t. injection of Paf-acether (1 µg/cavity) in animals pretreated with 1 daily injection of saline (open column), Paf-acether (1 µg/cavity) (hatched column) or Paf-acether (1 µg/cavity) plus WEB 2086 (6 µg/cavity) (cross-hatched column), for 3 days. Each column is the mean (with s.e.mean shown by vertical bars) from at least 6 animals. Statistically significant ($P < 0.05$) difference between treated and untreated Paf-stimulated animals is indicated by an asterisk.

lasting increase of the permeability of the endothelium (Bussolino *et al.*, 1987) of pleural vessels. Such an effect, a hallmark of acute inflammation, was probably accompanied by the acquisition of adhesive properties to infiltrated leucocytes, which should account for the reduction in their number, incoming cells becoming unavailable for counting, because of margination (Born & Planker, 1979). However, the possibility that leucocytes disappear as a conse-

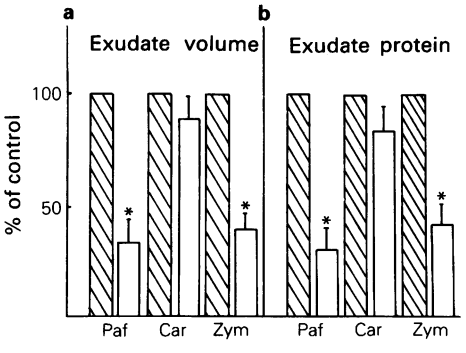

Figure 5 The effect of the i.t. injection of Paf-acether (1 µg/cavity), zymosan (zym, 1000 µg/cavity) or carrageenin (Car, 800 µg/cavity) on exudate volume (a) and extravasated protein (b) of animals pretreated with 1 daily injection of Paf-acether (1 µg/cavity) (open columns) or saline (hatched columns), for 4 days. The exudate volume was determined 30 min after the last injection of Paf-acether or 4 h after zymosan and carrageenin. Each column is the mean (with s.e.mean shown by vertical bars) from at least 6 animals. Statistically significant ($P < 0.05$) difference between Paf-treated and untreated animals is indicated by an asterisk.

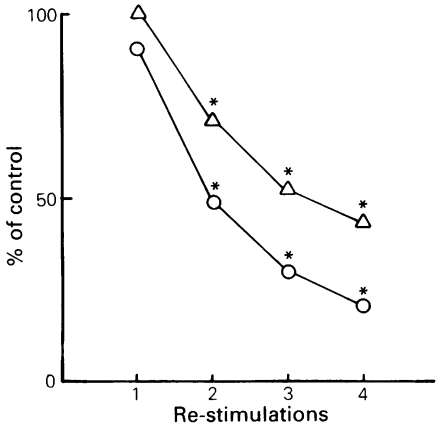

Figure 6 Modifications of the pleural exudation induced by 1 μg of Paf-acether (○) or 1000 μg of zymosan (△) observed in animals pretreated with 1 to 4 re-stimulations of Paf-acether (1 μg/cavity) or of saline in the control groups. The exudate volume was determined 30 min after the last injection of Paf-acether or 4 h after zymosan. Horizontal axis represents the number of pre-injections of Paf-acether. Each point is the mean of at least 6 animals. Statistical significances ($P < 0.05$) are indicated by an asterisk.

quence of cell aggregation and/or lysis cannot be ruled out.

The reduction in pleural leucocyte count was followed, at 6 h, by a marked cell infiltration into the pleural cavity. Since Paf-acether is a potent chemotactic agent for leucocytes, as indicated by *in vivo* (Colditz & Movat, 1984) and *in vitro* assays (Shaw *et al.*, 1981) it is likely that they invade the pleural cavity by active chemotaxis. Paf-acether has been shown to be highly selective for eosinophils (Wardlaw *et al.*, 1986; Lellouch-Tubiana *et al.*, 1987; Hakansson *et al.*, 1987) and indeed a four fold increase in their number was noted, under conditions where neutrophils and mononuclear cells infiltration into the pleural cavity was increased only 2 fold.

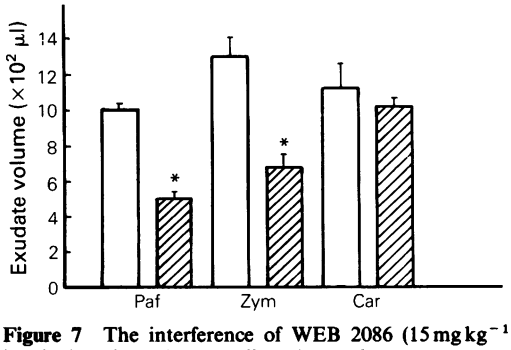

Figure 7 The interference of WEB 2086 (15 mg kg^{-1} hatched columns) or saline (control groups, open columns) with the pleurisy induced by Paf-acether (1 μg/cavity), zymosan (Zym, 1000 μg/cavity) or carrageenin (Car, 800 μg/cavity). The treatments were given, intraperitoneally, 1 h before the agonists. Each column represents the mean (with s.e.mean shown by vertical bars) from at least 6 animals and statistically significant ($P < 0.05$) differences are indicated by an asterisk.

Table 3 Zymosan-induced leucocyte accumulation after the desensitization to Paf-acether or pretreatment with WEB 2086

Treatment	*Total leucocytes* (× 10^5) *Saline*	*Zymosan*
None	50.8 ± 9.8	605.6 ± 19.8
Desensitization to Paf	68.0 ± 13.3	243.2 ± 42.0*
WEB 2086 (15 mg kg^{-1})		277.6 ± 22.3

WEB 2086 (15 mg kg^{-1}) was administered intraperitoneally 1 h before zymosan and the leucocyte counts was performed 4 h later. Values in the table represent the mean ± s.e.mean from at least 6 animals and statistically significant differences are indicated by an asterisk.

To clarify further the nature of the Paf-acether receptor which is involved in the development of pleurisy in rats, we used the receptor antagonists WEB 2086 (Casals-Stenzel *et al.*, 1986; 1987) and 48740 RP (Sédivy *et al.*, 1985; Lefort *et al.*, 1988). Over the dose-range of 2 to 15 μg/cavity, WEB 2086 dose-dependently inhibited both the volume of exudate and the extravasated protein 30 min after Paf-acether, whereas 48740 RP was inactive. However, both antagonists, WEB 2086 and 48740 RP, suppressed the reduction by Paf-acether of the number of free pleural leucocytes, strongly suggesting that exudate volume, extravasated protein and the drop in the leucocyte count are receptor-mediated phenomena. The close-relationship among the bell-shaped dose-response curves for these 3 events suggests that they may be linked via a single mechanism. However, since the pretreatment with 48740 RP abrogated the reduction in the leucocyte count without interfering with the volume of exudation and its content of proteins, these mechanisms are in fact distinct. It is noteworthy that compound 48740 RP is also inactive against the Paf-acether-induced rat paw oedema, under conditions where it blocks leucocytosis, thrombocytopenia and haemoconcentration induced by the lipid (Martins *et al.*, 1987). These observations are consistent with the interpretation that there are, at least two classes of receptors for Paf-acether which can be clearly distinguished by compound 48740 RP.

Since Paf-acether induces the release of arachidonate derivatives in several experimental models

(Voelkel *et al.*, 1982; Lefort *et al.*, 1984; Jancar *et al.*, 1987), the possibility must be considered that, at least part of the Paf-acether-induced exudative reaction is secondary to eicosanoid release. Previous studies by our group (Cordeiro *et al.*, 1986), using cyclo-oxygenase and lipoxygenase inhibitors, suggested the involvement of leukotrienes, but not of prostaglandins, in rat paw oedema induced by Paf-acether. These results are consistent with the data of Tarayre *et al.* (1986) who detected significant increase in the amount of leukotriene C_4, but not of prostaglandin-like materials, in the rat pleural exudate after Paf-acether. The hypothesis that the leukotrienes participate in the Paf-induced inflammatory reaction is now reinforced by the observation that the compound LY 171883 a peptido-leukotriene antagonist (Fleisch *et al.*, 1985) significantly suppressed Paf-induced rat pleurisy. The pleural exudatation was also significantly inhibited by the suppression of both cyclo- and lipoxygenase products with dexamethasone and BW 755C, whereas cyclo-oxygenase inhibitors, indomethacin, piroxicam and flurbiprofen only slightly inhibited the phenomenon. Together these data strongly suggest that Paf-induced rat pleurisy is largely dependent upon leukotrienes and to a lesser extent upon cyclo-oxygenase metabolites of arachidonate.

The desensitization to Paf-acether after prior exposure to this lipid is observed in several experimental models, such as platelet aggregation (Demopoulos *et al.*, 1979; Henson, 1981; Lalau-Keraly & Benveniste, 1982), smooth muscle contraction (Findlay *et al.*, 1981; Tokomura *et al.*, 1983; Detsouli *et al.*, 1985), macrophage secretion (Maridonneau-Parini *et al.*, 1985), neutrophil reactivity (Smith *et al.*, 1984; Bureau *et al.*, 1987) and lung mechanical changes (Halonen *et al.*, 1980; Lefort *et al.*, 1984). Topical and selective auto-desensitization to Paf-acether-induced rat paw oedema has been proposed previously as a tool to investigate its involvement in inflammation reactions (Cordeiro *et al.*, 1986). In this study we demostrated that daily repeated intrapleural injections of Paf-acether lead to a progressive state of auto-desensitization, under conditions where the responsiveness to 5-hydroxytryptamine was maintained. In addition, it was noted that the antagonist WEB 2086 suppressed Paf-induced auto-desensitization, clearly indicating that this phenomenon is selective, and depends upon the interaction of Paf-acether with specific receptors.

The auto-desensitization process and the receptor antagonist WEB 2086 were used to investigate the potential involvement of Paf-acether in the pleural inflammation triggered by carrageenin and zymosan in rats. Our findings showed that the response to carrageenin, unlike zymosan, was not modified after the treatment with WEB 2086 or after desensitization to Paf-acether. These results agree with the demonstration by Cordeiro *et al.* (1986) that neither desensitization to Paf-acether nor the Paf antagonist BN 52021 block carrageenin-induced oedema, which is thus independent of Paf-acether. In contrast, the pleural exudation and cell migration stimulated by zymosan were significantly inhibited by either WEB 2086 pretreatment or Paf-acether-induced desensitization, strongly suggesting that zymosan may trigger its inflammatory effects via generation of Paf-acether. It is of interest to point out that a marked generation of Paf-acether from rat peritoneal macrophages stimulated with zymosan has been demonstrated (Mencia-Huerta & Benveniste, 1981), thus indicating that indeed zymosan may trigger the *in vivo* release of Paf-acether.

In conclusion, our findings indicate that Paf-acether-induced rat pleurisy may be dependent on leukotrienes and to a lesser extent on cyclo-oxygenase metabolites. In addition, the results obtained with the Paf-acether desensitized animals, and also with WEB 2086, support the hypothesis that the inflammatory reaction triggered by zymosan but not that one induced by carrageenin, may be largely dependent of Paf-acether.

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Effect of selective agonists and antagonists on atrial adenosine receptors and their interaction with Bay K 8644 and [^{3}H]-nitrendipine

*Pier Andrea Borea, Laura Caparrotta, Mariella De Biasi, Giuliana Fassina, Guglielmina Froldi, Luisa Pandolfo & [1]Eugenio Ragazzi

Department of Pharmacology, Padua University, Largo E. Meneghetti 2, I-35131 Padova and *Institute of Pharmacology, University of Ferrara, Via Fossato di Mortara 61/B, I-44100 Ferrara, Italy

1 (−)-N^6-phenylisopropyladenosine (R-PIA) and N^6-cyclohexyladenosine (CHA), highly selective agonists at A_1-adenosine receptors, 5'-N-ethyl-carboxamidoadenosine (NECA), a non-selective agonist at A_1 and A_2 receptors, and 2-phenylaminoadenosine (CV-1808), a selective A_2 agonist, were compared in spontaneously beating and electrically driven atria. R-PIA, CHA and NECA inhibited contraction in both preparations. CV-1808 was not effective up to 500 nM.

2 1,3-Dipropyl-8-cyclopentylxanthine (DPCPX), a new selective A_1 receptor antagonist, competitively inhibited the effects of the adenosine agonists, at low concentrations ($IC_{50} < 1$ nM).

3 CHA and NECA were able to inhibit the positive inotropic effect of Bay K 8644 both in spontaneously beating and in electrically driven atria.

4 R-PIA, CHA and NECA (agonists), 8-phenyltheophylline (PT) and DPCPX (antagonists), failed to influence [^{3}H]-nitrendipine binding on microsomal membranes from guinea-pig atria and ventricles in a range of concentrations from 1 nM to 100 μM.

5 The data support the existence of A_1 receptors in atrial tissue. No evidence for a direct interaction between adenosine analogues and Bay K 8644 was found at the level of slow calcium channels. Adenosine analogues appear to antagonize the effects of Bay K 8644 indirectly by activation of A_1 receptors.

Introduction

Adenosine decreases the beating frequency and the force of contraction in isolated atrial preparations of different species (for reviews see Schütz & Freissmuth, 1985; Brückner *et al.*, 1985; Scholz *et al.*, 1987). The mechanism of these actions is still elusive and debatable. Most of the biological effects of adenosine appear to be mediated by membrane adenosine receptors that are coupled to adenylate cyclase via a G regulatory protein. Two receptors subtypes have been recognized: the A_1 subtype, which inhibits adenylate cyclase, and the A_2 subtype, which activates the enzyme (Van Calker *et al.*, 1979; Londos *et al.*, 1980; Stone, 1984). But this model does not hold regarding the effects of adenosine at the atrial level. It has been suggested that in mammalian atrial tissue a variant adenosine cell surface receptor exists, not detectably coupled to adenylate cyclase (Brückner *et al.*, 1985) but coupled instead to K^+ channels via a G-protein (Böhm *et al.*, 1986). An alternative hypothesis is that an adenosine A_3 receptor is present in cardiac tissue (Ribeiro & Sebastião, 1986). The activation of this A_3 receptor is postulated to induce electrophysiological effects directly by affecting transmembrane ion currents, mainly of calcium.

Recently discovered selective agonists and antagonists for adenosine receptors (see Bruns *et al.*, 1987b) have been used in the present paper to investigate the extracellular purinoceptors involved in atrial function. The recently reported, highly selective, A_1-receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), (Jacobson *et al.*, 1985; Haleen *et al.*, 1987; Bruns *et al.*, 1987a; Williams, 1987) and the A_2-selective agonist 2-phenylaminoadenosine (CV-1808) (Bruns *et al.*, 1986; 1987a,b) have been used. N^6-cyclohexyladenosine (CHA) and (−)-N^6-phenylisopropyladenosine (R-PIA), both A_1 selective agonists, and 5'-N-ethyl-carboxamidoadenosine (NECA), a non selective A_1 and A_2 agonist, were also studied for comparison. The

[1] Author for correspondence.

effects of these compounds were evaluated in spontaneously beating atria and in electrically driven isolated atria of the guinea-pig.

The mechanism of action of adenosine analogues at the atrial level was also investigated. We have recently shown that R-PIA antagonizes the positive inotropic effect of Bay K 8644 (Caparrotta *et al.*, 1985; 1987), a dihydropyridine slow calcium channel activator (Schramm *et al.*, 1983). The interaction of CHA and NECA with Bay K 8644 was studied and compared to that of R-PIA. In order to investigate if the antagonism by adenosine analogues to Bay K 8644 was dependent on A_1 receptors, or, instead, on a possible interaction with slow calcium channels, two approaches were used: (i) the interaction between CHA, R-PIA, NECA and Bay K 8644 was studied in the presence of the A_1-selective antagonist DPCPX; (ii) radioligand binding assays were carried out by use of [^{3}H]-nitrendipine to label the dihydropyridine binding site on microsomal fractions from guinea-pig atria and ventricles.

Methods

Tissue preparations for recording mechanical response

The hearts were removed from guinea-pigs of either sex (300–500 g) and placed in a physiological solution (29°C) of the following composition (mM): NaCl 120, KCl 2.7, $CaCl_2$ 1.36, $MgCl_2$ 0.09, $NaHCO_3$ 11.9, NaH_2PO_4 0.42, glucose 5.5 and gassed with 95% O_2 plus 5% CO_2. The atria were dissected, suspended in a 30 ml organ bath and connected to a transducer (Basile type DY0). An initial tension of 1 g was applied to the tissues and changes in isometric tension were recorded using an oscillograph (Basile, Unirecord System, Mod. 7050).

Left atria were mounted on punctate electrodes with a load of 0.5 g and stimulated by square wave electrical pulses (0.5–2 Hz, 3 ms, 0.5–1.2 V) provided by a Grass stimulator (Mod. S4 KR). The voltage was about 20% greater than threshold. The control developed tension ranged from 0.8 to 1.3 mN.

An equilibration period of 60 min was allowed before experiments were started. Concentration-response curves were constructed by a cumulative addition of agonist after the force had reached a new equilibrium. The effect of Bay K 8644 was slow in onset (20–30 min) and required a prolonged period of washing for reversal (at least 2 h). The effect of the stable analogues of adenosine reached equilibrium after 6–8 min. Reversibility of the effect of a single concentration of adenosine analogues was investigated by a 15 min washing with drug-free bathing solution at the end of experiments. The cumulative concentration-response curves to adenosine analogues were performed before and after treatment with Bay K 8644 and DPCPX. The concentration-response curves for the negative inotropic and chronotropic effects of adenosine analogues, were expressed as percentage decrease from the basal level. Cumulative concentration-response curves for the positive inotropic effects of Bay K 8644 were expressed as percentage of the maximum increase over control tension induced by Bay K 8644. The response to Bay K 8644 in the presence of adenosine antagonists was related to the maximum effect of Bay K 8644 (100%) in the absence of the inhibitor.

[^{3}H]-nitrendipine binding

Atria and ventricles were dissected, minced, and homogenized in 50 vol of 50 mM Tris HCl buffer, pH 7.4, with a Brinkman Polytron, PTA-10 probe at setting 6 for 30 s. After discarding a 3,500 g × 15 min pellet, a 48,000 g × 45 min pellet was prepared and resuspended in the Tris buffer to 0.5 mg protein ml^{-1}. The membranes were frozen in aliquots until use (−25°C) at which time they were washed once more. Two ml of the suspension was incubated with [^{3}H]-nitrendipine (78 Ci mmol^{-1}) 0.1 nM (a concentration close to its equilibrium K_d) in triplicate, and with increasing concentrations of R-PIA, CHA, NECA, 8-phenyltheophylline (PT), DPCPX. R-PIA, CHA and NECA were also tested in the presence of 10 μM PT. After 60 min at 23°C, the assays were terminated by filtration through Whatman GF/C glass filters under suction. The filters were washed four times with ice-cold buffer, dried and counted in 5 ml of acidified Instagel (Packard) by liquid scintillation counting. Non specific binding was determined by incorporation of 1 μM nifedipine in the assays and routinely represented about 30% of total binding. Our binding assay was similar to that employed by other groups (Bolger *et al.*, 1982). Different incubation media were also used, i.e., Tris HCl buffer pH 7.4 with the addition of 1.5 mM $MgCl_2$ or 10 mM $CaCl_2$.

Bay K 8644, in our experimental conditions, displaced [^{3}H]-nitrendipine with an IC_{50} of the order of 10 nM in accordance with binding data obtained by Janis *et al.* (1984a,b) in rabbit ventricular microsomes. Competition between nitrendipine and [^{3}H]-Bay K 8644 indicated a common high affinity binding site for Ca^{2+} channel activators and antagonists (Janis *et al.*, 1984a,b).

Drugs and compounds

The following were used: N^6-cyclohexyladenosine (CHA) (a kind gift of Prof. Luiz Belardinelli, Dept of Physiology, University of Virginia, U.S.A.); 1,3-

Table 1 Effects of stable analogues of adenosine on contractile tension and frequency in spontaneously beating and electrically driven guinea-pig isolated atria

Agonist	*Spontaneous*		*Electrically driven*
	Contractile tension $-\log EC_{50}$	*Frequency* $-\log EC_{50}$	*Contractile tension* $-\log EC_{50}$
R-PIA (A_1-agonist)	7.82 ± 0.07 ($EC_{50} = 15$ nM)	7.68 ± 0.06 ($EC_{50} = 20$ nM)	7.66 ± 0.06 ($EC_{50} = 21$ nM)
CHA (A_1-agonist)	7.64 ± 0.06 ($EC_{50} = 23$ nM)	6.93 ± 0.11 ($EC_{50} = 118$ nM)	7.12 ± 0.06 ($EC_{50} = 75$ nM)
NECA (A_1 and A_2 agonist)	7.63 ± 0.3 ($EC_{50} = 24$ nM)	6.94 ± 0.10 ($EC_{50} = 114$ nM)	7.46 ± 0.08 ($EC_{50} = 34$ nM)
CV-1808 (A_2-agonist)	ND	ND	ND

ND = not detectable.
Data are the mean ± s.e.mean of 5–10 preparations.
R-PIA = (−)-N^6-phenylisopropyladenosine; CHA = N^6-cyclohexyladenosine; NECA = 5′-ethyl-carboxamido-adenosine; CV-1808 = 2-phenylaminoadenosine.

dipropyl-8-cyclopentylxanthine (DPCPX) (Research Biochemical Inc.); 5′-N-ethyl-carboxamidoadenosine (NECA) (Sigma). Methyl-1,4,-dihydro-2,3-dimethyl-3-nitro-4-(2-trifluoro-methylphenyl)-pyridine-5-carb - oxylate (Bay K 8644), was generously supplied by Dr G. Franckowiak from Bayer AG (Wuppertal, F.R.G.). Bay K 8644, 1 mM, was freshly dissolved in absolute ethanol. This stock solution was diluted in appropriate amounts in bathing solution to achieve the desired final concentration. All experiments were carried out in a dark room using red light as the drug was sensitive to light. Nifedipine (4-(2′-nitrophenyl)-2,6-dimethyl-3,5-dicarbomethoxy-1,4 - dihy - dropyridine) (Pfizer). [^{3}H]-nitrendipine (2,6-dimethyl-3,5-dicarbomethoxy-4-(3-nitro)phenyl-1,4-dihydropyridine) (New England Nuclear, Boston, Ma) purity >97%, was stored protected from light at −20°C. 2-Phenylaminoadenosine (CV-1808) (Research Biochemical Inc.). (−)-N^6-phenylisopropyladenosine (R-PIA) (Boehringer, Mannheim, F.R.G.), was dissolved and diluted 50% ethanol-50% bathing solution. 8-Phenyltheophylline (PT) (Sigma) was dissolved in dimethylsulphoxide (DMSO).

All chemicals were of analytical or best commercial grade available. Deionized and twice-distilled water was used throughout.

Mathematical and statistical analysis of results

Values presented are the mean ± s.e.mean. The analysis of concentration-response curves was carried out from data in the region between 20% and 80% of maximal response. A linear least squares regression was constructed from individual values of effect versus log concentration.

The −log of concentration that produced half-maximal effect ($-\log EC_{50}$) and its standard error were determined by interpolation according to Tallarida & Murray (1987).

The log (dose ratio − 1) was plotted against −log antagonist concentration and a least squares regression analysis was used to determine pA_2 (Arunlakshana & Schild, 1959). Alternatively, K_i was determined according to the formula K_i = [inhibitor]/(dose ratio − 1) (Furchgott, 1967).

Results were tested for significance by Student's t test for unpaired data. A P value less than 0.05 was considered as significant.

Results

Effect of adenosine analogues on the contractile tension and frequency

CHA, NECA and R-PIA produced a concentration-dependent decrease of contractile tension and frequency in spontaneously beating atria at concentrations 3–300 nM (Table 1). CV-1808 did not show significant effects on isolated atria up to 500 nM. R-PIA, CHA and NECA were also effective in decreasing contractile tension in electrically driven atria (Table 1). The order of potency was R-PIA >= NECA > CHA.

Effect of a highly selective A_1 receptor antagonist, DPCPX, on the actions of R-PIA, CHA and NECA

In spontaneously beating atria, 1 to 50 nM DPCPX inhibited the negative effects of R-PIA on contractile

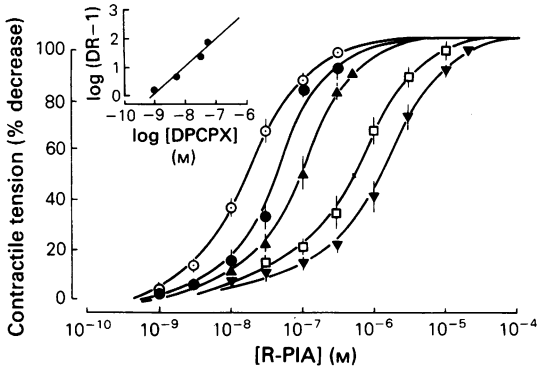

Figure 1 Inhibition by 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) of the negative inotropic effect of N^6-phenylisopropyladenosine (R-PIA) in spontaneously beating atria. Cumulative concentration-response curves for R-PIA alone (○) or in the presence of DPCPX 1 nM (●), 5 nM (▲), 30 nM (□) and 50 nM (▼). Each point is the mean of 4–6 experiments. Vertical lines indicate s.e.mean. Inset: Schild plot.

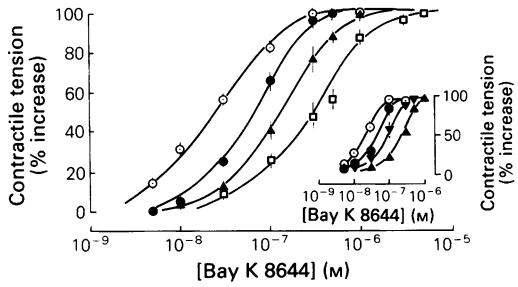

Figure 2 Inhibition by N^6-cyclohexyladenosine (CHA) of the positive inotropic effect of Bay K 8644 in electrically driven atria (larger figure) and in spontaneously beating atria (inset). Cumulative concentration-response curves for Bay K 8644 in the absence (○) and in presence of CHA 50 nM (●), 80 nM (▼), 100 nM (▲) and 500 nM (□). Each point is the mean of 6–10 experiments. Vertical lines indicate s.e.mean.

tension in a concentration-dependent manner and shifted the concentration-response curve of R-PIA to the right, indicating a competitive antagonism (Figure 1). The Schild plot (Figure 1, inset) was linear with a slope 0.96 ± 0.12 ($r = 0.98$). The pA_2 value of the antagonism of R-PIA by DPCPX was 9.18 ± 0.16 corresponding to an apparent K_i of 0.66 nM.

In the electrically driven left atria, DPCPX inhibited the negative inotropic effect of R-PIA and shifted the concentration-response curve of R-PIA to the right. In these experimental conditions, the apparent K_i of DPCPX was 0.67 nM (Table 2) as in spontaneously beating atria. DPCPX antagonized the negative effect on contraction by CHA and NECA in spontaneously beating and in paced atria with a similar potency (Table 2).

Table 2 Effects of (−)-N^6-phenylisopropyladenosine (R-PIA), N^6-cyclohexyladenosine (CHA) and 5′-N-ethyl-carboxamidoadenosine (NECA) on contractile tension in electrically driven atrial preparations alone or with 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) (5 nM)

Compound	[1]*Contractile tension* −log EC_{50} (M)	[2]*Apparent* K_i (nM)
R-PIA	7.66 ± 0.06	
R-PIA + DPCPX	6.75 ± 0.10*	0.67
CHA	7.12 ± 0.06	
CHA + DPCPX	6.04 ± 0.14*	0.45
NECA	7.46 ± 0.08	
NECA + DPCPX	6.46 ± 0.05*	0.54

[1] Data are the mean ± s.e.mean of 4–6 experiments.
[2] Apparent K_i was determined according to the formula: K_i = [Inhibitor]/(dose ratio − 1) (Furchgott, 1967).
* $P < 0.001$.

Effect of CHA and NECA on the positive inotropic effect of Bay K 8644

Bay K 8644 induced a concentration-dependent positive inotropic effect on spontaneously beating atria (−log EC_{50} = 7.68 ± 0.02 M) (Figure 2, inset). The positive chronotropic effect of Bay K 8644 became evident only at concentrations inducing the maximum increase in contractility, being very small (about 15% above control).

Stimulation frequency was found to modify the effect of Bay K 8644 on contraction. In electrically driven left atria, the positive inotropic effect of Bay K 8644 increased proportionally with the rate of stimulation from 0.5 to 1.5–2 Hz; at higher rates, over 2 Hz, the positive inotropic effect of Bay K 8644 decreased. Thus we used 1.5 Hz as a fixed rate of stimulation, when the effect of Bay K 8644 was at its maximum. In such experimental conditions, the −log EC_{50} was 7.61 ± 0.03 M.

CHA inhibited the positive inotropic effect of Bay K 8644 in a concentration-dependent manner, both in spontaneously beating and in paced atria (Figure 2). CHA shifted the concentration-response curve of Bay K 8644 to the right.

In both conditions the Schild plot was linear but the slope was different: 3.21 ± 0.75 in spontaneously

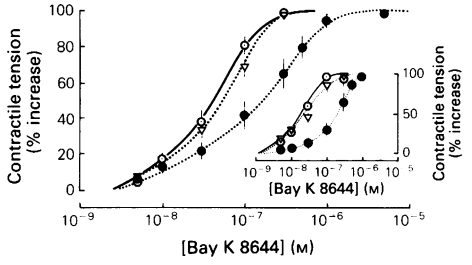

Figure 3 Inhibition by 5'-N-ethylcarboxamidoadenosine (NECA) on the positive inotropic effect of Bay K 8644 in electrically driven atria (larger figure) and in spontaneously beating atria (inset). Cumulative concentration-response curves for Bay K 8644 in the absence (○) and in presence of NECA 50 nM (▽) and 100 nM (●). Each point is the mean of 4–6 experiments. Vertical lines indicate s.e.mean.

beating atria and 0.79 ± 0.20 in paced atria. The pA_2 was 7.29 ± 0.02 in spontaneously beating and 7.72 ± 0.24 in paced atria. The antagonism by CHA of Bay K 8644 is qualitatively similar to that obtained previously with R-PIA (Caparrotta *et al.*, 1985; 1987). NECA also inhibited responses to Bay K 8644 (Figure 3) but was less potent than CHA.

The effects of Bay K 8644 were not antagonized in electrically driven atria by carbachol at concentrations of 50 nM and 100 nM, which reduced the atrial contractility by 40% and 70% respectively.

Effect of adenosine agonists and antagonists on [^3H]-nitrendipine binding in microsomal membrane fractions

In order to investigate the possibility of a direct interaction between adenosine analogues and Bay K 8644 at the level of dihydropyridine sites, studies were carried out with [^{3}H]-nitrendipine, a ligand for dihydropyridine binding sites in slow calcium channels of cardiac membranes (Janis *et al.*, 1984; Sarmiento *et al.*, 1987).

R-PIA, CHA and NECA (agonists), PT and DPCPX (antagonists), failed to influence [^{3}H]-nitrendipine binding up to a 100 μM concentration, both in atrial and ventricular membrane fractions. Similar negative results were also found when using Tris HCl buffer to which 1.5 mM $MgCl_2$ or 10 mM $CaCl_2$ was added, in order to affect the affinity status of extracellular adenosine receptors or dihydropyridine receptor in slow calcium channels (Yeung *et al.*, 1985; Ptasienski *et al.*, 1985).

Effect of DPCPX on the inhibition by adenosine agonists to Bay K 8644

The interaction of R-PIA and CHA with Bay K 8644 was studied in the presence of the antagonist

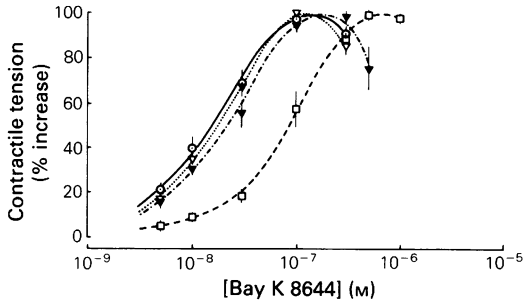

Figure 4 Effects of 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) on the antagonism between N^6-cyclohexyladenosine (CHA) and Bay K 8644 in spontaneously beating atria. Cumulative concentration-response curves for Bay K 8644 alone (○——○) in the presence of DPCPX 5 nM (▼–·–·–▼), CHA 80 nM (□– – –□), and both DPCPX 5 nM plus CHA 80 nM (▽···▽). Each point is the mean of 4–6 experiments. Vertical lines indicate s.e.mean.

DPCPX. The isolated atria, after stabilization, were incubated for 10 min in the presence of DPCPX 5 nM. This concentration of DPCPX did not modify the Bay K 8644 positive inotropism (Figure 4). The inhibition exerted by R-PIA 50 nM (IC_{50}) and by CHA 80 nM (IC_{50}) of the positive inotropic effect of Bay K 8644 was abolished in the presence of DPCPX (Figure 4).

Discussion

A major finding of this study was that data obtained by the use of stable adenosine agonists and antagonists support the existence of A_1 adenosine receptors in atrial tissue and their involvement in the negative effects of adenosine at atrial level. This is in accordance with previous studies (Evans *et al.*, 1982; Collis, 1983; Brückner *et al.*, 1985) on guinea-pig atria and in rat atria (Paton, 1983).

The data show that CHA and R-PIA, highly selective A_1 receptor agonists, and NECA, a non selective A_1 and A_2 agonist, are all able to decrease contractile tension and frequency in a concentration-dependent manner (3–300 nM). In contrast, CV-1808, an A_2-selective agonist, does not show significant effects on isolated atria up to 500 nM. The highly selective A_1 receptor antagonist DPCPX, competitively antagonized the negative inotropic and chronotropic effects of R-PIA, CHA and NECA at low concentrations. The apparent K_i values for contraction were 0.67, 0.45 and 0.54 nM, respectively, in accordance with the K_i (0.46 nM) found in central nervous system binding studies (Bruns *et al.*, 1987b).

It has been proposed that additional or variant

adenosine cell surface receptors exist in atrial tissue which are not coupled to adenylate cyclase, but are coupled instead via G-proteins to other effector systems such as K^+ channels (Böhm *et al.*, 1986) or Ca^{2+} currents (Ribeiro & Sebastião, 1986). A possible role of adenosine in modulating slow calcium channels was further suggested by results obtained in guinea-pig atria (Caparrotta *et al.*, 1987) showing that R-PIA inhibits the positive inotropic effect of Bay K 8644, a dihydropyridine slow calcium channel activator (Schramm *et al.*, 1983). This observation, which indicates a role for adenosine in the regulation of Ca^{2+} conductance and hence force of contraction of the atria, remained to be elucidated. Therefore, we investigated if CHA and NECA could also antagonize Bay K 8644. CHA and NECA were found to inhibit the Bay K 8644 positive inotropism both in spontaneously beating and in electrically driven atria. CHA was more potent than NECA at lower concentrations (50 nM) but equally potent with NECA at higher concentrations (100 nM). CHA produced, like R-PIA (Caparrotta *et al.*, 1987) a parallel rightward shift in the Bay K 8644 concentration-response curves; but in spontaneously beating atria the slope of the Schild regression was very high, indicating a temporal or thermodynamic disequilibrium (Kenakin, 1987) or more simply the influence of the reduced rate of contraction.

A possible competition between adenosine analogues and Bay K 8644 for a common receptor site in or near the slow calcium channel was investigated in radioligand binding studies. The failure of R-PIA, CHA and NECA (agonists), and of PT and DPCPX (antagonists) to influence [^{3}H]-nitrendipine binding in microsomal membrane fractions, provided no evidence for a competitive antagonism between adenosine analogues and the dihydropyridine calcium channel ligand in guinea-pig atria and ventricles. A possible interaction between adenosine analogues and Bay K 8644 at the level of a common receptor site is thus unlikely.

The antagonism by R-PIA and CHA of the effects of Bay K 8644 was eliminated by DPCPX. This indicates that the interaction between adenosine analogues and the slow calcium channel activator is dependent on the A_1 receptor.

An obvious question is how the signal sent by adenosine analogues through the A_1 receptor is transmitted to the final effector, the site of interaction with Bay K 8644. A likely hypothesis is that adenosine analogues send an inhibitory signal from the A_1 receptors by the involvement of a G-protein, not coupled to adenylate cyclase, directly to the site of action of Bay K 8644. This is still to be investigated, but it would be in agreement with results showing that a nucleotide binding protein is involved in the regulation of the receptor-mediated change in K^+ conductance and force of contraction by adenosine and R-PIA (Böhm *et al.*, 1986; Kurachi *et al.*, 1986). However, it should be pointed out that in atrial cells both adenosine (and R-PIA) and acetylcholine (and carbachol) share the same action and mechanism in increasing outflow K^+ currents (for reviews see, Nawrath *et al.*, 1985; Sperelakis, 1987; Isenberg *et al.*, 1987; West *et al.*, 1987). But the interaction of adenosine analogues with Bay K 8644 is apparently distinct from the above mentioned effect, as carbachol does not modify the positive inotropic effect of Bay K 8644 in spontaneously beating (Caparrotta *et al.*, 1987) and in paced atria.

In summary, the present data indicate that A_1 receptors are present in atrial tissue and mediate a negative inotropic response. The antagonism by R-PIA and CHA of the effects of Bay K 8644 is also mediated by A_1 receptors. It remains to be elucidated if the signal sent from A_1 receptors to the site of action of Bay K 8644 is mediated by the activation of a membrane bound G-protein.

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Characterization of 5-HT receptors mediating contraction of canine and primate basilar artery by use of GR43175, a selective 5-HT_1-like receptor agonist

[1]H.E. Connor, W. Feniuk & P.P.A. Humphrey

Department of Cardiovascular Pharmacology, Glaxo Group Research Ltd, Ware, Hertfordshire, SG12 0DP

1 The aim of this study was to characterize the 5-hydroxytryptamine (5-HT) receptor which mediates contraction of canine and primate isolated basilar artery by use of a variety of selective 5-HT agonists and antagonists.

2 5-HT, α-methyl 5-HT and the selective 5-HT_1-like receptor agonists, GR43175 and 5-carboxamidotryptamine (5-CT), each caused contraction of canine and primate basilar artery with a rank order of agonist potency of 5-CT ≥ 5-HT > GR43175 > α-methyl 5-HT. The 5-HT_1-like receptor agonists, GR43175 and 5-CT, produced maximum effects which were less than that produced by 5-HT or α-methyl 5-HT.

3 In canine basilar artery, ketanserin (0.1-1 μM) caused some depression of the maximum effect of 5-HT but produced little or no shift of the concentration-effect curve. The contractile effects of GR43175 were not modified by ketanserin (1 μM), MDL72222 (1 μM) or cyanopindolol (1 μM). However, the effects of 5-HT and GR43175 were specifically antagonized by methiothepin (0.1 μM); the mean agonist concentration-ratios were 33 and 48 respectively.

4 In primate basilar artery, ketanserin (1 μM) again caused a small depression of the 5-HT maximum response but had no effect against GR43175-induced contractions. In contrast, methiothepin (0.1 μM) antagonized both 5-HT- and GR43175-induced contractions; the mean agonist concentration-ratios were 35 for both.

5 These results demonstrate that a large component of the effects of 5-HT in canine and primate basilar artery is produced by stimulation of a 5-HT_1-like receptor. This receptor can be characterized by the high potency of the novel, selective agonist, GR43175, and susceptibility to blockade by methiothepin. However, there also appears to be a population of 5-HT_2 receptors in these prepAarations which contribute to the contractile effects of 5-HT.

Introduction

In comparison to its effects on peripheral vascular smooth muscle, 5-hydroxytryptamine (5-HT) is a highly potent spasmogen of isolated cerebral arteries (e.g. see Peroutka *et al.*, 1983). However, recent attempts to characterize the 5-HT receptor type which mediates contraction of cerebral arteries have resulted in varied conclusions. Thus, these effects of 5-HT have been ascribed to stimulation of 5-HT_1-like (Peroutka *et al.*, 1983; Bradley *et al.*, 1986b), 5-HT_{1A} (Peroutka *et al.*, 1986; Taylor *et al.*, 1986), or 5-HT_2 (Muller-Schweinitzer & Engel, 1983; Chang & Owman, 1987) receptors. Furthermore, Cohen & Colbert (1986) reported that the 5-HT receptor in canine basilar artery was neither 5-HT_1-like nor 5-HT_2.

Although species variation may account for some of these different findings, a major problem has been the lack of selective agonists or antagonists for the various 5-HT receptor subtypes. Recently, a novel 5-HT receptor agonist, GR43175, has been identified in our laboratories. This agent is inactive at 5-HT_2 and 5-HT_3 receptors and, furthermore, has no activity at the 5-HT_1-like receptor which mediates relaxation of vascular smooth muscle (Humphrey *et al.*, 1987). However, GR43175 is a potent and highly selective agonist at the 5-HT_1-like receptor which causes contraction of the dog isolated saphenous vein (Humphrey *et al.*, 1988).

[1] Author for correspondence.

Therefore, in the present studies, we have examined the effects of GR43175 on isolated canine and primate basilar arteries and, using other selective 5-HT agonists and antagonists, we have attempted to characterise the 5-HT receptors which mediate contraction of these cerebral arteries. A preliminary account of these findings has been presented to the British Pharmacological Society (Connor *et al.*, 1987).

Methods

Preparation of artery segments

Beagle dogs (7–10 kg, either sex) were killed with pentobarbitone sodium (100 mg kg^{-1}, i.v.) and basilar arteries were removed and stored overnight in modified Krebs solution (Apperley *et al.*, 1976) at 4°C. Cynomolgus monkeys (3–6 kg, either sex) were anaesthetized with alphaxalone (0.9%)/alphadolone (0.3%) (Saffan, 5 ml i.m.) and then killed with pentobarbitone sodium (100 mg kg^{-1}, i.v.). Basilar arteries were removed and placed in modified Krebs solution. Arteries were either used the same day or stored overnight in modified Krebs solution at 4°C.

Ring segments (3–4 mm) of canine or primate cerebral artery were placed in separate 10 ml organ baths containing modified Krebs solution (Apperley *et al.*, 1976) at 37°C, bubbled with 95% O_2, 5% CO_2. The segments were carefully suspended between 2 L-shaped, stainless steel wire supports (0.2 mm diameter) inserted into the lumen to record changes in isometric tension. No attempt was made to remove the endothelium. Preparations were maintained at an initial resting tension of 200–300 mg for 1 h and then tension was increased to 1 g. Preparations were allowed to equilibrate for a further hour and then the response to a submaximal concentration of potassium chloride (30 mM) was determined.

Determination of agonist potency

5-HT was added to the fluid bathing the basilar artery preparations using a cumulative concentration schedule, allowing sufficient time for the effects of each concentration to become fully established before adding the next concentration, until a maximum response was obtained or relaxation started to occur. After frequent washing, tissues were left for at least 30 min before re-challenging with either 5-HT or test agonist. On each preparation, one or two concentration-effect curves to 5-HT were obtained; preliminary experiments showed that at least three consecutive concentration-effect curves to 5-HT were highly reproducible. One preparation remained as a control in which the concentration-effect curve to 5-HT was again repeated while concentration-effect curves to various other agonists were constructed on 3 other preparations from the same animal. The EC_{50} value (concentration producing 50% of the maximum response) was calculated on individual curves and the agonist relative potency was calculated: EC_{50} value (test agonist)/EC_{50} value (5-HT, previous curve). Corrections were made for any spontaneous changes in sensitivity to 5-HT as assessed in the control preparation.

Determination of antagonist potency

In canine basilar artery, concentration-effect curves for agonists were established as described. One preparation remained as a control and a single concentration of antagonist was added to each of the 3 other baths. An antagonist equilibration time of 30 min was allowed and then the agonist concentration-effect curve was re-determined. Antagonist potency was assessed by calculating the agonist concentration-ratio (CR) in the absence and presence of antagonist at the level of 50% original agonist maximum response, except in experiments where the antagonist caused a non-parallel shift in the agonist concentration-effect curve when no quantitative assessment of antagonist potency was made. Experiments in which the antagonist potency of methiothepin was to be studied were carried out in the continued presence of a high concentration of ketanserin (1 μM) to negate any effects at 5-HT_2 receptors.

In experiments on primate basilar artery, due to the limited number of monkeys available, the effects of ketanserin and methiothepin were examined in the same preparation. The second agonist concentration-effect curve was constructed in the presence of ketanserin and then, after washings, a third agonist concentration-effect curve was redetermined in the presence of the same concentration of ketanserin plus methiothepin. Time matched control preparations allowed any spontaneous change in tissue sensitivity to be taken into account. The antagonist potency of methiothepin was assessed by calculating the agonist CR in the presence of ketanserin, and in the presence of ketanserin plus methiothepin (2nd and 3rd curve), at the level of 50% agonist maximum response obtained in the presence of ketanserin alone (2nd curve).

Drugs

The following drugs were used in this study: (±)-cyanopindolol (Sandoz), 5-hydroxytryptamine creatinine sulphate (Sigma), ketanserin (Janssen), methiothepin maleate (Roche) and MDL72222

hydrochloride (1αH,3α,5αH-tropan-3yl-3,5-dichlorobenzoate) (Merrell). 5-Carboxamidotryptamine maleate (5-CT), (±)-α-methyl 5-hydroxytryptamine maleate (α-Me 5-HT), GR43175 (3-[2-dimethyl amino] ethyl-N-methyl-1H-indole-5-methane sulphonamide), 8-hydroxy-2-(di-n-propylamino)-tetralin hydrobromide (8-OHDPAT) and 11,9 epoxymethano-prostaglandin H_2 (U46619) were synthesized by members of the Chemical Research Department, Glaxo Group Research Ltd, Ware. Cyanopindolol was a gift from Dr G. Engel, Sandoz, Basle.

Ketanserin, cyanopindolol and U46619 were initially dissolved in 0.1 M tartaric acid, distilled water with 0.01% 2N hydrochloric acid and 1% sodium bicarbonate, respectively; dilutions were made in distilled water. All other drugs were dissolved and diluted in distilled water.

Results

Effects of agonists

5-Hydroxytryptamine was a highly potent agonist causing contraction of canine and primate basilar artery with EC_{50} values of 45 (31–65) nM, and 67 (37–123) nM, respectively. Results are expressed as means (95% confidence limits) from 17 and 12 experiments. The maximum increase in tension produced by 5-HT was 0.99 ± 0.11 g ($n = 17$) and 1.12 ± 0.12 g ($n = 12$) in canine and primate basilar artery respectively (mean ± s.e.mean). The effects of 5-HT were highly reproducible, as illustrated in Figure 1.

The selective agonists, GR43175, 5-CT and α-methyl 5-HT also caused contraction of canine and primate basilar artery; results are shown in Figure 1. The rank order of potency for producing contraction was similar in both preparations (5-CT ⩾ 5-HT > GR43175 > α-methyl 5-HT); the relative

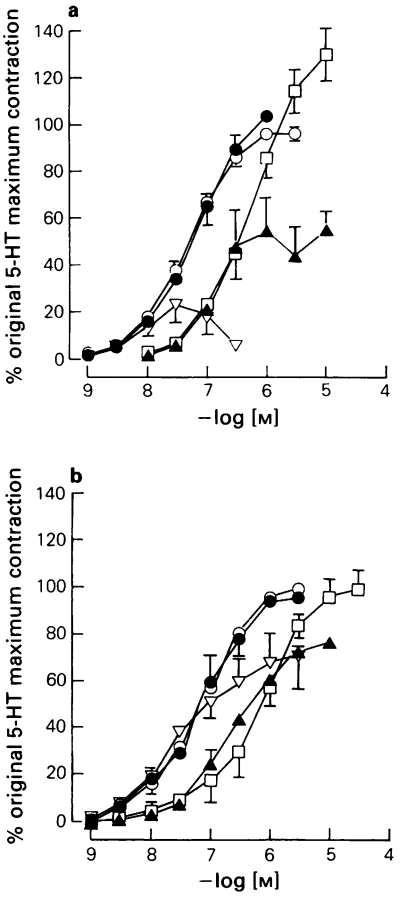

Figure 1 Concentration-effect curves to 5-HT, 1st curve (●), 2nd control curve (○), GR43175 (▲), 5-carboxamidotryptamine (▽) and α-methyl 5-HT (□) in canine (a) or primate (b) isolated basilar artery. Values are means with s.e.mean shown by vertical bars, $n = 3$–5 except for 1st 5-HT curve where $n = 17$ and 12 for canine and primate basilar artery, respectively.

Table 1 Relative potencies of 5-HT receptor agonists in isolated cerebral arteries

	Canine basilar artery		*Primate basilar artery*	
Agonist	Relative potency	% 5-HT max	Relative potency	% 5-HT max
5-HT	1	100	1	100
α-Me 5-HT	9.7 (6.4–17.8)	130.7 ± 10.9	6.1 (4.7–7.6)	99.3 ± 8.2
GR43175	1.7 (0.3–9.4)	56.3 ± 14.4	3.6 (2.6–5.1)	78.3 ± 1.9
5-CT	0.11 (0.06–0.31)	22.7 ± 7.2	0.95 (0.3–1.8)	74.3 ± 14.8

Relative potencies of 5-HT agonists (5-HT = 1) for contracting canine ($n = 3$–5) or primate ($n = 3$) basilar artery and relative maximum response produced (5-HT = 100); results are expressed as geometric mean (range) or arithmetic mean ± s.e.mean.

potency values for each agonist, compared to 5-HT, are shown in Table 1. However, these agonists differed in the magnitude of contraction produced. In canine basilar artery, α-methyl 5-HT produced a maximum effect greater than that of 5-HT (131% 5-HT max), GR43175 produced a smaller maximum (56% 5-HT max) and 5-CT caused a peak contraction equivalent to only 23% of the 5-HT maximum (Table 1). In primate basilar artery, the magnitude of contraction produced by α-methyl 5-HT was similar to 5-HT (99% 5-HT max), whilst GR43175 and 5-CT produced 78% and 74% of the 5-HT maximum, respectively (Figure 1b, Table 1).

In canine basilar artery, the effects of 8-OHDPAT were also examined. 8-OHDPAT, in concentrations up to 300 nM had little or no effect on resting tone. Higher concentrations (1–10 μM) caused contraction; the agonist potency, relative to 5-HT, was 38 (22–79), ($n = 3$, geometric mean and range). Although a maximum effect of 8-OHDPAT was not obtained, the highest concentration used (10 μM 8-OHDPAT) produced a contraction equivalent to 68% of the 5-HT maximum.

Effects of antagonists in canine basilar artery

The effects of some selective 5-HT antagonists were examined against contractions produced by GR43175 in canine basilar artery; neither ketanserin (1 μM) nor MDL72222 (1 μM) appeared to modify the effects of GR43175 (Figure 2). Cyanopindolol (1 μM) also produced little or no antagonism, although interestingly, this concentration of cyanopindolol produced a small contraction (Figure 2). In contrast to its lack of effect against GR43175, ketanserin attenuated 5-HT-induced contractions of canine basilar artery. This was not a competitive interaction, since ketanserin (0.1 μM) caused a depression of the 5-HT maximum response to about 50–60% of the original maximum with little or no rightward shift in the concentration-effect curve. A higher concentration of ketanserin (1.0 μM) caused no further antagonism (Figure 3).

The contractile effects of 5-HT and GR43175 in canine basilar artery were antagonized by methiothepin (0.1 μM). This antagonist caused an approximately parallel rightward displacement of the concentration-effect curve for both agonists (Figure 4); agonist concentration-ratios were 33 (20–49, $n = 3$) and 48 (25–92, $n = 4$) for 5-HT and GR43175, respectively (geometric mean and range). The antagonist effect of methiothepin was specific since the same methiothepin concentration (0.1 μM) did not antagonize contractions produced by the thromboxane A_2-mimetic, U46619 (CR 1.5 [1.1–2.0]; geometric mean and range, $n = 3$).

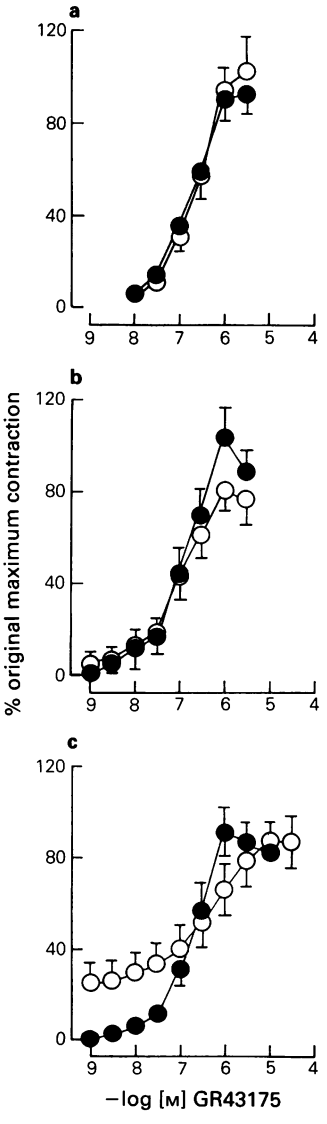

Figure 2 Concentration-effect curves to GR43175 in the canine isolated basilar artery in the absence (●) or presence (○ of (a) ketanserin (1 μM), (b) MDL72222 (1 μM) or (c) cyanopindolol (1 μM). Concentration-effect curves are for time-matched, paired preparations. Values are means with s.e.mean shown by vertical bars, $n = 3$–5. Note the contraction produced by cyanopindolol itself on which the 5-HT concentration-effect curve is superimposed.

Effects of antagonists in primate basilar artery

Three consecutive control concentration-effect curves for contraction to 5-HT and GR43175 were highly reproducible (Figure 5a). Ketanserin (1 μM)

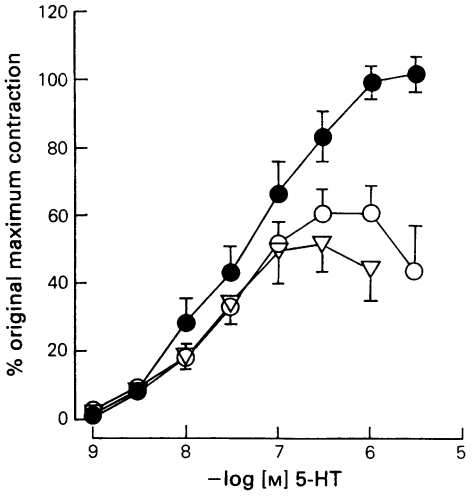

Figure 3 Concentration-effect curve to 5-HT in the canine isolated basilar artery in the absence (●) and presence of ketanserin 0.1 μM (▽) and 1.0 μM (○). Concentration-effect curves are for time-matched preparations. Values are means with s.e.mean shown by vertical bars from 4 experiments.

caused a depression of the 5-HT maximum contractile response, but did not change the threshold concentration for 5-HT-induced contractions of primate basilar artery. In contrast, the same concentration of ketanserin (1 μM) did not modify the contractions produced by GR43175 (Figure 5b). However, the further addition of methiothepin (0.1 μM) caused a rightward shift in the agonist concentration-effect curve for both 5-HT and GR43175 (Figure 5b); the calculated concentration-ratios were 35 (24–51) and 35 (8–69) for 5-HT and GR43175 respectively (geometric mean and range, $n = 4$).

Antagonist effects of 5-carboxamidotryptamine

In view of the small magnitude of contraction produced by 5-CT in canine basilar artery, the possibility that 5-CT could act as a partial agonist in this preparation was studied. 5-CT (10 nM–1 μM; in the presence of ketanserin 1 μM) caused a concentration-related attenuation of contractions to 5-HT (results not shown). This was not a competitive interaction since there was marked suppression of the maximum response to 5-HT. However, the highest concentration of 5-CT (1 μM) did not modify contractions to the thromboxane A_2-mimetic U46619, CR 0.64 (0.47–0.88), (geometric mean and range, $n = 3$).

Discussion

The aim of this study was to characterize the 5-HT receptor mediating contraction of canine and

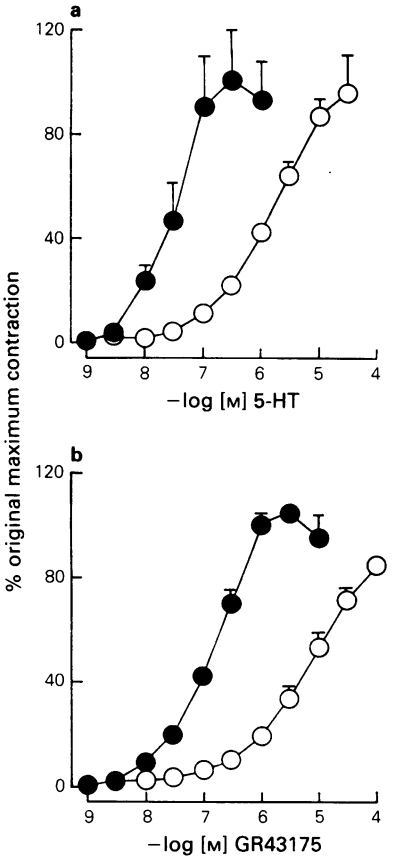

Figure 4 Concentration-effect curves to (a) 5-HT ($n = 3$) or (b) GR43175 ($n = 4$) in the canine isolated basilar artery in the absence (●) or presence (○) of methiothepin (0.1 μM); experiments were performed in the continued presence of ketanserin (1 μM). Concentration-effect curves are from time-matched preparations. Values are means with s.e.mean shown by vertical bars from n experiments.

primate basilar artery. 5-HT receptors have been classified into three broad groups, 5-HT_1-like, 5-HT_2 and 5-HT_3 on the basis of the differing selectivities of certain 5-HT receptor agonists and antagonists (see Bradley *et al.*, 1986a). However, it is now clear that 5-HT_1-like receptors are heterogeneous and can be further subdivided (Bradley *et al.*, 1986a; Humphrey & Feniuk, 1987; Humphrey *et al.*, 1988).

In view of conflicting reports concerning the nature of the 5-HT receptor mediating contraction of cerebral blood vessels, we have examined the ability of some recently identified, selective 5-HT mimetics which have differing selectivities for various 5-HT receptor subtypes, to cause contraction of canine and primate basilar arteries. In both preparations, GR43175, 5-CT and α-methyl 5-HT caused

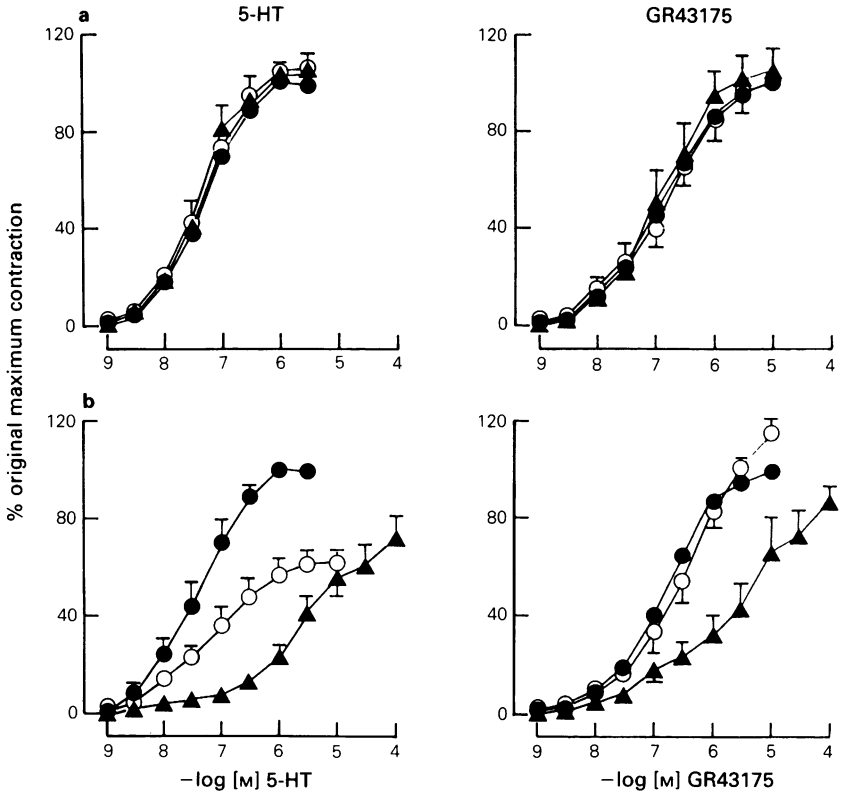

Figure 5 (a) Three consecutive control curves to 5-HT or GR43175, 1st (●), 2nd (○), 3rd (▲) in primate isolated basilar artery. Values are means with s.e.mean shown by vertical lines from 4 experiments. (b) Concentration-effect curves to 5-HT or GR43175 in the primate isolated basilar artery: 1st curve (●) in the absence of antagonists, 2nd curve (○) in the presence of ketanserin (1 μM) and 3rd curve (▲) in the presence of ketanserin (1 μM) and methiothepin (0.1 μM).

contraction with a rank order of agonist potency of 5-CT $\geqslant$ 5-HT > GR43175 > α-methyl 5-HT. However, the relative magnitude of contraction produced by the 5-HT_1-like agonists GR43175 and 5-CT was less than that produced by 5-HT or α-methyl 5-HT, particularly in canine basilar artery. The lower maximum effect produced by GR43175 could not be attributed to this compound acting as a partial agonist, since a high concentration of GR43175 (10–30 μM) did not prevent the additional contractile effects of 5-HT (1 μM, our unpublished observations).

These results demonstrate that the novel 5-HT receptor agonist GR43175 causes contraction of both canine and primate cerebral arteries. This agonist is highly selective for the 5-HT_1-like receptor mediating contraction of the dog isolated saphenous vein and has no activity at 5-HT_2 or 5-HT_3 receptors or at the 5-HT_1-like receptor mediating relaxation of certain vascular smooth muscle preparations (Humphrey *et al.*, 1988). Interestingly, the relative potency of GR43175, compared to 5-HT, in causing contraction of the dog saphenous vein (equipotent molar ratio (5-HT = 1) of 4.6; Humphrey *et al.*, 1988) is very similar to its potency in canine and primate basilar artery (relative potency (5-HT = 1) of 1.7 and 3.6, respectively) obtained in the present studies. This suggests that the 5-HT receptor type which mediates contraction of dog saphenous vein also mediates contraction in dog and primate basilar arteries. However, the greater contractile effects of 5-HT and α-methyl 5-HT in the cerebral artery preparations suggests that, in addition to the 5-HT_1-like receptor, another receptor is present in these tissues. It seems likely that this additional contractile effect of 5-HT is mediated via 5-HT_2 receptors since ketanserin caused a suppression of the 5-HT maximum response.

In canine basilar artery, the contractile effect of 5-CT, compared to GR43175, was small although both agonists produced a similar maximum response in primate basilar artery. In contrast to GR43175, 5-CT is also a potent agonist at the 5-HT_1-like

receptor mediating relaxation in cat saphenous vein and porcine vena cava (Feniuk *et al.*, 1984; Trevethick *et al.*, 1986) through a direct effect on the vascular smooth muscle. Furthermore, 5-HT can also produce relaxation of certain isolated blood vessels through an endothelium-dependent mechanism (Cocks & Angus, 1983; Leff *et al.*, 1987). Therefore, the small contractile effect of 5-CT in canine basilar artery could be explained if 5-HT receptors mediating relaxation were present, either on the cerebrovascular smooth muscle, or on the endothelium. The canine basilar artery preparations used in our studies retained a functional endothelium, since the endothelium-dependent dilator, substance P, caused marked relaxation when tone had been raised with prostaglandin $F_{2\alpha}$ (data not shown). However, despite using a variety of spasmogens, (e.g. prostaglandin $F_{2\alpha}$, U46619, potassium chloride), at submaximal concentrations to contract the tissue, we were unable to reveal a relaxant effect for 5-CT in endothelium-intact or endothelium-denuded canine isolated basilar artery (data not shown). We have previously reported that we can find no evidence for 5-HT or the other selective 5-HT receptor agonists causing an endothelium-dependent relaxation of this preparation (Connor & Feniuk, 1988).

Alternatively, the small contractile effect of 5-CT in canine basilar artery could indicate that it was acting as a partial agonist in this tissue. Indeed, in the dog saphenous vein, 5-CT produced a maximum effect equivalent to 75% of the 5-HT maximum (Feniuk *et al.*, 1985). The ability of 5-CT to cause a concentration-dependent, specific antagonism of 5-HT-induced contractions of canine basilar artery demonstrated that 5-CT and 5-HT interacted with a common receptor type and may suggest that 5-CT acted as a partial agonist. However, contractions to 5-CT were transiently maintained and thus, the antagonism, as well as the very low maximum response to 5-CT (even compared to that of GR43175), may be due to receptor desensitization rather than partial agonism.

Although a component of the contractile response to 5-HT can be attributed to 5-HT_2 receptor stimulation, we attempted to characterize further the 5-HT_1-like receptor which mediates contraction of canine and primate basilar artery by the use of some selective antagonists. In canine basilar artery, contractions produced by GR43175 were unaffected by relatively high concentrations of ketanserin or MDL72222. This rules out an action of GR43175 involving 5-HT_2 or 5-HT_3 receptors. Interestingly, a high concentration of cyanopindolol also caused little or no blockade of GR43175 contractions, thus excluding an action at 5-HT_{1A} or 5-HT_{1B} receptor subtypes (Hoyer *et al.*, 1985; Schlicker *et al.*, 1985). Further evidence against any involvement of 5-HT_{1A} receptors was provided by the weak contractile activity of 8-OHDPAT (Middlemiss & Fozard, 1983) in this preparation. This is in contrast to previous studies where 5-HT_{1A} receptors have been claimed to be involved in mediating contractions of canine basilar artery (Peroutka *et al.*, 1986; Taylor *et al.*, 1986). As regards other 5-HT_1 ligand binding subtypes, the present study has not excluded the possibility that 5-HT and GR43175 produce contractions of these cerebral blood vessels via an action on receptors which may be similar to the 5-HT_{1C} or 5-HT_{1D} sites (Heuring & Peroutka, 1987). However, the profile of action of both agonists and antagonists (see below) is identical in the cerebral arteries to that found in the dog isolated saphenous vein, where the contractile response to GR43175 is resistant to blockade by both mesulergine (1 μM) and metergoline (0.1 μM), excluding an action at these sites (Humphrey *et al.*, 1988).

In an attempt to gain some evidence that 5-HT and GR43175 interact with a common 5-HT receptor type, we have additionally examined the antagonist activity of methiothepin. These studies were carried out in the presence of ketanserin to negate any effects at 5-HT_2 receptors. The 5-HT_1-receptor antagonist methiothepin antagonized the effects of both GR43175 and 5-HT and, importantly, the degree of blockade produced was similar for each agonist and was specific, since the same concentration of methiothepin did not modify contractions to the thromboxane A_2-mimetic, U46619. This suggests that 5-HT and GR43175 are acting at the same receptor type to produce contraction and provides further evidence that this receptor is similar to the 5-HT_1-like receptor characterized in the dog saphenous vein (Humphrey *et al.*, 1988).

There are few reports in the literature concerning the nature of the 5-HT receptor mediating contraction of primate cerebral arteries. We examined the antagonistic effects of ketanserin and methiothepin against contractions produced by 5-HT or GR43175 in primate basilar artery. A high concentration of ketanserin had no effect on GR43175 contractions, but caused a small reduction in the maximum 5-HT response. However, methiothepin antagonized the effects of both agonists to an equal extent, with a potency similar to that seen in canine basilar arteries. Thus, our results suggest that the predominant component of the 5-HT contractile response in primate basilar artery, as in canine basilar artery, reflects activation of 5-HT_1-like receptors like those in the dog saphenous vein. This contrasts with the findings of Chang *et al.*, (1987) who reported that 5-HT_2 receptors mediate contraction of primate cerebral arteries from aethiops monkeys. Thus, the 5-HT receptor type may differ according to the strain of primate used.

In conclusion, our results have demonstrated that in canine and primate basilar artery, a large component of 5-HT-induced contraction is attributable to stimulation of 5-HT_1-like receptors. This receptor is characterized by the high agonist potency of GR43175, susceptibility to blockade by methiothepin and appears the same as the 5-HT_1-like receptor identified in the dog saphenous vein (Humphrey *et al.*, 1987). However, there also appears to be a population of 5-HT_2 receptors present in the cerebral arteries which contribute to the contractile effects of 5-HT. The presence of a mixed and probably varied 5-HT receptor population may explain some of the discrepancies in the literature. Furthermore the use of non-selective drugs undoubtedly complicates analysis of the receptors involved. Indeed, earlier reports have raised the possibility that more than one 5-HT receptor type may occur in cerebral arteries (Muller-Schweinitzer & Engel, 1983; Peroutka *et al.*, 1986).

The contractile action of GR43175 on canine and primate large cerebral arteries has been demonstrated. Whether or not GR43175 also causes contraction of human cerebral arteries remains to be demonstrated. However, it is possible that the efficacy of GR43175 in the alleviation of migraine headache (Doenicke *et al.*, 1988) may be due to selective constriction of those cranial arteries which become excessively dilated, distended and inflamed during an attack (Lance, 1973). Its apparent lack of generalized effects on the cardiovascular system in clinical studies presumably results from the inability of GR43175 to stimulate 5-HT_2 receptors. (Humphrey *et al.*, 1988).

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Endotoxin-induced hyperreactivity of the guinea-pig isolated trachea coincides with decreased prostaglandin E_2 production by the epithelial layer

[1]Gert Folkerts, Ferdi Engels & Frans P. Nijkamp

Department of Veterinary Pharmacology, University of Utrecht, P.O. Box 80176, 3508 TD Utrecht, The Netherlands

1 Pretreatment of guinea-pigs with endotoxin (1 mg kg^{-1} b.w., i.p., 4 days before the experiments) results in respiratory airway hyperreactivity *in vitro*. Dose-response curves with either arecoline or histamine on isolated tracheae from these animals display increased maximal contractions, and decreased EC_{50} values.

2 Tracheae denuded of epithelium respond with a similar hyperreactivity to histamine as observed in preparations from endotoxin pretreated animals. Removal of the epithelial layer of tracheae from endotoxin pretreated guinea-pigs did not additionally affect the histamine dose-response curve.

3 The cyclo-oxygenase inhibitor indomethacin (10 μM) induces histamine hyperreactivity which is equal in intact and epithelium-denuded tracheae from saline or endotoxin pretreated guinea-pigs. Similar results are obtained with the combined lipoxygenase/cyclo-oxygenase inhibitor nor-dihydroguaiaretic acid (10 μM).

4 Histamine (0.1 mM) induces an increase in prostaglandin E_2 (PGE_2) formation by the tracheal spiral *in vitro*, which is reduced by 34% by endotoxin pretreatment, and by about 60% following epithelium removal irrespective of endotoxin pretreatment.

5 Arachidonic acid (AA, 22 μM) stimulation of the guinea-pig trachea *in vitro* induces a relaxation, and an increase in PGE_2 production. In preparations lacking the epithelium, AA induces a contraction which coincides with a 60% reduced increase in PGE_2 formation. These effects are not altered by endotoxin pretreatment.

6 It is concluded that the endotoxin-induced respiratory airway hyperreactivity may be caused by a disturbed ability of epithelial cells to synthesize PGE_2. The decreased formation of this prostaglandin is rather the consequence of a diminished liberation of AA from the phospholipid stores than a dysfunction of the cyclo-oxygenase enzyme.

Introduction

Morphological abnormalities of epithelial cells lining the respiratory tract have been described in man after fatal asthmatic attacks (Dunnill, 1975) and in patients with asthma (Laitinen *et al.*, 1985). Epithelial damage is considered to be associated with the non-specific bronchial hyperreactivity observed in asthmatic patients (Laitinen *et al.*, 1985). Consistent with this, removal of the epithelium from isolated tracheal preparations *in vitro* causes an increased responsiveness to contractile agents (Flavahan *et al.*, 1985; Barnes *et al.*, 1985). Prostaglandins may have a modulating role in the induction of airway smooth muscle hyperresponsiveness. Prostaglandin E_2 (PGE_2), in particular, is released during contractions of animal and human tracheal preparations (Orehek *et al.*, 1973; Steel *et al.*, 1979), and can be synthesized by rabbit tracheal epithelial cells (Butler *et al.*, 1987). This prostaglandin relaxes respiratory airway smooth muscle and inhibits contractile responses evoked in intact and epithelium-denuded tracheal preparations (Mathé *et al.*, 1977; Anderson *et al.*, 1983; Braunstein *et al.*, 1988), whereas prostaglandin synthesis inhibitors augment the responses of trachea to contractile agents (Orehek *et al.*, 1975).

In our laboratory, bronchial hyperreactivity is induced in guinea-pigs by intraperitoneal administration of the Gram-negative bacterium *Haemophilus influenzae* or the active cell constituent endotoxin. These animals show an altered responsiveness of the respiratory airways *in vitro* and *in vivo* (Schreurs *et*

[1] Author for correspondence.

al., 1983; Folkerts & Nijkamp, 1985; Van Heuven-Nolsen *et al.*, 1986; Van Oosterhout *et al.*, 1988).

The present study investigates whether epithelial cells and/or an altered arachidonic acid metabolism are involved in the endotoxin-induced respiratory airway hyperreactivity in the guinea-pig. Some of these results have been presented to the British Pharmacological Society (London, 1988).

Methods

Animals

The animals used in this study were specified pathogen-free male Dunkin Hartley guinea-pigs (Olac Ltd., Bicester, England) weighing 400–450 g, which received water and commercial chow *ad libitum*. They were without infections of the respiratory tract as judged from histological examination and the low number of neutrophils in pulmonary lavage fluid (Folkerts *et al.*, 1988). Guinea-pigs were injected intraperitoneally with endotoxin ($1\,mg\,kg^{-1}$ body weight, LPS from *Escherichia coli* $O_{111}:B_4$) 4 days before the experiment (Engels *et al.*, 1987). Control animals were injected with $1\,ml\,kg^{-1}$ sterile non-pyrogenic saline. Histological examination of the epithelial layer revealed no structural damage or morphological changes in the endotoxin pretreated group. Animals were killed with pentobarbitone sodium ($30\,mg\,100\,g^{-1}$ body weight) intraperitoneally.

Tracheal smooth muscle reactivity

The trachea was removed from the animals, trimmed free of connective tissue and blood vessels, and divided into two parts. The proximal and distal parts of the trachea were used alternately for epithelial denudation, which was achieved by pulling a cotton swab through the lumen. The effectiveness of this procedure was determined by histological examination of formaldehyde (4%) fixed 5 μm sections stained with haematoxylin-eosin.

Each tracheal segment was cut in a spiral of 7 cartilage rings, and mounted in an organ bath (8.5 ml) filled with Krebs-bicarbonate buffer (37°C, pH 7.4) gassed with 95% O_2, 5% CO_2. The buffer composition was as follows (mmol l^{-1}): NaCl 118.1, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and glucose 8.3. Changes in length of the tracheal spirals were measured isotonically under a constant load of 0.8 g. Tissues were washed three times at 15 min intervals and after a stable tone was attained, cumulative dose-response curves were constructed with either histamine or the muscarinic receptor agonist, arecoline. Where indicated, tracheal spirals were preincubated with the cyclo-oxygenase inhibitor indomethacin (10 μM) for 30 min, or with the dual lipoxygenase and cyclo-oxygenase inhibitor nordihydroguaiaretic acid (NDGA, 10 μM) for 15 min, before construction of dose-response curves.

In separate experiments arachidonic acid (AA, 22 μM) was added to spirals precontracted with KCl (final bath concentration 20 mM). The contractile and relaxant effects of AA were investigated with drugs that inhibit either the formation or action of AA-metabolites. Indomethacin (10 μM) was added to the organ bath 30 min before precontraction with KCl. NDGA (10 μM) and the leukotriene D_4 receptor antagonist FPL 55712 (30 μM) were added to KCl precontracted tracheae 15 min before addition of AA. Piriprost (15 μM), a leukotriene synthesis inhibitor, was added to tracheal segments 5 min before addition of AA. Each tracheal spiral was used for one dose-response curve or exposed to one antagonist. When appropriate, aminophylline (0.01 M) was added to the organ bath to produce maximal relaxation.

Radioimmunoassay of prostaglandin E_2

The radioimmunoassay (RIA) procedure was carried out according to the manufacturer's instructions. Determinations were made in duplicate and expressed as pg of PGE_2 released per 100 μl bath fluid. Samples from the organ bath were taken (a) 2 min before the addition of histamine or AA, (b) when the maximal response was achieved after a complete histamine dose-response curve and (c) after the maximal response to AA (22 μM). Samples from the organ bath were diluted 10 times with Krebs-buffer and 100 μl of this solution was used in the RIA. Krebs-buffer did not interfere with the assay, but data have been corrected for interference by histamine and AA.

Materials

E. coli $O_{111}:B_4$ lipo-polysaccharide (Phenolic extraction), arecoline hydrobromide, AA, and NDGA were from Sigma Chemical Company, St. Louis, U.S.A. AA was stored as a free acid in hexane under N_2 at −20°C. To obtain the salt an appropriate volume was evaporated under N_2 and dissolved in 0.5 M KOH in ethanol, and evaporated again under N_2. The salt was finally dissolved in 0.5 mM Tris-HCl buffer. NDGA was dissolved in 0.01 N NaOH. Nembutal, containing $60\,mg\,ml^{-1}$ pentobarbitone sodium, was obtained from Abbott Laboratories, North Chicago, IL, U.S.A. Histamine phosphate was obtained from the Onderlinge Pharmaceutische Groothandel, Utrecht, The Netherlands. Indomethacin was obtained from Merck Sharp & Dohme, Haarlem, The Netherlands and was dissolved in 1 M Tris-HCl buffer (pH 8.0). Piriprost (U-

60,257) and FPL 55712 (sodium 7-3-(4-acetyl-3-hydroxy-2-propylphenoxy)-2-hydroxy propoxy-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylate) were generous gifts of Dr M.K. Bach (Upjohn Company, Kalamazoo, U.S.A.) and Dr P. Sheard (Fisons, Loughborough, U.K.), respectively. Piriprost was dissolved with an equal weight of Tris-base in distilled water. All drug solutions were kept on ice until use. Throughout the experiments only plastics and siliconized glassware were used in order to minimize adherence of drugs or mediators to the walls of the tubes and organ baths. The [^{125}I]-PGE_2 RIA-kit was from New England Nuclear, Boston, MA, U.S.A.

Statistical analysis

Dose-response curves were analyzed by means of a computerized curve fitting technique based on a 4-parameter logistic equation. Parameters defining the sigmoidal curve, i.e. the maximal response, the EC_{50}-value (the concentration that induces a half maximal response), and the slope were determined for each dose-response curve. The slope can be interpreted as the Hill coefficient. Subsequently, the respective parameters were averaged for the various experimental groups and statistically evaluated with Student's unpaired t test. Student's unpaired t test was also used to determine changes in the AA-induced relaxations and contractions of the trachea, and release of PGE_2 in the organ bath. Data were considered significantly different when $P < 0.05$. The results are expressed as the mean ± standard error of the mean (s.e.mean).

Results

Reactivity of isolated tracheae

Pretreatment of guinea-pigs with endotoxin increased the responsiveness of tracheae stimulated *in vitro* with either arecoline or histamine. The dose-response curves of tissues from endotoxin-pretreated animals had significantly greater maxima ($P < 0.01$) and lower EC_{50} values ($P < 0.05$ for arecoline) when compared with tissues from untreated animals (Figures 1 and 2). Similarly, removal of the tracheal epithelium increased the maximal responses ($P < 0.01$) and decreased the EC_{50} values ($P < 0.05$ for histamine, and $P < 0.01$ for arecoline) for both agonists. The increased responsiveness was of similar magnitude following either epithelium removal or endotoxin pretreatment and when combined in the same tissue the effects of these two treatments were not additive (Figures 1 and 2). The slope of the dose-response curves obtained with arecoline, or histamine, did not differ between the experimental groups.

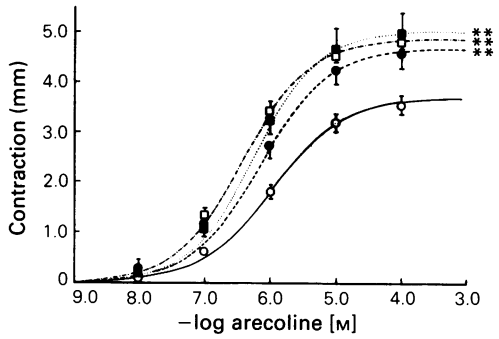

Figure 1 Mean dose-response curves to arecoline in intact and epithelium-denuded tracheal spirals from saline and endotoxin-treated guinea-pigs: (○) intact saline ($EC_{50} = 1.10 \pm 0.12\,\mu M$, $n = 10$); (●) intact endotoxin ($EC_{50} = 0.70 \pm 0.14\,\mu M$, $P < 0.05$, $n = 10$); (□) epithelium-denuded saline ($EC_{50} = 0.39 \pm 0.05\,\mu M$, $P < 0.01$, $n = 10$); (■) epithelium-denuded endotoxin ($EC_{50} = 0.58 \pm 0.09\,\mu M$, $P < 0.01$, $n = 10$). The responses are shown as mean with s.e.mean indicated by vertical bars. ** Maximal responses are significantly increased ($P < 0.01$) as compared to the intact saline group. The slopes did not differ between the experimental groups.

Preincubation of tracheal spirals with indomethacin or NDGA increased the maximal response ($P < 0.01$) and decreased the EC_{50} values ($P < 0.05$)

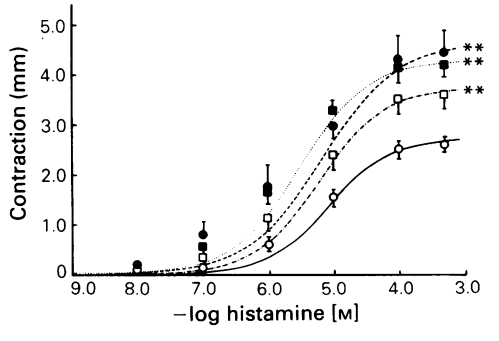

Figure 2 Mean dose-response curves to histamine in intact and epithelium-denuded tracheal spirals from saline and endotoxin-treated guinea-pigs: (○) intact saline ($EC_{50} = 8.60 \pm 1.70\,\mu M$, $n = 10$); (●) intact endotoxin ($EC_{50} = 6.70 \pm 1.79\,\mu M$, $n = 12$); (□) epithelium-denuded saline ($EC_{50} = 6.20 \pm 1.44\,\mu M$, $n = 13$); (■) epithelium-denuded endotoxin ($EC_{50} = 3.10 \pm 0.59\,\mu M$, $P < 0.05$, $n = 14$). The responses are shown as mean with s.e.mean indicated by vertical bars. ** Maximal responses are significantly increased ($P < 0.01$) as compared to the intact saline group. The slopes did not differ between the experimental groups.

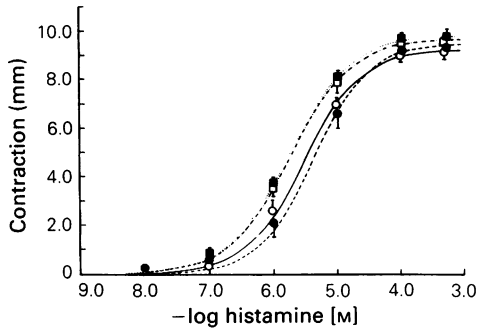

Figure 3 Mean dose-response curves to histamine, after indomethacin (10 μM) preincubation for 30 min, in intact and epithelium-denuded tracheal spirals from saline and endotoxin-treated guinea-pigs: (○) intact saline ($EC_{50} = 3.10 \pm 0.54\,\mu$M, $n = 5$); (●) intact endotoxin ($EC_{50} = 4.40 \pm 0.72\,\mu$M, $n = 7$); (□) epithelium-denuded saline ($EC_{50} = 1.90 \pm 0.25\,\mu$M, $n = 6$); (■) epithelium-denuded endotoxin ($EC_{50} = 1.90 \pm 0.24\,\mu$M, $n = 7$). The responses are shown as mean with s.e.mean indicated by vertical bars. Maximal responses are increased ($P < 0.01$) and EC_{50} values decreased ($P < 0.05$) as compared to the intact saline group (Figure 2). The slopes did not differ between the experimental groups.

of dose-response curves to histamine in all groups (Figures 3 and 4). Indomethacin induced a relaxation of the tracheae. This loss of tone was partly the cause of the increased maximal response to histamine. NDGA induced a small relaxation in all preparations also, but after correction of this effect an increased response was still observed (Figure 4). When either endotoxin pretreatment or epithelium removal (or both) was combined with indomethacin or NDGA preincubation, the maximal response to histamine was not greater than for preparations treated with indomethacin or NDGA alone (Figures 3 and 4).

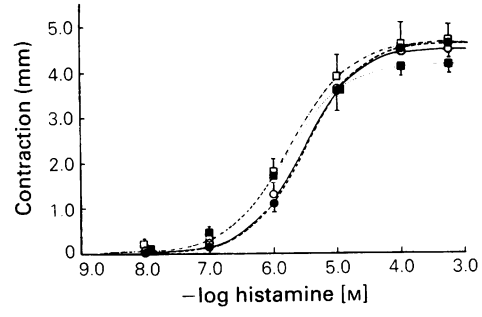

Figure 4 Mean dose-response curves to histamine, after nordihydroguaiaretic acid (NDGA, 10 μM) preincubation for 15 min, in intact and epithelium-denuded tracheal spirals from saline and endotoxin-treated guinea-pigs: (○) intact saline ($EC_{50} = 2.80 \pm 0.44\,\mu$M, $n = 7$); (●) intact endotoxin ($EC_{50} = 3.20 \pm 0.17\,\mu$M, $n = 7$); (□) epithelium-denuded saline ($EC_{50} = 1.80 \pm 0.17\,\mu$M, $n = 6$); (■) epithelium-denuded endotoxin ($EC_{50} = 1.50 \pm 0.15\,\mu$M, $n = 7$). The responses are shown as mean with s.e.mean indicated by vertical bars. Maximal responses are increased ($P < 0.01$) and EC_{50} values decreased ($P < 0.05$) as compared to the intact saline group (Figure 2). The slopes did not differ between the experimental groups.

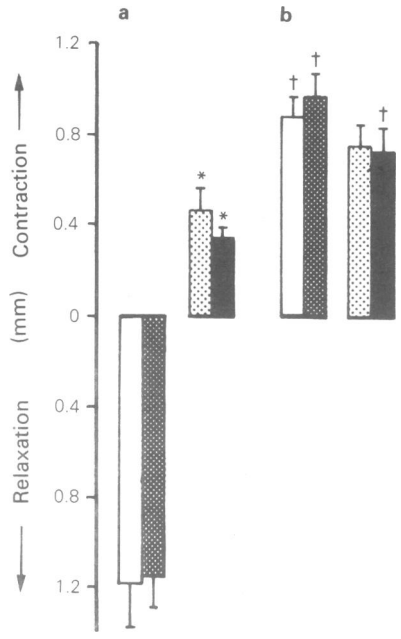

Figure 5 (a) Arachidonic acid (22 μM)-induced relaxations and contractions of intact and epithelium-denuded tracheal spirals from saline and endotoxin pretreated guinea pigs ($n \geq 8$). (b) Arachidonic acid (22 μM)-induced contractions, after preincubation with indomethacin (10 μM) for 30 min, on isolated and epithelium-denuded tracheal spirals from saline and endotoxin pretreated guinea-pigs ($n \geq 7$). Open column = intact control; hatched column = intact endotoxin; dotted column = epithelium-denuded saline; filled column = epithelium-denuded endotoxin. Responses are shown as the mean with s.e.mean indicated by vertical bars. * $P < 0.01$ as compared to the saline and endotoxin groups with epithelium. † $P < 0.01$ as compared to the respective control group without indomethacin.

Arachidonic acid-induced relaxations and contractions

Arachidonic acid (22 μM) was added to KCl (20 mM) precontracted tracheal spirals in order to investigate whether metabolism of AA by epithelial cells contributed to the endotoxin-induced hyperreactivity of

the trachea. The KCl-induced precontractions did not differ between the experimental groups. In intact tracheae, AA induced a relaxation and no difference was observed between preparations from saline and endotoxin pretreated guinea-pigs (Figure 5a). In the epithelium-denuded spirals, AA caused a contraction and again no difference was detected between saline and endotoxin pretreated animals (Figure 5a). Contractions induced by AA in the epithelium-denuded saline and endotoxin pretreated groups were significantly inhibited by piriprost (15 μM, 65% and 35% respectively, $P < 0.05$). NDGA (10 μM, 83% and 76% respectively, $P < 0.01$), and FPL 55712 (30 μM, 93%, $P < 0.01$, and 71%, $P < 0.05$).

When intact preparations were preincubated with indomethacin (10 μM, 30 min) the AA-induced relaxation was reversed into a contraction. In epithelium-denuded preparations the AA-induced contraction was augmented. The contractions were not significantly different between the four experimental groups (Figure 5b).

The aminophylline (0.01 M)-induced maximal relaxation, after correction for the AA-induced relaxations or contractions, did not differ between the four experimental groups. Therefore differences in basal tone were not responsible for the observed effects of AA.

Prostaglandin E_2 production after histamine or arachidonic acid stimulation

The release of PGE_2 from tracheal preparations was measured before and after stimulation with histamine (0.1 mM) or AA (22 μM). Basal production of PGE_2 by preparations from control animals was 38.3 ± 5.6 pg per 100 μl bath fluid (Table 1) and this level was not significantly different in tissues from endotoxin pretreated guinea-pigs. Removal of the epithelium decreased the basal release of PGE_2 to less than half of the control level ($P < 0.01$, Table 1): this change was also observed in tissues from endotoxin pretreated guinea-pigs.

When control tracheal preparations were stimulated with histamine the amount of PGE_2 released in the organ bath increased (Table 1). Histamine-stimulated PGE_2 release was significantly decreased in tracheae from endotoxin pretreated guinea-pigs (34%, $P < 0.05$), and also in tissues of which the epithelium had been removed (65%, $P < 0.01$). Endotoxin pretreatment did not further reduce PGE_2-formation in epithelium-denuded preparations (Table 1).

Addition of AA (22 μM) to tracheal spirals *in vitro* also led to the release of PGE_2 into the organ bath. The amount of PGE_2 released by tracheae from endotoxin-pretreated guinea-pigs was not different from control. In tracheae where the epithelium had been removed, the formation of PGE_2 was decreased by 67% (saline pretreated guinea-pigs) and by 52% (endotoxin pretreated animals) when compared to intact preparations (Table 1). There was no difference between the epithelium-denuded saline and endotoxin pretreated groups.

Discussion

Results strongly suggest that loss of PGE_2 released from epithelial cells augments tracheal responses to contractile stimuli. The loss of PGE_2 occurred after indomethacin treatment or when epithelial cells were physically removed *in vitro*: both procedures augmented responses to contractile stimuli. Consistent with this, the tracheal relaxant action of AA was converted to a contractile response after physical removal of the epithelium. Interestingly, this AA-induced contraction was blocked by lipoxygenase inhibitors and the leukotriene D_4 (LTD_4) antagonist FPL 55712, suggesting that the AA-induced contraction was due to its metabolism to peptidoleukotrienes.

Tracheae from guinea-pigs injected with endotoxin showed exaggerated responses *in vitro* to two different contractile stimuli, namely histamine and arecoline. Such exaggerated responses were not associated

Table 1 Prostaglandin E_2 (PGE_2) production of intact and epithelium-denuded tracheal spirals from saline and endotoxin-treated guinea-pigs

Treatment		*Basal value*	*Histamine* (0.1 mM)	*Arachidonic acid* (22 μM)
Control	+epi	38.3 ± 5.6† (8)	123.7 ± 15.9 (7)	133.9 ± 13.4 (7)
Endotoxin	+epi	25.1 ± 4.8 (9)	81.8 ± 6.3* (8)	151.3 ± 17.0 (7)
Control	−epi	15.0 ± 2.3** (7)	44.7 ± 10.4** (6)	44.6 ± 5.6** (6)
Endotoxin	−epi	14.6 ± 2.2** (8)	40.0 ± 5.5** (8)	64.4 ± 6.2** (7)

† Data are presented as pg PGE_2 per 100 μl bath fluid and expressed as the mean ± s.e.mean.
+epi = epithelium intact, −epi = epithelium denuded.
The number of observations is given in parentheses.
* $P < 0.05$, ** $P < 0.01$ as compared to the control group (+epi).

with physical damage to epithelial cells, as judged by histological examination. The magnitude of responses of tissues taken from endotoxin-treated animals was not increased further by removal of epithelial cells. The exaggerated response to histamine *in vitro* observed after endotoxin pretreatment did not occur in indomethacin pretreated tissues. These observations strongly suggest that endotoxin pretreatment leads to exaggerated tracheal responses to contractile stimuli through the diminished capacity of epithelial cells to generate the relaxant prostaglandin, PGE_2. This interpretation was supported by RIA measurements of the amounts of PGE_2 released into the organ bath following histamine-induced contractions. The amount of PGE_2 released was lower in tissues from endotoxin-treated guinea-pigs, compared with controls. Further evidence for a role for prostaglandins, and not for lipoxygenase-derived products, in the endotoxin-induced respiratory hyperreactivity was obtained by preincubating the tissues with NDGA. NDGA is an inhibitor of the 5-lipoxygenase enzyme ($IC_{50} = 2\,\mu M$) and cyclo-oxygenase enzyme ($IC_{50} = 5\,\mu M$) and it also possesses inhibitory properties towards 15-lipoxygenase enzymes ($IC_{50} = 10\,\mu M$, Ku *et al.*, 1985). 15-Lipoxygenase metabolites may be important since it was shown that these products induce bronchial hyperreactivity to histamine in the guinea-pig (Folkerts *et al.*, 1983). As with indomethacin, NDGA ($10\,\mu M$) induced a hyperresponsiveness of intact control preparations to histamine. Again, endotoxin-induced hyperresponsiveness and the NDGA-effect were not additive. Obviously, the lipoxygenase-inhibitory properties of NDGA did not affect the endotoxin-induced tracheal hyperresponsiveness, indicating that lipoxygenase products play a subservient role, if any at all. Indeed, contractions induced by AA in epithelium-denuded tracheal segments from saline and endotoxin pretreated guinea-pigs did not differ from each other and were inhibited to the same extent by lipoxygenase inhibitors and the peptidoleukotriene antagonist.

During contractions of guinea-pig and human tracheal preparations the predominant eicosanoid released is PGE_2 (Orehek *et al.*, 1973; 1975; Grodzinska *et al.*, 1975; Steel *et al.*, 1979). This might have physiological significance since addition of PGE_2 *in vitro*, in the nanomolar range, inhibits contractions of intact and epithelium-denuded tracheae (Anderson *et al.*, 1983; Braunstein *et al.*, 1988). In the present study the concentration of PGE_2 in the organ bath after histamine stimulation was 3.5 nM and the local concentration might be even higher because of the close proximity of epithelial cells and smooth muscle cells. This study and others suggest that epithelial cells are responsible for the formation of PGE_2. It was found that the rabbit mucosal epithelial cells contain the AA cyclo-oxygenase enzyme (Butler *et al.*, 1987). Furthermore, we and others (Nijkamp & Folkerts, 1986; Farmer *et al.*, 1987; Brunelleschi *et al.*, 1987) found that AA- or platelet activating factor-induced relaxations of isolated guinea-pig tracheae were dependent on the epithelial layer and PGE_2 formation. Furthermore, the participation of epithelial cells and PGE_2 in modulating airway hyperreactivity has recently been emphasized by the observation that the increased responsiveness of isolated, epithelium-denuded tracheae to contractile agents could be inhibited by adding chopped tracheal epithelium or PGE_2 to the organ bath (Aizawa *et al.*, 1988; Braunstein *et al.*, 1988).

Tracheae from endotoxin pretreated guinea-pigs were stimulated with AA in order to investigate whether the decreased formation of PGE_2 after histamine stimulation was caused by a diminished activity of cyclo-oxygenase. The results suggest that the function of this enzyme was not changed since no decreased relaxation, or production of PGE_2 was observed in these tissues after AA stimulation. However, histamine strongly stimulates phospholipase A_2 activity (Blackwell *et al.*, 1978), and the results shown in Table 1 suggest that the activity of this enzyme in tracheal epithelial cells from endotoxin-treated animals is reduced, leading to a lower concentration of free AA and hence PGE_2. This hypothesis deserves further investigation.

In light of the observed epithelial damage in asthmatic patients (Laitinen *et al.*, 1985), it is suggested that the absence of cells or enzymes responsible for the formation of PGE_2, could be a prime cause of bronchial hyperresponsiveness. Several lines of evidence obtained from the results described above indicate that endotoxin-induced respiratory airway hyperreactivity in the guinea-pig is also due to a loss of modulation by PGE_2. Furthermore, in this animal model, the decreased production of PGE_2 is probably a consequence of a diminished phospholipase A_2 activity in the epithelial layer.

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Inhibitory effect of a selective thromboxane A_2 receptor antagonist, EP 092, on platelet aggregation in whole blood *ex vivo* and *in vivo*

[2]R.F.G. Booth, [2]A.C. Honey, [3]N. Lad, [1]D.P. Tuffin & P.J. Wade

Searle Research and Development, High Wycombe, Buckinghamshire, HP12 4HL

1 The inhibitory effect of a selective prostaglandin H_2 (PGH_2)/thromboxane A_2 receptor antagonist, EP 092, on platelet aggregatory responses in whole blood *ex vivo* (guinea-pig: Rhesus monkey) and intravascular aggregation *in vivo* (rabbit) has been investigated.

2 Collagen (0.1–10.0 $\mu g\,ml^{-1}$) caused a concentration-dependent decrease in single platelet count in samples of both guinea-pig and Rhesus monkey citrated whole blood incubated *ex vivo*. EP 092 administered to guinea-pigs by intravenous (0.1–3.0 $mg\,kg^{-1}$) or oral (1.0–10.0 $mg\,kg^{-1}$) routes significantly inhibited the platelet responses to collagen (ED_{50} values 1.3 ± 0.2 and $1.4 \pm 0.2\,mg\,kg^{-1}$ respectively). Similar potency against collagen-induced whole blood aggregation was observed in Rhesus monkey blood samples following EP 092 given orally (ED_{50} $0.9 \pm 0.3\,mg\,kg^{-1}$).

3 The duration of action of EP 092 against collagen aggregatory responses *ex vivo* in both guinea-pigs and Rhesus monkeys was between 3 and 6 h following oral administration at 3.0 $mg\,kg^{-1}$.

4 The inhibitory activity demonstrated by EP 092 against collagen-induced aggregation of Rhesus monkey whole blood *ex vivo* was not accompanied by any significant reduction in thromboxane A_2 formation except at the highest dose tested (10 $mg\,kg^{-1}$).

5 The intravascular aggregatory response induced by collagen or thrombin in the anaesthetized rabbit was significantly inhibited by an intravenous infusion of EP 092 (10 $mg\,kg^{-1}$). EP 092 appeared less potent and its effect was of shorter duration in this preparation compared with its inhibitory effect on *ex vivo* aggregation, being evident immediately after infusion of drug but not after a further 30 min.

6 It is concluded that collagen-induced platelet aggregatory responses in guinea-pig and Rhesus monkey whole blood *ex vivo* and rabbit *in vivo* exhibit a thromboxane-dependent component which can be inhibited in a dose-related fashion by pretreatment with the thromboxane antagonist EP 092. In the rabbit, moreover, the data support the possibility of a role for thromboxane in the intravascular aggregatory response to thrombin.

Introduction

The potent platelet aggregatory activity of the cyclic endoperoxide prostaglandin H_2 (PGH_2; Hamberg & Samuelsson, 1974) and its product thromboxane A_2 (TXA_2) formed by the enzyme thromboxane synthase (Svensson *et al.*, 1976) has been recognised for more than a decade. Despite this, it is only relatively recently that the structure of TXA_2 has been unequivocably confirmed by total synthesis (Bhagwat *et al.*, 1985a), enabling the pharmacology of pure material to be finally described (Bhagwat *et al.*, 1985b; Bunting *et al.*, 1987). Recent evidence strongly suggests that TXA_2, PGH_2 and PGH_2-mimetics each bind with varying affinity to the same platelet receptor population (Armstrong *et al.*, 1983; Halushka *et al.*, 1987), and that TXA_2 is an order of magnitude more potent than PGH_2 at inducing aggregation of human platelet-rich plasma (PRP) *in vitro* (Bhagwat *et al.*, 1985b). Consequently,

[1] Present address and author for correspondence: Bioscience II Department, ICI Pharmaceuticals, Mereside, Alderley Park, Macclesfield, Cheshire, SK10 4TG.
Present address: [2]Department of Biochemical Sciences, Wellcome Research Laboratories, Langley Court, Beckenham, Kent, BR3 3BS. [3]Department of Biology, Roche Products Ltd., P.O. Box, 8, Welwyn Garden City, Herts, AL7 3AY.

inhibition of the aggregatory activity of both potent endogenous arachidonic acid pathway products might be achieved by a single agent via selective blockade of the platelet PGH_2/TXA_2 receptor. This strategy has been widely viewed as therapeutically attractive, and the use of platelet radioligand binding assays in conjunction with aggregation experiments in washed platelet suspensions or PRP has been instrumental in the identification of several putative antagonists (Cross & Dickinson, 1987).

One such compound, EP 092 ((±)-5-endo-(6'-carboxyhex-2'Z-enyl)-6-exo-[1'-[N-(phenylthiocarbamoyl)-hydrazono]-ethyl]-bicyclo[2,2,1]-heptane), was initially identified amongst members of a chemical series first synthesized by Wilson *et al.*, (1982) and evaluated by Wilson & Jones (1985). The compound is a potent inhibitor of [^{3}H]-15(S)-9,11-expoxymethano-PGH_2 ([^{3}H]-U44069) binding to washed human platelets, and of aggregatory responses induced by TXA_2, PGH_2, or stable PGH_2-mimetics including 11,9-epoxymethano-PGH_2 and 9,11-azo-PGH_2 in diluted PRP or plasma-free platelet suspensions (Armstrong *et al.*, 1985a). EP 092 also causes a parallel shift to the right of log concentration-effect curves obtained in response to 15(S)-11,9-epoxymethano-PGH_2 (U46619) on isolated rabbit aorta, dog saphenous vein and guinea-pig trachea smooth muscle preparations *in vitro* (Jones, 1984).

In vivo, partial inhibition of U46619 and collagen-induced thrombocytopoenia associated with elevated pulmonary inflation pressure in the guinea-pig has been noted (Jones *et al.*, 1983). Furthermore, administration of EP 092 prevents the marked rise in pulmonary artery pressure, the fall in cardiac output, and the respiratory distress which follows intravenous injection of *E. coli* endotoxin in the anaesthetized sheep (Armstrong *et al.*, 1985b). However, the inhibitory activity of this compound against platelet aggregatory responses in whole blood, rather than in PRP or washed cell suspensions, and the potency and duration of any such activity observed following parenteral or oral dosing to experimental animals, remains to be firmly established.

In the present study, we chose to investigate the ability of EP 092 to inhibit collagen-induced whole blood platelet aggregatory responses *ex vivo* using a single platelet counting technique (Lad *et al.*, 1987) in citrated blood samples obtained from guinea-pigs or Rhesus monkeys pretreated with EP 092. These experiments prompted an examination of the effect of EP 092 on collagen and thrombin-induced intravascular aggregation in the anaesthetized rabbit by continuous platelet count monitoring *in vivo* (Honey *et al.*, 1986). A preliminary account of these experiments has been given to the British Pharmacological Society (Lad *et al.*, 1988).

Methods

Collection of blood

EP 092 was administered to male Dunkin Hartley guinea-pigs (350–500 g) by either bolus intravenous injection (0.1–3.0 mg kg^{-1}) in sodium bicarbonate buffer pH 8.5 or oral gavage (1.0–10.0 mg kg^{-1}) as a PEG 400/2% carboxymethylcellulose (CMC) aqueous suspension (1:5 v/v). Treatment groups ($n \geqslant 5$ animals in all cases) were bled at various time intervals after dosing as detailed in the text. Vehicle controls were included for both dosing routes and all time points, to allow comparison with drug-treated guinea-pigs. For blood collection, the guinea-pigs were anaesthetized with pentobarbitone sodium (Sagatal) by intraperitoneal injection. Blood samples (5.0 ml) were obtained from the abdominal aorta via a butterfly cannula, and drawn gently into a syringe containing 3.1% trisodium citrate anticoagulant at a final citrate:blood ratio of 1:9 v/v.

Male Rhesus monkeys (*Macaca mulatta*; 11–16 kg) were dosed by oral gavage with EP 092 (0.3–10.0 mg kg^{-1}) in PEG 400/2% CMC vehicle. Prior to collection of blood, each animal was lightly sedated with intramuscular ketamine. Blood samples were taken at the times specified in the text by venepuncture into a syringe containing 3.1% trisodium citrate anticoagulant as described above. A group of six animals were used to complete the study, with any one animal being dosed and bled on up to three occasions at one month intervals.

The values obtained for baseline citrated whole blood platelet count expressed as the mean (95% confidence limits) × 10^6 μl^{-1} were: guinea-pig 502 (270–734; $n = 67$) and Rhesus monkey 269 (102–436; $n = 23$). In any experiment where the correct volume of blood with respect to anticoagulant was not obtained, visible haemolysis was present, or a low baseline platelet count outside the normal range was recorded, the blood sample was discarded.

Platelet aggregation in whole blood

The method employed for monitoring platelet aggregatory responses in guinea-pig and Rhesus monkey whole blood was a modification of the single platelet counting technique first described by Nunn & White (1980) which we have described previously (Lad *et al.*, 1987). Briefly, each 0.5 ml aliquot of citrated blood was stirred in a 1 ml aggregation cuvette at 1000 r.p.m. and equilibrated to 37°C for 2 min before addition of either collagen at final concentrations in the range 0.1–3.0 μg ml^{-1} or buffer alone as control (dose volume always 5.0 μl). Sequential 50 μl samples of blood were removed from each incubation immediately prior to, and at 1 min intervals up to 4 min following the addition of the aggregatory stimulus.

These samples were transferred to 5 ml centrifugation tubes containing 950 μl of counting diluent (Coulter Isoton II), mixed, and immediately centrifuged (2 min at 150 g) in a Coulter Electronics Thrombofuge to obtain a dilute (1:19 v/v) platelet supernatant. This supernatant was then diluted a second time, 66 μl being taken and added to 20 ml Isoton II diluent (1:303 v/v) with thorough roller mixing, before being counted in a Coulter Thrombocounter (each sample was counted twice and the mean value used in subsequent data analysis).

The original blood platelet count in the aggregation cuvette at the time of sampling was derived by correction for the overall dilution factor in processing ($\times$ 6080). Agonist concentrations and responses to collagen obtained in whole blood have not been corrected for haematocrit. All samples were diluted, processed and counted immediately. Aggregation experiments were completed within 2 h of obtaining a blood sample.

The results of the blood platelet aggregation experiments are expressed in the text as overall decrease in single platelet count (mean% $\pm$ s.e.mean) either with time or at the end of the incubation (4 min after addition of agonist). Attenuation of the aggregatory response to collagen in those samples from EP 092 treated animals is expressed as inhibition (mean% $\pm$ s.e.mean) at 4 min, compared to decreases in single platelet count observed at an identical collagen concentration in blood samples from vehicle (control) dosed animals. Data have been analysed by Student's t test with $P < 0.05$ being taken as indicative of a significant result. All determinations were performed in duplicate for each blood sample and at each agonist concentration.

Intravascular platelet aggregation

The effect of EP 092 on collagen and thrombin-induced intravascular platelet aggregation *in vivo* was determined in male NZW rabbits (2.5–4.0 kg) anaesthetized with a combination of 5,5'-diallyl barbituric acid and urethane administered by intraperitoneal injection (Banerjee *et al.*, 1985). Each animal preparation was artifically ventilated with air via a tracheal cannula throughout the experiment using a Harvard respirator. Indwelling cannulae in the left femoral artery and vein were used to monitor arterial blood pressure via a Statham pressure transducer, and to administer collagen or thrombin. Finally, a specially designed double lumen indwelling cannula (Smith & Freuler, 1973) was inserted into the right external carotid artery, and was connected to a Technicon continuous flow platelet autocounter.

Anticoagulant (3.2% trisodium citrate; 0.015 ml min^{-1}) was pumped through the outer lumen of the tube to the tip of the cannula, which on mixing with carotid arterial blood at the cannula tip, was drawn out of the vessel via the inner cannula sleeve at a flow rate of 0.1 ml min^{-1} into the autocounter. Following a two stage treatment of the blood with a mixture of ammonium oxalate (1% w/v) and saponin (0.002% w/v) during which the erythrocytes were lysed and the blood diluted by a factor of 2880, the number of single platelets present was determined by continuous passage of the diluted platelet suspension through a flow cell focussed in a photo-multiplier tube particle counter. Platelet count was corrected for dilution and continuously recorded on chart paper which had been precalibrated with a standard of known cell count immediately prior to each experiment.

The platelet agonists collagen (30 μg kg^{-1}) or thrombin (10 units kg^{-1}) were administered as bolus intravenous injections via the femoral vein indwelling cannula, with a minimum period of 30 min being allowed to elapse between successive agonist doses. The first intravenous dose of agonist served to (a) ensure all dosing and monitoring cannulae were patent, (b) assess the robustness of the preparation on challenge (particularly with respect to mean arterial blood pressure, a fall in which accompanies the platelet response (Lad *et al.*, 1986)), and (c) confirm sensitive detection of platelet count changes with acceptably low background noise on the autocounter. However, this initial platelet response was frequently found to be inconsistent between preparations, and atypical of those obtained subsequently in any one preparation, being variously of greater or lesser magnitude. It was not clear why this should be so except in those cases when it reflected problems with (a)–(c) above, but consequently, the data were not included for analysis. Thereafter, each agonist elicited reproducible successive platelet responses in controls (see text) and was administered 30 min prior to, immediately following, and finally at 30 min after infusion of EP 092.

The results in the text are derived from the maximum fall in circulating platelet count achieved in response to a particular dose of collagen or thrombin, and are expressed either as a percentage decrease relative to the mean platelet count determined over the 3 min period immediately before injection of agonist, or as the percentage inhibition of the decrease in circulating single platelet count when EP 092 was present compared to pre-drug controls.

EP 092 (3 or 10 mg kg^{-1}) or vehicle alone (sodium bicarbonate buffer, pH 8.5) were administered intravenously to separate groups of rabbits over a 10 min infusion period (1.0 ml min^{-1}) via a lateral ear vein butterfly cannula. Responses obtained to either thrombin or collagen at 0, 30 and 60 min after drug

were analysed both by comparison with the pre-drug control response in the same animal (paired Student's t test), and with responses obtained in the vehicle alone treated group (unpaired Student's t test).

Estimation of thromboxane formation

The effect of EP 092 on the formation of thromboxane A_2 (TXA_2) which accompanies collagen-induced platelet aggregation in citrated whole blood *ex vivo* was investigated in Rhesus monkey samples. Each aggregation incubation was terminated at 4 min after addition of collagen as stated above, by addition of indomethacin (10 μM) and rapid cooling by immersion in an ice bath. The incubation mixture was transferred to an Eppendorf 1 ml capped tube, centrifuged (1 min, 10000 g), the plasma removed, and stored frozen (−20°C) until assay. Estimation of plasma TXA_2 formation was carried out by use of a specific radioimmunoassay procedure for TXB_2, the stable breakdown product of TXA_2. The radioimmunoassay contained commercially available [^{3}H]-TXB_2 (150 Ci mmol^{-1}; Amersham International plc) and rabbit antibody to TXB_2 (Calbiochem-Behring, California). The presence of EP 092 in plasma samples did not interfere with measurement of TXB_2 in the radioimmunoassay. Cross reaction with the antibody was found to be <0.01% at final EP 092 concentrations in the assay between 3.2 and 405 μM. The data shown in the text are expressed as specific collagen-induced increases in plasma TXB_2 (mean ± s.d.) derived by subtraction of basal (buffer incubated) control values.

Chemicals

Agents used in these studies were purchased from the following: collagen (Hormon-Chemie, Munich, F.R.G.), thrombin (Parke-Davis freeze dried material), urethane and 5,5'-diallylbarbituric acid (Sigma Chem. Co.). EP 092, (±)-5-endo-(6'carboxyhex-2'z'enyl)-6-exo-1'-[N-(phenyl thio carbamoyl)-hydrazono]-ethyl-bicyclo-(2,2,1)-heptane), was a generous gift from Drs R.L. Jones and N.H. Wilson (Department of Pharmacology, University of Edinburgh).

Results

Aggregatory responses to collagen in citrated whole blood

The collagen-induced platelet aggregatory response observed in samples of guinea-pig and Rhesus monkey citrated whole blood obtained from control animals is shown in Figure 1. In both cases, collagen (0.03–10.0 μg ml^{-1}) caused a rapid, concentration-

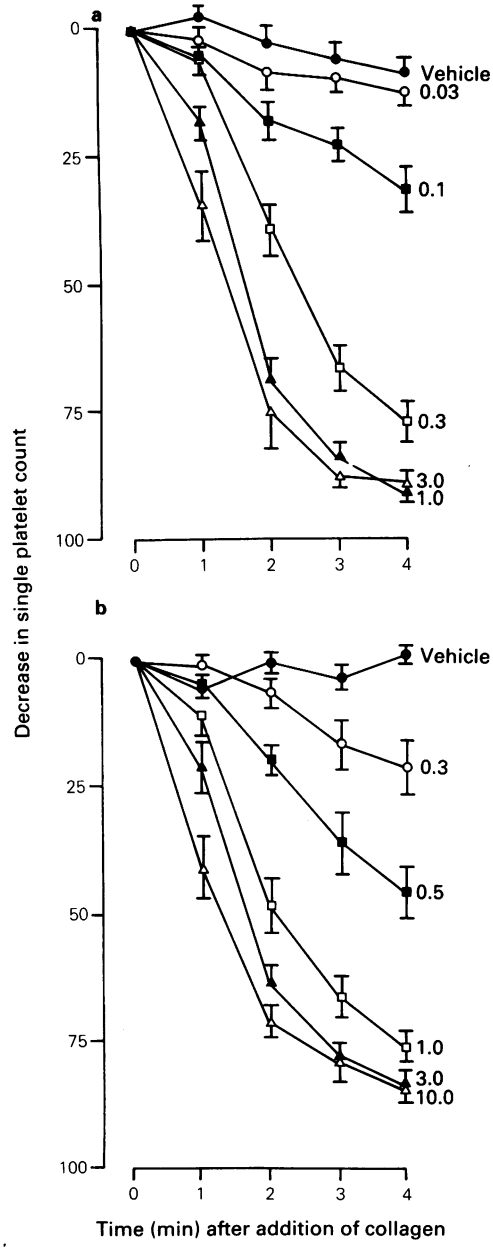

Figure 1 The effect of collagen on single platelet count over a 4 min incubation period in samples of citrated (a) guinea-pig (n = 18) and (b) Rhesus monkey (n = 22) whole blood *in vitro*. Concentrations of collagen added at 0 min are shown in μg ml^{-1}; the vehicle alone treatment was glycine buffer (pH 3.5) at the standard dose volume (5 μl). The data are expressed as the mean percentage decrease in count (s.e.mean shown by vertical bars) from baseline values obtained prior to the addition of collagen.

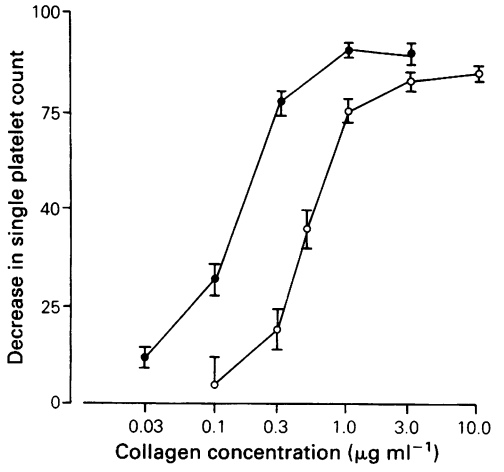

Figure 2 Concentration-effect curves for the platelet aggregatory response to collagen in guinea-pig (●) and Rhesus monkey (○) citrated whole blood *in vitro*. Results shown are decreases in single platelet count (mean % with s.e.mean shown by vertical bars) measured at 4 min after addition of agonist.

dependent decrease in single platelet count during the 4 min incubation period. Both the rate and extent of the decrease in platelet count were directly related to the initial collagen dose. In both species, the greatest decrease (approximately 90%) in platelet count was achieved at the higher collagen concentrations ($\geqslant 3\,\mu g\,ml^{-1}$). Although concentration-effect curves plotted for the response at 4 min in blood from both species were parallel (Figure 2), guinea-pig platelets in whole blood appeared threefold more sensitive to collagen than those from the Rhesus monkey. EC_{50} values obtained were 0.14 ± 0.02 and $0.48 \pm 0.4\,\mu g\,ml^{-1}$ (mean ± s.e.mean; $n > 15$) for guinea-pig and Rhesus monkey respectively. When vehicle alone was present, no consistent change in platelet count from baseline occurred, with an overall decrease in count being observed in guinea-pig blood samples of $8.7 \pm 3.1\%$ (509 ± 34 vs $465 \pm 32 \times 10^6\,\mu l^{-1}$; NS) and an increase in Rhesus monkey blood samples of $2.0 \pm 2.3\%$ (248 ± 19 vs $253 \pm 20 \times 10^6\,\mu l^{-1}$; NS) at 4 min after addition of collagen buffer. The addition of reagent vehicle and stirring of samples for the standard incubation period thus caused no significant non-specific platelet aggregation.

Effect of EP 092 on collagen-induced blood platelet aggregation ex vivo

Guinea-pig In these experiments EP 092 was administered either by intravenous injection (0.1–$3.0\,mg\,kg^{-1}$) as its sodium salt or by oral gavage

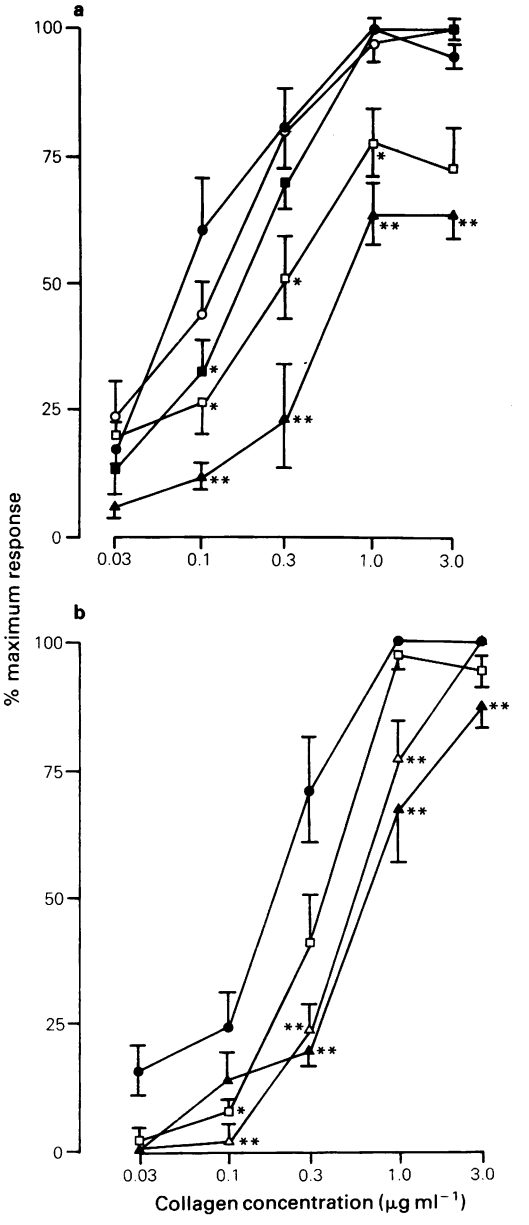

Figure 3 Effect of EP 092 on collagen-induced aggregation of citrated guinea-pig whole blood *ex vivo*. Drug was administered as an intravenous bolus injection and the animal killed at 30 min (a) or by oral gavage and the animals killed at 1 h (b). The results are expressed as the mean percentage of the maximum control (●) response (vertical bars show s.e.mean) ($n = 4$–6 animals per group). Doses of EP 092 shown were 0.1 (○), 0.3 (■), 1.0 (□), 3.0 (▲) and 10.0 (△) $mg\,kg^{-1}$ respectively, with statistical significance relative to controls indicated at the $P < 0.05$ (*) and $P < 0.01$ (**) levels.

(1.0–10.0 mg kg^{-1}) in PEG/CMC vehicle to groups of conscious male guinea-pigs. Initially, collagen-induced platelet aggregation was determined in individual samples of citrated blood obtained at 0.5 h (i.v.) and 1 h (orally) after dosing. EP 092 significantly inhibited the aggregatory response to collagen in a dose-related fashion when the compound was administered by either route (Figure 3). In both cases, the concentration-effect curves constructed to collagen were shifted in a progressive rightward manner with each increase in antagonist dose. EP 092 was a more potent inhibitor of the platelet responses to lower concentrations of collagen (0.03–0.3 μg ml^{-1}). Following intravenous (but not oral) administration of EP 092, a significant decrease in the maximum response achieved by collagen was observed at the higher antagonist doses (Figure 3a). ED_{50} values (mean ± s.e.mean) for EP 092 against responses to a submaximal collagen concentration of 0.3 μg ml^{-1} were 1.3 ± 0.2 mg kg^{-1} (i.v.) and 1.4 ± 0.2 mg kg^{-1} (orally) respectively.

In a second series of experiments, the duration of action of EP 092 (taken as the time after dose during which >50% inhibition of submaximal 0.3 μg ml^{-1} collagen responses was maintained) was investigated following intravenous (1.0 and 3.0 mg kg^{-1}) or oral (3.0 and 10.0 mg kg^{-1}) administration. Again, onset of inhibition of platelet responses *ex vivo* was rapid with pronounced inhibitory activity evident at 30 min after dosing and peak inhibitory effects by 1 h with either route of administration (Figure 4). Duration of action of the compound was dose-dependent. Determination of an accurate pharmacodynamic half life was not attempted from the limited data, but whilst the effect of 1.0 mg kg^{-1} i.v. was weak and transient, inhibitory activity following 3.0 mg kg^{-1} i.v. was evident for at least 3 h. Indeed, although the onset of effect (30 min) was more rapid following i.v. dosing, thereafter, the time-response curves for the 3.0 mg kg^{-1} dose level (i.e. approximately 2.0 × ED_{50}) by i.v. or oral route were remarkably similar (no significant difference at 1, 3 or 6 h time points). At the highest oral dose used (10 mg kg^{-1}) the duration of action was >6 h.

Rhesus monkey Platelet aggregation induced by collagen was monitored in citrated blood samples obtained at 1 and 3 h after dosing with EP 092 (0.3–10.0 mg kg^{-1}). EP 092 inhibited the aggregatory responses to collagen in a dose-dependent manner (Table 1). Estimated mean ED_{50} values for the compound against a submaximal collagen concentration (0.5 μg ml^{-1}) were 0.9 ± 0.3 mg kg^{-1} at 1 h and 1.8 ± 0.4 mg kg^{-1} at 3 h. Duration of action appeared to be between 1 and 3 h at 1.0 mg kg^{-1}, and greater than 3 h at 3.0 and 10.0 mg kg^{-1}. In both the Rhesus monkey and the guinea-pig, the degree of inhibition achieved was comparable at 3.0 and 10.0 mg kg^{-1} dose levels, with approximately 70% inhibition being observed at 3 h following either dose.

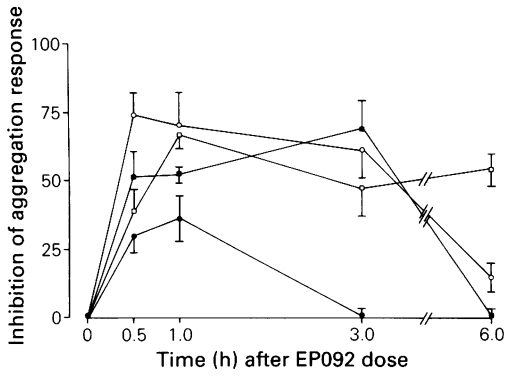

Figure 4 Duration of action of the inhibitory effect of EP 092 against collagen-induced guinea-pig citrated whole blood platelet aggregation *ex vivo*. Drug was administered either intravenously at 1.0 mg kg^{-1} (●) and 3.0 mg kg^{-1} (○) or orally at 3.0 mg kg^{-1} (■) and 10.0 mg kg^{-1} (□). Treatment groups ($n = 4$) were killed at the times shown, and blood platelet aggregation determined. The results are expressed as inhibition (mean % with s.e.mean shown by vertical bars) of the decrease in single platelet count induced by a submaximal collagen concentration (0.3 μg ml^{-1}) relative to the response in groups of control (vehicle alone) guinea-pigs killed at identical times.

In the *ex vivo* experiments with Rhesus monkey whole blood, plasma samples were obtained at termination of the 4 min aggregation incubation period to determine the effect of EP 092 on TXB_2 synthesis. Collagen-induced whole blood aggregation was accompanied by an agonist concentration-dependent

Table 1 Effect of EP 092 on collagen-induced Rhesus monkey blood platelet aggregation *ex vivo*

EP 092 (mg kg^{-1})	(n)	*Inhibition at time after oral EP 092 dose (h)* 1	3
0.3	4	22 ± 8	20 ± 15
1.0	4	52 ± 7*	33 ± 9
3.0	5	60 ± 12*	76 ± 8*
10.0	5	87 ± 7*	73 ± 6*

Values are mean ± s.e.mean
Inhibition data are expressed relative to the aggregatory response induced by 0.5 μg ml^{-1} collagen in blood samples from control (vehicle dosed) animals, where * = significance at the $P < 0.05$ level. Vehicle alone had no significant effect at either time interval after dosing.

Table 2 Collagen stimulated thromboxane B_2 (TXB_2) formation in Rhesus monkey blood

EP 092 ($mg\,kg^{-1}$)	n	*Sample Time*	*Increase in plasma TXB_2 ($ng\,ml^{-1}$) at collagen concentration:* $0.5\,\mu g\,ml^{-1}$	$1.0\,\mu g\,ml^{-1}$	$3.0\,\mu g\,ml^{-1}$	$10.0\,\mu g\,ml^{-1}$
		Pre-Drug	6.3 ± 1.6	20.4 ± 3.9	68.5 ± 13.8	255.3 ± 24.2
1.0	4	1 h	4.2 ± 2.1	15.6 ± 3.5	52.7 ± 14.2	255.2 ± 24.8
		3 h	5.3 ± 2.4	16.0 ± 6.0	66.9 ± 17.8	258.9 ± 41.6
		Pre-Drug	3.4 ± 0.9	20.5 ± 5.6	61.3 ± 12.8	338.1 ± 57.1
3.0	5	1 h	2.9 ± 0.9	16.8 ± 5.8	55.6 ± 15.0	281.9 ± 39.1
		3 h	3.8 ± 2.1	17.4 ± 5.2	45.6 ± 12.4	355.6 ± 65.2
		Pre-Drug	8.5 ± 3.4	24.7 ± 5.4	80.2 ± 19.2	390.2 ± 56.4
10.0	5	1 h	8.3 ± 2.8	27.5 ± 5.1	93.4 ± 18.3	347.7 ± 42.2
		3 h	7.1 ± 2.6	17.9 ± 2.3*	52.5 ± 18.8*	248.6 ± 50.5*

Platelet aggregation in citrated Rhesus monkey blood was induced by the addition of collagen at the concentrations stated. Basal levels of TXB_2 in collagen buffer-incubated samples for each treatment sample time ranged between 0.3–10.9 $ng\,ml^{-1}$ (mean ± s.d. 3.8 ± 0.5 $ng\,ml^{-1}$). The data shown in the table are specific collagen-stimulated increases in plasma TXB_2 (also mean ± s.d.) derived by subtraction of individual basal values, where * = $P < 0.05$ compared to pre-drug controls (paired t test).

increase in plasma TXB_2 levels. No significant effect of orally administered EP 092 on collagen-stimulated TXB_2 formation was observed at the 1.0 and 3.0 $mg\,kg^{-1}$ dose levels. Despite pronounced inhibition of the platelet aggregatory response to collagen (Table 1) plasma TXB_2 levels were unchanged compared to pre-dose samples (Table 2). However, at the highest dose of EP 092 (10 $mg\,kg^{-1}$) partial inhibition of TXB_2 formation was evident in the 3 h (but not 1 h) samples ranging 27–36% at 1.0–10.0 $\mu g\ ml^{-1}$ collagen concentrations in the incubation ($P < 0.05$; paired Student's t test).

Rabbit intravascular platelet aggregation in vivo

The effect of EP 092 on collagen and thrombin-induced transient thrombocytopoenia (intravascular platelet aggregation) in the anaesthetized rabbit preparation was examined by the circulating platelet count continuous monitoring technique. Baseline circulating platelet counts observed at the start of each experiment were in the range 3.5–7.0 × $10^8\,ml^{-1}$. In the case of collagen, the pretreatment control 3.0 $\mu g\,kg^{-1}$ i.v. dose (excluding the initial dose, see methods) caused decreases in platelet count ranging 19–34% (mean ± s.e.mean 29 ± 4%; $n = 22$). Further doses of collagen given immediately after and 30 min following administration of drug vehicle caused successive falls of 26 ± 7 and 25 ± 4% respectively (no significant difference from pre-dose controls). Similarly, for thrombin (10 units kg^{-1} i.v.), successive doses administered to vehicle-dosed preparations induced decreases in platelet count of 54 ± 8%, 49 ± 6% and 51 ± 8% respectively ($n = 14$). Thus, for each agonist, three successive responses to either collagen or thrombin were consistent for the duration of the experiment. However, the recovery in circulating platelet count which occurred in the period between agonist doses was slow (up to 15 min being necessary to establish a new baseline), and seldom complete, such that pre-dose counts decreased progressively throughout the experiment (Figure 5).

The administration of drug vehicle had no effect on platelet responses to collagen or thrombin. In

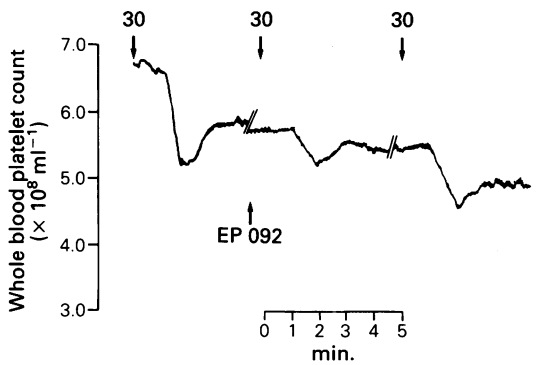

Figure 5 Effect of EP 092 on collagen-induced intravascular aggregation in the anaesthetized rabbit. The trace shows a representative experiment in which three successive responses to bolus i.v. injections of collagen (30 $\mu g\,kg^{-1}$) administered 30 min before, immediately following and a further 30 min after infusion of EP 092 (10 $mg\,kg^{-1}$) were observed. The recovery period between responses (30 min) is not shown in full and is represented by breaks in the trace.

Table 3 Effect of EP 092 on collagen and thrombin-induced intravascular platelet aggregation in the anaesthetized rabbit

Treatment	*Dose* ($mg\,kg^{-1}$)	n	*Inhibition of response to collagen* 0 min	30 min
Vehicle	0	11	-7 ± 5	5 ± 5
EP 092	3.0	6	-12 ± 6	-13 ± 6
EP 092	10.0	5	46 ± 8*	20 ± 8
			Inhibition of response to thrombin 0 min	30 min
Vehicle	0	14	-6 ± 7	-4 ± 13
EP 092	3.0	6	12 ± 17	20 ± 17
EP 092	10.0	4	35 ± 7**	26 ± 8

Responses to collagen ($30\,\mu g\,kg^{-1}$) and thrombin (10 units kg^{-1}) were determined in separate preparations (n) before and at 0 and 30 min after administration of EP 092. Drug was given by slow intravenous infusion over a 10 min period at rates of 0.3 and $1.0\,mg\,kg^{-1}\,min^{-1}$ respectively. Data shown are mean ± s.e.mean; statistical significance with respect to pre-drug and vehicle alone controls as indicated: $*P < 0.05$, $**P < 0.01$.

contrast (Table 3), immediately following the infusion of EP 092 at $10\,mg\,kg^{-1}$ but not $3.0\,mg\,kg^{-1}$, platelet responses to both collagen and thrombin were partially but significantly inhibited (Table 3). However, even at the highest EP 092 dose level, the inhibitory effect was of short duration, since no significant effect was evident after a further 30 min.

Discussion

Although the relative potency of PGH_2 and TXA_2 as platelet agonists *in vitro* has been clearly defined (Bhagwat *et al.*, 1985b), the respective contribution of each as secondary mediators in platelet aggregatory responses initiated by collagen or thrombin *in vitro*, and particularly *in vivo*, remains less clear. In PRP, collagen-induced aggregatory responses appear largely dependent on two pathways; ADP secretion from dense granules and the formation of PGH_2 and TXA_2 from platelet phospholipids following activation of phospholipase (Kinlough-Rathbone *et al.*, 1977; Vargaftig *et al.*, 1980; Lewis & Watts, 1982). TXA_2 synthase inhibitors such as dazoxiben or OKY1581 inhibit threshold responses to collagen in human PRP, but are ineffective against aggregation induced by higher concentrations of collagen (Randall *et al.*, 1981; Ito *et al.*, 1983). This latter observation coupled with the more pronounced inhibitory activity of cyclo-oxygenase inhibitors and PGH_2/TXA_2 receptor antagonists (Patscheke & Stegmeier, 1984; Ogletree *et al.*, 1985) at higher collagen concentrations implies that in these circumstances, PGH_2 may play an increasingly important role (Hornby & Skidmore, 1982). However, in whole blood, the activity of both agonists and inhibitors may vary, and may also be influenced by other cells present and potentially also by other mediators such as prostaglandin I_2 or platelet activating factor (Morley *et al.*, 1979; Kennedy *et al.*, 1980; Abbate *et al.*, 1986).

In the *ex vivo* studies with guinea-pig and Rhesus monkey citrated whole blood, collagen caused concentration-dependent aggregatory responses reflected in a rapid decrease in single platelet count detectable within 1–2 min. The range of collagen concentrations at which an aggregatory response was observed was similar to that observed previously for both rodent and human blood (Saniabadi *et al.*, 1983; Lad *et al.*, 1987). Administration of EP 092 to guinea-pigs or Rhesus monkeys potently inhibited collagen-induced aggregatory responses in whole blood *ex vivo*, indicating a significant role for PGH_2 and TXA_2 in these responses. However, whilst a similar magnitude of inhibitory effect (between 70 and 80% inhibition) was achieved in both species at comparable oral doses of EP 092, full inhibition was not achieved in either case, indicating that additional mediators such as ADP are important in whole blood. The highest oral dose of EP 092 tested in the guinea-pig ($10\,mg\,kg^{-1}$) merely extended the duration of the inhibitory effect of EP 092 to >6 h, rather than its magnitude. Also in the guinea-pig, the ratio of the intravenous and oral ED_{50} values for EP 092 (1.3 ± 0.2 and $1.4 \pm 0.2\,mg\,kg^{-1}$ at 30 min and 1 h after dosing respectively) was close to unity, which suggests that the compound had been rapidly and efficiently absorbed from the gastrointestinal tract following oral dosing.

In the Rhesus monkey, a similar oral ED_{50} was obtained ($0.9 \pm 0.3\,mg\,kg^{-1}$), and the inhibitory effect of EP 092 on aggregation was not accompa-

nied by any significant reduction in collagen stimulated TXB_2 formation except at 3 h after the highest EP 092 dose ($10\,mg\,kg^{-1}$). These data suggest that aggregation is not a prerequisite for collagen-induced TXB_2 synthesis in platelets, and equally, that EP 092 causes substantial inhibition of the platelet response to collagen, independent of any effect on TXB_2 formation until high doses of the compound ($10 \times ED_{50}$) are administered. Our results closely mirror *in vitro* platelet data previously obtained for EP 092 (Armstrong *et al.*, 1985a), where a partial inhibitory effect on TXB_2 formation from arachidonic acid was noted only at higher *in vitro* concentrations of EP 092. The present data therefore support the earlier conclusion that with EP 092, inhibition of the endogenous thromboxane component of aggregation is likely to be due to thromboxane receptor antagonism rather than inhibition of biosynthesis. Furthermore, Darius *et al.* (1985) have also found that with the PGH_2/TXA_2 receptor antagonist SQ29548, an attenuation of the aggregatory response of rabbit platelets to arachidonic acid was not accompanied by any significant reduction in the formation of TXB_2.

In the dog, collagen-induced aggregatory responses in whole blood *ex vivo* have been shown to be potently inhibited by administration of the selective PGH_2/TXA_2 antagonists, AH23848 and GR32191 (Humphrey & Lumley, 1984; Lumley *et al.*, 1988). The present results demonstrate that platelet aggregatory responses to collagen can also be routinely monitored in citrated whole blood samples from a non human primate and that a selective PGH_2/TXA_2 antagonist is active in this species. Reports in the literature concerning platelet behaviour in blood or PRP samples obtained from Rhesus monkeys are few. It has been claimed that platelets in PRP from this species respond to agonists in a similar way to human platelets (Addonizio *et al.*, 1978). Rhesus monkey platelets synthesize TXB_2 in response to addition of arachidonic acid (Gorman *et al.*, 1983) or collagen (our data), and are susceptible to inhibition of U-44069-induced aggregation in PRP responses *ex vivo* by the PGH_2/TXA_2 receptor antagonist L-655240 (Hall *et al.*, 1987). In the present experiments, Rhesus monkey platelets in whole blood responded with a sensitivity to collagen (EC_{50} $0.48 \pm 0.4\,\mu g\,ml^{-1}$) almost identical to that of human whole blood under the same experimental conditions (EC_{50} $0.53 \pm 0.1\,\mu g\,ml^{-1}$; Lad *et al.*, 1987).

In the anaesthetized rabbit preparation, EP 092 also demonstrated inhibitory activity against collagen-induced intravascular aggregation (transient thrombocytopoenia) assessed by continous platelet count monitoring. EP 092 was, however, less effective in this model such that an intravenous dose of $10\,mg\,kg^{-1}$ was necessary to achieve a significant inhibitory effect compared to an intravenous ED_{50} of $1.3\,mg\,kg^{-1}$ in the guinea-pig *ex vivo* test. The apparent difference in the potency of EP 092 between species may be due to the different test design employed (*in vivo* or *ex vivo*), but this seems unlikely, since EP 092 has been reported to be as effective at 0.25 and $1.0\,mg\,kg^{-1}$ in the guinea-pig against collagen-induced intravascular aggregation and bronchoconstriction (Jones *et al.*, 1983). Furthermore, a close analogue, EP 045, is active at $1.0\,mg\,kg^{-1}$ against collagen responses in the guinea-pig (Mallarkey & Smith, 1985). It seems improbable that the rate of metabolism and excretion of EP 092 should differ to such an extent in the rabbit that the observed inhibitory potency of EP 092 against collagen-induced platelet responses *in vivo* is reduced by almost an order of magnitude, and its duration of action decreased from >6 h to <0.5 h at $10\,mg\,kg^{-1}$. More plausible is the possibility that the relative affinity of the antagonist for the platelet PGH_2/TXA_2 receptors varies between species due to receptor heterogeneity. Some support for this hypothesis is provided by the observation that EP 092 is significantly less active against 11,9-epoxymethano-PGH_2-induced aggregation of rabbit washed platelet preparations compared to those of rat or man (Jones *et al.*, 1987). Moreover, it has also been reported that rabbit PRP is relatively insensitive to the aggregatory action of the TXA_2 analogue STA_2 (Narumiya *et al.*, 1986). Clarification would be provided by comparative EP 092 receptor-binding studies with animal platelets, but these have yet to be carried out. Finally, it is conceivable that the amounts of intraplatelet PGH_2 and TXA_2 generated in response to collagen, and the relative contribution of these agonists to the observed aggregatory response varies between species. It is noteworthy that whereas up to 80% inhibition of the collagen platelet response was observed with EP 092 in the guinea-pig, the maximal inhibitory effect achieved *in vivo* in the rabbit was between 40 and 50%. A maximal inhibitory effect of approximately 50% has previously been reported in the rabbit collagen-induced intravascular aggregation model with indomethacin (Honey *et al.*, 1986) and with two other selective PGH_2/TXA_2 antagonists SQ29548 and BM13177 (Darius & Lefer, 1985).

Perhaps most surprising was the partial inhibitory activity observed with EP 092 against thrombin-induced intravascular aggregation in the rabbit. Although administration of intravenous bolus doses of thrombin causes a substantial increase in plasma TXB_2 levels in this model, PGH_2 and TXB_2 do not appear to contribute directly to the thrombin-induced aggregatory response since indomethacin is inactive *in vivo* (Honey *et al.*, 1986) and *in vitro*

(Kinlough-Rathbone *et al.*, 1977). Since thrombin is also a potent stimulant of PGI_2 formation in vascular endothelial cells (Weksler *et al.*, 1978), it is conceivable that in the presence of a selective inhibitor of the action of PGH_2 and TXA_2, a weak inhibitory effect on platelet aggregation *in vivo* due to release of vascular tissue derived PGI_2 may be unmasked.

In conclusion, the potent and selective platelet PGH_2/TXA_2 receptor antagonist EP 092 exhibited marked inhibitory activity against collagen-induced whole blood aggregation *ex vivo* in the guinea-pig and Rhesus monkey, and against collagen and thrombin-induced *in vivo* intravascular aggregatory responses in the rabbit. The present data and results reported previously (Jones *et al.*, 1983) suggest that its activity as an inhibitor of PGH_2/TXA_2 dependent platelet responses varies between species with a relative potency order: guinea-pig $\simeq$ Rhesus monkey > rat > rabbit. As the compound is also a potent inhibitor of human platelet aggregation *in vitro* (Armstrong *et al.*, 1985a), we suggest these findings support evaluation of its therapeutic potential as an anti-thrombotic agent in man.

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A local circuit neocortical synapse that operates via both NMDA and non-NMDA receptors

[1]Alex M. Thomson, Debbie Girdlestone & David C. West

Department of Physiology, University College, P.O. Box 78, Cardiff CF1 1XL

1 In slices of rat neocortex, spike triggered averaging was employed to record in one neurone the excitatory postsynaptic potential (e.p.s.p.) generated by a spike in another, neighbouring neurone. When recorded at different membrane potentials, some of these e.p.s.ps exhibited a voltage relation typical of neuronal responses to N-methyl-D-aspartate (NMDA).

2 Selective NMDA antagonists reduced the amplitude of these e.p.s.ps, but had little effect on their early rising phase. In contrast, a less selective excitatory amino acid antagonist reduced all phases of the e.p.s.p.

3 By analyzing single axon e.p.s.ps we have been able to establish that the synaptic input to one cortical cell, delivered by a single presynaptic cortical cell, operates simultaneously via NMDA and non-NMDA amino acid receptors.

Introduction

N-methyl-D-aspartate (NMDA) receptors have been implicated in synaptic plasticity during cortical development (Rauscheker & Hahn, 1987), in memory (Morris *et al.*, 1986) and epilepsy (Meldrum, 1984). We have previously demonstrated that one small component of the input to cortical pyramidal neurones was mediated by these receptors. It was not, however, clear in these early experiments whether afferent inputs acted directly through NMDA receptors, or whether an intracortical neurone was interposed in the pathway (Thomson, 1986). An equally important question that arose (Forsythe & Westbrook, 1988) was whether transmission at a given synapse could be mediated by NMDA receptors alone, or whether other excitatory amino acid receptors were involved. Electrical stimulation of afferent pathways activates many presynaptic elements and cannot be used to answer this question. Accordingly, spike triggered averaging (Jankowska & Roberts, 1972) and electrophoretic pharmacology were combined, to study the synaptic connections between pairs of cortical neurones. The excitatory postsynaptic potential (e.p.s.p.) generated in one neurone by an action potential in another was recorded and challenged with amino acid antagonists.

[1] Author for correspondence at present address: Dept. Physiology, Royal Free Hospital Medical School, Rowland Hill Street, London NW3 2PF.

Methods

Coronal slices of cingulate/sensorimotor cortex, 400 μm thick, from young adult female rats were maintained at 35°C at the interface between artificial cerebrospinal fluid (composition mM: NaCl 124, $NaHCO_3$ 25.5, KCl 3.3, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgCl_2$ 1.0 and D-glucose 10) and 95% O_2 : 5% CO_2. Conventional intracellular recordings were made from putative postsynaptic neurones in layer III with 3 M K acetate filled micropipettes (resistance 60–130 MΩ). Membrane potential was altered by intracellular current injection and electrode balance monitored by observation of voltage responses to small injected current pulses. Extracellular recordings were obtained from putative presynaptic neurones in layers III/IV, 50 to 200 μm from the intracellular recording site, with a multibarrel pipette containing, 3 M NaCl (recording barrel), 200 mM L-glutamate (pH 8) and 200 mM NaCl (balance barrel). These action potentials were used to trigger averages of the simultaneous intracellular recording. Amino acid antagonists, D-2-amino-5-phosphonovaleric acid (AP5), 3-(2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid (CPP) and γ-D-glutamylglycine (γDGG) (5 mM in 200 mM NaCl, pH8) were applied electrophoretically from a second multibarrel pipette placed within 50 μm of the intracellular recording site. The selectivity of these antagonists against

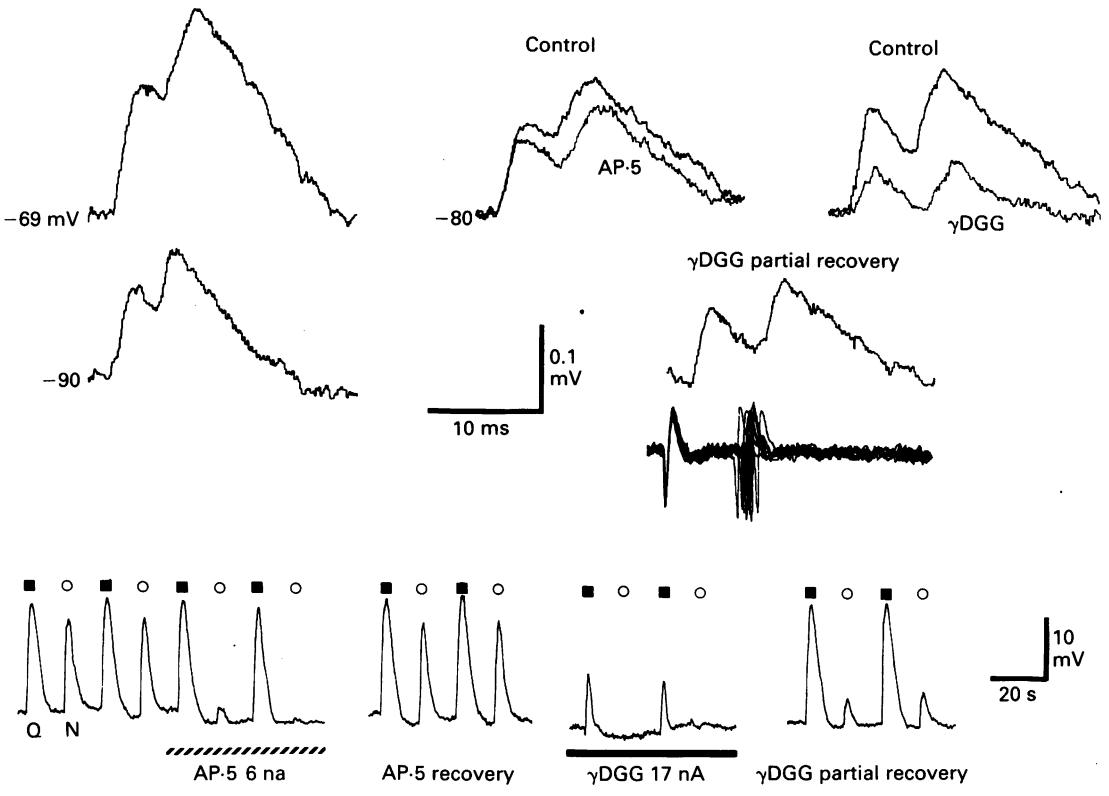

Figure 1 Spike triggered averages of an excitatory postsynaptic potential recorded at three membrane potentials (−69, −80 and −90 mV) under control conditions, and during electrophoretic ejection of D-2-amino-5-phosphonovaleric acid (AP5, 6 nA) and γ-D-glutamylglycine (γDGG, 17 nA) at −80 mV. Each record is an average of 512 sweeps. Ten superimposed sweeps of the extracellular recording from the presynaptic neurone are shown and demonstrate that this cell fired pairs of spikes, at short intervals, evoking a double e.p.s.p. in the postsynaptic cell. All averaged sweeps were triggered by spike pairs. The variability in the latency and rise time of the second e.p.s.p. is due to inevitable small changes in the duration and constancy of the interspike interval during the 3 h recording period. Although AP5 reduced the e.p.s.p. amplitude, it had little effect on the early rising phase. In contrast, γDGG reduced all phases of the e.p.s.p. The lower records illustrate the responses of the postsynaptic neurone to N-methyl-D-aspartate (NMDA, N: 14 nA, 1s) and quisqualate (Q: 60 nA, 1s) and their sensitivity to AP5 (6 nA) and γDGG (17 nA). Similar reductions in both the response to NMDA and in the e.p.s.p. are seen during application of AP5 and during partial recovery following γDGG. More complete blockade of the e.p.s.p., particularly its rising phase, was always associated with blockade of responses to quisqualate.

responses of the postsynaptic cell to electrophoretically applied NMDA and quisqualate (200 mM, pH 8) or α-amino-3-hydroxy-5-methylisoxalone-4-propionic acid (AMPA) (5 mM, pH 8) was tested.

Results

Simultaneous recordings were made from 780 pairs of cortical neurones comprising 133 intracellularly recorded neurones in layer III and 768 extracellularly recorded neurones in layers III and IV. The extracellularly recorded neurones were generally silent at rest, but responded to electrophoretically applied glutamate, indicating that recorded activity was due to activation of the soma/dendrites of layer III/IV cells, not to activity in axons en passage. Of the simultaneously recorded pairs of neurones, 35 were demonstrated to be synaptically connected. Presynaptic neurones were between 50 and 200 μm ventral and up to 50 μm lateral or medial to the postsynaptic neurones.

Single axon e.p.s.ps averaged at resting potential (RP) displayed peak amplitudes between 0.04 and 1.61 mV. Twenty four were recorded at more than

one membrane potential and of these, 19 had voltage relations characteristic of neuronal responses to NMDA, i.e. they increased in amplitude and duration, when recorded at more depolarized membrane potentials (Nowak *et al.*, 1984). To confirm that these were indeed mediated by NMDA receptors, their sensitivity to the antagonists AP5 (Watkins & Evans, 1981) and CPP (Davies *et al.*, 1986) was tested. AP5 ($n = 7$) and CPP ($n = 3$) reduced the amplitude and duration of the e.p.s.ps tested. Currents of antagonist that blocked NMDA responses by >80%, but had little effect on quisqualate responses, reduced e.p.s.p. amplitudes by between 16 and 65% (mean 27.5%) and widths at half amplitude by between 20 and 50% (mean 29.2%). Figure 1 shows that NMDA antagonists typically reduced the later component of the e.p.s.p., leaving the early rising phase relatively unaffected. A similar, partial blockade of unitary e.p.s.ps was seen in immature central neurones in culture, by Forsythe & Westbrook (1988) who concluded that the early fast component was mediated by non-NMDA amino acid receptors and the later slower component by NMDA receptors.

Three of the e.p.s.ps were therefore also challenged with the less selective amino acid antagonist, γDGG, which reduced NMDA and quisqualate responses by >80 and >60% respectively. γDGG produced a larger decrease in the e.p.s.p. than either AP5 or CPP and had a pronounced effect on its early rising phase resulting in a reduced rate of rise, but no significant change in time to peak (see Figure 1).

Discussion

These results demonstrate that NMDA receptor-mediated synapses received by layer III pyramidal neurones arise from neurones within the cortex. The greater sensitivity of these e.p.s.ps to the less selective amino acid antagonist γDGG, than to selective NMDA antagonists indicates that they consist of both late NMDA and earlier non-NMDA receptor-mediated components. Since the antagonists were applied focally, close to the soma of the postsynaptic neurone, it is unlikely that the pharmacologically distinct components involve wide spatial separation of the different postsynaptic receptor types. The possibility arises that a single presynaptic bouton may act via both NMDA and non-NMDA receptors. Whether a single transmitter activates both receptor subtypes, or whether two transmitters are released by a single presynaptic neurone remains to be determined.

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Modulation of the $GABA_A$ receptor complex by steroids in slices of rat cuneate nucleus

[1]J.P. Turner & [2]M.A. Simmonds

MRC Neuropharmacology Research Group, Department of Pharmacology, The School of Pharmacy, 29/39 Brunswick Square, London WC1N 1AX

1 Several derivatives of pregnane and androstane that have hypnotic properties have been investigated for their ability to potentiate responses to the $GABA_A$ receptor agonist muscimol and to reduce the effect of the non-competitive $GABA_A$ antagonist picrotoxin.

2 Depolarizing responses to muscimol in slices of rat cuneate nucleus were potentiated most potently by 3α-hydroxy,5α-pregnane-11,20-dione (alphaxalone), which gave half-maximal potentiation at 0.15 μM. The 5β isomer of alphaxalone had little effect up to 3 μM but in analogues lacking an 11-keto substituent (pregnanolones), both the 5α- and 5β-isomers potentiated with potencies 20 and 10 times lower, respectively, than that of alphaxalone. The α configuration of the 3-hydroxy was essential in alphaxalone, the 3β-hydroxy isomer being inactive. However, there was little difference between the potencies of the 3α- and 3β-hydroxy configurations in the pregnanolones, although the maximal effects of the 3β-hydroxy isomers were rather lower than those of the 3α-hydroxy isomers.

3 Reductions in the effect of picrotoxin as an antagonist of muscimol were caused most potently by the 3α-hydroxy pregnanolones, with a ten fold reduction in picrotoxin potency at 1 μM concentrations of these steroids. Alphaxalone and its 5β-isomer were about half as potent. Androsterone was about 10 times less potent and the 3β-hydroxy pregnanolones were ineffective.

4 This difference in the structure-activity relationships for steroidal potentiation of muscimol and reduction in picrotoxin antagonism of muscimol is reminiscent of an analogous distinction found with the barbiturates.

Introduction

The steroid anaesthetic alphaxalone potentiates the $GABA_A$ receptor-mediated effects of GABA and muscimol and enhances their binding to central neurones in a pentobarbitone-like manner (Harrison & Simmonds, 1984). Alphaxalone also prolongs the open times of GABA-operated chloride channels in cultured spinal cord neurones from rat and mouse (Barker *et al.*, 1986; 1987; Cottrell *et al.*, 1987a), again resembling the effects of pentobarbitone (Mathers & Barker, 1980; Mathers, 1985). In high concentrations, both alphaxalone and pentobarbitone can themselves cause long-duration openings of the same population of chloride channels. Similar observations have been made in bovine chromaffin cells in culture (Cottrell *et al.*, 1986; 1987b).

Other steroids closely related to alphaxalone, including some that are endogenously occurring, also have hypnotic properties (Atkinson *et al.*, 1965; Holzbauer, 1976) and act in a similar way. For example, two of the pregnanolones which are reduced metabolites of progesterone, namely, the 5α- and 5β-configurations of 3α-hydroxy,pregnane-20-one, have been shown to potentiate GABA and muscimol (Callachan *et al.*, 1986; 1987; Majewska *et al.*, 1986; Harrison *et al.*, 1987; Kirkness *et al.*, 1987; Simmonds & Turner, 1987) as has 3α,5α-tetrahydrodeoxycorticosterone, the reduced metabolite of deoxycorticosterone, (Majewska *et al.*, 1986; Harrison *et al.*, 1987) and androsterone, a reduced analogue of testosterone (Simmonds *et al.*, 1984). In contrast, progesterone, deoxycorticosterone and testosterone were each reported to be inactive in this respect.

Another feature of barbiturate interactions with the $GABA_A$ receptor complex is the ability to reduce the potency of the non-competitive GABA antagonist picrotoxin in slices of rat cuneate nucleus (Harrison & Simmonds, 1983) and to reduce ligand

[1] Present address: Department of Neurology, Institute of Psychiatry, De Crespigny Park, London SE5 8AF.
[2] Author for correspondence.

binding at the picrotoxin site in neuronal membranes (Olsen *et al.*, 1979; Ticku & Rastogi, 1986). It is clear that this is a distinct effect from the potentiation of muscimol, with a different structure-activity relationship across a series of barbiturates (Harrison & Simmonds, 1983). Preliminary experiments with alphaxalone and androsterone suggested that steroids too can reduce picrotoxin potency on the cuneate nucleus slice (Simmonds *et al.*, 1984). This has been supported by a study of a wide range of steroids that were found to reduce the binding of *t*-butylbicyclophosphorothionate (TBPS) to the picrotoxin site, although there was no evidence that the structural requirements for this effect were any different from those of potentiation of GABA (Harrison *et al.*, 1987). In this latter respect, the present work on the cuneate nucleus slice preparation leads to a different conclusion.

Methods

Experiments were performed on slices of rat medulla oblongata containing the dorsal funiculus and cuneate nucleus (Simmonds, 1978; 1980). Each slice was placed in a two-compartment bath so that the dorsal funiculus projected through a grease-filled gap in the barrier separating the two compartments. The slices were superfused with Krebs medium at room temperature and drugs were incorporated into the medium that perfused the compartment containing the terminals of the dorsal funiculus within the cuneate nucleus. The potential difference between the two compartments was recorded continuously and negativity induced in the drug-perfused compartment was interpreted as a depolarization of the terminals of dorsal funiculus fibres projecting through the barrier. Responses were measured at their peak amplitudes.

Muscimol (Fluka) was used as the $GABA_A$ receptor agonist. To minimize the problem of densensitization, control responses in the lower half of the log dose-response curve were used and these were routinely obtained from 2 min superfusions of 2.5 and 5 μM muscimol. The steroids to be tested were perfused for 30–40 min before and the perfusion continued during the re-determination of agonist responses. Concentrations of muscimol were found that produced responses overlapping the range of the control responses. Parallel shifts of the log dose-response lines allowed equi-effective dose-ratios for muscimol to be calculated as measures of potentiation or antagonism.

The effect of steroids on picrotoxin potency was determined by comparing the Schild plots of log (muscimol dose-ratio − 1) versus log picrotoxin concentration in the presence and absence of the steroids. The experiments followed a paired design on the two slices obtained from each rat. On one slice, a control Schild plot for 3, 30 and 300 μM picrotoxin was constructed in the absence of steroid. On the other slice, the effect of a steroid on responses to muscimol was first determined and then, in the continued presence of the steroid, a Schild plot for picrotoxin was constructed. A few experiments were performed with bicuculline in the place of picrotoxin.

The Krebs medium contained (mM): NaCl 118, KCl 2.1, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 2.2, $NaHCO_3$ 25 and glucose 11 and was continuously gassed with 95% O_2 : 5% CO_2. Picrotoxin (Sigma) was dissolved directly in the Krebs medium and bicuculline (Sigma) was prepared as a 10 mM solution in 0.01 M HCl for dilution into the Krebs medium just before use. The steroids listed in Table 1 were prepared as 1–100 mM solutions in ethanol for dilution into the Krebs medium. The final concentration of ethanol never exceeded 0.1% which we have shown not to affect responses to muscimol on this preparation. All the steroids were obtained from Sigma except alphaxalone which was a gift from Glaxo.

Results

Direct effects of steroids

The initial exposure of cuneate nucleus slices to low concentrations of steroid tended to produce small and inconsistent direct depolarization responses that faded within a few minutes. With several of the steroids, however, concentrations were reached at which more consistent and sustained direct depolarization responses occurred (e.g. Figure 1). Table 1 shows the threshold concentrations of steroid that caused statistically significant depolarizations with mean values greater than 0.1 mV in replicate experiments. Some degree of reversal of the responses could be obtained after washing out the steroid for 3 h. Although the pregnanolone 3α-hydroxy,5β-pregnane-20-one caused a direct depolarization at much lower concentrations than the other steroids, the threshold response did not further increase in amplitude as the concentration of this pregnanolone was increased up to 100 fold.

Modulation of muscimol responses

With increasing concentrations of steroid from 0.03 μM, the lowest concentration tested, the first statistically significant effect on the responses to muscimol was a potentiation. This was seen as an approximately parallel leftwards shift of the musci-

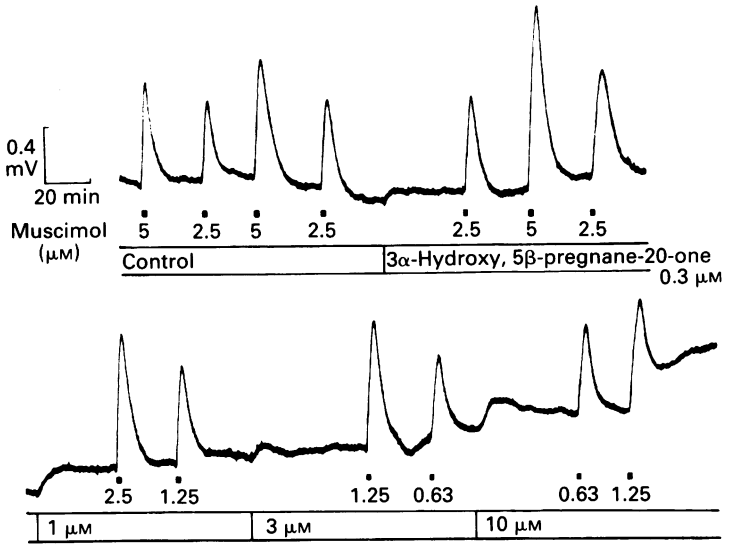


Figure 1 Original tracing of typical responses to muscimol on the cuneate nucleus slice and the effect of 3α-hydroxy,5β-pregnane-20-one, 0.3–10 μM. Applications of muscimol were for periods of 2 min. The two segments of trace are continuous.

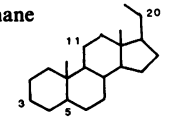
mol log dose-response curve but it was often accompanied by a reduction in the maximal response to muscimol. An example of this with 3α-hydroxy,5β-pregnane-20-one is shown in Figure 2. The reduction in maximal response was not systematically studied but the potentiating phenomenon was routinely measured from the shift of the lower part of the muscimol log concentration-response curve.

Table 1 Equi-effective concentrations of steroid for (A) direct effect, (B) potentiation of muscimol and (C) reduction in picrotoxin potency; also shown (D) is the extent of the reduction in picrotoxin potency by the steroid concentrations under B

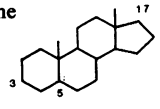
	Concentrations (μM) for			
*Steroids**	*A*	*B*	*C*	*D*
3α-Hydroxy,5α-pregnane-11,20-dione (alphaxalone)	3	0.15	1.75	NS
3α-Hydroxy,5β-pregnane-11,20-dione	3	>3	0.98	1.05 (at 3 μM)
3α-Hydroxy,5α-pregnane-20-one	10	3.2	0.78	≈1.38
3α-Hydroxy,5β-pregnane-20-one	0.1	1.4	0.51	1.38
3β-Hydroxy,5α-pregnane-20-one	>10	2.4	>10	NS
3β-Hydroxy,5β-pregnane-20-one (4 isomers of pregnanolone)	>10	2.4	>10	NS
3α-Hydroxy,5α-androstane-17-one (androsterone)	100	1.7	9.0	0.32
3β-Hydroxy,5α-androstane-17-one	>10	>10		
17-Hydroxy,5α-androstane-3-one (stanolone)	>30	>100	>30	
5α-Androstane-3,17-dione	>30	>100	>30	

A: Threshold concentration for a statistically significant depolarization of 0.1 mV or more.
B: Concentration that potentiates muscimol by 0.15 log unit.
C: Concentration that shifts the picrotoxin Schild plot to the right by 0.8 log unit.
D: Shift to the right (log unit) of the picrotoxin Schild plot by concentrations of steroid that potentiate muscimol by 0.15 log unit (B). (NS = no shift).

* Pregnane



Androstane



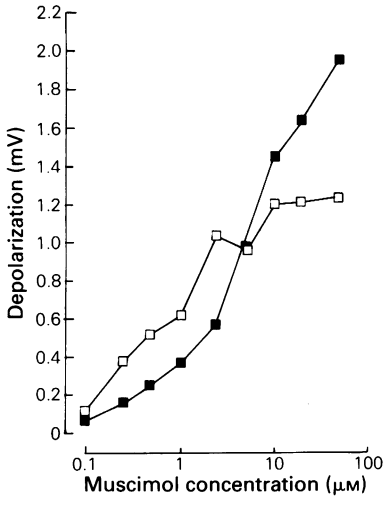


Figure 2 Dose-response curves for muscimol in the absence (■) and presence (□) of 1 μM 3α-hydroxy,5β-pregnane-20-one, showing a parallel leftward shift and simultaneous depression of the maximum. The single data points are from one experiment.

Several of the steroids proved capable of causing dose-related potentiations of muscimol up to a maximum shift of 0.3–0.4 log unit whilst others caused smaller potentiations that did not increase much above threshold levels with increasing dose of steroid. The data are shown in Figure 3a–c for three isomeric pairs of 5α- and 5β-steroids based on pregnane and in Figure 3d for four steroids based on androstane. With the 3α-hydroxy,pregnane-11,20-dione isomers, it is clear that the 5α-configuration is much more favourable for muscimol potentiation than is the 5β-configuration (Figure 3a). However, with the 3α-hydroxy,pregnane-20-one isomers that have no 11-keto substituent, the 5-configuration makes less difference and, indeed, 5β- appears more favourable than 5α- at threshold levels of muscimol potentiation (Figure 3b). Likewise, with the 3β-hydroxy,pregnane-20-one isomers, there was little difference in potency between the 5α- and 5β- configurations except that the potentiating activity of the 5α- isomer was lost at high concentrations (Figure 3c). Compared with the 3α-hydroxy, pregnane-20-one isomers, the 3β-hydroxy,pregnane-

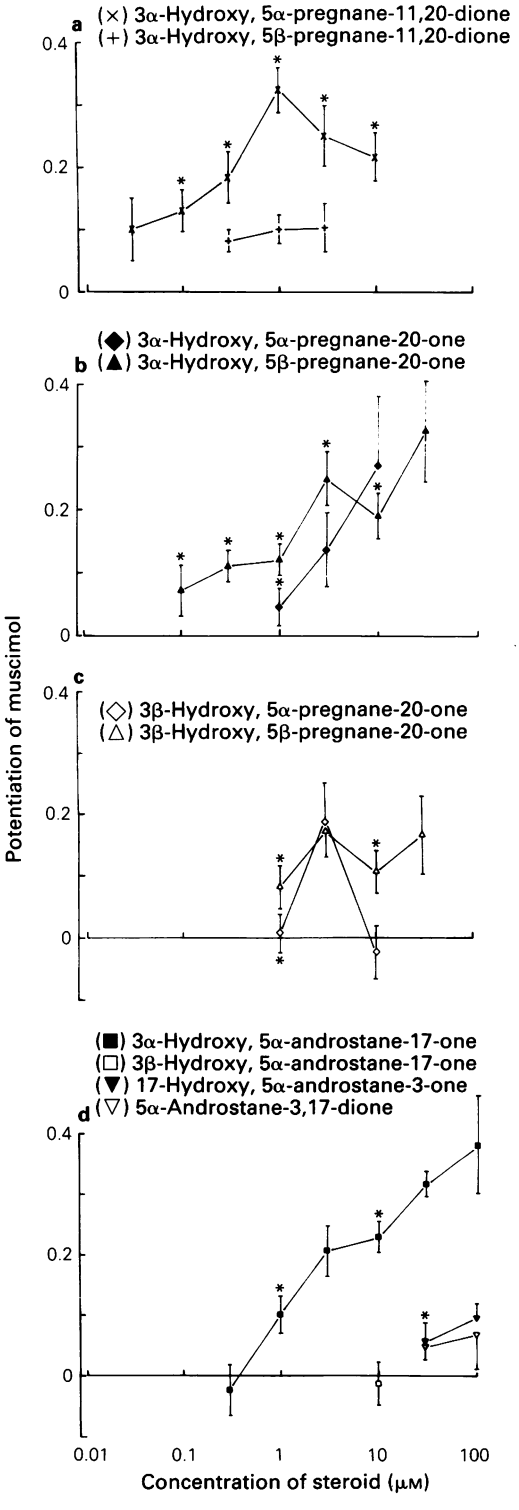


Figure 3 Potentiation of muscimol, expressed as the leftwards shit (log unit) of the muscimol log dose-response line, due to various concentrations of steroids. Values shown are the mean (s.e.mean indicated by vertical bars) from groups of 4–34 experiments; groups of 10 or more experiments are indicated by a * above or below the error bar.

20-one isomers caused a rather lower maximal potentiation of muscimol but the concentrations required for threshold effects were not very different. With the androstane steroids, the requirement for a 3α-hydroxy substituent was much stronger. Compared with 3α-hydroxy,5α-androstane-17-one, which had a similar potency to its pregnanolone analogue, the 3β-hydroxy isomer was at least 30 fold less potent as a potentiator of muscimol (Figure 3d). Also, replacement of the 3α-hydroxy with a keto group in both 17-hydroxy,5α-androstane-3-one and 5α-androstane-3,17-dione resulted in a 50–100 fold loss of potency.

For a more precise comparison of the potencies of those steroids that caused a clear potentiation of muscimol, the concentrations required to shift the muscimol dose-response curve to the left by 0.15 log unit were interpolated from the plots in Figure 3. This degree of potentiation was chosen because it was about half the maximal potentiation that could be obtained. The results are given in Table 1.

Interaction with picrotoxin

Despite the non-competitive nature of the antagonism of muscimol by picrotoxin, it has consistently been found in the cuneate nucleus slice preparation that picrotoxin causes approximately parallel shifts to the right of the muscimol log dose-response line. Thus, the antagonism can conveniently be expressed as muscimol dose-ratio and, in these terms, several steroids caused substantial reductions in the potency of picrotoxin as an antagonist of muscimol. Excerpts from a typical experiment are shown in Figure 4. The upper trace, obtained in the absence of steroid, indicates that the muscimol dose-ratio for 3 μM picrotoxin was about 4. In the lower trace, obtained from a paired slice in the same experiment, 1 μM 3α-hydroxy,5β-pregnane-20-one had potentiated the responses to muscimol (control responses not shown) and a concentration of 30 μM picrotoxin was now required for a muscimol dose-ratio of about 4. The direct hyperpolarizing effect of picrotoxin occurred both in the absence and presence of the steroids. Any effect this hyperpolarization itself may have had on the responses to muscimol would, therefore, have been little affected by the steroids.

To quantify the reduction in picrotoxin potency, Schild plots for picrotoxin were constructed in the absence and presence of steroid in a paired slice design. Such plots can be regarded as log concentration-effect relationships for picrotoxin and any reduction in the potency of picrotoxin would be expected to depress the plot and/or shift it to the right. The accumulated data for the effects of 0.1, 0.3, 1 and 3 μM 3α-hydroxy,5β-pregnane-20-one on the Schild plots for picrotoxin are shown in Figure 5. This steroid caused concentration-related shifts of

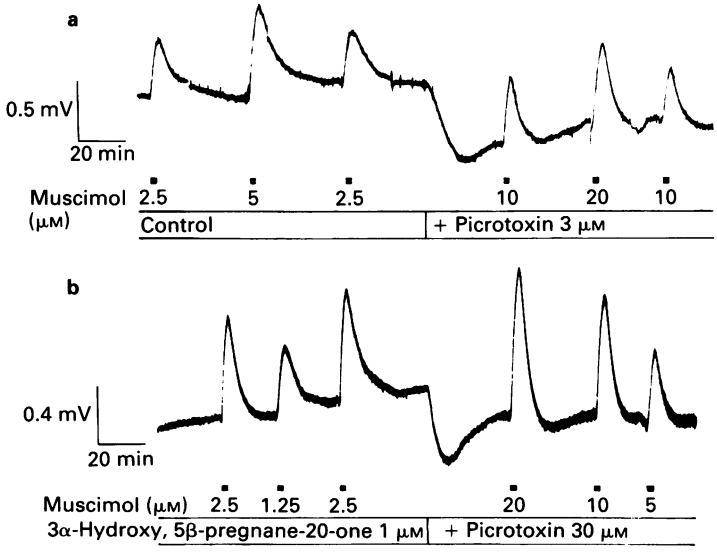


Figure 4 Original tracings of responses to 2 min applications of muscimol and their antagonism by picrotoxin in a pair of slices in the absence (a) and presence (b) of 1 μM 3α-hydroxy,5β-pregnane-20-one. Note that, in the presence of steroid, about 10 times as much picrotoxin was required as in the absence of steroid to cause similar degrees of antagonism of muscimol. The direct hyperpolarizing response to picrotoxin was a consistently observed effect. The transient deflections in (a) are artefacts.

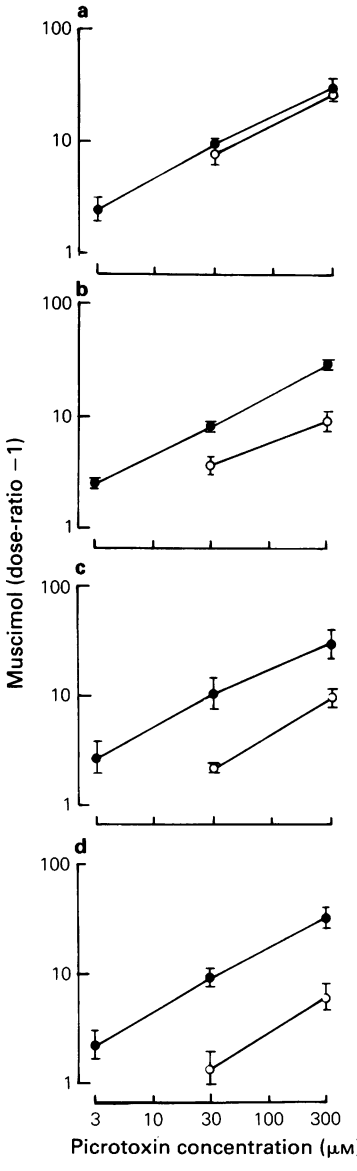


Figure 5 Schild plots for picrotoxin antagonism of muscimol in the absence (●) and presence (○) of 3α-hydroxy,5β-pregnane-20-one at concentrations of (a) 0.1 μM, (b) 0.3 μM, (c) 1 μM and (d) 3 μM. Each point is the mean of 5–8 values; s.e.mean shown by vertical bars.

the plots to the right. There were slight reductions in slope but they were not concentration-related. Similar analyses for all the concentrations of the pregnane steroids that caused a significant reduction in picrotoxin potency showed a change in slope of -0.080 ± 0.040 (mean ± s.e.mean, $n = 9$), which was not significant ($P > 0.05$), and there was no significant correlation ($r = 0.195$, $P > 0.05$) between changes in slope and rightward shift of the Schild plots for picrotoxin. Thus, the reduction in picrotoxin potency could reasonably be expressed in terms of the parallel rightwards shift of the Schild plot and such measurements are presented in Figure 6.

With the 3α-hydroxy,pregnane-20-one isomers, the configuration at the 5-position made little difference to their ability to cause substantial reductions in picrotoxin potency (Figure 6b). The addition of an 11-keto substituent to these steroids slightly lessened the effect but, again, there was little difference between the 5α- and 5β- isomers (Figure 6a). In contrast, there was no effect on picrotoxin potency of either the 5α- or the 5β- isomers of 3β-hydroxy, pregnane-20-one (Figure 6c). With the androstane steroids, a substantial reduction in picrotoxin potency was obtained with 3α-hydroxy,5α-androstane-17-one (Figure 6d) but it was considerably less potent than the pregnane-20-one derivatives containing a 3α-hydroxy substituent. The only other androstane steroid tested was 17-hydroxy,5α-androstane-3-one and it was about 30 times less potent than 3α-hydroxy,5α-androstane-17-one, again pointing to the importance of the 3α-hydroxy substituent.

As a control, 3α-hydroxy,5α-pregnane-20-one and its 5β-isomer, the two steroids that were most potent in reducing the effect of picrotoxin, were tested for their ability to reduce the effect of the competitive antagonist bicuculline. At 1 μM concentrations, 3α-hydroxy,5β-pregnane-20-one reduced bicuculline potency by 0.20 ± 0.16 log unit which was not statistically significant and 3α-hydroxy,5α-pregnane-20-one reduced it by 0.26 ± 0.07 log unit which was significant ($P < 0.05$). These effects were much smaller than the reductions in picrotoxin potency caused by these steroids.

For the five steroids that were shown to cause substantial reductions in picrotoxin potency, equieffective concentrations were determined at the level of 0.8 log unit reduction in picrotoxin potency, which was about half the maximum effect achieved. The results of this comparison are shown in Table 1. It is clear that the potencies of the steroids relative to alphaxalone differed considerably from the analogous profile for potentiation of muscimol. It was of interest, therefore, to compare the degree of reduction in picrotoxin potency at concentrations of the steroids that were equi-effective in causing a small potentiation of muscimol. The concentrations listed in Table 1 for potentiation of muscimol by 0.15 log unit were used as the basis for such a comparison and estimates of the reductions in picrotoxin potency at these concentrations were made from

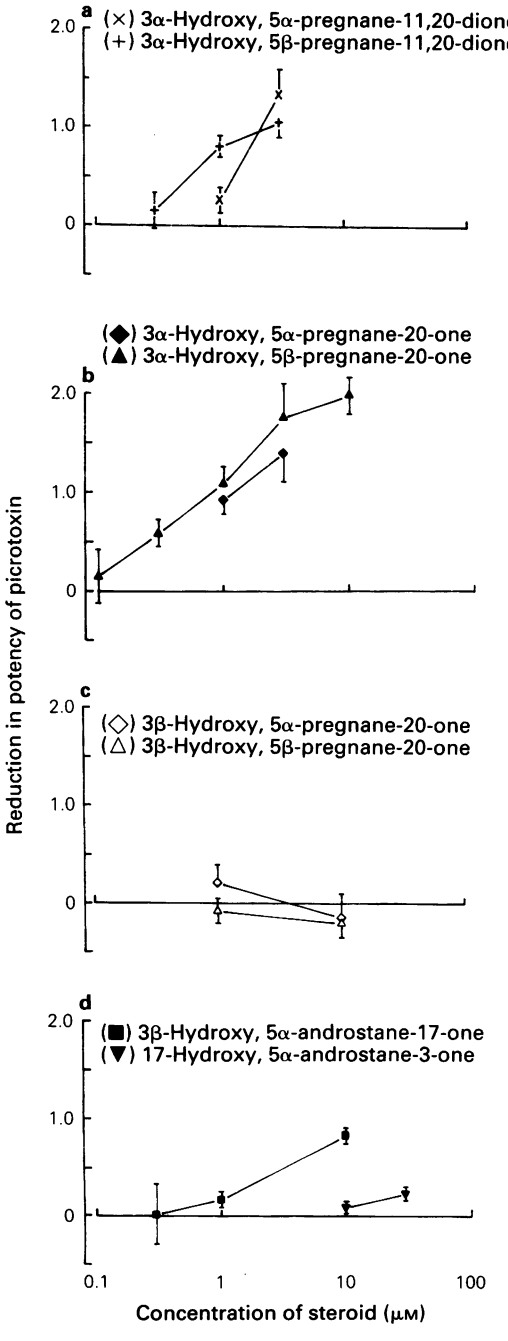


Figure 6 Reduction in potency of picrotoxin as an antagonist of muscimol, expressed as the rightwards shift (log unit) of the Schild plot for picrotoxin, due to various concentrations of steroids. Values shown are the mean from groups of 4–11 experiments; s.e.mean shown by vertical bars.

Figure 6. The results are presented in the last column of Table 1 and show that both the 5α- and 5β-isomers of 3α-hydroxy,pregnane-20-one at 3.2 μM and 1.4 μM, respectively, caused more than a 20 fold reduction in picrotoxin potency. 3α-Hydroxy,5β-pregnane-11,20-dione at 3 μM caused a 10 fold reduction but its 5α- isomer (alphaxalone) had no effect at the much lower concentration of 0.15 μM required to potentiate muscimol. 3α-Hydroxy,5α-androstane-17-one at 1.7 μM caused a 2 fold reduction in picrotoxin potency.

Discussion

The two important aspects of steroidal interaction with the $GABA_A$ receptor complex described in this study are potentiation of responses to the agonist muscimol and reduction in the potency of picrotoxin as an antagonist of muscimol. A third effect, the direct depolarizing response to the steroids, occurred at higher concentrations than those generally required for the other two effects and, therefore, should not have interfered with them.

With regard to the potentiation of muscimol, the steroidal structure-activity profile obtained in the present experiments is consistent in several respects with the observations of others (Harrison *et al.*, 1987; Cottrell *et al.*, 1987b; Kirkness *et al.*, 1987). There is agreement that, in the series of four pregnanolones tested, the considerable change in the shape of the molecule determined by the configuration at the 5-position is not crucial for potentiation of GABA and muscimol but that, when an 11-keto substituent is added, the 5α-isomer (alphaxalone) is substantially more potent than the 5β-isomer. Additionally, it is agreed that the 3β-hydroxy isomer of alphaxalone (betaxalone) does not potentiate GABA and muscimol (Harrison & Simmonds, 1984; Cottrell *et al.*, 1987b) and our results with androsterone and its 3β-hydroxy isomer show a similar requirement for 3α-hydroxy in these analogues.

There are, however, certain differences between the present results and the observations of others. The clearly greater potency of alphaxalone over 3α-hydroxy, 5α-pregnane-20-one and 3α-hydroxy,5β-pregnane-20-one shown here was not seen in studies with GABA on rat cultured hippocampal and spinal cord neurones (Harrison *et al.*, 1987), where all three steroids were approximately equi-potent. Nor was it seen in bovine cultured adrenomedullary cells (Cottrell *et al.*, 1987b; Kirkness *et al.*, 1987), where alphaxalone was reported to be less potent than the other two steroids. A further difference is the lack of effect at concentrations up to 10 μM of 3β-hydroxy, 5α-pregnane-20-one and 3β-hydroxy,5β-pregnane-20-one in the single cell recordings (Harrison *et al.*,

1987; Kirkness *et al.*, 1987) compared with their approximately equal potency to the 3α-hydroxy isomers for threshold potentiations of muscimol in the present experiments. We did, however, find that the maximal potentiation of muscimol by these 3β-hydroxy pregnanolones was less than the maximal potentiation by the 3α-hydroxy isomers. This is in keeping with some binding data reported by Kirkness *et al.* (1987) who found that, at 100 μM, the 3β-hydroxy pregnanolones did increase [^{3}H]-muscimol binding to synaptosomal membranes, but by only 10–15%, whereas the 3α-hydroxy isomers caused a maximal 55% increase in [^{3}H]-muscimol binding.

One factor that could contribute to these discrepancies is a reduction in maximal response to agonist such as that shown for 3α-hydroxy,5β-pregnane-20-one and muscimol in Figure 2. It is possible that this might be due to enhanced desensitization to the agonist but, whatever the cause, it restricts the potentiations to the lower end of the agonist dose-response curve. In the single cell recordings, potentiations of GABA were recorded as increases in response to a given concentration of GABA rather than a leftward shift of a log dose-response curve (Harrison *et al.*, 1987; Kirkness *et al.*, 1987), thus, allowing the possibility that potentiations were obscured by a reduced maximum response. Since we did not consistently study the reduction in maximal response by different steroids, it is difficult to tell whether such a phenomenon might underlie the discrepancies. However, the low maximal potentiations by the 3β-hydroxy isomers of the pregnanolones could well have made the effects of these steroids difficult to detect.

The other major effect of the steroids on the $GABA_A$ receptor system, the selective reduction in potency of picrotoxin as a non-competitive antagonist of muscimol, has not so far been studied on $GABA_A$ responses in other systems. There is, however, evidence that the steroids will displace the binding of [^{35}S]-TBPS from the picrotoxin site in rat brain synaptosomal membranes (Harrison *et al.*, 1987). Four of the steroids that we studied were considerably more potent than the others in reducing the effect of picrotoxin (Table 1). These same four steroids were also among the five found to be most potent at displacing [^{35}S]-TBPS binding (Harrison *et al.*, 1987). Thus, there is good agreement between these two studies and a clear structural requirement emerges for a 3-hydroxy in the α configuration. Both 5α- and 5β-configurations of 3α-hydroxy,pregnane-20-one were similarly effective at the picrotoxin site and the 5β-isomer of alphaxalone as well as alphaxalone itself were each only a little less effective. It is apparent, therefore, that this structure-activity profile differs from that for potentiation of muscimol on the cuneate nucleus slice in respect of the lack of effect of the pregnanolones containing a 3β-hydroxy and the clear effect of the 5β-isomer of alphaxalone.

From the structure-activity data presented here, it may be concluded that the potentiation of muscimol by the steroids is a separate phenomenon from the reduction in picrotoxin potency. This distinction is reminiscent of the effects of barbiturates on the cuneate nucleus slice (Harrison & Simmonds, 1983) although the distinction found with the steroids is much more substantial. Thus, 3α-hydroxy,5α-pregnane-20-one, 3α-hydroxy,5β-pregnane-20-one and 3α-hydroxy,5β-pregnane-11,20-dione caused reductions in picrotoxin potency of more than 10 fold at concentrations that potentiated muscimol by no more than a factor of 1.4. Whereas, for similar degrees of potentiation of muscimol, barbiturates caused a maximum 2 fold reduction in picrotoxin potency (Harrison & Simmonds, 1983).

A physiological role for certain of these steroids as potentiators of GABA at the $GABA_A$ receptor complex may be envisaged. However, the functional significance of an ability to reduce picrotoxin potency cannot be assessed until the physiological role, if any, of the picrotoxin site itself is elucidated.

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A specific inhibitor of nitric oxide formation from L-arginine attenuates endothelium-dependent relaxation

Daryl D. Rees, Richard M.J. Palmer, Harold F. Hodson & Salvador Moncada

Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS

1 The role of L-arginine in the basal and stimulated generation of nitric oxide (NO) for endothelium-dependent relaxation was studied by use of N^G-monomethyl L-arginine (L-NMMA), a specific inhibitor of this pathway.

2 L-Arginine (10–100 μM), but not D-arginine (100 μM), induced small but significant endothelium-dependent relaxations of rings of rabbit aorta. In contrast, L-NMMA (1–300 μM) produced small, endothelium-dependent contractions, while its enantiomer N^G-monomethyl-D-arginine (D-NMMA; 100 μM) had no effect.

3 L-NMMA (1–300 μM) inhibited endothelium-dependent relaxations induced by acetylcholine (ACh), the calcium ionophore A23187, substance P or L-arginine without affecting the endothelium-independent relaxations induced by glyceryl trinitrate or sodium nitroprusside.

4 The inhibition of endothelium-dependent relaxation by L-NMMA (30 μM) was reversed by L-arginine (3–300 μM) but not by D-arginine (300 μM) or a number of close analogues (100 μM).

5 The release of NO induced by ACh from perfused segments of rabbit aorta was also inhibited by L-NMMA (3–300 μM), but not by D-NMMA (100 μM) and this effect of L-NMMA was reversed by L-arginine (3–300 μM).

6 These results support the proposal that L-arginine is the physiological precursor for the basal and stimulated generation of NO for endothelium-dependent relaxation.

Introduction

The release of nitric oxide (NO) by endothelial cells in culture accounts for the relaxation of vascular strips (Palmer *et al.*, 1987) and for the inhibition of platelet aggregation and adhesion (Radomski *et al.*, 1987a,b) attributed to endothelium-derived relaxing factor. Nitric oxide is also released from the perfused rabbit aorta and in the rabbit coronary circulation in amounts sufficient to account for the relaxation of aortic rings and the fall in coronary perfusion pressure induced by acetylcholine (ACh; Chen *et al.*, 1989; Amezcua *et al.*, 1988). These and other data (Moncada *et al.*, 1988) have established that NO mediates endothelium-dependent relaxation (Furchgott & Zawadzki, 1980).

We have recently demonstrated that aortic endothelial cells of the pig in culture synthesize NO from the terminal guanidino nitrogen atom(s) of the amino acid L-arginine (Palmer *et al.*, 1988a). In addition, the L-arginine analogue, N^G-monomethyl-L-arginine (L-NMMA), inhibits both the release of NO from these cells and the endothelium-dependent relaxation of rings of rabbit aorta (Palmer *et al.*, 1988b). As a result, we have proposed that L-arginine is the physiological precursor for NO synthesis by vascular endothelial cells.

In order to investigate this NO-generating system further and to characterize its substrate specificity, we have now examined the actions of L-arginine, L-NMMA and some other arginine analogues on the endothelium-dependent relaxations of rings of rabbit aorta. Furthermore, we have studied the effects of L-arginine and L-NMMA on the release of NO from perfused segments of this tissue.

Methods

Organ bath studies

Endothelium-dependent relaxation of rings of rabbit aorta was studied as described previously (Rees *et al.*, 1988). Briefly, male New Zealand White rabbits (2.0–2.3 kg) were killed with an overdose of sodium

pentobarbitone. The thoracic aorta was removed, trimmed free of adhering fat and connective tissue and cut into 4 mm rings. The endothelium was removed from some rings by gently rubbing the intimal surface with a pipe cleaner. The failure of ACh (1 μM) to induce relaxation of these rings was taken as an indication of endothelium removal. This was confirmed in some experiments by scanning electron microscopy.

The rings were mounted under 2.5 g resting tension on stainless steel hooks in 20 ml organ baths containing Krebs buffer at 37°C, gassed with 95% O_2 and 5% CO_2. Tension was recorded with Grass FT03 isometric transducers on a 6-channel multipen recorder (Rikadenki). The tissues were allowed to equilibrate for 40 min before being contracted submaximally by addition of phenylephrine (750 nM). Cumulative relaxation curves to ACh, substance P, A23187, glyceryl trinitrate (GTN) or sodium nitroprusside were obtained in each ring. After washout, L-arginine or its analogues were added to the organ bath 10 min before a second cumulative relaxation curve was obtained. After further washout, a third curve was obtained to demonstrate the reversibility of any effects observed.

Cascade bioassay

A segment (6 cm) of the rabbit thoracic aorta (donor aorta) was removed, cleaned and placed in a perspex chamber and perfused intraluminally with Krebs buffer (5 ml min^{-1}). For the bioassay of NO, the effluent from this aorta was used to superfuse 3 spiral strips of rabbit aorta denuded of endothelium (bioassay tissues) in a cascade (Gryglewski *et al.*, 1986a; Palmer *et al.*, 1987). The bioassay tissues were contracted submaximally with either 9,11-dideoxy-9α, 11α methanoepoxy-prostaglandin $F_{1\alpha}$ (U46619; 30 nM) or phenylephrine (750 nM). Atropine sulphate (0.2 μM) was infused over the bioassay tissues in order to block any direct action of ACh. The amplification of the recorder was adjusted so that similar relaxations to GTN (50 nM) were observed in each tissue.

Chemiluminescence

The release of NO from the perfused rabbit aorta was also detected by chemiluminescence (Palmer *et al.*, 1987). Briefly, the effluent of a perfused donor aorta was infused continuously into a reaction vessel containing 75 ml 1% sodium iodide in glacial acetic acid under reflux. Nitric oxide was removed under reduced pressure in a stream of N_2, mixed with ozone and the chemiluminescent product quantified by reference to NO standards.

Chemicals

Nitric oxide (>99.98% pure, British Oxygen Corporation) solutions were prepared as described previously (Palmer *et al.*, 1987). Glyceryl trinitrate (Wellcome), phenylephrine, ACh, substance P, L- and D-arginine, L-homoarginine, L-α-amino-γ-guanidinobutyric acid, L-α-amino-β-guanidinopropionic acid, L-canavanine, L-argininamide, L-citrulline, urea, L-arginine methyl ester, L-arginyl-aspartate, sodium nitroprusside, atropine sulphate (Sigma), agmatine sulphate (Aldrich), the calcium ionophore A23187 (Calbiochem), U46619 (Cayman Chemicals) and sodium pentobarbitone (May and Baker) were obtained as indicated. L-NMMA, D-NMMA and N^G, N^{1G}-dimethyl-L-arginine were synthesized as described previously (Patthy *et al.*, 1977). 5-Guanidino valeric acid was synthesized according to the published method (Kobayashi, 1947).

Statistics

Results are expressed as mean $\pm$ s.e.mean for n separate experiments. Student's unpaired t test was used to determine the significance of differences between means and $P < 0.05$ was taken as statistically significant.

Results

Endothelium-dependent relaxation

L-Arginine (10–100 μM) caused small but significant endothelium-dependent relaxations of rings of rabbit aorta pre-contracted with 750 nM phenylephrine (maximum relaxation = 9 $\pm$ 1% of the contraction induced by phenylephrine, $P < 0.05$, $n = 3$). These relaxations were concentration-dependent (ED_{50} = 28 $\pm$ 5 μM) and reversible. In contrast, L-NMMA (1–300 μM) caused small but significant endothelium-dependent contractions (maximum 17 $\pm$ 2% of the contraction induced by phenylephrine; $P < 0.05$, $n = 3$) which were also concentration-dependent (ED_{50} = 4 $\pm$ 2 μM) and reversible (Figure 1). The relaxations induced by L-arginine (100 μM) were completely reversed by L-NMMA (30 μM) and the contractions induced by L-NMMA (30 μM) were completely reversed by L-arginine (100 μM; $n = 3$). The tone of the rings and the effects of L-arginine or L-NMMA ($n = 3$) were not affected by D-arginine or D-NMMA (both at 100 μM).

Acetylcholine (1 μM), substance P (10 nM) and A23187 (0.1 μM) caused endothelium-dependent relaxation of rings of rabbit aorta which were

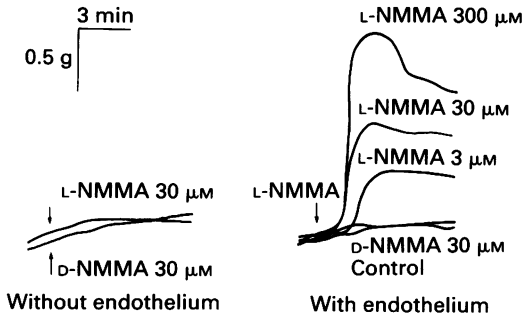

Figure 1 Effect of N^G-monomethyl-L-arginine (L-NMMA) and N^G-monomethyl-D-arginine (D-NMMA) on the basal tone of rabbit aortic rings pre-contracted with phenylephrine (750 nM), with and without endothelium. Trace representative of three experiments.

76 ± 3, 47 ± 9 and $67 \pm 5\%$ ($n = 3$) respectively of the contraction induced by phenylephrine. The relaxations induced by ACh (1 μM) were marginally increased ($9 \pm 5\%$; $n = 3$; $P > 0.05$) by L-arginine (100 μM). The relaxations induced by ACh, substance P and A23187 were inhibited in a concentration-dependent manner by L-NMMA (1–300 μM) but not D-NMMA (100 μM; $n = 3$; see Figure 2). The IC_{50}s were 10 ± 2, 5 ± 3 and 12 ± 2 μM ($n = 3$) for ACh, substance P and A23187 respectively. The maximum degree of inhibition by L-NMMA (300 μM) of these relaxations was 64 ± 1, 55 ± 5 and $76 \pm 5\%$ ($n = 3$ for each compound). The endothelium-independent relaxations induced by GTN or sodium nitroprusside were not affected by L-NMMA (300 μM, $n = 3$).

The inhibition by L-NMMA (1–300 μM) of endothelium-dependent relaxation induced by ACh was fully reversible after washing (90 min). Concomitant addition of L-arginine (3–300 μM), but not D-arginine (300 μM), overcame, in a concentration-dependent manner, the inhibition of ACh-induced endothelium-dependent relaxation produced by L-NMMA (30 μM; Figure 3). The ED_{50} against L-NMMA (30 μM) was 25 ± 3 μM ($n = 3$) and approximately three times more L-arginine was required to abolish the effect of a given concentration of L-NMMA.

The tone of contracted aortic rings was not affected by N^G, N^{1G}-dimethyl-L-arginine, L-homoarginine, L-α-amino-γ-guanidinobutyric acid, L-α-amino-β-guanidinopropionic acid, L-canavanine, L-argininamide, agmatine, 5-guanidino valeric acid, L-citrulline, urea, L-arginine methyl ester and L-arginyl-aspartate (all at 100 μM; $n = 3$ for each). Of these compounds, only L-arginine methyl ester (100 μM) caused a partial ($37 \pm 3\%$) reversal of the

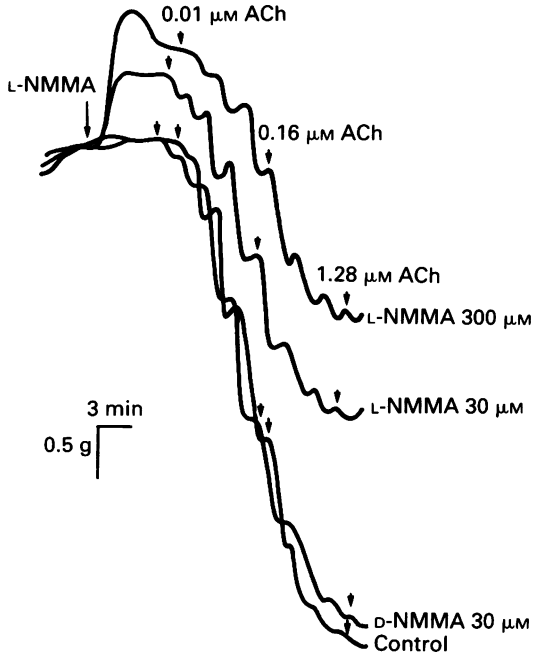

Figure 2 Inhibition by N^G-monomethyl-L-arginine (L-NMMA), but not by N^G-monomethyl-D-arginine (D-NMMA), of ACh-induced endothelium-dependent relaxation. Cumulative relaxation curves were obtained by addition of ACh (0.01–1.28 μM) to the organ bath; the concentration of ACh doubled at each addition. The points of addition of 0.01, 0.16 and 0.128 μM ACh are indicated by the arrows; those of intermediate concentrations of ACh are not shown. Trace representative of three experiments.

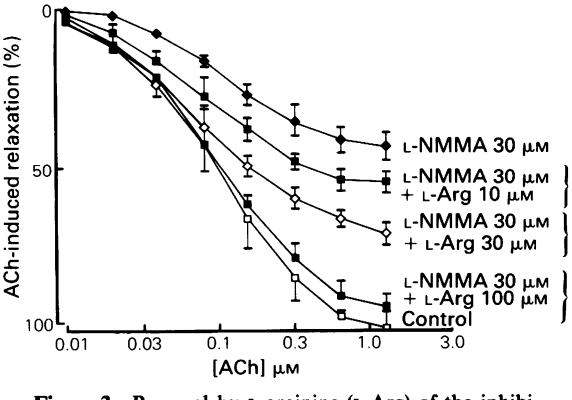

Figure 3 Reversal by L-arginine (L-Arg) of the inhibitory effect of N^G-monomethyl-L-arginine (L-NMMA) on ACh-induced endothelium-dependent relaxation. Each point is the mean of 3 experiments; vertical bars show s.e.mean.

inhibition by L-NMMA (30 μM) of ACh-induced endothelium-dependent relaxation.

Cascade bioassay

The tone of the bioassay tissues in the cascade and their response to NO was not affected by infusions over the tissues of ACh (30 μM), L- and D-arginine (300 μM) or L-NMMA (300 μM; $n = 3$ for each). However, when ACh (0.1–30.0 μM) was infused for 1 min through the donor aorta, it induced a concentration-dependent release of NO detected by bioassay ($ED_{50} = 0.6 \pm 0.2$ μM, $n = 3$).

Nitric oxide release induced by ACh (1 μM) was enhanced by L-arginine (100 μM) to a small ($21 \pm 3\%$, $n = 3$) but significant ($P < 0.05$) extent and was inhibited in a concentration-dependent manner ($IC_{50} = 16 \pm 5$ μM, $n = 3$) by L-NMMA (3–300 μM; Figure 4), but not by D-NMMA (100 μM; TD, $n = 3$). The inhibition by L-NMMA (30 μM) of NO release induced by ACh was slowly reversible over a period of 90 min. The response to ACh could also be fully restored by a 10 min infusion of L-arginine (100 μM) following termination of the infusion of L-NMMA (Figure 4). The inhibition of NO release by L-NMMA (30 μM) was reversed by L-arginine (3–300 μM), but not by D-arginine (300 μM; $n = 3$), in a concentration-dependent manner ($ED_{50} = 28 \pm 3$ μM, $n = 3$).

Chemiluminescence

Infusion of ACh (0.1–30.0 μM) through the donor aorta for 1 min induced a concentration-dependent release of NO (4 ± 2–93 ± 15 pmol) detected by chemiluminescence ($ED_{50} = 2 \pm 1$ μM, $n = 3$). The release of NO (41 ± 5 pmol) induced by ACh (1 μM), was inhibited in a concentration-dependent manner by L-NMMA (3–300 μM) with an IC_{50} of 15 ± 3 μM ($n = 3$), but not by D-NMMA (100 μM; $n = 3$). The effect of L-NMMA was reversed by L-arginine (3–300 μM; Figure 5) but not by D-arginine (300 μM) and L-arginine caused a small ($18 \pm 5\%$, $n = 3$) but significant ($P < 0.05$) potentiation of the release of NO induced by ACh (1 μM; Figure 5).

Discussion

Vascular endothelial cells synthesize NO from the terminal guanidino nitrogen atom(s) of L-arginine by an enzymic process which exhibits strict substrate specificity (Palmer *et al.*, 1988a). Furthermore, we have suggested that NO release from endothelial

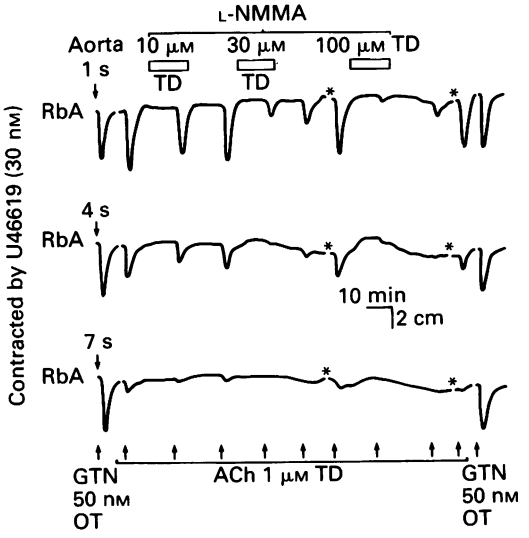

Figure 4 Effect of N^G-monomethyl-L-arginine (L-NMMA; 10–100 μM), infused through the donor rabbit aorta (TD), on the relaxation of the bioassay tissues in the cascade by ACh- (1 μM, TD) induced NO release. Asterisks denote L-arginine infusion (100 μM; TD) for 10 min followed by washout (10 min). The responses of the tissues were standardized by an infusion of glyceryl trinitrate administered over the tissues (GTN; 50 nM, OT). Trace representative of three experiments.

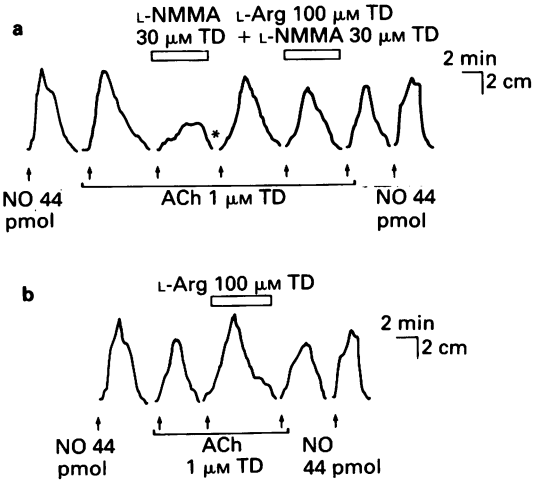

Figure 5 (a) Reversal by L-arginine (L-Arg) of the inhibition by N^G-monomethyl-L-arginine (L-NMMA) of ACh-induced release of NO and (b) potentiation of ACh-induced release of NO by L-arginine. Asterisk denotes L-arginine infusion (100 μM; TD) for 10 min followed by washout (10 min). The release of NO, determined by chemiluminescence, was quantified as the area under the peak compared with those of NO standards. Traces representative of three experiments.

cells is a two-step process involving mobilisation of endogenous substrate and subsequent formation of NO. In addition, we have shown that the formation of NO from L-arginine and the endothelium-dependent relaxation induced by ACh is inhibited by the arginine analogue L-NMMA, but not by its D-enantiomer (Palmer *et al.*, 1988b).

We now show that L-arginine but not D-arginine, marginally enhances NO release induced by ACh from segments of rabbit aorta and induces concentration-related, endothelium-dependent relaxation of vascular rings without significantly affecting endothelium-dependent relaxation induced by ACh. Furthermore, L-arginine does not affect endothelium-independent relaxation induced by GTN or sodium nitroprusside. These results are consistent with the synthesis of NO from L-arginine; however, the enhancement of NO release and the relaxations induced by L-arginine are small. This suggests that exogenous L-arginine is unable to compete with endogenous L-arginine which may be presented to the enzyme more efficiently. In addition, it is possible that during stimulation there is activation of the NO-forming enzyme and sufficient mobilisation of substrate to saturate the enzyme.

The release of NO from fresh vascular tissue and the endothelium-dependent relaxation induced by different agonists were inhibited by L-NMMA, but not by its D-enantiomer. Furthermore, L-NMMA induces an endothelium-dependent contraction. All the effects of L-NMMA can be antagonized by L- but not by D-arginine. These data indicate that endogenous L-arginine is utilized for the generation of NO during endothelium-dependent relaxation. Moreover, they show that there is continuous use of L-arginine for the basal release of endothelium-derived relaxing factor (NO) which has been demonstrated in fresh vascular tissue (Martin *et al.*, 1985) and vascular endothelial cells in culture (Gryglewski *et al.*, 1986b).

Our data also suggest a competitive interaction between L-NMMA and L-arginine, for equimolar concentrations of L-arginine are required to reverse the effects of L-NMMA by 50%. Furthermore, the concentration of L-NMMA which produces 50% contraction of the rings (4 μM) is approximately 6 times lower than that required to inhibit ACh-induced relaxation by 50%. This, together with the fact that the IC_{50} of L-NMMA against substance P is lower than that required to inhibit the greater relaxations induced by ACh or A23187 by 50%, is consistent with an enhanced mobilisation of substrate during activation of the endothelial cell which may vary according to the degree of stimulation.

Unlike L-arginine, the effect of L-NMMA on NO release is slow to disappear unless accelerated by exogenous L-arginine. Whether the 90 min required for the complete reversal of the effect of L-NMMA in the absence of exogenous L-arginine reflects simple dissociation or is the result of a gradual displacement by small amounts of endogenous L-arginine should be investigated.

The NO-forming enzyme system has a strict structural requirement for the substrate, as indicated by the inability of arginine analogues to reverse or mimic the effects of L-NMMA and to affect endothelium-dependent relaxation. None of the analogues studied altered the tone of aortic rings and only L-arginine methyl ester, which may contain some unesterified L-arginine, reversed the effects of L-NMMA. The studies with these analogues show that alterations in the guanidino or the amino acid function of L-arginine or in the length of the methylene chain of the molecule are sufficient to modify the interaction with the enzyme site profoundly. The fact that L-citrulline does not induce endothelium-dependent relaxation or compete with L-NMMA is consistent with the possibility that this compound needs to be converted to L-arginine prior to formation of NO as has been suggested to occur in endothelial cells in culture (Palmer *et al.*, 1988a).

The maximum degree of inhibition by L-NMMA of endothelium-dependent relaxation was 55–76% with the three agonists studied. When determined by bioassay or chemiluminescence, L-NMMA causes complete inhibition of NO release. Endothelium-dependent relaxation detects the abluminal release of NO and is more sensitive than the cascade bioassay or the chemiluminescence methods (Chen *et al.*, 1989) which detect luminal release of NO. It is, therefore, possible that there is a site for the abluminal synthesis of NO which is inaccessible to L-NMMA. Alternatively, there may be factors other than NO involved in endothelium-dependent relaxation (Feletou & Vanhoutte, 1988). The existence of such factors will be determined with the help of L-NMMA in perfusion experiments where biologically active materials are transferred from donor to detector systems.

Our finding that L-canavanine does not affect endothelium-dependent relaxation is at variance with a recent observation that this compound inhibited endothelium-dependent relaxation in rings of the rat aorta (Schmidt *et al.*, 1988). Whether the effect reported is due to endothelial damage by the very large concentrations of L-canavanine used or is due to a specific effect on the NO-generating system, remains to be established.

The oxidation products of NO, NO_2^- and NO_3^-, are formed from the terminal guanidino nitrogen atom(s) of L-arginine by activated macrophages (Hibbs *et al.*, 1987a). There seem to be some differences between the NO-generating system in the endothelium and in the activated macrophage. For

example, L-homoarginine, L-argininamide and L-arginyl-aspartate are substrates and L-canavanine is an inhibitor of the generation of NO_2^- and NO_3^- by macrophages (Iyengar *et al.*, 1987; Hibbs *et al.*, 1987b) and yet are inactive in the endothelium.

The vascular endothelial cell has cytotoxic and antitumour properties that have been ascribed to the generation of superoxide ions (Ryan & Vann, 1987). It is possible that, as in the macrophage (Granger *et al.* 1988; Hibbs *et al.*, 1987b), some of these effects are associated with the generation of NO. If this is indeed the case, the generation of NO by the vascular endothelium may have biological consequences beyond endothelium-dependent relaxation.

A relaxing factor, likely to be NO, is released by rat peritoneal neutrophils (Rimele *et al.*, 1988). In addition, EMT-6 adenocarcinoma cells (Amber *et al.*, 1988) and haemopoietic cells (Schneider *et al.*, 1988) form L-citrulline directly from L-arginine, a process probably associated with NO generation. It is likely that other cells will be shown to have the ability to generate NO. The significance of the generation of NO in different biological systems remains to be established. For this purpose, L-NMMA, a specific inhibitor of NO_2^- and NO_3^- release in the macrophage (Hibbs *et al.*, 1987a) and of NO generation in the vascular endothelium may prove invaluable.

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Adenosine enhancement of adrenergic neuroeffector transmission in guinea-pig pulmonary artery

[1]N. Peter Wiklund, Bo Cederqvist & *Lars E. Gustafsson

Department of Physiology, Karolinska Institutet, and the *National Institute of Environmental Medicine, S-104 01 Stockholm, Sweden

1 Adenosine and its derivatives N^6-[(**R**)-1-methyl-2-phenylethyl]adenosine (R-PIA) or 5'-N-ethylcarboxamideadenosine (NECA) enhanced nerve-induced contractile responses and augmented the basal smooth muscle tone in transmurally stimulated isolated strips of the guinea-pig pulmonary artery.

2 Adenosine, R-PIA and NECA enhanced contractile responses induced by noradrenaline, whereas N^6-[(S)-1-methyl-2-phenylethyl]-adenosine (S-PIA) was virtually inactive.

3 Adenosine, R-PIA and NECA inhibited the nerve stimulation evoked release of [^{3}H]-noradrenaline. However, the total release of [^{3}H]-noradrenaline during the periods of NECA application was increased.

4 The nucleoside effects were blocked by the adenosine receptor antagonist 8-*p*-sulphophenyltheophylline.

5 8-*p*-Sulphophenyltheophylline inhibited nerve-induced contractions and lowered basal muscle tone in preparations not having received any exogenous purines.

6 It is suggested that the observed stimulatory effects on muscle tone and on contractile responses to transmural nerve stimulation are mainly due to action at postjunctional stimulatory A_1 adenosine receptors. In addition, actions at prejunctional inhibitory A_1 and stimulatory A_2 adenosine receptors are evident in this preparation.

Introduction

Adenosine and adenine nucleotides are potent vasodilators in many vascular beds (Drury & Szent-Györgyi, 1929; Berne *et al.*, 1983), although in the kidney adenosine exhibits a vasoconstrictor effect (Osswald 1975; Hedqvist & Fredholm 1976; Hedqvist *et al.*, 1978). The concept of adenosine as a local regulator of blood flow has been extensively studied, but the physiological importance of adenosine in this respect is still uncertain (Berne *et al.*, 1987; Sparks & Gorman 1987).

Ligand binding studies have shown that the actions of adenosine are mediated via at least two extracellular receptor types: A_1 and A_2 (Daly, 1982). The subclasses of adenosine receptors may be distinguished by the relative agonist potencies of certain adenosine analogues (Daly, 1982). At the adenosine A_2 receptor, 5'-N-ethylcarboxamideadenosine (NECA) is more potent than N^6-[(**R**)-1-methyl-2-phenylethyl]-adenosine (R-PIA), whereas R-PIA is equi- or more potent than NECA at the A_1 receptor. R-PIA is 50–100 times more potent than its stereoisomer S-PIA at the A_1 receptor. The difference in potency between R-PIA and S-PIA at the A_2 receptor is at most 5 fold (Bruns *et al.*, 1980). Recently a further subclassification of A_2 receptors has been suggested (Bruns *et al.*, 1987). All receptor subtypes are blocked by methylxanthines. We have used 8-*p*-sulphophenyltheophylline as an adenosine receptor antagonist, since it has been identified as a competitive adenosine antagonist without direct smooth muscle inhibitory action (Gustafsson, 1984; Wiklund *et al.*, 1985).

Events that impair oxygen availability or enhance oxygen consumption may result in increased adenosine release in several tissues (Berne *et al.*, 1983), and adenosine can be released in the lungs during hypoxia (Mentzer *et al.*, 1975). The present study demonstrates stimulatory effects by adenosine and its analogues on both smooth muscle and noradrenergic nerves in the guinea-pig pulmonary

[1] Author for correspondence.

artery, and thus raises the question whether adenosine might be involved in the regulation of blood flow in the pulmonary circulation.

Methods

General procedure

Guinea pigs (300–500 g) of either sex were stunned and bled. The lungs and heart were removed and placed on ice. The pulmonary artery was isolated under a dissection microscope.

Motor activity

Nerve-smooth muscle preparations were obtained by cutting the main pulmonary artery and its two proximal branches in spirals and suspending them vertically in 6 ml organ baths containing Tyrode solution (composition, mM: Na 149, K 2.9, Ca 1.8, Mg 0.5, Cl 144, HCO_3 11.9, H_2PO_4 0.4 and glucose 5.5) kept at 37°C and continuously aerated with 5% CO_2 in O_2. The organ baths were equipped with platinum electrodes 10 mm in length and 10 mm apart. Motor activity was recorded isometrically by Grass force displacement transducers (FT03), and tracings were made on a Grass polygraph. The preparations were given an initial tension of 2.5 mN and were allowed to accommodate for 30 min before experiments started. Transmural nerve stimulation was by monophasic pulses at 10–20 Hz, 0.5–1 ms, 60 V effective over bath electrodes, 100–200 pulses at 2–3 min intervals.

Noradrenaline release

The main pulmonary artery was dissected and incubated at 37°C for 1 h in Tyrode solution containing 2.5 μCi ml^{-1} (−)-[7,8-^{3}H]-noradrenaline (34 Ci mmol^{-1}, Amersham) and 0.1 mM ascorbic acid. After incubation, the segments were mounted in 1 ml organ baths with platinum electrodes (12 mm long, 6 mm apart) and perifused with Tyrode solution at a rate of 1 ml min^{-1}. Contractile activity was recorded isometrically as above.

Transmural nerve stimulation was intermittently applied (5 Hz, 1 ms, 60 V, 50 s) at 10 min intervals. During an initial 30 min rinsing period, including two periods of transmural stimulation, the effluent was discarded. The effluent was then collected at 2 min intervals and analyzed for radioactivity in a Packard liquid scintillation spectrometer. Quenching was monitored by the channels ratio method. Three control stimulations (S1, S2, S3), were performed before drugs were added (S4, S5). The radioactivity remaining in the tissue was determined at the end of experiments. Stimulus-evoked noradrenaline release was calculated as total ^{3}H overflow during a 2 min period with a 50 s stimulation and its following 2 min poststimulatory collection period minus the expected basal ^{3}H overflow, estimated from the ^{3}H overflow obtained during the collection period immediately before stimulation. Evoked release of ^{3}H was matched against the calculated total ^{3}H present in the tissue at the beginning of each stimulation. The fractional ^{3}H release thus calculated was expressed as a percentage of that obtained during the first control stimulation (denoted as S1 = 100%).

The total ^{3}H efflux during the 10 min periods between and including each one individual stimulation (thus consisting of the summed ^{3}H overflow obtained in three prestimulatory, one stimulation and one poststimulatory sampling period) was also determined, and expressed as a percentage of the first such 10 min period (denoted as R1 = 100%).

In a set of experiments 15 Hz, 1 ms, 60 V, 17 s was applied at 10 min intervals in order to study purine effects on ^{3}H release at higher stimulation frequencies.

Drugs

Adenosine, (−)-noradrenaline-(+)-bitartrate, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and tetrodotoxin were purchased from Sigma Co. (St. Louis, U.S.A.). Other drugs used were phentolamine hydrochloride (CIBA AG, Basel, Switzerland), (−)-[7,8-^{3}H]-noradrenaline (34 Ci mmol^{-1}, Amersham), R-PIA (N^6-[(**R**)-1-methyl-2-phenylethyl]-adenosine, formerly called L-PIA), S-PIA (N^6-[(**S**)-1-methyl-2-phenylethyl]-adenosine, formerly called D-PIA) (both from Boehringer Mannheim GmbH, Mannheim, West Germany) and NECA (5′-N-ethylcarboxamide-adenosine; Byk-Gulden Lomberg Chem. Fabr., Konstanz, West Germany). 8-*p*-sulphophenyltheophylline was synthesized according to Daly *et al.* (1985).

Statistics

Experimental data are expressed as mean values ± s.e.mean. Statistical significance was tested according to Student's *t* test for unpaired variates: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Results

Transmural stimulation (10–20 Hz, 0.5–1 ms pulse duration, 100–200 pulses) of isolated spirals of guinea-pig pulmonary artery elicited contractile responses which were abolished by tetrodotoxin 3×10^{-7} M, antagonized by phentolamine 3×10^{-7} M and accompanied by ^{3}H overflow from

preparations previously incubated with [^{3}H]-noradrenaline, indicating activation of noradrenergic nerves. Adenosine (10^{-6}–10^{-4} M) enhanced the nerve-induced contractile responses and augmented the basal smooth muscle tone in isolated spirals of guinea-pig pulmonary artery (Figures 1, 3, 4). Both the enhancement of contractile responses to nerve stimulation as well as the contractile effect of adenosine were antagonized by 8-*p*-sulphophenyltheophylline (3×10^{-5}–10^{-4} M) (Figure 1). Application of the adenosine analogues R-PIA or NECA (10^{-8}–10^{-6} M) also enhanced the nerve-induced contractile responses and increased the basal muscle tone of the preparations (Figures 2, 3, 4). R-PIA tended to be more potent than NECA in inducing contraction, although this difference was not statistically significant. 8-*p*-Sulphophenyltheophylline (3×10^{-5}–10^{-4} M) antagonized the effects of R-PIA ($n = 5$, not shown) and NECA ($n = 6$) (Figure 2b). The effect of S-PIA was very variable, yielding stimulatory effects only at times.

To discover whether there was a relationship between increased basal tone and enhancement of nerve-induced contractions, the effects of noradrenaline and $PGF_{2\alpha}$ were studied. Thus, both noradrenaline (10^{-6}–10^{-5} M, $n = 4$) and $PGF_{2\alpha}$ (10^{-7}–10^{-6} M, $n = 4$) increased the basal tone and concomitantly enhanced the nerve-induced contractions.

The contractile effect of the nucleosides, leading to an increase in the basal muscle tone, might suggest a postjunctional effect of adenosine and its analogues. To investigate this we studied the effect of adenosine (10^{-6}–10^{-4} M), R-PIA (10^{-8}–10^{-6} M), S-PIA (10^{-8}–10^{-6} M) and NECA (10^{-8}–10^{-6} M) on contractile

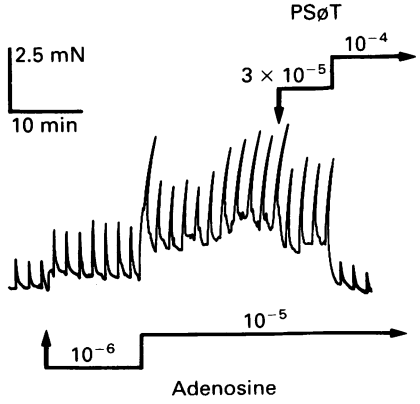

Figure 1 Contractile responses to transmural stimulation (20 Hz, 1 ms, 200 pulses at 2 min intervals) in guinea-pig pulmonary artery. Enhancement of nerve-induced contractile responses and increased basal muscle tone by adenosine (10^{-6}–10^{-5} M) and its antagonism by 8-*p*-sulphophenyltheophylline (PSØT) (3×10^{-5}–10^{-4} M).

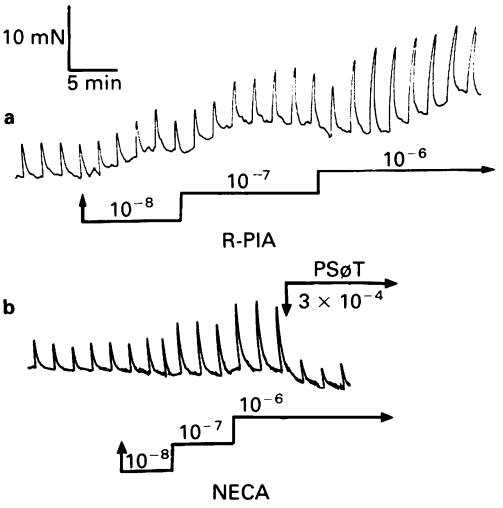

Figure 2 Contractile responses to transmural nerve stimulation (20 Hz, 1 ms, 200 pulses at 2 min intervals) in guinea-pig pulmonary artery. Enhancement of nerve-induced contractile responses and increased basal muscle tone by (a) N^6-[(R)-1-methyl-2-phenyl]-adenosine (R-PIA, 10^{-8}–10^{-6} M) and (b) 5'-N-ethylcarboxamideadenosine NECA, (10^{-8}–10^{-6} M). Note antagonism of the NECA-induced effects by 8-*p*-sulphophenyltheophylline (PSØT) (10^{-4} M).

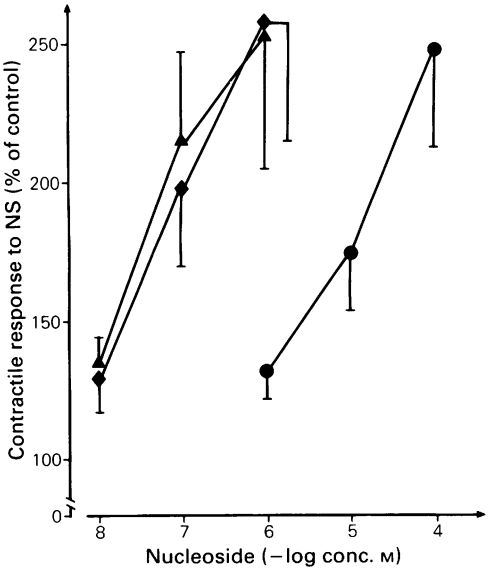

Figure 3 Concentration-dependent enhancement of contractile responses to transmural stimulation (NS) (10–20 Hz, 1 ms, 100–200 pulses at 2 min intervals) in guinea-pig pulmonary artery during application of adenosine ($n = 8$–10) (●), NECA ($n = 7$) (◆) or R-PIA ($n = 7$–8) (▲). Symbols represent means, vertical bars indicate s.e.mean. n = number of experiments.

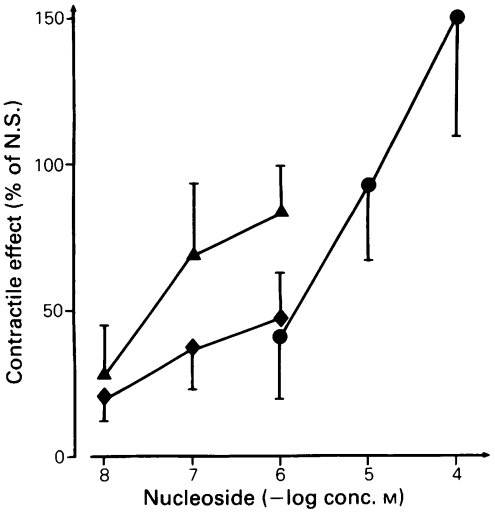

Figure 4 Guinea-pig pulmonary artery: concentration-dependent contractile effect induced by adenosine ($n = 10$) (●), by NECA ($n = 8$) (◆) or by R-PIA ($n = 10$) (▲) on basal muscle tone, as compared to the contractile effect of a standard transmural nerve stimulation (20 Hz, 1 ms, 200 pulses at 2 min intervals). n = number of experiments.

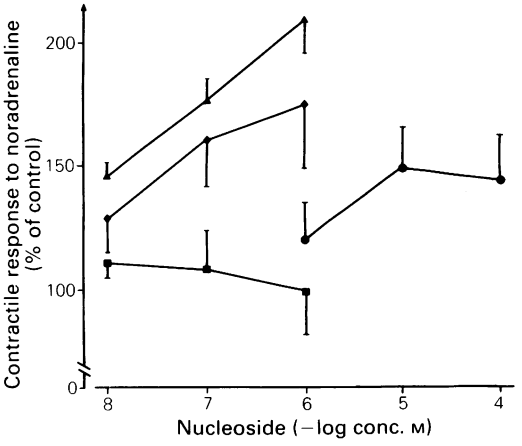

Figure 5 Contractile responses to application of noradrenaline (10^{-6}–10^{-5} M) in guinea-pig pulmonary artery. Concentration-dependent enhancement of contractions by adenosine ($n = 6$) (●), NECA ($n = 5$) (◆), R-PIA ($n = 10$) (▲) and S-PIA ($n = 4$) (■). Symbols represent means, vertical bars indicate s.e.mean, n = number of experiments. R-PIA 10^{-8} M was more potent than S-PIA 10^{-8}–10^{-6} M, $P < 0.05$.

responses induced by application of noradrenaline (10^{-6}–10^{-5} M), in preparations that were not transmurally stimulated. Adenosine, R-PIA or NECA enhanced the noradrenaline-induced contractile responses, whereas S-PIA was virtually inactive (Figure 5). There was no significant difference in potency between R-PIA and NECA. However, R-PIA 10^{-8} M ($n = 10$) was significantly more potent

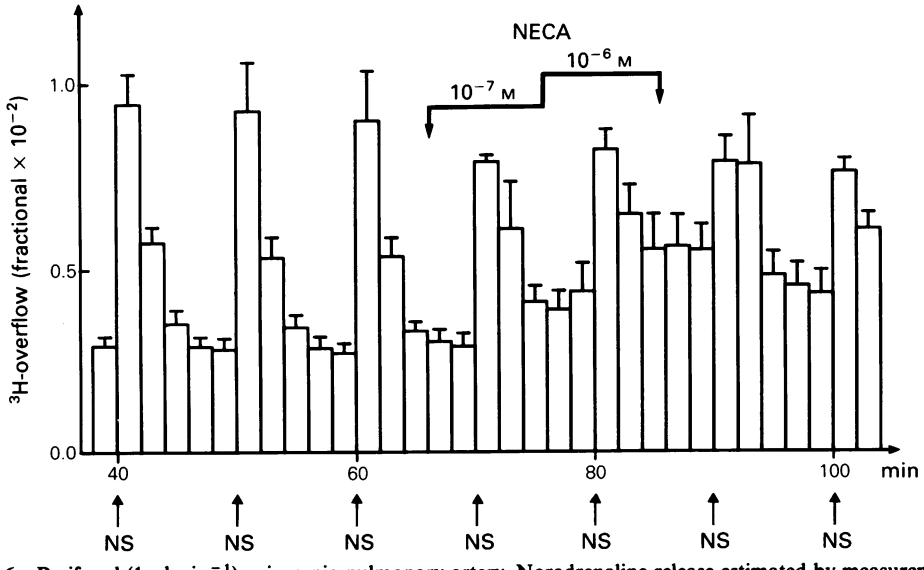

Figure 6 Perifused (1 ml min^{-1}) guinea-pig pulmonary artery. Noradrenaline release estimated by measurement of fractional ^{3}H overflow into perifusate collected at 2 min intervals from preparations preincubated with [^{3}H]-noradrenaline. NS indicates transmural nerve stimulation (5 Hz, 1 ms, 250 pulses) at 10 min intervals. Effect of NECA on ^{3}H overflow. Columns indicate mean values of 5 experiments, vertical bars denote s.e.mean.

than S-PIA 10^{-6} M ($n = 6$) ($P < 0.001$) indicating a more than 100 fold difference in potency between the two stereoisomers (Figure 5).

Possible prejunctional effects of adenosine analogues were studied by determination of noradrenaline release through monitoring ^{3}H overflow from preparations preincubated in [^{3}H]-noradrenaline. After the initial washout period, the overflow of ^{3}H was stable, and reproducible increments were obtained during intermittent transmural (5 Hz, 1 ms, 250 pulses at 10 min intervals) nerve stimulation (Figures 6 and 7). Adenosine, R-PIA and NECA inhibited the stimulation-evoked noradrenaline release. Thus, in the presence of adenosine 10^{-4} M (period S5) the stimulation-evoked release of ^{3}H was reduced to 68% of control (±3%, $P < 0.05$, $n = 4$) (Figure 7b). During R-PIA 10^{-6} M (period S5) the stimulation-evoked release of ^{3}H was decreased to 56% of control (±5%, $P < 0.01$, $n = 5$) (Figure 7c). Similarly, during NECA 10^{-6} M (period S5) the stimulation-evoked release of ^{3}H was reduced to 60% of control (±9%, $P < 0.05$, $n = 5$) (Figure 7d). The inhibitory effect of NECA 10^{-6} M on ^{3}H release was abolished in the presence of 8-*p*-sulphophenyltheophylline 10^{-4} M (^{3}H release 94 ± 6% of control, $n = 5$, not shown). To discover whether the inhibitory effect of the purines was still evident at higher stimulation frequencies, the effect of NECA 10^{-7}–10^{-6} M on stimulation-evoked release of ^{3}H was studied at 15 Hz, 1 ms, 60 V, 250 pulses at 10 min

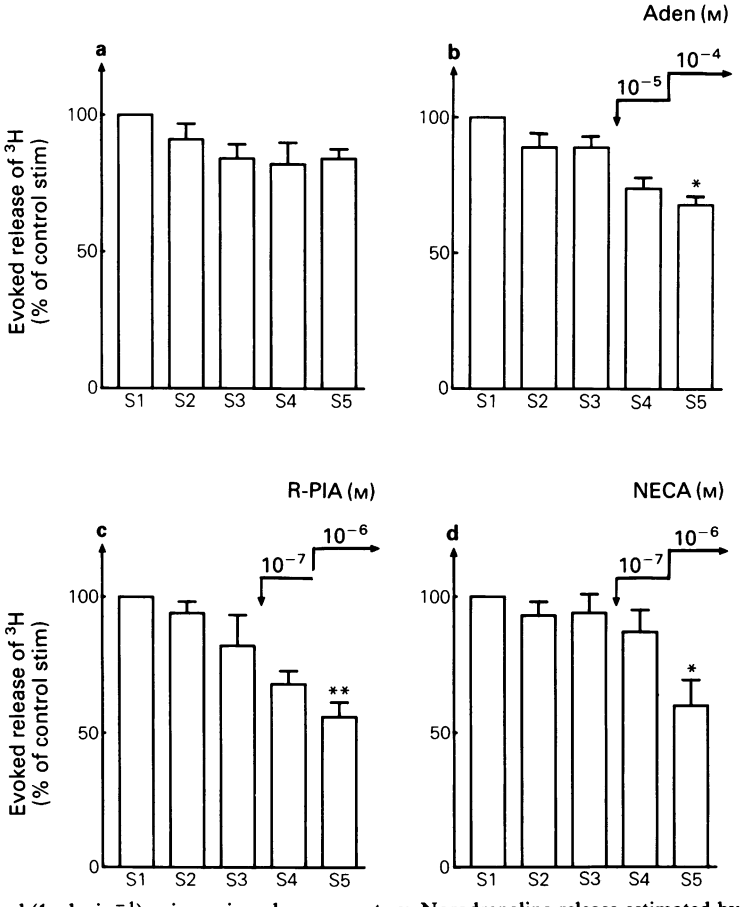

Figure 7 Perifused (1 ml min^{-1}) guinea-pig pulmonary artery. Noradrenaline release estimated by measurement of fractional ^{3}H overflow into perifusate collected at 2 min intervals from preparations preincubated with [^{3}H]-noradrenaline. Inhibition of stimulus-evoked (5 Hz, 1 ms, 250 pulses at 10 min intervals) fractional release of ^{3}H by (b) adenosine (Aden, 10^{-5}–10^{-4} M, $n = 4$), (c) R-PIA (10^{-7}–10^{-6} M, $n = 5$) and (d) NECA (10^{-7}–10^{-6} M, $n = 5$). Stimulus-evoked fractional release of ^{3}H was calculated as denoted in Methods. Statistical analysis (Student's *t* test for unpaired variates) was made for the comparison with the corresponding stimulation period in the control series (a, $n = 5$). Means with s.e.mean shown by vertical bars. *$P < 0.05$, **$P < 0.01$. n = number of experiments.

intervals. During this type of stimulation, NECA at 10^{-6} M inhibited the evoked release of ^{3}H to 65% of control ($\pm$7%, $P < 0.05$, $n = 4$) (not shown).

However, during NECA application a stimulatory effect on noradrenaline overflow was also observed. Thus, although the nerve stimulation-evoked release was markedly depressed (Figures 6, 7d), the total release of ^{3}H was increased during 10 min of NECA application (Figures 6, 8d), probably as a result of an increased basal overflow of ^{3}H. During 10 min of NECA 10^{-6} M application, the total ^{3}H overflow (period R5) was 130% of control ($\pm$9%, $P < 0.05$, $n = 5$) (Figures 6, 8d). In the presence of 8-*p*-sulphophenyltheophylline 10^{-4} M NECA 10^{-6} M failed to cause an increase of the ^{3}H overflow (total ^{3}H release during period R5 was 97 $\pm$ 4%, $n = 5$, not shown). R-PIA at 10^{-6} M decreased the total overflow of ^{3}H to 68% of control ($\pm$8%, $P < 0.05$, $n = 5$) (Figure 8c), whereas adenosine at 10^{-4} M was without significant effect (81 $\pm$ 4% of control, $n = 4$) (Figure 8b).

8-*p*-Sulphophenyltheophylline (3×10^{-5}–3×10^{-4} M) was added to pulmonary arteries not having received any exogenous purines, in order to study whether endogenous purines could modulate nerve-induced contractions or ^{3}H release. Thus, at

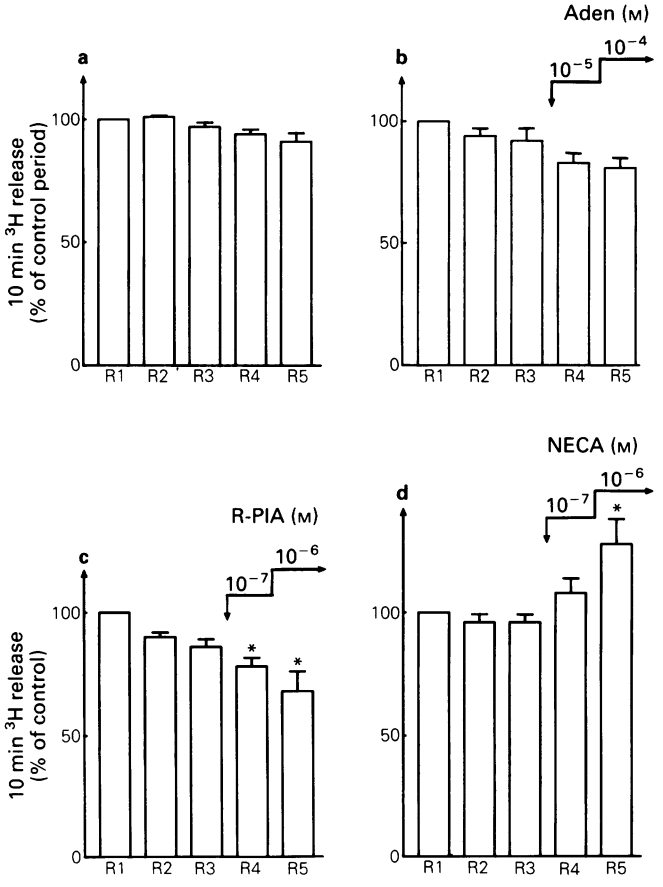

Figure 8 Perifused (1 ml min^{-1}) guinea-pig pulmonary artery: noradrenaline release estimated by measurement of fractional ^{3}H overflow into perifusate collected at 2 min intervals from preparations preincubated with [^{3}H]-noradrenaline. Total release of ^{3}H during the 10 min periods (R1, R2 etc) each consisting of three prestimulation, one stimulation and one poststimulation sampling period (see Methods). (a) Control experiments ($n = 6$); (b) effect on total ^{3}H efflux by adenosine (Aden, 10^{-5}–10^{-4} M, $n = 4$); (c) inhibition of the total ^{3}H efflux by R-PIA (10^{-7}–10^{-6} M, $n = 5$); (d) enhancement of the ^{3}H efflux by NECA (10^{-7}–10^{-6} M, $n = 5$). Statistical analysis of drug effects (Student's t test for unpaired variates) was made (as indicated in c and d) by comparison with the corresponding 10 min period in the control series (a). Means with s.e.mean shown by vertical bars. *$P < 0.05$, n = number of experiments.

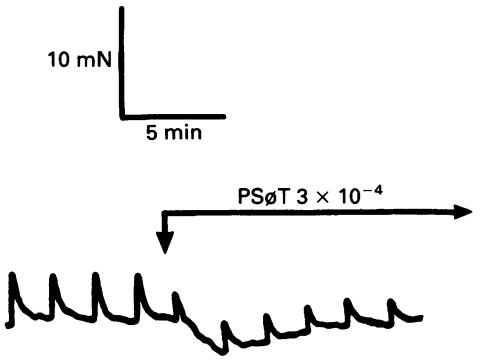

Figure 9 Contractile responses to transmural nerve stimulation (20 Hz, 1 ms, 200 pulses at 2 min intervals) in guinea-pig pulmonary artery. Inhibition of nerve-induced contractile responses and lowering of basal muscle tone by 8-*p*-sulphophenyltheophylline (PSØT) (3×10^{-4} M).

3×10^{-5} M the nerve-induced contractile responses were inhibited by 39% (±5, $n = 27$) and the basal tone was decreased by 20% (±6, $n = 27$) of the control response to transmural stimulation. 8-*p*-Sulphophenyltheophylline at 3×10^{-4} M showed the same effects (Figure 9). The stimulation-evoked release of ^{3}H or the total overflow of ^{3}H was not significantly altered by 8-*p*-sulphophenyltheophylline (10^{-4} M) (83 ± 3% and 90 ± 3% of control, respectively, $n = 4$) (not shown).

Discussion

The major part of the contractile response to transmural stimulation in the guinea-pig pulmonary artery was probably due to activation of postganglionic adrenergic nerves, since the contractile response was mimicked by noradrenaline, antagonized by phentolamine, abolished by tetrodotoxin and accompanied by [^{3}H]-noradrenaline release.

In contrast to purine effects in most other vascular beds, adenosine and its derivatives enhanced the nerve-induced contractile responses and augmented the basal muscle tone in the guinea-pig pulmonary artery. This is similar to findings in the renal arteries where adenosine is known to enhance the nerve-induced contractile responses although it inhibits noradrenaline release (Hedqvist & Fredholm 1976; Hedqvist *et al.*, 1978). The finding that noradrenaline and $PGF_{2\alpha}$ increased the basal muscle tone and concomitantly enhanced the nerve-induced contractions indicates a relationship between muscle tone and contractile responses to transmural stimulation.

From the present data, three distinct patterns of purine action may be discerned. First: action at stimulatory postjunctional receptors, most likely of the A_1 subtype since the nucleosides enhanced contractile responses to exogenous noradrenaline, and where R-PIA was at least 100 fold more potent than S-PIA. Second: action at inhibitory prejunctional receptors, most likely of the A_1 subtype since R-PIA and NECA were equipotent at this receptor as judged from their effect on stimulus-evoked ^{3}H overflow. Equipotency of NECA and R-PIA has been observed at other prejunctional adenosine A_1 receptors (Paton, 1981; Gustafsson *et al.*, 1985a; Wiklund, 1985; Wiklund *et al.*, 1986). Third: action at stimulatory prejunctional receptors, leading to the observed increase in total [^{3}H]-noradrenaline overflow. These receptors were probably of the A_2 subtype since NECA was more effective than R-PIA.

Regarding the modulation of motor responses, the stimulatory effects (possibly both pre- and postjunctional) of adenosine seemed to dominate over the prejunctional inhibitory effect. Furthermore, it is likely that of the two stimulatory effects, the postjunctional A_1 receptor-mediated stimulation was more important than the prejunctional A_2 receptor-mediated stimulation, since the structure-activity data for the adenosine analogues on enhancement of nerve-induced contractile responses and enhancement of the basal muscle tone were more compatible with action at A_1 receptors.

The inhibitory effect of purines on noradrenaline and acetylcholine release is well established (Wiklund, 1985; Fredholm *et al.*, 1987), whereas a prejunctional effect of NECA at A_2 receptors mediating enhancement of noradrenaline release has to our knowledge previously never been shown. Adenosine had no stimulatory effect on ^{3}H overflow. This might suggest that the enhancing effect of NECA on ^{3}H overflow might be unrelated to adenosine effects. However, since the NECA effect was antagonized by 8-*p*-sulphophenyltheophylline, it is highly probable that the effect was via adenosine receptors, but required a selective agonist such as NECA to be revealed. Since adenosine is non-selective, this enhancing effect on ^{3}H overflow might not be of physiological importance, but might be of importance in future pharmacological exploration of highly selective A_2 receptor agonists. In support of the present findings it has, from studies on effector responses in rabbit bronchial smooth muscle and in rabbit iris, been suggested that adenosine could act to enhance cholinergic and non-adrenergic non-cholinergic transmitter release, respectively, via prejunctional adenosine A_2 receptors (Wiklund, 1985; Gustafsson & Wiklund, 1986; Gustafsson *et al.*, 1985b; 1986).

8-*p*-Sulphophenyltheophylline inhibited nerve-induced contractions and lowered basal muscle tone in preparations not having received any exogenous

purines. This indicates that under these experimental conditions, endogenous purines were present in amounts sufficient to enhance the neuroeffector transmission. It is well known that the pulmonary arteries respond with vasoconstriction during alveolar hypoxia (the Euler-Liljestrand mechanism). Since adenosine is released during hypoxia (Mentzer *et al.*, 1975) and adenosine showed contractile effects in the pulmonary artery in our experiments we would suggest the possibility that adenosine, or other purines acting on adenosine receptors, might be involved in the pulmonary artery pressor response to alveolar hypoxia. Possibly in support of this notion it has been found that the methylxanthine aminophylline (also an adenosine antagonist, Daly, 1982; Gustafsson, 1984) blocks the pulmonary pressor response to alveolar hypoxia in humans, although the aminophylline effect was ascribed to an interference with cyclic AMP mechanisms (Sill *et al.*, 1973). However, since adenosine infusion can evoke vasodilatation in the pulmonary circulation (Mentzer *et al.*, 1975), possible dual effects of purines on the vasculature should be considered for future study. At any rate, the guinea-pig pulmonary artery should prove to be a useful *in vitro* model for stimulatory effects of purine nucleosides on blood vessels, as can be seen in the kidney (Osswald, 1975; Hedqvist & Fredholm, 1976).

In conclusion, adenosine and its derivatives enhanced nerve-induced contractile responses and augmented the basal smooth muscle tone in isolated spirals of the guinea-pig pulmonary artery. These effects were mainly due to action at postjunctional stimulatory A_1 receptors, but actions at prejunctional inhibitory A_1 and stimulatory A_2 receptors were also evident. The results therefore lend support for a prejunctional stimulatory adenosine action not only on cholinergic and peptidergic autonomic nerves (Gustafsson *et al.*, 1985b; 1986; Gustafsson & Wiklund, 1986), but also on noradrenergic neurotransmission as distinct from the well-known prejunctional inhibitory effect by adenosine. The present findings may suggest a role for adenosine, or other purines acting on adenosine receptors, in the regulation of pulmonary blood flow.

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5-HT_1-like receptors mediate 5-hydroxytryptamine-induced contraction of human isolated basilar artery

[1]Andrew A. Parsons, Eric T. Whalley, *Wasyl Feniuk, *Helen E. Connor & *Patrick P.A. Humphrey

Department of Physiological Sciences, University of Manchester, Oxford Road, Manchester M13 9PT and *Department of Cardiovascular Pharmacology, Glaxo Group Research Limited, Ware, Herts., SG12 0DJ

1 The 5-hydroxytryptamine (5-HT) receptor mediating contraction of endothelium denuded human basilar artery has been characterized *in vitro*.

2 5-HT and a variety of 5-HT agonists contracted human isolated basilar artery with a rank order of agonist potency, 5-carboxamidotryptamine (5-CT) > 5-HT ≡ methysergide > GR43175 ≫ 8-OHDPAT ≫ 2-methyl-5-HT. The maximum response produced by these agonists differed.

3 None of the agonists relaxed human basilar artery when tone was elevated with prostaglandin $F_{2\alpha}$, indeed further contraction was seen.

4 The contractile responses of human basilar artery to 5-HT and the selective 5-HT_1-like agonist GR43175 were highly reproducible whilst those to 5-CT were not.

5 The contractile response to both 5-HT and GR43175 was resistant to antagonism by ketanserin and GR38032, thus excluding activation of 5-HT_2 and 5-HT_3 receptors. The contractile action of 5-HT and GR43175 was also not antagonized by (±)-cyanopindolol, excluding the activation of receptors similar to 5-HT_{1A} and 5-HT_{1B} recognition sites identified in ligand binding studies.

6 In marked contrast, methiothepin was a potent antagonist of the contractile actions of both 5-HT and GR43175, with a pA_2 value of 8.8 against both agonists. Methiothepin (100 nM) had no effect on the contractile response to the thromboxane A_2-mimetic U46619.

7 We conclude that 5-HT and GR43175 contract the human isolated basilar artery by activating the same receptor type. This receptor appears identical to the 5-HT_1-like receptor causing contraction of the dog isolated saphenous vein and cerebral blood vessels from the dog and primate.

Introduction

5-Hydroxytryptamine (5-HT) has been localised in platelets as well as perivascular nerves in cerebral blood vessels (Tranzer *et al.*, 1972; Edvinsson *et al.*, 1984). Although the functional significance of 5-HT in the cerebral circulation is still unclear, the amine has been implicated in a variety of cerbrovascular disorders including migraine and cerebral vasospasm (Sicuteri, 1967; Allen *et al.*, 1974). Understanding of the role that 5-HT may play in the cerebral circulation can only be achieved by a greater understanding of the receptors that mediate the many and varied biological actions of this amine.

Recently 5-HT receptors have been characterized into three main types according to the actions of some recently identified 5-HT agonists and antagonists. These receptors have been named 5-HT_1-like, 5-HT_2 and 5-HT_3 (Bradley *et al.*, 1986). Previous studies (Forster & Whalley, 1982) have shown that the potent contractile effect of 5-HT in the human isolated basilar artery is mediated via activation of a receptor which is not blocked by a variety of 5-HT_2 receptor blocking drugs.

In the present study we have further characterized the 5-HT receptor mediating contraction of human isolated basilar artery by examining the action of some recently identified 5-HT agonists and purported antagonists. In particular we have investigated the effect of the highly selective 5-HT_1-like agonist, GR43175 which causes contraction of dog isolated saphenous vein (Humphrey *et al.*, 1988). and dog and primate cerebral blood vessels (Connor *et al.*, 1987) via activation of 5-HT_1-like receptors. Interestingly,

[1] Author for correspondence at present address: Physiologisches Institut, Universität München, Pettenkoferstr. 12, D-8000, München, B.R.D.

GR43175 has recently been shown to be efficacious in the treatment of actute migraine headache (Doenicke *et al.*, 1988).

Methods

Human basilar arteries from either sex, were obtained *post-mortem* (6–18 h) from patients who had died from non-neurological causes. Usually, the vertebral arteries, basilar and part or all of the circle of Willis were removed and placed in isotonic saline. In the laboratory the arteries were placed immediately in Krebs-Henseleit solution of the following composition: (mM) NaCl 118, KCl 4.75, $CaCl_2 \cdot 6H_2O$ 2.55, $MgSo_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.19, $NaHCO_3$ 25 and glucose 11, bubbled with 95% O_2 and 5% CO_2 and maintainined at 0–4°C until use.

The basilar artery was then dissected and cut into vascular rings of approximately 4–6 mm width and the endothelium was removed by gentle rubbing with a shaved wooden cocktail stick. The vascular rings were suspended on small stainless steel hooks in 4 ml tissue baths and bathed with Krebs-Henseleit solution containing 2.8 μM indomethacin, bubbled with 95% O_2 and 5% CO_2 and maintained at 37°C.

Vascular rings were placed under a resting isometric tension of 1 g. Changes in isometric tension were measured by a transducer (Pye Ether, 2 oz) and recorded on a Grass Polygraph Model 79D. The rings were allowed a 2 h equilibration period with repeated washing every 10–20 min. After this period two concurrent cumulative concentration effect (C-E) curves to 5-HT (0.1 nM to 100 μM) were constructed with a time interval of 60–90 min between each. After this time, up to 3 C-E curves were constructed to a variety of agents at 60–90 min intervals under resting or sustained tension, or in the absence and presence of a range of antagonists (see below).

Test for functional endothelium

The functional integrity of the endothelium was assessed by application of 1 μM acetylcholine (Furchgott *et al.*, 1984) on sustained tension to 5-HT. On some preparations *en face* silver staining (Poole *et al.*, 1958) was also used to confirm the presence or absence of endothelium.

Determination of rank orders of agonist potency

Basal tone After the construction of C-E curves to 5-HT, cumulative C-E curves to 5-CT (0.1 nM to 100 μM), GR43175 (10 nM to 100 μM), methysergide (0.1 nM to 100 μM), 8-OH-DPAT (10 nM to 100 μM) or 2-methyl–5-HT (2-Me–5-HT) (0.1 nM to 100 μM) were constructed. One preparation received only 5-HT as a concurrent control in order to assess spontaneous changes in agonist sensitivity.

Elevated tone After construction of the C-E curve to 5-HT, cumulative C-E curves to $PGF_{2\alpha}$ (0.1 nM–100 μM) were constructed and an EC_{50} selected; 60–90 min later this EC_{50} of $PGF_{2\alpha}$ was applied to the tissues and when sustained tension had been achieved, 5-HT (0.1 nM–100 μM) was added cumulatively to the tissue. Subsequently, C-E curves were constructed under sustained tension to either 5-CT, GR43175, 8-OH-DPAT, 2-Me-5-HT or 5-HT (concurrent control).

Antagonist studies

Concentration-effect curves were constructed to either 5-HT or GR43175, in the absence and in the presence of either methiothepin (1 nM and 100 nM), ketanserin (100 nM), GR38032 (100 nM), (±)-cyanopindolol (100 nM) or phentolamine (1 μM). Antagonist contact time was 30 min. A cumulative C-E curve to U-46619 was also constructed in the presence and absence of methiothepin. In each case at least one preparation received no antagonist and acted as concurrent control.

Analysis of data

Geometric mean EC_{50} (that concentration producing 50% of its own maximal response) for each agonist was calculated on individual curves. Changes in EC_{50} and in maximal response from control were assessed by a paired Student's t test (two tailed) at a significance level of $P = 0.05$. Where appropriate, concentration-ratio shifts for agonists in the presence of antagonists and a pA_2 value were calculated (Arunlakshana & Schild, 1959).

Drugs

The drugs used were: GR38032 (1,2,3,9-tetrahydro-9-methyl-3[2-methyl 1H-imidazol-1-yl)methyl]4H-carbazol-4-one, $HCl \cdot 2H_2O$), GR43175(3-[2](dimethylamino)-ethyl-N-methyl-1H indole 5 methane sulphonamide), 2-methyl-5-hydroxytryptamine HCl. H_2O, (±)-cyanopindolol butendioate, 8-OH-DPAT (8-hydroxy-2-(di-n-propylamino)tetralin) HBr, 5-carboxamidotryptamine (5-CT; 3-(2-aminoethyl)-1-H-indole-5-carboxamide maleate) (all synthesized by the Chemistry Department, Glaxo Group Research Limited, Ware), 5-hydroxytryptamine creatinine sulphate (Sigma), methiothepin maleate (Hoffman La Roche), phentolamine mesylate (Ciba), methysergide hydrogenmaleinate (Sandoz). All the above drugs were dissolved in isotonic saline. Ketanserin tartate (Janssen) was dis-

solved in distilled water. Acetylcholine chloride (Sigma), prostaglandin $F_{2\alpha}$ Tris salt (Sigma), U-46619 (9,11 dideoxy 9, 11_{α} methaneopoxy prostaglandin $F_{2\alpha}$, Cayman Chemicals), indomethacin (Sigma) were dissolved in absolute alcohol. $PGF_{2\alpha}$ and U-46619 were made into stock solutions of 10 mM. All dilutions were in isotonic saline and made on the day of use. All stocks and dilutions were kept on ice.

Results

Rank order of agonist potency

Basal tension All compounds tested, except 2-Me-5-HT which was inactive, produced concentration-related contractions of the human isolated basilar artery under resting tension. Concentration-effect curves are plotted in Figure 1 and the pD_2 ($-\log EC_{50}$) values and potency ratios compared to 5-HT are shown in Table 1. Thus, the rank order of agonist potency was found to be 5-CT > 5-HT ≡ methysergide > GR43175 ⩾ 8-OH- DPAT ⩾ 2-Me-5-HT. Compared to 5-HT all the other compounds produced maximum responses which were significantly less than that seen to 5-HT, the order being 5-HT > 5-CT > GR43175 > methysergide ≡ 8-OH-DPAT ⩾ 2-Me-5-HT.

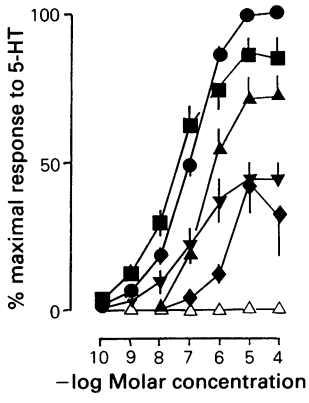

Figure 1 Concentration-effect curves to 5-hydroxytryptamine (●), 5-carboxamidotryptamine (5-CT, ■), methysergide (▼), GR43175 (▲), 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT, ◆) and 2-Me-5-HT (△) on the human isolated basilar artery under resting tension. Only one agonist was compared with 5-HT on any given tissue and individual potency ratios determined, the data being presented in Table 1. The curve to 5-HT represents the pooled data obtained with all tissues. Vertical bars represent s.e.means of $n \geqslant 4$.

Table 1 Geometric mean, pD_2 ($-\log EC_{50}$) and geometric mean potency ratios with 95% confidence intervals (5-HT assigned a potency = 1) for 5-carboxamidotryptamine (5-CT), 5-HT, methysergide, GR43175, 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) and 2-methyl-5-hydroxytryptamine (2-Me-5-HT) on the human isolated basilar artery under resting tension; also shown are values for the degree of contraction (E_{max}) produced for each agent expressed as the percentage of the 5-HT maximum

Agonist	pD_2	*Potency ratio* (95% CI)	E_{max} (% 5-HT max)
5-CT	7.59* ±0.16	0.14* (0.05–0.4)	86.9* ±5.8
5-HT	6.99 ±0.07	1	100
Methysergide	6.87 ±0.28	3.0 (0.3–30.6)	45.3* ±5.4
GR43175	6.45* ±0.06	3.1* (1.9–5.0)	71.4* ±6.4
8-OH-DPAT	5.73* ±0.05	22.39* (11.8–42.6)	43.5* ±10.3
2-Me-5-HT	<4.0	—	—

Values of pD_2 and E_{max} are mean ± s.e.mean. Only one agonist was compared with 5-HT on any given tissue. The data presented for 5-HT are those pooled from all preparations; $n \geqslant 4$. *$P < 0.05$ compared with respective 5-HT data.

Elevated tension At no concentration of any agonist used was relaxation of the $PGF_{2\alpha}$ precontracted artery observed.

Antagonist study

The effect of a variety of antagonists at different 5-HT receptors was initially assessed against 5-HT, GR43175 and 5-CT. Preliminary experiments indicated that concentration-effect curves to 5-HT and GR43175, but not 5-CT, were reproducible at 60–90 min intervals (Figure 2). For this reason antagonists were assessed only against 5-HT and GR43175.

Methiothepin produced concentration-related rightward shifts of the concentration-effect curves to 5-HT and GR43175 (Figure 3a and b respectively) compared to concurrent controls. From a Schild plot of methiothepin (1 nM, 10 nM and 100 nM) versus 5-HT a pA_2 value of 8.8 (8.3–9.3; 95% confidence intervals) and a slope of 0.9 (0.7–1.25; 95% confidence intervals) ($n = 5$) were calculated.

Full concentration-effect curves to GR43175 in the presence of the highest concentration of methiothepin were not determined. Thus a Schild plot for methiothepin against GR43175 was constructed using an equi-effective concentration-ratio calculated

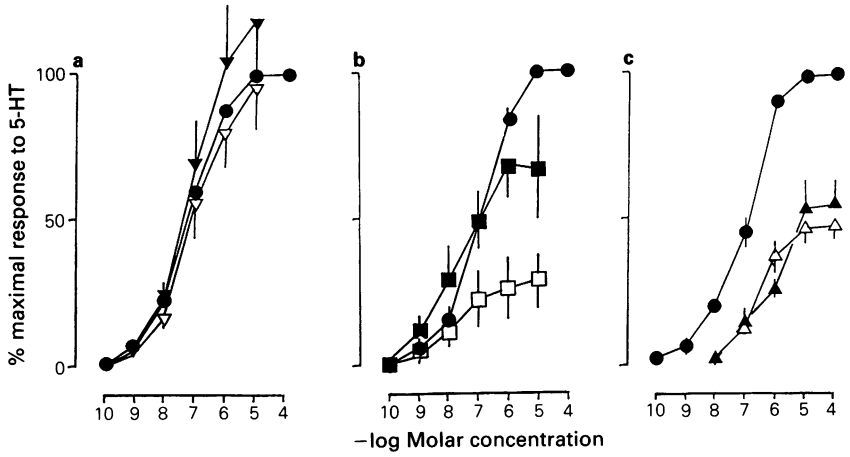

Figure 2 Concentration-effect curves to (a) 5-hydroxytryptamine (5-HT, ▼, 1st curve; ▽ 2nd curve) (b) 5-carboxamidotryamine (5-CT ■, 1st curve; □, 2nd curve) and (c) GR43175 (▲, 1st curve; △, 2nd curve) constructed at intervals of 60–90 min after construction of an initial concentration-effect curve to 5-HT (●) on human isolated basilar artery. Vertical bars represent s.e.means of $n \geqslant 4$.

at the 40% of maximum response level. From this plot a pA_2 value of 8.8 (7.8–9.9, 95% confidence interval) and a slope of 0.8 (0.5–1.1, 95% confidence interval) ($n = 5$) were obtained. In contrast, the highest concentration of methiothepin used (100 nM) had no effect on C-E curves to U-46619.

In the presence of phentolamine (1 μM) a concentration-ratio shift of 2.95 (0.98–8.85; 95% confidence interval; $n = 5$) in the concentration-effect curve to 5-HT ($P = 0.052$) was obtained. A similar but significant ($P < 0.05$) rightward shift (2.95 with 95% confidence limits being 2.46–3.54; $n = 4$) occurred with GR43175. No significant differences were observed in maximal responses to 5-HT or GR43175 between control and test C-E curves (Figure 4a).

Concentration-effect curves to 5-HT or GR43175 were unaffected in the presence of either ketanserin (100 nM), or GR38032 (100 nM), (Figure 4b,c) compared to concurrent controls. (±)-Cyanopindolol at 100 nM was seen to produce a small contraction of the tissues when added to the bath, however no sig-

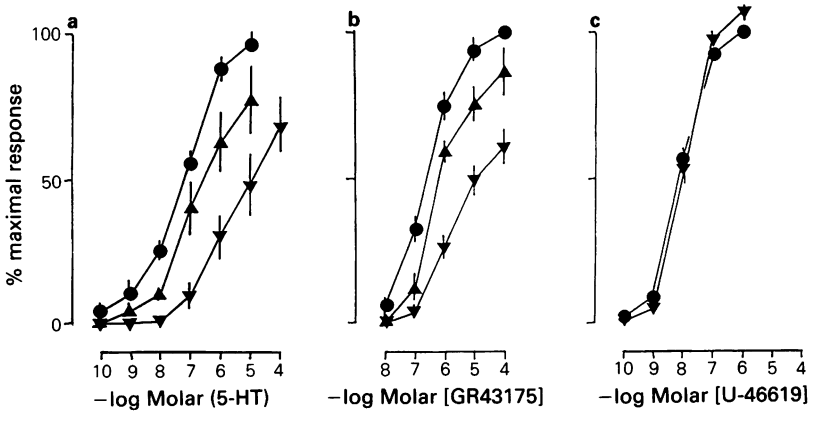

Figure 3 Concentration-effect curves to (a) 5-hydroxytryptamine (5-HT), (b) GR43175 and (c) U-46619 on the human isolated basilar artery in the absence (●) and presence of methiothepin, 1 nM (▲) or 100 nM (▼). Vertical bars represent s.e.means of $n = 3$–6.

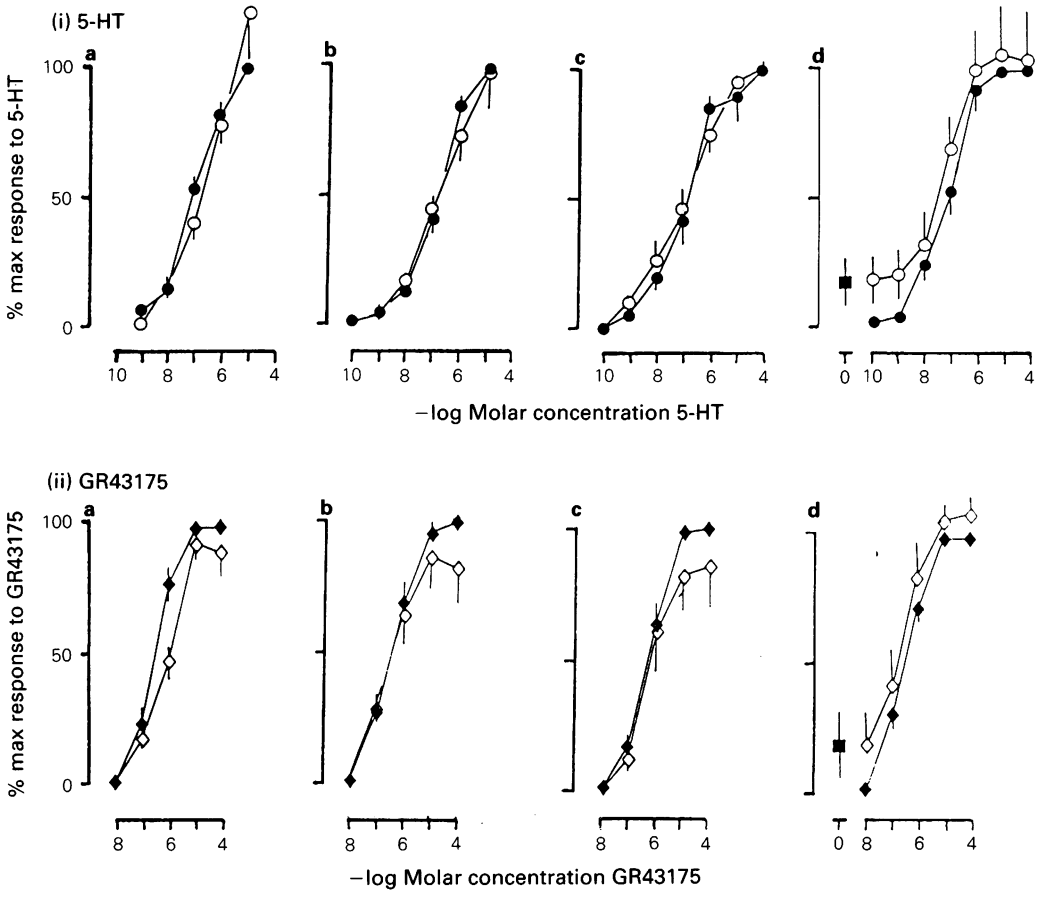

Figure 4 Concentration-effect curves to (i) 5-hydroxytryptamine (5-HT) and (ii) GR43175 on the human isolated basilar artery in the absence (solid symbols) and presence (open symbols) of (a) phentolamine, 1 μM, (b) ketanserin, 0.1 μM, (c) GR38032, 0.1 μM and (d) (±)-cyanopindolol, 0.1 μM. The response produced by cyanopindolol alone at 0.1 μM (■) is also shown for comparison. Vertical bars represent s.e.means of $n = 3$–6.

nificant differences were seen between C-E curves to 5-HT or GR43175 compared to concurrent controls in the presence of this antagonist (Figure 4d).

Discussion

The results from the present study have demonstrated that 5-HT is a potent agonist causing contraction of human basilar artery. The aim of the study was to characterize the receptor involved in this action using some recently identified 5-HT agonists and antagonists. Previous studies have shown that 5-HT-induced contractions of human basilar artery are resistant to blockade by 5-HT_2 receptor blocking drugs (Forster & Whalley, 1982). Indeed this observation has been confirmed in the present study by the absence of antagonist effects of the 5-HT_2 receptor blocking drug, ketanserin. Furthermore, activation of 5-HT_3 receptors can also be excluded since 2-Me-5-HT was devoid of agonist activity and GR38032 had no effect on the contractile response to 5-HT (see Bradley *et al.*, 1986).

It was previously suggested (Forster & Whalley, 1982) that the 5-HT receptor mediating contraction of human basilar artery had some similarity with the 5-HT_1-like receptor mediating contraction of the dog isolated saphenous vein (Apperley *et al.*, 1980). This conclusion was largely based on the agonistic action of methysergide which we have again confirmed. The results from the present study provide further evidence that the characteristics of the 5-HT receptor mediating contraction of human cerebral blood vessels are similar to the 5-HT_1-like receptors

causing contraction of the dog isolated saphenous vein (Humphrey *et al.*, 1988). This conclusion is based upon several experimental observations.

The selective 5-HT_1-like agonists, 5-CT and GR43175 (Humphrey *et al.*, 1988) were potent agonists causing contraction of the human basilar artery and were potent agonists causing contraction of the dog isolated saphenous vein (Feniuk *et al.*, 1985; Humphrey *et al.*, 1988). It is however noteworthy that in human basilar artery the maximum response to these selective agonists was slightly less than that observed with 5-HT. Since 5-HT has been shown to relax cerbrovascular smooth muscle when tone is increased (Edvinsson *et al.*, 1978), it could be argued that the lower maximum response to 5-CT and GR43175 may reflect an action of these compounds at 5-HT receptors mediating relaxation (Feniuk *et al.*, 1983; Trevethick *et al.*, 1986). Such an explanation seems unlikely in view of the fact that GR43175 is devoid of activity at 5-HT_1-like receptors mediating relaxation of vascular smooth muscle (Humphrey *et al.*, 1988) and in the present study neither 5-CT nor GR43175 relaxed the human basilar artery when tone was increased with $PGF_{2\alpha}$. Although we have no direct evidence to explain this small difference in the maximum response to 5-CT and GR43175 when compared with 5-HT in the human basilar artery it is possible that 5-CT and GR43175 may be acting as partial agonists in this blood vessel. Such a difference in maxima was not seen in the dog isolated saphenous vein (Humphrey *et al.*, 1988), and may reflect differences in receptor coupling/density (Black & Leff, 1983).

The experimental studies with methiothepin were also consistent with the view that the contractile response to 5-HT and GR43175 was due to the activation of 5-HT_1-like receptors since methiothepin caused a concentration-dependent and specific antagonism of the effects of both agonists. The effect of 5-HT and GR43175 was not mediated via the activation of α-adrenoceptors since phentolamine was only a weak antagonist of the effect of 5-HT and GR43175 which is probably a reflection of its weak 5-HT receptor blocking activity (Feniuk, 1984).

In order to investigate whether the 5-HT_1-like receptor mediating contraction of human basilar artery was similar to either of the 5-HT_{1A} or 5-HT_{1B} recognition sites identified from ligand binding studies, experiments were carried out with the 5-HT_{1A} ligand 8-OH-DPAT (Middlemiss & Fozard, 1983) and (±)-cyanopindolol which has a high affinity at both 5-HT_{1A} and $_{1B}$ recognition sites (Hoyer *et al.*, 1985). 8-OH-DPAT was a weak agonist at causing contraction of human basilar artery, an effect which would be inconsistent with its high affinity for 5-HT_{1A} receptors. Cyanopindolol also had no effect on the contractile response to either 5-HT or GR43175 in the human isolated basilar artery, despite causing a small contraction itself. Similar results have been found in the dog isolated saphenous vein (Humphrey *et al.*, 1988). These results clearly demonstrate that the 5-HT_1-like receptor mediating contraction of human basilar artery is dissimilar to the 5-HT_{1A} and $_{1B}$ recognition sites identified in ligand binding studies and is different from the 5-HT_{1A} receptor mediating contraction of the canine basilar artery (Peroutka *et al.*, 1986; Taylor *et al.*, 1986). Indeed, the contractile action of 8-OH-DPAT on the human basilar artery can be blocked by phentolamine (unpublished observations) suggesting that this compound has agonist activity at α-adrenoceptors. The present study has not excluded the possibility that the contractile effect of 5-HT and GR43175 may be mediated by receptors similar to the 5-HT_{1C} and 5-HT_{1D} recognition sites identified in ligand binding studies (Pazos *et al.*, 1984; Heuring & Peroutka, 1987). However, the similarity between the 5-HT receptor causing contraction of human basilar artery and dog saphenous veins has already been emphasied. In the latter preparation an action of GR43175 at such sites has been excluded (Humphrey *et al.*, 1988).

Although the precise mechanism of the antimigraine action of GR43175 (Doenicke *et al.*, 1988) is not known, the present study has clearly demonstrated its ability to constrict human cerebral arteries. Since dilatation of intracranial blood vessels is known to produce pain (Lance, 1973) it is possible that the antimigraine action of GR43175 is a reflection of its ability to constrict excessively dilated cranial arteries. One site implicated on the basis of current concepts are the meningeal blood vessels (Markowitz *et al.*, 1988). An action of GR43175 on cerebral blood flow seems unlikely since GR43175 does not decrease cerebral flow in animal studies (Feniuk *et al.*, 1987).

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Time course and extent of α_1-adrenoceptor density changes in rat heart after β-adrenoceptor blockade

[1]Volker Steinkraus, Monika Nose, Hasso Scholz & Katrin Thormählen

Abteilung Allgemeine Pharmakologie, Universitäts-Krankenhaus Eppendorf, Universität Hamburg, Martinistrasse 52, D-2000 Hamburg 20, F.R.G.

1 It has been suggested that impaired β-adrenoceptor stimulation is a condition under which the functional role of cardiac α_1-adrenoceptors is enhanced. We therefore investigated the extent and time course of changes in α_1-adrenoceptor characteristics after chronic treatment with the β-adrenoceptor blocker propranolol in rat heart. For comparison β-adrenoceptors were also studied. The mechanism of the changes in adrenoceptor density was investigated with cycloheximide, an inhibitor of protein synthesis. The functional significance of an increased α_1-adrenoceptor density was tested by measuring isometric force of contraction in the presence of phenylephrine or isoprenaline in right ventricular papillary muscles.

2 Rats were treated with propranolol ($9.9\,mg\,kg^{-1}$ daily) or 0.9% NaCl, applied with osmotic minipumps for 1, 2, 3 or 7 days. Propranolol treatment resulted in a maximally 28% increase of α_1-adrenoceptor density after 3 days (NaCl 95.9 ± 3.5 vs. propranolol $123.0 \pm 1.6\,fmol\,mg^{-1}$ protein, $n = 6$, $P < 0.01$). This up regulation reached significant levels after 2 days of treatment and was reversible after cessation of treatment within two days. K_D-values were the same for NaCl- and propranolol-treated rats. Changes of B_{max} and K_D in β-adrenoceptor binding assays did not reach significant levels.

3 Cycloheximide ($1.5\,mg\,kg^{-1}$ i.p. daily for 3 days) inhibited the propranolol-induced increase in B_{max} of α_1-adrenoceptors completely. In addition, cycloheximide also decreased the density of α_1- and β-adrenoceptors also under control conditions.

4 pD_2-values for the positive inotropic effect of phenylephrine and isoprenaline in isolated electrically driven papillary muscle were similar in NaCl- and propranolol-treated rats (phenylephrine: 5.41 ± 0.11 vs. 5.41 ± 0.19, $n = 7$; isoprenaline: 6.31 ± 0.18 vs. 6.65 ± 0.19, $n = 7$). The observed increase in α_1-adrenoceptor density in healthy rat heart may therefore not be high enough to enhance the phenylephrine-induced increase in force of contraction.

5 In conclusion, time course and effects of cycloheximide indicate that the increase in B_{max} of myocardial α_1-adrenoceptors is due to *de novo* synthesis of receptors. However, at least for the rat heart model, a functional significance of this increase could not be demonstrated.

Introduction

Radioligand binding studies and functional experiments have given evidence for the coexistence of myocardial α_1- and β-adrenoceptors in hearts of various species, including rat (Alexander *et al.*, 1975; Williams & Lefkowitz, 1978; Wagner & Brodde, 1978; Winek & Bhalla, 1979; Mukherjee *et al.*, 1983; Brückner *et al.*, 1985). Though different effector enzymes and different second messengers seem to be involved (Endoh, 1986; Schmitz *et al.*, 1987; Scholz *et al.*, 1988), both adrenoceptors mediate positive inotropic effects (Benfey, 1973; 1987; Scholz, 1980). Since during β-adrenoceptor blockade the importance of β-adrenoceptors for regulation of cardiac performance is greatly reduced, it is conceivable that this enhances the significance of α_1-adrenoceptor-mediated effects. Indeed, previous studies have

[1] Author for correspondence.

demonstrated increased α_1-adrenoceptor density in rat heart after chronic propranolol treatment (Mügge *et al.*, 1985; 1986). But the time course and the functional importance of the increase in α_1-adrenoceptor density under chronic β-adrenoceptor blockade is unknown. Our aim was to study the extent and the time course of changes in α_1-adrenoceptor density (B_{max}) and affinity (K_D), measured by radioligand binding in rat heart ventricular membranes, after chronic propranolol-treatment. For comparison, the influence of propranolol on B_{max} and K_D of β-adrenoceptors was also studied. Since changes in receptor characteristics may be accompanied by altered responses to sympathomimetic drugs, the influence of the α_1-adrenoceptor agonist phenylephrine and the β-adrenoceptor agonist isoprenaline, respectively, on myocardial force of contraction was also investigated. The mechanism of the possible changes in adrenoceptor density was studied with cycloheximide, an inhibitor of protein synthesis.

Methods

Male Wistar rats (250–300 g), fed with altromin 1324 (Altromin, Lage, FRG) and water *ad libitum*, were treated with (±)-propranolol (9.9 mg kg^{-1} body weight daily) or 0.9% NaCl for control, supplied by osmotic minipumps (OMPS, ALZET, Palo Alto U.S.A., model 2ML2) for 1, 2, 3 or 7 days. OMPS were implanted on day zero under the neck skin during short ether anaesthesia. Assays were performed after 1, 2, 3 or 7 days of treatment. In addition, OMPS from rats receiving 7 days of either propranolol- or NaCl-treatment were explanted and these animals were killed 1, 2, 3 or 7 days following explantation. The effectiveness of propranolol-treatment was controlled by heart rate measurements with an ECG Cardioscript III (Schwarzer, Munich, FRG). The rats were killed by cervical dislocation, bled from the carotid arteries, the hearts quickly removed and dissected free of pericardium, atria, fat and large vessels. Ventricles (0.65–0.75 g) were weighed and placed in 20 volumes of ice-cold incubation buffer (50 mmol l^{-1} Tris-HCl, 10 mmol l^{-1} $MgCl_2$, pH 7.5). Crude membrane homogenates were prepared according to the method of Baker *et al.* (1980) with minor modifications. Briefly, the hearts were minced and homogenized in a precooled Ultra-Turrax tissue homogenizer (3 × 10 s bursts). The suspension was diluted with an equal volume of ice-cold 1 mol l^{-1} KCl solution and left on ice for 10 min before centrifugation at 48,000 *g* for 10 min at 4°C. Pellets were resuspended and homogenized with a motor driven glass-teflon homogenizer (Colora, Lorch, FRG) for 1 min at setting 6 and recentrifuged. This procedure was repeated twice. The final pellet was resuspended in 14 volumes of incubation buffer (to adjust the protein content in incubation assays to approximately 0.250 mg), passed through 4 layers of gauze and kept on ice under stirring. The radioligands [^{3}H]-prazosin and (−)-[^{3}H]-CGP-12177 were used to label myocardial α_1- and β-adrenoceptor binding sites, respectively. Assays were carried out in duplicate. Aliquots of 250 µl of the membrane preparations were incubated in a total volume of 1000 µl with either (−)-[^{3}H]-CGP-12177 (0.05–11.0 nmol l^{-1}) or [^{3}H]-prazosin (0.025–1.0 nmol l^{-1}). Time and temperature during incubation was 90 min at 35°C for β- and 60 min at 25°C for α_1-adrenoceptor binding, respectively. Conditions chosen allowed complete equilibration of radioligands with binding sites. Incubation was terminated by rapid vacuum filtration through Whatman GF/C filters and two 5 ml washes with ice-cold incubation buffer. Filters were dried at room temperature and radioactivity was counted by liquid scintillation spectrometry. Specific binding was calculated by subtracting non-specific binding (in the presence of 0.1 µmol l^{-1} prazosin for α_1- or 5 µmol l^{-1} propranolol for β-adrenoceptor binding) from total binding. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

The contraction experiments were performed on electrically driven papillary muscles isolated from the right ventricles of propranolol- and NaCl-treated rats, respectively. One of two papillary muscles from each rat was exposed to isoprenaline (concentration range from 1 nmol to 10 µmol l^{-1}) and the other to phenylephrine (concentration range from 0.1 µmol to 100 µmol l^{-1}). Preparations were attached to a bipolar platinum stimulating electrode and mounted individually in 10 ml glass tissue chambers for recording isometric contractions (for details see Böhm *et al.*, 1984). The bathing solution was a modified Tyrode solution containing (mmol l^{-1}): NaCl 119.8, KCl 5.4, $MgCl_2$ 1.05, $CaCl_2$ 1.8, NaH_2PO_4 0.42, $NaHCO_3$ 22.6, glucose 5.0, ascorbic acid 0.28 and Na_2-EDTA 0.05. The bathing solution was continuously aerated with 95% O_2 and 5% CO_2 (pH 7.4). Temperature was kept constant at 35°C. Force of contraction was measured with an inductive force transducer (W. Fleck, Mainz, FRG) attached to a Hellige Helco Scriptor recorder. Each muscle was stretched to the length at which force of contraction was maximal. The resting force (approximately 5 mN) was kept constant throughout the experiment. Stimulation frequency was 1 Hz (duration 5 ms, intensity 10–20% above threshold). All preparations were allowed to equilibrate for at least 60 min until

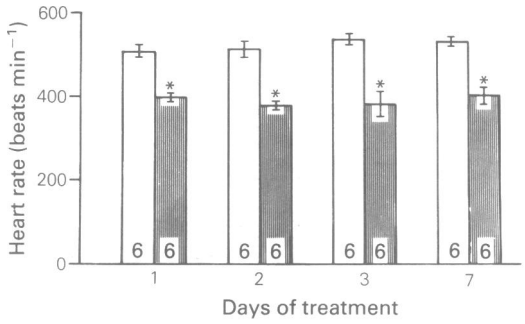

Figure 1 Effect of propranolol-treatment on heart rate of rats, determined by ECG-measurement. Ordinate scale: heart rate in beats min^{-1}; abscissa scale: days of propranolol- or NaCl-treatment (control), respectively. Open columns, control; hatched columns, (±)-propranolol 9.9 mg kg^{-1} daily. Numbers of animals are displayed in the columns. *$P < 0.01$.

complete mechanical stabilization before any drug addition. Concentration-response curves were obtained cumulatively. Time of exposure to each concentration of isoprenaline and phenylephrine was

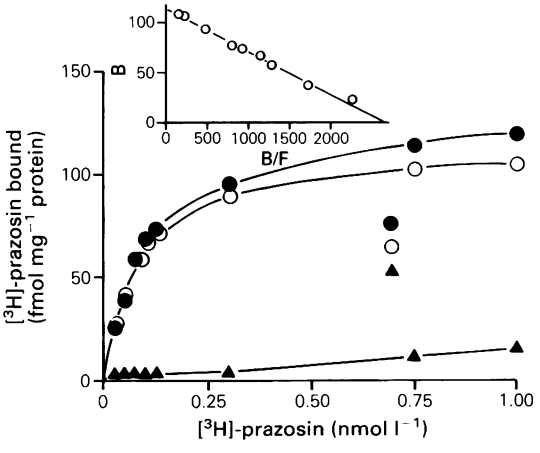

Figure 2 Total (●), specific (○) and non-specific (▲) [^{3}H]-prazosin binding in a single saturation experiment of rat heart ventricular membranes after propranolol-treatment for 3 days. Ordinate scale: [^{3}H]-prazosin bound in fmol mg^{-1} protein; abscissa scale: concentration of radioligand in nmol l^{-1}. Inset: Linearized binding plot of specific binding. [^{3}H]-prazosin bound in fmol mg^{-1} protein (B) was plotted as a function of the ratio (B/F) of bound [^{3}H]-prazosin to free [^{3}H]-prazosin in nmol l^{-1}. The intercept with the ordinate scale (108.1) represents the maximal number of binding sites in fmol mg^{-1} protein (B_{max}), the slope represents the K_D (0.04 nmol l^{-1}).

5 and 15 min, respectively. To avoid any interference with β-adrenoceptor activation, the effects of phenylephrine were studied in the presence of 1 μmol l^{-1} propranolol added 30 min before phenylephrine. In all experiments, only one concentration-response curve was determined in each preparation.

In some experiments NaCl- and propranolol-treated rats were additionally treated with cycloheximide (1.5 mg kg^{-1} body weight daily, i.p.) for 3 days, starting on the morning of OMPS implantation. This dose was shown to inhibit protein synthesis markedly (Yeh & Shils, 1969). Animals were killed on day 3 and binding assays performed as described above.

Drugs

The drugs used were from the following sources: (±)-propranolol hydrochloride (Rhein-Pharma, Heidelberg, FRG); prazosin hydrochloride (Pfizer, Karlsruhe, FRG); (±)-isoprenaline hydrochloride (Boehringer, Ingelheim, FRG); (−)-phenylephrine hydrochloride (Boehringer, Ingelheim, FRG); cycloheximide (Sigma, Deisenhofen, FRG); [^{3}H]-prazosin (specific activity 80.9 Ci $mmol^{-1}$, New England Nuclear, Dreieich, FRG); (−)-[^{3}H]-CGP-12177 ((−)-4-(3-*t*-butylamino-2-hydroxypropoxy)-[5,7-^{3}H]benzimidazol 2-one); specific activity 53.1 Ci $mmol^{-1}$, Amersham Buchler, Braunschweig, FRG).

Statistics

Values presented are means ± s.e.mean. Statistical significance was estimated by Student's *t* test for unpaired observations. A *P* value of less than 0.05 was considered significant. The maximal density (B_{max}) and apparent affinity (K_D) of binding sites were obtained in individual experiments from Scatchard plots determined by linear regression analysis as described by Winek & Bhalla (1979). In contraction experiments, drug concentrations that produced 50% of the maximal effect (EC_{50}) were determined graphically in each experiment and are presented as pD_2-values.

Results

Heart rate

Compared to control animals, heart rates of propranolol-treated rats were reduced by 25%

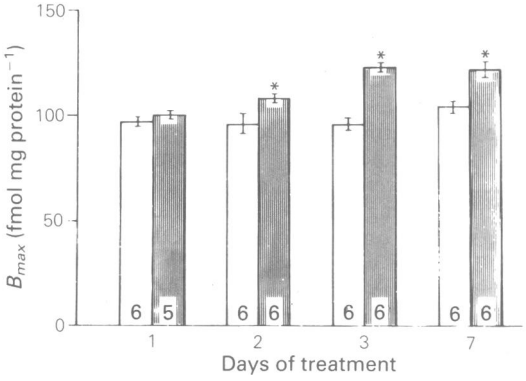

Figure 3 Time course of α_1-adrenoceptor density (B_{max}) on rat heart ventricular membranes, recorded on days 1, 2, 3 and 7 after beginning of propranolol- or NaCl-treatment, respectively. Ordinate scale: [^{3}H]-prazosin bound in fmol mg^{-1} protein; abscissa scale: days of treatment. Numbers of animals are displayed in the columns; open columns, control; hatched columns, (±)-propranolol 9.9 mg kg^{-1} daily. K_D-values (nmol l^{-1}) of control and propranolol-treated rats, respectively, were (n = 5–6): day 1: 0.05 ± 0.002 vs. 0.05 ± 0.003; day 2: 0.08 ± 0.024 vs. 0.05 ± 0.003; day 3: 0.07 ± 0.018 vs. 0.05 ± 0.005; day 7: 0.06 ± 0.013 vs. 0.05 ± 0.001. *P < 0.05.

(Figure 1). The reduction remained constant on day 1, 2, 3 or 7 after beginning of propranolol-treatment indicating effective β-adrenoceptor blockade during the entire treatment period.

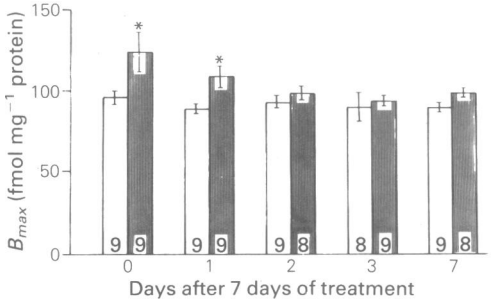

Figure 4 Time course of α_1-adrenoceptor density (B_{max}) changes in rat heart ventricular membranes, recorded on day 0, 1, 2, 3 and 7 after withdrawal of a 7 day treatment period with propranolol or NaCl, respectively. Ordinate scale: [^{3}H]-prazosin bound in fmol mg^{-1} protein; abscissa scale: days after end of treatment. Numbers of animals are displayed in the columns: open columns, control; hatched columns, (±)-propranolol 9.9 mg kg^{-1}, daily. K_D values (nmol l^{-1}) of control and propranolol-treated rats, respectively, were (n = 8–9): day 0: 0.04 ± 0.002 vs. 0.04 ± 0.002: day 1: 0.04 ± 0.003 vs. 0.04 ± 0.004; day 2: 0.04 ± 0.002 vs. 0.04 ± 0.002; day 3: 0.04 ± 0.002 vs. 0.04 ± 0.004; day 7: 0.04 ± 0.003 vs. 0.04 ± 0.003. *P < 0.05.

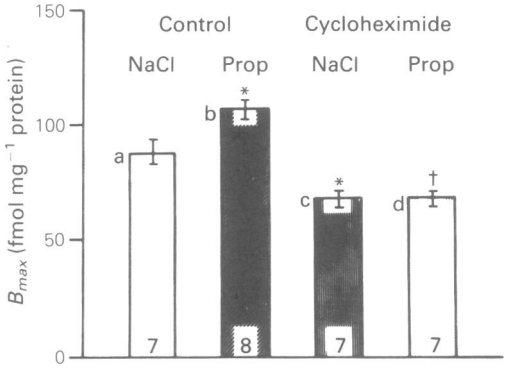

Figure 5 Influence of cycloheximide (1.5 mg kg^{-1} daily) on α_1-adrenoceptor density in rat heart ventricular membranes (B_{max}) without and with propranolol(Prop)-treatment (9.9 mg kg^{-1} daily) over a period of 3 days. Numbers of animals are displayed in the columns. K_D values (nmol l^{-1}; n = 7–8) were: column (a): 0.04 ± 0.003; column (b): 0.04 ± 0.001; column (c): 0.04 ± 0.002; column (d): 0.04 ± 0.002. *P < 0.05 vs. (a); †P < 0.001 vs. (b).

α_1-Adrenoceptor binding

Scatchard analysis of the saturation curves of all experiments showed a single component, high affinity binding of [^{3}H]-prazosin in NaCl- and propranolol-treated rats. Non-specific binding at the K_D-value averaged 4.0% of total binding. Figure 2 shows an example of concentration-dependent (0.025–1.0 nmol l^{-1}) [^{3}H]-prazosin-binding to cardiac α_1-adrenoceptors after 3 days of propranolol-treatment. B_{max} and K_D in this experiment, calculated from linearized binding plot (inset of Figure 2), were 108.1 fmol mg^{-1} protein and 0.04 nmol l^{-1}, respectively. Whereas propranolol-treatment did not affect K_D, α_1-adrenoceptor density was increased following propranolol-treatment. The time course of this propranolol-induced up regulation is shown in Figure 3. The effect became significant after 2 days and reached a maximal increase of 28% after 3 days of treatment. Thereafter α_1-adrenoceptor density remained constant throughout 7 days of treatment.

Withdrawal of propranolol was followed by decreased α_1-adrenoceptor density reaching pretreatment levels two days after cessation of propranolol-uptake (Figure 4). Thus, the time courses for up- and down regulation of myocardial α_1-adrenoceptors were similar.

Influence of cycloheximide

Impairment of protein synthesis by cycloheximide led to a significant decrease in the α_1-adrenoceptor

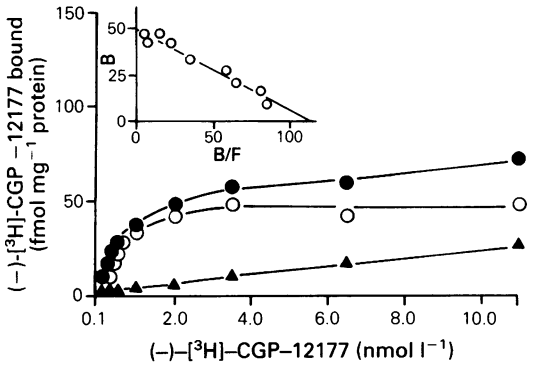

Figure 6 Total (●), specific (○) and non-specific (▲) (−)-[^{3}H]-CGP-12177 ([^{3}H]-CGP) binding in a single saturating experiment of rat heart ventricular membranes after propranolol-treatment (3 days). Ordinate scale: [^{3}H]-CGP bound in fmol mg^{-1} protein; abscissa scale: concentration of radioligand in nmol l^{-1}. Inset: linearized binding plot of specific binding. [^{3}H]-CGP bound in fmol mg^{-1} protein (B) was plotted as a function of the ratio (B/F) of bound [^{3}H]-CGP to free [^{3}H]-CGP in nmol l^{-1}. The intercept with the ordinate scale (49.9) represents the maximal number of binding sites in fmol mg^{-1} protein (B_{max}), the slope represents the K_D (0.45 nmol l^{-1}).

density (Figure 5). When NaCl-treated rats were also given cycloheximide, α_1-adrenoceptor binding sites were reduced by 22.9% (Figure 5c), indicating a marked influence of cycloheximide on the turnover process of α_1-adrenoceptors. The propranolol-induced increase of α_1-adrenoceptors was inhibited completely by cycloheximide (Figure 5d).

β-Adrenoceptor binding

Scatchard analysis of the saturation curves of all experiments showed a single component, high affinity binding of (−)-[^{3}H]-CGP-12177 in NaCl- and propranolol-treated rats. Non-specific binding at the K_D-concentration averaged 10.8% of total binding. Figure 6 shows an example of concentration-dependent (0.1–11.0 nmol l^{-1}) (−)-[^{3}H]-CGP-12177-binding to cardiac β-adrenoceptors after 3 days of propranolol-treatment with the linearized binding plot depicted in the inset. Treatment with propranolol did not yield significant increases of B_{max} nor did it influence K_D. Table 1 lists B_{max} and K_D values of β-adrenoceptor binding assays after the beginning and the end of propranolol treatment. Similar results were obtained in the presence of cycloheximide (Table 2).

Force of contraction

Phenylephrine produced increases in force of contraction in control rats from a threshold concentration of 3 μmol l^{-1} to a maximal concentration of 30 μmol l^{-1}. Similar results were obtained for propranolol-treated rats (Figure 7a). The pD_2 values were the same for both groups (5.41 ± 0.11 in

Table 1 B_{max} and K_D values from β-adrenoceptor binding experiments with (−)-[^{3}H]-CGP-12177 in rat heart ventricular membranes after beginning or end of propranolol-treatment (Prop: 9.9 mg kg^{-1} daily)

	After beginning of treatment				*After end of treatment*			
	n	*Day*	*Control*	*Prop*	n	*Day*	*Control*	*Prop*
B_{max} (fmol mg^{-1} protein)					7	0	38.1 ±3.3	46.0 ±7.0
	6	1	33.3 ±1.3	30.8 ±1.4	5	1	34.7 ±2.0	40.5 ±6.2
	6	2	34.1 ±2.1	36.8 ±1.8	5	2	36.2 ±2.7	33.7 ±4.1
	6	3	31.6 ±2.0	35.6 ±1.9	5	3	31.8 ±3.7	31.8 ±5.4
	6	7	32.2 ±1.2	36.1 ±2.4	5	7	35.0 ±2.2	33.0 ±5.5
K_D (nmol l^{-1})					7	0	0.32 ±0.09	0.38 ±0.13
	6	1	0.27 ±0.03	0.23 ±0.01	5	1	0.27 ±0.10	0.33 ±0.11
	6	2	0.24 ±0.05	0.25 ±0.04	5	2	0.25 ±0.02	0.27 ±0.02
	6	3	0.18 ±0.02	0.19 ±0.03	5	3	0.21 ±0.04	0.20 ±0.04
	6	7	0.28 ±0.10	0.30 ±0.07	5	7	0.43 ±0.15	0.39 ±0.18

n = number of experiments.

Table 2 B_{max} and K_D values of β-adrenoceptor binding experiments with (−)-[^{3}H]-CGP-12177 in rat heart ventricular membranes after propranolol-treatment (Prop: 9.9 mg kg^{-1} daily) without or with additional treatment with cycloheximide (1.5 mg kg^{-1} daily): both drugs were given simultaneously for 3 days

B_{max} (fmol mg^{-1} protein)				K_D (nmol l^{-1})			
Control		*Cycloheximide*		*Control*		*Cycloheximide*	
NaCl(7)	Prop(8)	NaCl(7)	Prop(6)	NaCl(7)	Prop(8)	NaCl(7)	Prop(6)
41.5 ±1.4	44.8 ±1.6	35.4* ±2.0	39.1* ±2.1	0.22 ±0.02	0.26 ±0.04	0.23 ±0.02	0.22 ±0.02

Numbers of animals are shown in parentheses. (*$P < 0.05$ vs. control).

control rats, $n = 7$; 5.41 ± 0.19 in propranolol-treated rats, $n = 7$).

Isoprenaline produced increases in force of contraction in control rats from a threshold concentration of 1 μmol l^{-1} to a maximal concentration of 10 μmol l^{-1}. In propranolol-treated rats the positive inotropic effect started at 0.1 μmol l^{-1} and was also maximal at 10 μmol l^{-1} (Figure 7b). The pD_2 values were not significantly different in both groups (6.31 ± 0.18 in control rats, $n = 7$; 6.65 ± 0.19 in propranolol-treated rats, $n = 7$).

Discussion

Catecholamine-induced increase in myocardial force of contraction is predominantly a result of β-adrenoceptor stimulation (Black & Prichard, 1973; Neil, 1975; Kaumann *et al.*, 1978; Kenakin, 1984). However, stimulation of myocardial α_1-adrenoceptors also leads to a positive inotropic response (Benfey, 1973; 1987; Scholz, 1980; Endoh, 1986). Whereas agonist occupation of β-adrenoceptors leads to activation of adenylate cyclase and to a subsequent increase in cyclic AMP content, no elevation of cyclic AMP has been detected upon α_1-adrenoceptor stimulation (Osnes & Øye, 1975; Schümann *et al.*, 1975; Brückner *et al.*, 1978; Homcy & Graham, 1985; Endoh, 1986; Scholz *et al.*, 1986). Since both receptor systems act via different pathways (Endoh, 1986; Schmitz *et al.*, 1987; Scholz *et al.*, 1988), it is conceivable that in the diseased state they may also be influenced in a different manner. Thus chronic heart failure with down regulation of β-adrenoceptors (Bristow *et al.*, 1982; 1986; Brodde *et al.*, 1986; Fowler *et al.*, 1986) or chronic β-adrenoceptor blockade with impaired β-adrenoceptor stimulation could enhance the significance of myocardial α_1-adrenoceptors in order to maintain cardiac function (Osnes *et al.*, 1985).

In this study, we observed in rats chronically treated with propranolol an increase in α_1-adrenoceptor density two days after beginning of treatment without change in affinity. Since this elevation was maintained throughout the duration of a seven day treatment period it may be considered as an adaptive process that is maintained for the same period of time that β-adrenoceptors are blocked with propranolol.

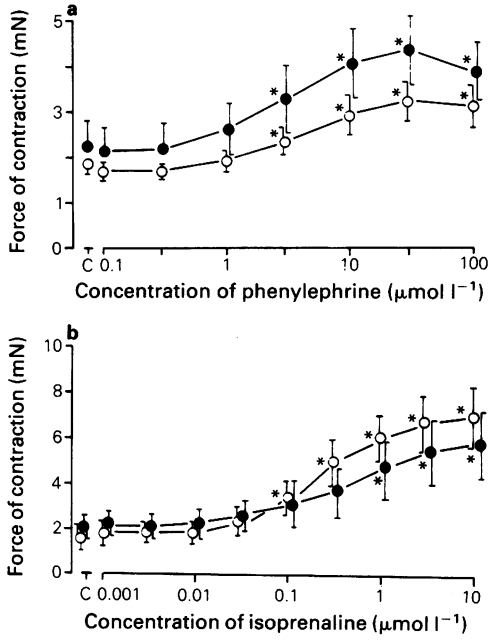

Figure 7 Cumulative concentration-response curves for the effects of phenylephrine (a) and isoprenaline (b) on isometric force of contraction in right ventricular papillary muscles isolated from control rats (●) and from rats after propranolol-treatment (○) 9.9 mg kg^{-1} daily for 3 days. Ordinate scale: force of contraction in mN; abscissa scale: concentration range of phenylephrine and isoprenaline, respectively, expressed in μmol l^{-1}. Values for phenylephrine were obtained in the presence of 1 μmol l^{-1} propranolol. In (a), pD_2 for control = 5.41 ± 0.11 ($n = 7$); pD_2 for propranolol-treatment = 5.41 ± 0.19 ($n = 9$); *$P < 0.05$ vs. predrug value. In (b) pD_2 for control = 6.31 ± 0.18 ($n = 7$); pD_2 for propranolol-treatment = 6.65 ± 0.19 ($n = 7$). *$P < 0.05$ vs. predrug value.

The observed increase in the density of α_1-adrenoceptors was reversible within two days after withdrawal of propranolol. This demonstrates similar time courses for up- and down regulation of myocardial α_1-adrenoceptors.

The increase of α_1-adrenoceptors in rat heart after chronic propranolol-treatment could either be the result of new synthesis of adrenoceptors or externalization of adrenoceptors located within the cell (Lefkowitz *et al.*, 1983; Sibley & Lefkowitz, 1985; Fratelli & De Blasi, 1987). We therefore treated rats under identical experimental conditions with cycloheximide. This substance is a strong inhibitor of protein synthesis (Trakatellis *et al.*, 1965; Korner, 1966; Jondorf & Grünberger, 1967; Yeh & Shils, 1969). The propranolol-induced increase of myocardial α_1-adrenoceptor density was inhibited completely by cycloheximide. This indicates that new receptor synthesis is responsible for the increase in receptor density. Furthermore, the basal turnover rate of α_1-adrenoceptor synthesis in rat heart may be decreased by cycloheximide since hearts from NaCl-treated rats also revealed a decreased number of binding sites after cycloheximide.

In contraction experiments, one might expect an increased potency or efficacy of the α-sympathomimetic drug phenylephrine in ventricular heart preparations of propranolol-treated rats, but no significant differences from preparations from NaCl-treated rats were obtained. The increase in α_1-adrenoceptor density in rat heart after chronic propranolol-treatment can therefore not be correlated to higher potency or efficacy of phenylephrine in our experiments, and the biological significance of this effect thus remains an open question. However, it is also conceivable that the observed increase of α_1-adrenoceptor density may have been too small to be of functional relevance.

Whether a similar adaptation in density of α_1-adrenoceptors after chronic β-adrenoceptor blockade also occurs in other species has not been investigated. Species differences in the number of myocardial α-adrenoceptors are well known (Mukherjee *et al.*, 1983) and it remains speculative, whether our results are applicable e.g. to the human heart, where only a very low α_1-adrenoceptor density was found (Ferry & Kaumann, 1987; Bevilaqua *et al.*, 1987). But the reason for the clinical improvement of certain patients with dilated cardiomyopathy after treatment with β-adrenoceptor blocking drugs is still uncertain (Waagstein *et al.*, 1975; Shanes, 1987) and it seems worth investigating whether the myocardial α_1-adrenoceptor density is also increased in human heart after chronic β-adrenoceptor blockade.

The results from the β-adrenoceptor binding studies show a slight increase in binding sites after propranolol-treatment without reaching significant levels. Other results have been reported by Glaubiger & Lefkowitz (1977) and Aarons & Molinoff (1982) who found a significantly increased number of β-adrenoceptor binding sites in rat heart after chronic treatment with propranolol. The reason for this discrepancy remains unclear. However, our results are in agreement with Baker & Potter (1980) who also did not detect significant increases of β-adrenoceptor binding sites in rat ventricular membranes after chronic propranolol-treatment. Similar results were presented for guinea-pig ventricular membranes by Chess-Williams & Broadley (1984).

In NaCl- and propranolol-treated rats, β-adrenoceptor densities were depressed to a similar extent by cycloheximide. Compared to α_1-adrenoceptor binding, cycloheximide influence was less pronounced in β-adrenoceptor binding experiments and our results denote a stronger influence of cycloheximide on myocardial α_1-adrenoceptors than on myocardial β-adrenoceptors. This is in agreement with studies on turnover rate of adrenoceptors in cell cultures (Bouhelal *et al.*, 1987) showing a considerable shorter half life for α_1-adrenoceptors than for β-adrenoceptors. It is thus conceivable that the α_1-adrenoceptor may have a faster turnover rate and hence is more sensitive to inhibition of protein synthesis.

Contraction experiments with isoprenaline did not display a significant difference between propranolol- and NaCl-treated rats although the concentration-response curve for propranolol-treated rats is shifted slightly to the left. These results are in agreement with the data obtained from our β-adrenoceptor binding where the difference in binding sites did not reach significance for propranolol-treated rats and where an unchanged affinity was found for the ligand used.

In conclusion, chronic β-adrenoceptor blockade with propranolol led to a compensatory increase in the number of myocardial α_1-adrenoceptors which was apparently due to a new synthesis of receptors. Since this increase was not accompanied by an increased positive inotropic effect of the α_1-adrenoceptor agonist phenylephrine it may have been too small to be of functional importance. It was therefore not possible to determine an increased functional role of myocardial α_1-adrenoceptors during chronic β-adrenoceptor blockade, at least not for the rat heart. Whether myocardial α_1-adrenoceptors are of greater importance during the development of cardiac failure with down regulation of β-adrenoceptors remains to be investigated.

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The interaction of benzodiazepines with thyrotropin-releasing hormone receptors on clonal pituitary cells

Lesley A. Joels & [1]Alan H. Drummond

Department of Pharmacology, School of Pharmacy, 29/39 Brunswick Square, London WC1N 1AX

1 Seven benzodiazepines were investigated for their ability to interact with receptors for thyrotropin-releasing hormone (TRH) on GH_3 and GH_4C_1 pituitary tumour cells.

2 Midazolam and chlordiazepoxide were the most potent inhibitors of TRH-induced [^{3}H]-inositol phosphate formation with K_i values in the low micromolar range. The antagonism was competitive in nature and was increased in potency at sub-physiological temperatures.

3 None of the agents examined antagonized bombesin-induced [^{3}H]-inositol phosphate formation in GH_4C_1 cells.

4 While the ability of benzodiazepines to interact with the GABA receptor-chloride channel ionophore is markedly stereospecific, little difference was evident in the ability of (+)- and (−)-4-methylmidazolam (Ro 21-5656 and Ro 21-5657) to compete with TRH at its receptor.

5 Recently it has been suggested that, in contrast to phosphatidylinositol hydrolysis, the TRH-induced breakdown of phosphatidylinositol polyphosphates is transient in clonal pituitary cells. Addition of chlordiazepoxide to TRH-stimulated GH_3 cells up to 60 min after initiating the reaction leads, however, to an immediate decline in the cellular content of inositol trisphosphate. This indicates that TRH-induced phosphatidylinositol 4,5-bisphosphate hydrolysis is not transient.

Introduction

The elucidation of the physiological role of thyrotropin-releasing hormone (TRH) has been significantly hindered by the absence of a specific antagonist. Binding of TRH to its receptor in the CNS has recently been demonstrated to be inhibited by a number of benzodiazepines, in particular, chlordiazepoxide (Sharif *et al.*, 1983; Simasko & Horita, 1984). A similar conclusion has been reached from work on the TRH receptor of pituitary cells (Sharif *et al.*, 1983; Gershengorn & Paul, 1986) and, in the GH_3 cell line, additional studies indicate that the benzodiazepine-TRH receptor interaction is of an antagonist nature (Drummond, 1985; Gershengorn & Paul, 1986; Martin *et al.*, 1986). However, chlordiazepoxide fails to satisfy the requirement for a specific TRH antagonist because of its powerful interaction with the GABA system. Nevertheless, the finding raises the possibility that other benzodiazepines or related substances might retain TRH receptor blocking activity while being devoid of a GABAergic action.

The cellular effects of TRH are now recognised to be the result of stimulated inositol lipid hydrolysis, a process which has been widely characterized in clonal pituitary cells (for recent reviews, see Drummond, 1986; Gershengorn, 1986). In an attempt to characterize further the molecular requirements for the benzodiazepine-TRH receptor interaction, we have investigated the ability of a number of related benzodiazepines to act as inhibitors of TRH-stimulated inositol phosphate formation in two pituitary cell lines. Of the substances tested, only the anaesthetic agent, midazolam, exceeds chlordiazepoxide in activity.

Methods

Cell culture

GH_3 and GH_4C_1 cells were grown as previously described for GH_3 cells (Macphee & Drummond,

[1] Author for correspondence at present address: Department of Pharmacology, British Bio-technology Ltd., Brook House, Wallington Road, Cowley, Oxford OX4 5LY.

1984) in 100 mm petri-dishes (Nunc, Gibco, Paisley, Scotland); 7–10 days after plating, [^{3}H]-inositol (7μCi per dish; 9 ml) was added and the cells allowed to grow for a further 2–3 days. Previous work has shown that, under those conditions, labelling of the inositol lipids in GH_3 cells has reached isotopic equilibrium (Drummond *et al.*, 1984). On the day of the experiment, cell suspensions were prepared (Drummond *et al.*, 1984) in a balanced salt solution containing (mM): lithium chloride 10, sodium chloride 125, potassium chloride 4.5, calcium chloride 1.5, magnesium chloride 0.5, glucose 5.6, HEPES 10, sodium pyruvate 1, disodium hydrogen phosphate 1.86, 0.1% (w/v) BSA pH 7.4 with sodium hydroxide.

Cell stimulation and isolation of [^{3}H]-inositol phosphates

Aliquots (0.5 ml) of cell suspension (containing around 250 μg cell protein) were incubated at 37°C (unless otherwise stated) with various concentrations of TRH, bombesin and the benzodiazepines for the times indicated in the table or figure legends. The presence of lithium ions (10 mM) has been shown to amplify considerably agonist-induced inositol phosphate formation in a wide range of cells and tissues including GH_3 cells (Berridge *et al.*, 1982; Drummond *et al.*, 1984). The reactions were stopped by addition of an equal volume of 20% (w/v) TCA. After 15 min on ice, precipitated proteins were removed by centrifugation and the remaining TCA extracted by three washes with 3.5 ml of water-saturated ether.

[^{3}H]-inositol and its mono-, bis-, tris-, and tetrakisphosphates in the neutralised cell extract were separated by sequential elution from Biorad AG 1-X8 (200-400 mesh, formate form) anion-exchange resin as described by Downes *et al.* (1986).

Calculation of K_i value

K_i values were calculated using the equation:

$$K_i = \frac{[\text{antagonist}]}{DR - 1}$$

where DR = ratio of equiactive concentrations of agonist in the presence and absence of a given [antagonist].

Materials

Culture media, sera and antibiotics were from Gibco, Paisley, Scotland. GH_3 cells were from Flow Labs, Irvine, Scotland. GH_4C_1 cells were kindly provided by Dr W. Schlegel, Fondation pour Recherches Medicales, University of Geneva, Geneva, Switzerland. myo-[2-^{3}H]-inositol (14.3 Ci mmol^{-1}) was from Amersham International, Amersham, Bucks. TRH and bombesin were from Bachem (U.K.), Saffron Waldon, Essex and Peninsula Labs., St Helens, Lancs., respectively. Chlordiazepoxide hydrochloride, midazolam maleate, demoxepam, (±)-4-hydroxy-midazolam, (−)-8-chloro-1,4-dimethyl-6-2-fluorophenyl)-4H-imidazo[1,5a][1,4]benzodiazepine-l-tartrate (Ro 21-5656; (−)-4-methylmidazolam) and (+)-8-chloro-1,4-dimethyl-6-(2-fluorophenyl)-4H-imidazo[1,5a][1,4]-benzodiazepine l-tartrate (Ro 21-5657; (+)-4-methylmidazolam) were kindly provided by Mr D.N. Davison, Roche Products Ltd., Welwyn Garden City, Herts. Adinazolam mesylate was a gift from The Upjohn Co., Kalamazoo, Michigan, U.S.A. All other reagents were of the highest quality available.

Results

The effect of benzodiazepines on TRH-induced inositol phosphate formation in lithium treated GH_3 cells

The structures of the benzodiazepines investigated in this study are outlined in Figure 1. Incubation of lithium-treated GH_3 cells with TRH (10^{-9} M) for 15 min leads to the accumulation of a variety of inositol phosphates (Drummond *et al.*, 1984). The data in Table 1 show the effect of a number of agents (at 10^{-4} M) on [^{3}H]-inositol monophosphate levels in

Table 1 The effect of benzodiazepines on TRH-induced [^{3}H]-InsP_1 formation in lithium-treated GH_3 cells

Benzodiazepine	*Control*	*TRH* (10^{-9} M)
	InsP_1 (d.p.m./sample)	
None	1023 ± 124	4995 ± 373
Adinazolam	1079 ± 45	5172 ± 179
Chlordiazepoxide	867 ± 29	1646 ± 113
Demoxepam	990 ± 10	4257 ± 49
Midazolam	1053 ± 89	1443 ± 86
4-Hydroxymidazolam	1269 ± 67	3213 ± 438

GH_3 cells, prelabelled with [^{3}H]-inositol (7 μCi per dish for 48 h) were stimulated for 15 min at 37°C with 10^{-4} M of the indicated benzodiazepine and/or TRH. [^{3}H]-InsP_1 was extracted and measured as described in the Methods section. Values shown are the means ± s.e.mean for triplicate samples from a representative experiment. In all of the experiments of this type, there was a significant inhibition of TRH stimulation ($P < 0.05$) by each benzodiazepine apart from adinazolam.

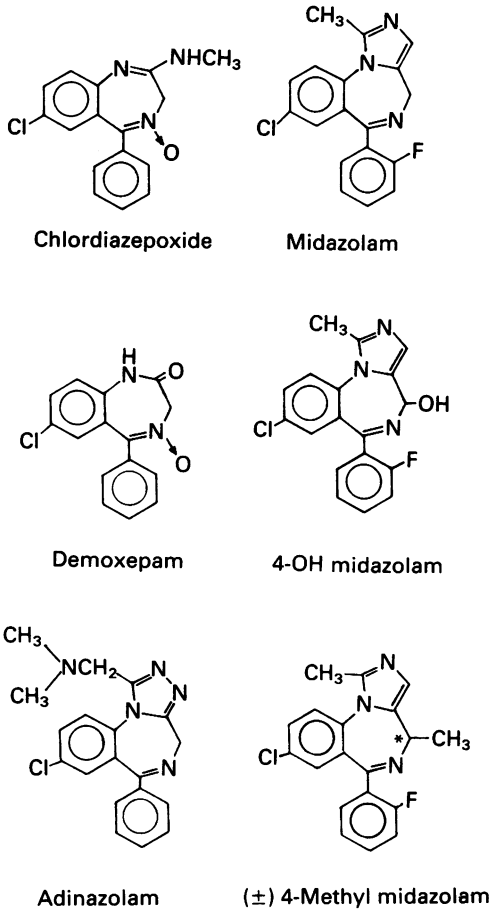

Figure 1 Structures of the benzodiazepines studied for their abilities to interact with TRH at its receptor in GH pituitary cells. * denotes the position of the assymmetric carbon atom for the enantiomers of 4-methyl midazolam.

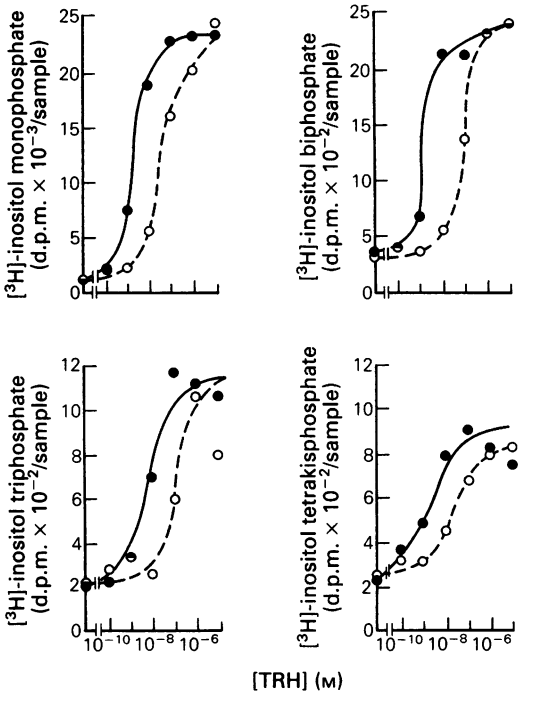

Figure 2 Antagonism of TRH-induced inositol phosphate accumulation in lithium-treated GH_3 cells by midazolam (5×10^{-5} M). Cells, prelabelled with [^{3}H]-inositol (7μCi per dish for 48 h), were incubated with various concentrations of TRH in the presence (●) or absence (○) of midazolam (5×10^{-5} M). Ordinates show levels of [^{3}H]-$InsP_1$. (a); [^{3}H]-$InsP_2$, (b); [^{3}H]-$InsP_3$, (c) and [^{3}H]-$InsP_4$, (d) expressed as d.p.m./sample, which were extracted and measured as described in the Methods section. It should be noted that these levels indicate the total amount of each inositol phosphate fraction with no separation of the individual isomers. Values plotted are the means of duplicate determinations from a representative experiment.

TRH-stimulated cells. None of the agents examined significantly altered basal [^{3}H]-InsP levels with the exception of (±)-4-hydroxymidazolam which caused a small increase of around 20% in this and two other experiments. All, however, except adinazolam caused an attenuation of the TRH-induced response, with midazolam and chlordiazepoxide being the most potent. The nature of the antagonism of TRH action by midazolam was further investigated by compiling concentration-response curves for TRH in the presence and absence of midazolam (5×10^{-5} M) (Figure 2). In the presence of midazolam, the ability of TRH to increase the cellular content of the various inositol phosphates was shifted by around 25 fold to the right in a parallel manner, indicating competitive antagonism. A mean K_i value of 3.2×10^{-6} M could be calculated from this and a further similar experiment. The corresponding value for chlordiazepoxide, obtained previously (Drummond, 1985) was 1.4×10^{-5} M, indicating that the imidazobenzodiazepine was the more potent receptor antagonist.

Interestingly, when the incubation temperature was reduced to 25°C, midazolam and chlordiazepoxide were more potent antagonists. At the lower temperature, the K_i for midazolam, for example, against the TRH-induced responses was 7×10^{-7} M (data not shown). A similar increase in antagonist potency has recently been observed at 25°C in [^{3}H]-TRH

ligand-binding studies using intact GH_3 cells (Joels & Drummond, unpublished work).

(±)-4-Hydroxy-midazolam, a metabolite of midazolam, was a less potent inhibitor of TRH-induced inositol phosphate formation, when analysed using a similar experimental design, having a mean K_i of 3×10^{-5} M (data not shown). One notable feature of these experiments, which confirms and extends previous work (Drummond, 1985), was the finding that while the K_i for a particular benzodiazepine against the TRH-induced accumulation of the different inositol phosphates was very similar, the potency of TRH in stimulating their accumulation was not. In particular, the EC_{50} for TRH-induced $InsP_1$ and $InsP_4$ formation ($1 \pm 0.3 \times 10^{-9}$ M; four experiments) was significantly less than that for the accumulation of $InsP_3$ ($6 \pm 0.2 \times 10^{-9}$ M). The implication may be that the Ins(1,4,5)P_3 kinase may saturate with substrate during stimulation with high concentrations of the tripeptide.

The availability of two stereoisomers of 4-methylmidazolam allowed us to investigate whether the TRH receptor, like the $GABA_A$ receptor-ionophore complex (Mohler & Okada, 1977), exhibited a steric preference for benzodiazepines. The data presented in Figure 3 indicate that both (+)- and (−)-4-methylmidazolam were active as TRH antagonists, although in three experiments of this type, the (+)-isomer was about twice as active.

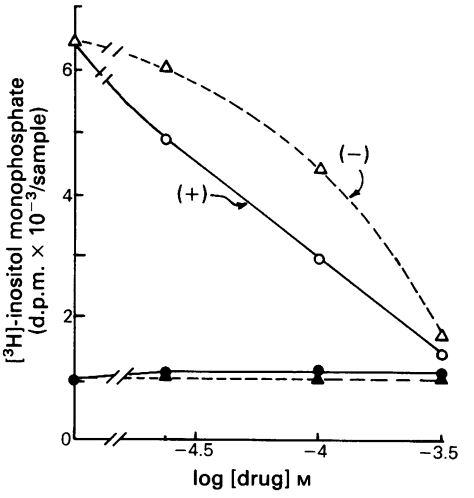

Figure 3 Antagonism of TRH-induced inositol monophosphate accumulation in lithium-treated GH_3 cells by the enantiomers of 4-methylmidazolam. Cells, prelabelled with [^{3}H]-inositol (7 μCi per dish for 48 h), were incubated with the concentrations of (+)-4-methylmidazolam (○---○) and (−)-4-methylmidazolam (△---△) indicated on the abscissa scale, in the presence (●▲) or absence (○△) of TRH (3×10^{-9} M) for 15 min at 37°C. The cellular content of [^{3}H]-$InsP_1$, expressed as d.p.m. × 10^{-3}/sample, was extracted and measured as described in the Methods section. Values plotted are the means of duplicate determinations from a representative experiment.

Specificity of benzodiazepines for the TRH receptor

The bombesin receptor on GH_3 cells is also coupled to changes in inositol lipid metabolism (Sutton & Martin, 1982). Most GH_3 cell clones, however, have only a small number of such receptors and the magnitude of the bombesin-induced rise in inositol phosphates is difficult to measure (Drummond, 1985). For this reason, the demonstration that benzodiazepines do not act at some post-receptor stage in the cellular signalling process required the use of a related pituitary cell clone, the GH_4C_1 line, which is much more responsive to bombesin. Table 2 represents results obtained from incubating lithium-treated GH_4C_1 cells with a low concentration of TRH or bombesin in the presence or absence of various benzodiazepines at a fixed concentration. With all three drugs, only the TRH response was inhibited. In order to confirm that this lack of effect of the benzodiazepines on the bombesin response was not due to the inappropriate selection of a receptor-saturating bombesin

Table 2 The effect of benzodiazepines on peptide-induced [^{3}H]-$InsP_1$ formation in lithium-treated GH_4C_1 cells

Benzodiazepine	*Control*	*TRH* (10^{-9} M)	*Bombesin* (10^{-9} M)
		$InsP_1$ (d.p.m./sample)	
None	13105 ± 1361	90258 ± 909	47122 ± 13501
Chlordiazepoxide	14293 ± 114	35883 ± 1076	45404 ± 3358
4-hydroxymidazolam	14914 ± 79	84914 ± 1164	44150 ± 725
Midazolam	13194 ± 367	20606 ± 368	51275 ± 2145

GH_4C_1 cells, prelabelled with [^{3}H]-inositol (7 μCi per dish for 48 h), were stimulated for 60 min at 37°C with the peptides and 10^{-4} M of the indicated benzodiazepine. [^{3}H]-$InsP_1$ was extracted and measured as described in the Methods section. Values shown are the means ± s.e.mean for triplicate samples from a representative experiment.

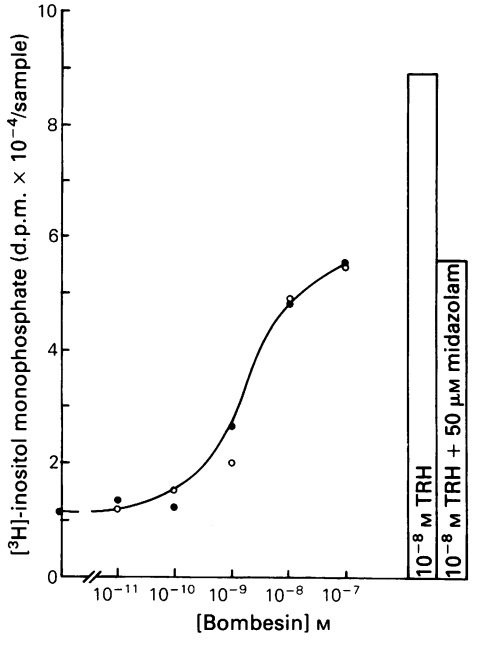

Figure 4 Effect of midazolam (5×10^{-5} M) on bombesin-induced accumulation of inositol monophosphate in lithium-treated GH_4C_1 cells. Cells, prelabelled as with GH_3 cells, were incubated with various concentrations of bombesin (10^{-7}–10^{-11} M) in the presence (●) and absence (○) of midazolam for 60 min at 37°C. The ordinate scale shows accumulation of cellular $InsP_1$ expressed as d.p.m. × 10^{-4}/sample, measured as described earlier. The bar graphs to the right hand side of this figure indicate the response of these cells when incubated with TRH (10^{-8} M) in the presence and absence of midazolam. Values are the means of duplicate determinations from a representative experiment.

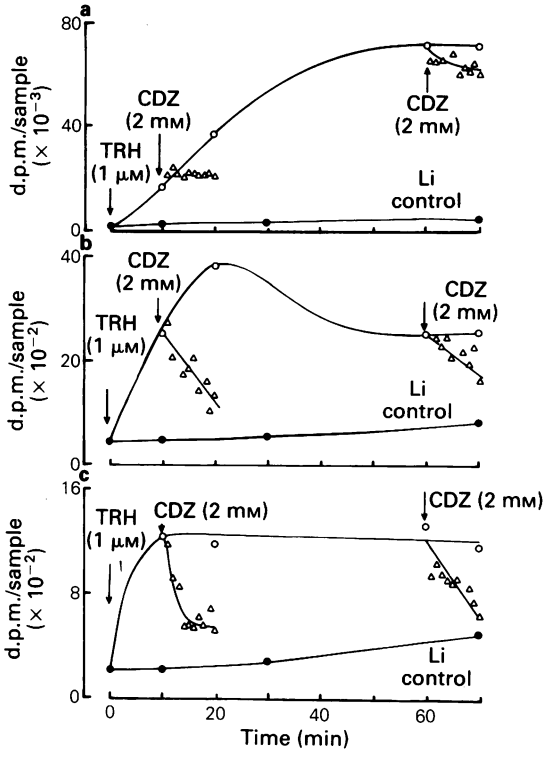

Figure 5 Antagonism of TRH-induced inositol phosphate accumulation in lithium-treated GH_3 cells by chlordiazepoxide (CDZ) (2×10^{-3} M). Cells, prelabelled as before, were incubated either with the agonist, 10^{-6} M TRH (○---○), or without the agonist (●) for 70 min at 37°C (○). Chlordiazepoxide was added 10 min and 60 min (△) after stimulation with TRH and the levels of $InsP_1$ (a), $InsP_2$ (b) and $InsP_3$ (c) were measured, as described in the Methods section, at the times indicated on the abscissae. Values are the means for duplicate determinations from a representative experiment.

concentration, full concentration-response curves for bombesin-induced InsP formation in GH_4C_1 cells were constructed in the absence or presence of a fixed benzodiazepine concentration. This work, presented in Figure 4 for midazolam, shows that the drug was without effect at any bombesin concentration, despite its evident ability to inhibit TRH in the same experiment. Similar results were obtained for chlordiazepoxide and (±)-4-hydroxymidazolam (both at 2×10^{-4} M).

Chlordiazepoxide as a pharmacological tool

Characteristically, TRH-induced changes in inositol phosphates in GH_3 cells are long-lived. This steady-state level, however, gives no information as to the rate at which these substances are produced and metabolised following prolonged receptor stimulation. As is demonstrated in Figure 5, the elevated levels of $InsP_3$ and $InsP_2$ in GH_3 cells stimulated by TRH represent a dynamic equilibrium, addition of the TRH receptor antagonist, chlordiazepoxide (2×10^{-3} M), at times up to 60 min after initiation of the reaction, leads to an immediate decline in the cellular content of these metabolites. Similar data were found in studies using lower concentrations of chlordiazepoxide (2×10^{-4} M) in which the same ratio of [agonist] to [antagonist] was maintained (data not shown). These data contradict the report of Imai & Gershengorn (1986) which suggested that $InsP_3$ production was a transient phenomenon. The data in

Figure 5 also demonstrate that the cellular content of InsP falls rather slowly following displacement of TRH from its receptor. This is probably due to the ability of lithium to inhibit the metabolism of InsP more markedly than that of its phosphorylated derivatives.

Discussion

This work was conducted with the general aim of developing further the pharmacology of the benzodiazepine-TRH receptor interaction. It has demonstrated that a number of 1,2-annelated benzodiazepines show varying degrees of ability to interact with the TRH receptor as competitive antagonists. The anaesthetic agent, midazolam, in particular, is the most potent TRH receptor blocker so far identified, thus confirming the TRH receptor binding data of Rinehart *et al.* (1986). Although around 20 structurally related substances have now been examined in CNS binding assays and in studies of TRH-induced cellular effects, there is still no substantial breakthrough in identifying a benzodiazepine which has potent TRH receptor blocking action in the absence of GABA-potentiating effects. What is now abundantly clear, however, is that the benzodiazepine pharmacology at the TRH receptor and at the $GABA_A$ receptor-ionophore is markedly different. For example, while GABA-related effects are clearly dependent on the stereochemistry at the 3-position (this is analogous to the 4-position in the imidazobenzodiazepine series) such that (+)-isomers of 3-substituted benzodiazepines are >100 fold more potent than their corresponding (−)-isomers (Mohler & Okada, 1977), this is not the case for the TRH receptor, as seen with the isomers of 4-methylmidazolam. A number of structurally advantageous and deleterious substitutions on the basic benzodiazepine nucleus are now, however, evident. In general, drugs substituted in the 1 and 2 positions are more potent TRH antagonists than their unsubstituted counterparts: demoxepam is much less potent than chlordiazepoxide or diazepam (Drummond, 1985) and certain 1,2-annelated compounds e.g. midazolam, are even more active than chlordiazepoxide.

There is a rather marked discrepancy between the potency of a number of benzodiazepines as TRH antagonists measured in the rat brain TRH receptor binding assay and in studies of cellular responses in GH_3 cells. For example, Rinehart *et al.* (1986) using rat amygdala membranes, found IC_{50} values for midazolam and chlordiazepoxide of 0.63 and 3.68×10^{-7} M respectively. This contrasts with K_i values measured in GH_3 cells of 3.2 and 15×10^{-6} M for midazolam and chlordiazepoxide respectively. One piece of information to emerge from the present study is that the affinity of the benzodiazepine for the TRH receptor is markedly dependent on the reaction temperature: midazolam was 4–5 times more potent as a TRH antagonist at 25°C than at 37°C. Although the reasons for this remain unknown, it seems that the low temperature (4°C) used in the CNS binding assays may have overestimated receptor affinity for physiological conditions. In support of this, it is noteworthy that Gershengorn & Paul (1986) report a K_i for chlordiazepoxide-TRH receptor binding in GH cells of 5×10^{-6} M at 37°C; this is significantly higher (10–20 fold) than that found in CNS binding studies conducted at 4°C.

As previously argued for chlordiazepoxide (Drummond, 1985), it seems unlikely that the interaction of midazolam with the TRH receptor will have significant therapeutic ramifications. The blood concentration of this drug achieved after administration of an anaesthetic dose to an adult will be in the range $1–4 \times 10^{-7}$ M (Puglisi *et al.*, 1978). This is below a midazolam concentration that would compete effectively with TRH for binding to its receptor. Moreover, although we were unable to test its major human metabolite, α-hydroxymidazolam, for TRH receptor blocking activity, another metabolite, 4-hydroxymidazolam, was much weaker than the parent molecule.

The use of chlordiazepoxide has been informative in probing the mechanisms underlying the formation and metabolism of inositol phosphates following TRH receptor stimulation in GH_3 cells. Measurements of changes in the specific activity of the inositol phospholipids in ^{32}P-labelled GH_3 cells stimulated by TRH led Imai & Gershengorn (1986) to propose that PtdIns(4,5)P_2 hydrolysis is transient. The data presented here contradict this view for the following reasons. Firstly, we and others (Schlegel *et al.*, 1984; Drummond & Raeburn, 1984) have shown that increases in $InsP_3$ and $InsP_2$ are long-lived following TRH stimulation of GH_3 cells. Secondly, antagonism of the TRH-induced inositol phosphate response by adding chlordiazepoxide once an elevated steady-state level has been established, leads to an immediate fall in the levels of $InsP_2$ and $InsP_3$. Thirdly, formation of Ins4P, a product of polyphosphoinositide but not PtdIns metabolism, continues for a considerable length of time (>20 min) in TRH-stimulated GH_3 cells (Dean & Moyer, 1987; Hughes & Drummond, 1987). Taken together, these data indicate that the elevated level of the inositol polyphosphates is maintained by continued rather than transient biosynthesis.

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Affinity of muscarinic receptor antagonists for three putative muscarinic receptor binding sites

Ronald E. Delmendo, [1]Anton D. Michel & Roger L. Whiting

Institute of Pharmacology, Syntex Research, 3401 Hillview Avenue, Palo Alto, CA, 94303, U.S.A.

1 A range of muscarinic receptor antagonists were examined for affinity at the M_1 muscarinic binding site, present in rat cerebrocortical membranes and the M_2 muscarinic binding sites of rat cardiac and submaxillary gland membranes.

2 The results obtained were consistent with the presence of three classes of muscarinic binding site.

3 Both the M_1 binding site, labelled by [^{3}H]-pirenzepine ([^{3}H]-Pir) in rat cerebrocortical membranes, and the M_2 gland binding site, labelled by [^{3}H]-N-methyl scopolamine ([^{3}H]-NMS) in rat submaxillary gland membranes, displayed higher affinity for pirenzepine, dicyclomine, 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) and cyclohexylphenyl (2-piperidinoethyl) silanol (CPPS) than did the M_2 binding sites of cardiac membranes labelled by [^{3}H]-NMS.

4 The M_2 cardiac sites displayed higher affinity for methoctramine, himbacine and AF-DX 116 than did either the M_1 binding site of cerebrocortical membranes or the M_2 gland binding site present in rat submaxillary gland membranes.

5 The M_1 and M_2 gland binding sites could only be distinguished by considering the absolute affinity of compounds for these two sites. Thus, all compounds, with the exception of 4-DAMP, displayed between a 2 and 8 fold higher affinity for the M_1 than for the M_2 gland binding site. There were no antagonists with higher M_2 gland than M_1 affinity.

Introduction

Within the past decade results obtained from both functional and ligand binding studies has provided evidence for the existence of multiple subtypes of the muscarinic receptor (Burgen, 1984; Eglen & Whiting, 1986b). On the basis of ligand binding studies which demonstrated a tissue selectivity for pirenzepine (Hammer *et al.*, 1980) the terms M_1 and M_2 were introduced to describe muscarinic receptors with high and low pirenzepine affinity, respectively (Hammer & Giachetti, 1982). This binding classification has been supported by the demonstration of receptors displaying high affinity for pirenzepine in functional studies (Brown *et al.*, 1980; Gil & Wolfe, 1985).

In functional studies considerable evidence has accumulated to indicate that there may be heterogeneity of the M_2-muscarinic receptor (Eglen & Whiting, 1986b). Thus, the muscarinic receptors present in cardiac and ileal tissue, which are defined as belonging to the M_2 subtype on the basis of their low affinity for pirenzepine (Eglen & Whiting, 1986b), display marked differences in their pharmacology. The non-competitive antagonist gallamine (Riker & Wescoe, 1951; Clark & Mitchelson, 1976) and the competitive antagonists AF-DX 116 (Hammer *et al.*, 1986), himbacine (Gilani & Cobbin, 1986) and methoctramine (Melchiorre *et al.*, 1987) have been shown to display a higher affinity for the cardiac than for ileal muscarinic receptors, while 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) (Barlow *et al.*, 1976), cyclohexylphenyl (2-piperidinoethyl) silanol (CPPS) (Eglen & Whiting, 1986a) and hexahydrosilaprocyclidine (Mutschler & Lambrecht, 1984) display the converse selectivity.

Similar heterogeneity of the M_2 receptor has been shown in direct binding studies. Thus, AF-DX 116 (Hammer *et al.*, 1986) has been shown to differentiate between cardiac and exocrine gland M_2-type muscarinic receptors. Differences between cardiac and exocrine gland M_2 muscarinic binding sites have also been demonstrated by De Jonge *et al.* (1986).

One of the major difficulties in differentiating putative subtypes of the muscarinic receptor in binding studies has been the limited number of truly

[1] Author for correspondence.

selective antagonists that can differentiate the three putative subtypes of the muscarinic receptor. With the introduction of two novel muscarinic receptor antagonists, himbacine (Gilani & 1986) and methoctramine (Melchiorre *et al.*, 1987) the aim of the present study was to determine the affinity of these compounds for the three putative subtypes of muscarinic binding site identified in ligand binding studies in order to determine whether these agents can aid in defining the pharmacology of muscarinic receptor binding sites.

In addition, since buffer composition has been reported to affect antagonist affinity for muscarinic receptors (Birdsall *et al.*, 1979; Burgen, 1986) and since many direct binding studies are conducted in non-physiological hypotonic buffers, the present study was also extended to compare the influence of buffer composition on the pharmacology of the muscarinic binding sites.

Methods

Membrane preparation

In all studies disodium edetate (EDTA) washed membrane preparations (Cheung *et al.*, 1982) were used. The cerebral cortex and heart were removed from 200–300 g male Sprague-Dawley rats. Rat submaxillary glands were obtained from Pel Freez (Arkansas). Identical results with regard to receptor density, [^{3}H]-N-methyl scopolamine ([^{3}H]-NMS) K_d and pirenzepine affinities were obtained with fresh tissues. The preceding and all subsequent membrane preparation procedures were conducted at 4°C. The tissues were separately homogenized (1 g wet weight tissue to 30 ml buffer; Polytron P10: setting 10: 2 × 20 s bursts) in ice cold Tris-EDTA homogenizing buffer (50 mM Tris; 5 mM disodium EDTA: pH 7.4 at 4°C). The cardiac and submaxillary gland homogenates were passed through a double layer of cheesecloth and centrifuged at 500 *g* for 10 min in order to remove cell debris. The supernatants were reserved and the pellets rehomogenized in buffer, filtered and centrifuged at 500 *g*. The supernatants from this centrifugation were combined with the original supernatants and centrifuged, along with the cortical homogenate at 30,000 *g* for 15 min. The pellets were superficially washed with 2 × 40 ml aliquots of homogenizing buffer before resuspending (Polytron P10; 1 × 5 s burst; setting 5) in homogenizing buffer and centrifuging at 30,000 *g* for 15 min. The membrane pellets so obtained were washed twice more using the same protocol but substituting ice cold Tris-EDTA buffer (composition: 50 mM Tris; 0.5 mM EDTA; pH 7.4 at 4°C) for homogenizing buffer. The final membrane pellet was resuspended in 50 mM Tris buffer (pH 7.4 at 32°C) and stored under liquid nitrogen until required.

Binding assays

The assay buffers used in all binding experiments were either a Tris-EDTA-magnesium (TEM) assay buffer (50 mM Tris, 0.5 mM EDTA, 5 mM magnesium, pH 7.4 at 32°C) or a modified Tris-Krebs solution of the following composition (mM): NaCl 144, KCl 4.7, KH_2PO_4 1,2, $CaCl_2$ 2.5, $MgCl_2$ 1.1, D-glucose 10, Tris 10, pH 7.4 at 32°C.

Saturation experiments

In saturation studies, [^{3}H]-NMS and [^{3}H]-pirenzepine ([^{3}H]-Pir) were incubated to equilibrium with membranes (4–20 pM with respect to [^{3}H]-Pir or [^{3}H]-NMS binding sites) in polypropylene tubes containing the appropriate assay buffer at 32°C. Preliminary kinetic experiments indicated that 3 h was sufficient for equilibration of the lowest concentrations of [^{3}H]-NMS (5 pM) and [^{3}H]-Pir (0.1 nM) used in these studies.

The assay volume was 3 ml in studies using [^{3}H]-NMS and 1 ml in studies using [^{3}H]-Pir. In all studies with [^{3}H]-NMS, 10–13 concentrations of radioligand, ranging from at least 10 fold lower to 10 fold greater than the K_d value, were employed. For [^{3}H]-Pir saturation binding studies, the maximal radioligand concentration tested in TEM buffer systems was 10 fold higher than the radioligand K_d. However, in the Tris-Krebs buffer the maximal radioligand concentration was only 3 times greater than the radioligand K_d. At each concentration of radioligand, total binding was determined in triplicate and non-specific binding (NSB), defined as radioligand binding in the presence of 1 μM atropine, was determined in duplicate. Extensive studies established that this concentration of atropine adequately defined the NSB of both [^{3}H]-Pir and [^{3}H]-NMS at the concentrations used in the present study.

At equilibrium, bound ligand was separated from free ligand by vacuum (22 in Hg) filtration over double SKATRON receptor binding filtermats using a SKATRON cell harvester. The filtermats were pretreated with polyethyleneimine, by soaking in a 0.1% solution 18–24 h before use, in order to reduce filter binding of both [^{3}H]-Pir and [^{3}H]-NMS. After filtration the filtermats were washed for 10 s with 5 ml of either ice cold ([^{3}H]-Pir studies) or room temperature ([^{3}H]-NMS studies) distilled water.

Competition experiments

In these studies fixed concentrations of either [^{3}H]-NMS (80–150 pM) or [^{3}H]-Pir (200–500 pM) were

incubated with membranes (4–10 pM with respect to both [^{3}H]-NMS and [^{3}H]-Pir binding sites) in a final volume of 3 ml of the appropriate assay buffer for 3 h at 32°C in the presence or absence of the competing compounds. For all compounds studied at least 15 concentrations (ranging from 1000 times lower to 1000 times greater than the IC_{50} value for the compound under study) were employed. Bound ligand was separated from free ligand by vacuum filtration as previously described. In some experiments a Brandel 48 well cell harvester was used. In these later studies filtration was over 0.1% polyethyleneimine pretreated Whatman GF/B filter strips.

Data analysis

All binding data were analyzed by iterative curve fitting procedures. Saturation and competition binding data were analyzed using LIGAND (Munson & Rodbard, 1980). Competition binding data were also analyzed using an iterative curve fitting programme (Michel & Whiting, 1984) to provide IC_{50} values and Hill coefficients (nH) for competing compounds. When data were analyzed by the latter method, the IC_{50} values obtained were corrected for the presence of radioligand according to the method of Cheng & Prusoff (1973). Unless otherwise stated all values are the geometric mean and standard error of the mean (s.e.mean), derived from between 4 and 6 experiments. In those tables where pKi values are presented, the mean and standard error of the mean were calculated from the individual pKi values.

Protein assays

Protein was assayed by the dye binding method (Bio-rad) with bovine serum albumin used as standard.

Drugs and radiochemicals

[^{3}H]-NMS (specific activity 72 Ci mmol^{-1}) and [^{3}H]-Pir (specific activity 76 Ci mmol^{-1}) were obtained from Amersham and New England Nuclear, respectively. Pirenzepine hydrochloride was obtained from Boehringer Ingelheim. Adiphenine, atropine sulphate, and (−)-scopolamine were purchased from Sigma Chemical Company as were all chemicals and reagents used. AF-DX 116 (11-[[2-(diethylamino)methyl]-1-piperidinyl]acetyl-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine-6-one), CPPS, 4-DAMP and methoctramine were synthesized by J. Berger, Dr R. Clark and D. Repke (IOC, Syntex, Palo Alto). Himbacine was obtained from Dr Taylor, University of Sydney, Australia.

Results

Saturation binding of [^{3}H]-NMS *and* [^{3}H]-Pir *in rat EDTA-washed membranes*

In the present study [^{3}H]-NMS was used to label M_2-muscarinic receptors present in rat cardiac and submaxillary gland membranes whereas [^{3}H]-Pir was used to label M_1-muscarinic receptors present in rat cerebral cortex. In the two buffer systems used both [^{3}H]-NMS and [^{3}H]-Pir bound reversibly (data not shown) and with high affinity in the three tissues studied (Table 1). In cardiac and submaxillary gland membranes good ratios (90–96%) of specific to total binding were obtained when using concentrations of [^{3}H]-NMS up to the K_d value (data not shown). For [^{3}H]-Pir binding in rat cortical membranes, the ratio of specific to total binding was between 70 and 90% when using concentrations of radioligand up to the K_d value.

In all saturation studies, Scatchard plots

Table 1 Binding parameters of muscarinic radioligands in rat membrane preparations

Radioligand	*Tissue*	*Buffer*	K_D	B_{max}
[^{3}H]-Pir	C	TEM	4.7 ± 0.3	1302 ± 102
[^{3}H]-Pir	C	TK	18.2 ± 1.6*	1525 ± 78
[^{3}H]-NMS	H	TEM	0.21 ± 0.02	376 ± 28
[^{3}H]-NMS	H	TK	0.46 ± 0.07*	365 ± 25
[^{3}H]-NMS	S	TEM	0.11 ± 0.02	456 ± 32
[^{3}H]-NMS	S	TK	0.27 ± 0.04*	421 ± 34

Values shown are the mean ± s.e.mean for the K_D(nM) or B_{max} (fmol mg^{-1} protein) of the indicated radioligands for binding sites located in cerbrocortical (C), cardiac (H) or submaxillary gland (S) membranes. Binding parameters were determined both in Tris-EDTA-magnesium (TEM) and Tris-Krebs (TK) assay buffer.

With the exception of the data for [^{3}H]-pirenzepine ([^{3}H]-Pir) binding to cerebral cotex in TEM assay buffer, the values shown are best fit values which were obtained from fitting specific binding isotherms to a model describing an interaction of the radioligand with a homogenous population of binding sites. For [^{3}H]-Pir binding to cerebral cortex in TEM assay buffer, the data could be best described by assuming an interaction of this ligand with a saturable binding site (values shown in Table) and a non-saturable component of binding.

* Value significantly different ($P < 0.05$) from that obtained using a TEM assay buffer.

(Scatchard, 1949) of specific [^{3}H]-NMS binding were linear and were consistent with [^{3}H]-NMS identifying homogeneous populations of binding sites (Table 1). This homogeneity of [^{3}H]-NMS binding was also confirmed by a direct analysis of total radioligand binding isotherms in which NSB was estimated together with the K_d and B_{max} (data not shown). Furthermore, the binding parameters arrived at by this second method of analysis were in close agreement with those obtained when specific binding data were analyzed assuming a single site model of binding (data not shown).

In Tris-Krebs buffer [^{3}H]-Pir bound to an apparently homogeneous population of binding sites. In TEM assay buffer the Scatchard plots of specific [^{3}H]-Pir binding to cerebrocortical membranes displayed signs of upward concave curvature. This was most marked when using high ligand concentrations (30–60 nM). The specific binding data could be better described by models which assumed [^{3}H]-Pir to bind either to two populations of saturable binding sites or two components of specific binding, one a saturable binding site and the other a component of displaced NSB. Since there was no statistical improvement between the two site and the two component models of binding the latter, more simple, model was selected. It should be noted that the second component of binding comprised only 5% of specific binding in competition studies when a low concentration of [^{3}H]-Pir (0.5 nM) was used (data not shown).

B_{max} values obtained for [^{3}H]-NMS in cardiac and gland tissue and for [^{3}H]-Pir in cerebral cortex were not affected by the choice of assay buffer. However, the radioligand affinities were significantly higher in TEM buffer than in Tris-Krebs buffer (Table 1).

Pharmacology of ^{3}H-radioligand binding sites

The affinity estimates of a series of compounds for the sites labelled by [^{3}H]-NMS in rat cardiac and gland tissue and by [^{3}H]-Pir in cortical tissue using TEM buffer are shown in Table 2. Affinity estimates obtained with Tris-Krebs buffer are shown in Table 3.

Irrespective of the buffer used the pharmacology of both the [^{3}H]-NMS and [^{3}H]-Pir binding sites was consistent with that of the muscarinic receptor.

In the competition binding studies nearly all of the compounds examined displayed Hill coefficients close to unity and the data could only be analyzed by assuming competition with a homogeneous population of [^{3}H]-NMS binding sites (Tables 2 and 3). The only exception to this was methoctramine which displayed a low Hill coefficient in the submaxillary gland membranes. With the exception of AF-DX 116 and secoverine, compound affinity estimates were generally 1.5 to 3 fold lower in Tris-Krebs than in TEM buffer. The greatest effect of buffer composition was exerted on methoctramine which displayed up to a 10 fold higher affinity estimate in TEM than

Table 2 Muscarinic antagonist affinity estimates obtained in TEM assay buffer

Compound	[^{3}H]-*Pir Cortex* pKi	[^{3}H]-*NMS Heart* pKi	[^{3}H]-*NMS Gland* pKi	*Fold selectivity** A	B	C
Adiphenine	7.82 (0.10)	6.73 (0.04)	7.11 (0.05)	12	5	0.4
AF-DX 116	6.26 (0.08)	6.52 (0.06)	5.72 (0.05)	0.6	3	6
CPPS	8.97 (0.05)	7.70 (0.07)	8.21 (0.09)	19	6	0.3
4-DAMP	9.09 (0.07)	8.16 (0.06)	8.91 (0.08)	9	2 +	0.2
Dicyclomine	8.87 (0.09)	7.42 (0.06)	8.32 (0.04)	28	4	0.1
Hexahydroadiphenine	8.97 (0.04)	7.83 (0.04)	8.42 (0.04)	14	4	0.3
Himbacine	7.37 (0.06)	7.70 (0.08)	6.86 (0.08)	0.5	3	7
Methoctramine	7.68 (0.08)	8.85 (0.05)	6.82 (0.03)	0.07	7	107
Pirenzepine	8.17 (0.05)	6.63 (0.06)	7.29 (0.11)	35	8	0.2
Secoverine	8.21 (0.05)	7.73 (0.09)	7.44 (0.07)	3	6	2

All values are the mean (± s.e.mean) from 5–9 experiments. Selectivity values were calculated in columns A, B and C. Column A is the antilog of the difference between the pKi values obtained at the [^{3}H]-Pir binding site in cerebral cortex and the [^{3}H]-NMS binding site in cardiac tissue. Column B is the antilog of the difference between the pKi values obtained at the [^{3}H]-Pir binding site in cerebral cortex and the [^{3}H]-NMS binding site in gland tissue. Column C is the antilog of the difference between the pKi values obtained at the [^{3}H]-NMS binding site in cardiac tissue and the [^{3}H]-NMS binding site in submaxillary gland.

Hill coefficients were not significantly different from unity except for methoctramine in the submaxillary gland membranes (0.85 ± 0.06).

All fold selectivities were significantly different from unity except for those indicated (+).

* Constraining the Hill slopes to unity did not significantly affect the observed selectivities.

Table 3 Muscarinic antagonist affinity estimates obtained in Tris-Krebs assay buffer

Compound	[^{3}H]-*Pir cortex* pKi	[^{3}H]-*NMS heart* pKi	[^{3}H]-*NMS gland* pKi	*Fold selectivity** A	B	C
Adiphenine	7.25 (0.06)	6.58 (0.07)	6.68 (0.04)	5	4	0.8 +
AF-DX 116	6.59 (0.05)	7.02 (0.03)	5.99 (0.08)	0.4	4	11
CPPS	8.36 (0.06)	7.55 (0.06)	7.70 (0.07)	6	5	0.7 +
4-DAMP	8.51 (0.08)	7.97 (0.06)	8.74 (0.07)	3	0.6 +	0.2
Dicyclomine	8.46 (0.07)	7.22 (0.06)	8.02 (0.02)	17	3	0.2
Hexahydroadiphenine	8.64 (0.06)	7.35 (0.06)	7.97 (0.05)	20	5	0.2
Himbacine	7.00 (0.04)	7.53 (0.03)	6.66 (0.06)	0.3	2	7
Methoctramine	6.86 (0.05)	7.76 (0.03)	6.29 (0.08)	0.1	4	30
Pirenzepine	7.72 (0.06)	6.38 (0.06)	6.79 (0.05)	22	9	0.4
Secoverine	8.37 (0.04)	7.98 (0.08)	7.72 (0.09)	2	4	1.82 +

All values are the mean (± s.e.mean) from 5–9 experiments. Selectivity values were calculated in columns A, B and C. Column A is the antilog of the difference between the pKi values obtained at the [^{3}H]-Pir binding site in cerebral cortex and the [^{3}H]-NMS binding site in cardiac tissue. Column B is the antilog of the difference between the pKi values obtained at the [^{3}H]-Pir binding site in cerebral cortex and the [^{3}H]-NMS binding site in gland tissue. Column C is the antilog of the difference between the pKi values obtained at the [^{3}H]-NMS binding site in cardiac tissue and the [^{3}H]-NMS binding site in submaxillary gland.
Hill coefficients were not significantly different from unity except for methoctramine in the submaxillary gland membranes (0.87 ± 0.04).
All fold selectivities were significantly different from unity except for those indicated (+).
* Constraining the Hill slopes to unity did not significantly affect the observed selectivities.

in Tris-Krebs. In contrast to the other compounds studied, AF-DX 116 and secoverine were between 1.8 fold and 3 fold more potent in Tris-Krebs buffer than in TEM assay buffer.

Muscarinic receptor subtype selectivity

Comparison of cardiac [^{3}H]-NMS and cortical [^{3}H]-Pir binding sites From Tables 2 and 3 (column A) it can be seen that AF-DX 116, himbacine and methoctramine displayed a higher affinity for the cardiac [^{3}H]-NMS binding sites than for the cerebrocortical [^{3}H]-Pir binding sites. The remainder of the ligands were selective for the cortical [^{3}H]-Pir binding sites. The degree of selectivity ranged from the approximately 30 fold selectivity of pirenzepine for the [^{3}H]-Pir binding sites in rat cortex to the 15 fold selectivity of methoctramine for the [^{3}H]-NMS binding sites of rat cardiac membranes.

Comparison of cardiac and glandular [^{3}H]-NMS binding sites AF-DX 116, himbacine, methoctramine and secoverine were selective for the cardiac muscarinic binding site (Tables 2 and 3, column C). In particular, methoctramine was nearly 100 fold selective. The remainder of the compounds were all marginally selective for the submaxillary gland muscarinic binding site. Dicyclomine was the most selective of these agents displaying a 5–6 fold higher affinity for the gland as opposed to cardiac binding sites.

Comparison of cerebrocortical [^{3}H]-Pir and submaxillary gland [^{3}H]-NMS binding sites As can be seen from Tables 2 and 3 (column B) most of the compounds studied were between 1.5 and 8.5 fold selective for the cortical [^{3}H]-Pir binding sites. The only exception to this was 4-DAMP which appeared to be non-selective in both the TEM and Tris-Krebs assay buffer. It should be noted that there were no compounds that were selective for the submaxillary gland muscarinic [^{3}H]-NMS binding sites.

Discussion

The two aims of the present study were to define more precisely the pharmacology associated with the three putative muscarinic receptor binding sites and to examine the influence of buffer composition on the affinity and selectivity of muscarinic antagonists for subtypes of the muscarinic receptor.

In terms of the affinity of ligands for the muscarinic binding sites, the effects of buffer composition were in general agreement with previous studies in cerebral cortex and cardiac membranes (Birdsall *et al.*, 1979; Burgen, 1986) in that for most compounds affinity estimates were lower in the more physiological, high ionic strength, Krebs-based buffer than in the TEM buffer. The present study extended these

findings to the M_2 gland muscarinic binding site. Furthermore we were able to demonstrate that while the affinity of most compounds was 2–3 fold higher in TEM assay buffer, methoctramine was much more sensitive to ionic composition and displayed between a 3 and 10 fold higher affinity in TEM than in Tris-Krebs. In addition, AF-DX 116 and secoverine were actually more potent in a Tris-Krebs buffer than in the low ionic strength TEM assay buffer.

The reason for the greater susceptibility of methoctramine affinity estimates to buffer composition, especially at the M_2 receptor of cardiac tissue, and the differential behaviour of both AF-DX 116 and secoverine are at present unknown. AF-DX 116 and secoverine do not share any obvious structural similarities. Furthermore, AF-DX 116 is structurally a close analogue of pirenzepine, yet pirenzepine displayed a higher affinity in TEM than in Tris-Krebs.

It should be stressed that the differences in affinity between the two buffer systems used were generally small and were of similar magnitude at the three subtypes of muscarinic binding site examined. Nevertheless these small differences could affect apparent antagonist selectivity and may, especially in the case of compounds such as methoctramine, also hamper attempts to correlate binding data with functional data.

Considering the issue of multiple subtypes of muscarinic receptor, the data obtained in the present study are in general agreement with those of Doods *et al.* (1987) and provide additional evidence to support the existence of at least two and possibly three classes of muscarinic binding sites. Furthermore, it would appear that the overall pharmacological profile of these three muscarinic receptor binding sites was not markedly affected by buffer composition.

There were distinct pharmacological differences between the M_2 muscarinic binding sites in cardiac and gland membranes. In both tissues the binding sites could be described as belonging to the M_2 subtype on the basis of their low affinity for pirenzepine, although the gland muscarinic binding sites, as has been reported by others (Hammer *et al.*, 1980), displayed intermediate rather than low affinity for pirenzepine.

In agreement with previous studies (Hammer *et al.*, 1986; De Jonge *et al.*, 1986; Doods *et al.*, 1987), a range of ligands in addition to pirenzepine, could differentiate between the M_2 muscarinic receptors present in cardiac and gland tissues. Most compounds studied were marginally (3 to 10 fold) selective for the [^{3}H]-NMS binding site in submaxillary gland membranes. The most important finding was that secoverine, himbacine and AF-DX 116 exhibited a 2 to 10 fold higher affinity for cardiac than for gland [^{3}H]-NMS binding sites while methoctramine, depending on the buffer system used, displayed between a 30 and 107 fold selectivity for cardiac muscarinic receptors (Tables 2 and 3).

It is perhaps interesting to note that all of the compounds that were cardiac selective in these binding studies have also been described as displaying atrial selectivity, as opposed to ileal selectivity, in functional studies. Furthermore the degree of atrial selectivity obtained in functional studies for methoctramine (275 fold by Melchiorre *et al.*, 1986: 53 fold by Mutschler *et al.*, 1987), himbacine (10 fold by Gilani & Cobbin, 1986), AF-DX 116 (7 fold by Giachetti *et al.*, 1986) and secoverine (7 fold by Clague *et al.*, 1985) are similar to the selectivity of these compounds for cardiac as opposed to gland [^{3}H]-NMS binding sites (Tables 2 and 3). This indicates that the cardiac binding sites may be analogous to the atrial muscarinic receptors identified in functional studies and that the submaxillary gland binding sites may be similar to the ileal muscarinic receptors involved in contraction of the smooth muscle. It is perhaps worth noting in this connection that 4-DAMP which in functional studies is 10 fold ileal-selective (Clague *et al.*, 1985) was 5–6 fold gland-selective in the present binding studies. The only evidence against such a correlation is the finding that pirenzepine, which does not differentiate between ileal and atrial muscarinic receptors in functional studies (Eglen & Whiting, 1986b), has been shown in previous binding studies to differentiate between gland and cardiac M_2 muscarinic binding sites (Hammer *et al.*, 1980; De Jonge *et al.*, 1986) and in the present study possessed between a 2 and 6 fold higher affinity at the submaxillary gland [^{3}H]-NMS binding sites than at the cardiac sites.

Irrespective of whether gland [^{3}H]-NMS binding sites are analogous to ileal muscarinic receptors, it would appear that, given the present and previous ligand binding data (De Jonge *et al.*, 1986; Doods *et al.*, 1987), the muscarinic binding sites present in cardiac and gland tissue differ substantially. In terms of nomenclature this may make the use of the terms M_2-cardiac and M_2-gland misleading in addition to being cumbersome. Although, it may be better to adopt the classification proposed by De Jonge *et al.* (1986), and refer to cardiac binding sites as M_2 and glandular binding sites, or sites that display a similar pharmacology, as M_3 binding sites it is perhaps prudent to use the existing nomenclature until a concensus opinion is reached as to the terminology to be used for describing muscarinic receptors.

In addition to the considerable difference between the M_2-cardiac and M_2-gland [^{3}H]-NMS binding sites, there were also marked differences between the M_2 muscarinic binding sites in cardiac tissue and the M_1 muscarinic binding sites labelled using [^{3}H]-Pir in rat cerebral cortex.

These differences were as marked as those found between the M_2-cardiac and M_2-gland binding sites. Thus a wide range of both M_1 and M_2 cardiac selective ligands could be described. As previously reported both pirenzepine (Hammer *et al.*, 1980) and dicyclomine (Luber-Narod & Potter, 1983) were highly M_1-selective. In addition a range of other compounds were also shown to be selective for the M_1 muscarinic binding site. These included the two ileal selective antagonists 4-DAMP and CPPS as well as adiphenine and its analogue, hexahydroadiphenine.

As previously reported by Hammer *et al.* (1986), AF-DX 116 displayed higher affinity for the M_2-cardiac than for the M_1 binding site although in our studies the degree of selectivity obtained, 1.8–2.7 fold, was not as great as the selectivity reported by these authors. More importantly, in the present study, two recently described compounds, himbacine and methoctramine, were shown to be selective for M_2-cardiac as opposed to M_1 muscarinic binding sites. The demonstration of a wide range of both M_1 and M_2-cardiac selective compounds would indicate that these two binding sites can be defined as different entities from a pharmacological standpoint.

While the present binding studies could clearly differentiate between M_1 and M_2-cardiac binding sites and between M_2-cardiac and M_2-gland binding sites it was evident that the M_1 and the M_2-gland binding sites could not be so readily differentiated. Indeed nearly all compounds examined could be classified as marginally M_1 selective. The only exception was 4-DAMP which was non-selective. The degree of M_1 selectivity for those compounds that were selective amounted to no more than 2–8.5 fold (Tables 2 and 3).

The reason for the similarity between the M_1 and M_2-gland binding site is not known at present. It may simply be that none of the compounds examined was selective for M_1 or M_2-gland binding sites. It should however be noted that a wide range of other compounds that we have examined have not been able to differentiate M_1 from M_2-gland binding sites. These include benzhexol, trihexyphenidyl, scopolamine, gallamine, N-methyl atropine and NMS (unpublished data). It may also be worth noting that we find the kinetics of [^{3}H]-NMS binding in gland and cortex are indistinguishable with both a slow association and dissociation that contrasts markedly with the rapid association and dissociation of [^{3}H]-NMS at cardiac-M_2 binding sites (unpublished observations). This once again points to considerable differences in the M_2-cardiac binding site from both the M_1 and M_2-gland binding site but indicates the similarities between M_1 and M_2-gland binding sites.

These findings may indicate that the M_1 and M_2-gland binding sites are similar entities and may represent either different states of the same muscarinic receptor site or may be different due to the assay conditions employed (membrane fractions, tissue environment, different radioligands). Clearly further studies of this aspect of muscarinic receptor classification are essential to determine whether the M_1 and M_2-gland binding sites are truly different receptors. This is especially important since the degree of selectivity of the compounds examined for the M_1 binding site was generally less than or marginally greater than the three fold difference in affinity suggested by Furchgott (1972) as a criterion for defining receptor subtypes.

In conclusion, the present study has indicated that at least three classes of muscarinic binding site can be identified. M_2-cardiac receptors can be defined on the basis of several selective antagonists including methoctramine, AF-DX 116 and himbacine. In addition both M_1 and M_2-gland binding sites can be defined pharmacologically as possessing low affinity for the aforementioned drugs and possessing high affinity for pirenzepine, dicyclomine, hexahydroadiphenine, 4-DAMP and CPPS. These binding sites can be clearly differentiated from the M_2 binding site present in cardiac tissue. Although the M_1 and M_2-gland binding sites can be distinguished in terms of their absolute affinity for muscarinic antagonists, they share many pharmacological similarities which make a pharmacological differentiation between these two sites difficult at present.

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Monoamine oxidase activity and triiodothyronine biosynthesis in human cultured thyroid cells

Z. Kraiem, O. Sadeh & *[1]M.B.H. Youdim

Endocrine Research Unit, Carmel Hospital, Haifa 34362, and *Rappaport Family Research Institute, Dept. of Pharmacology, Faculty of Medicine, Technion, Haifa 31096, Israel

1 The proposal that monoamine oxidase (MAO) is a source of peroxide in thyroid hormone biosynthesis has been examined by use of isolated cultured human thyroid cells which retain the ability to secrete triiodothyronine (T_3) in response to thyroid stimulating hormone (TSH).

2 The results demonstrated the presence of MAO A and B in human thyroid cells which oxidized 5-hydroxytryptamine and 2-phenylethylamine, respectively, and were selectively inhibited by the MAO inhibitors clorgyline and (−)-deprenyl.

3 Addition of propylthiouracil to the culture system induced a 61% reduction in TSH-stimulated T_3 secretion, indicating that the bulk of such secretion apparently derives from *de novo* iodothyronine synthesis.

4 The MAO A and B substrate, tyramine, was ineffective in stimulating T_3 secretion.

5 The selective MAO inhibitors, clorgyline and (−)-deprenyl, alone and in combination, and in the presence and absence of tyramine, failed to inhibit basal as well as TSH-stimulated T_3 secretion in cultured human thyrocytes.

6 It is therefore apparent that even though thyroid MAO A and B enzyme reactions result in the generation of H_2O_2, this H_2O_2 does not seem to play a significant role in T_3 biosynthesis.

Introduction

Formation of thyroid hormones involves iodination of the phenolic ring of tyrosyl residues in the thyroglobulin molecule and coupling of the iodinated tyrosyls to form iodothyronines. Both of these processes are catalysed by thyroperoxidase in the presence of H_2O_2 (Taurog, 1970; DeGroot & Niepomniszcze, 1977).

It has been demonstrated that the thyroid has the capacity of generating H_2O_2 but little is known about the exact mechanism of this production (DeGroot & Niepomniszcze, 1977). Several enzyme systems have been proposed as sources of H_2O_2 in the thyroid (DeGroot & Niepomniszcze, 1977; Ekholm, 1981; Nunez & Pommier, 1982). Thus, the reduced pyridine nucleotides NADPH and NADH have been regarded as likely intracellular H_2O_2 generating systems. Xanthine oxidase has also been proposed as a possible source of H_2O_2. Finally, formation of H_2O_2 via oxidation of monoamines by monoamine oxidase (MAO) has been considered to be directly involved (Schulz & Oliner, 1967; Fischer *et al.*, 1968). It has not yet been conclusively demonstrated which of these H_2O_2-generating enzyme systems is involved in thyroid hormone biosynthesis.

We have established an *in vitro* assay system of cultured human thyroid cells which retain the ability to synthesize and secrete triiodothyronine (T_3) in response to thyroid stimulating hormone (TSH). Using such a system, we have examined MAO, types A and B, activities (Youdim & Finberg, 1982), and tested whether they play a significant role in H_2O_2 generation required for T_3 biosynthesis.

[1] Author for correspondence at: Technion-Faculty of Medicine, Department of Pharmacology, Bat Galim, POB 9649, Haifa, Israel.

Methods

Thyroid cell culture

Human thyroid cells were isolated, cultured and preserved in liquid nitrogen by the procedure of Rapoport *et al.* (1982; 1984). For individual experiments, the thyroid cells were thawed and plated onto 24-well microtiter plates at a density of 10^5 cells/well in a medium consisting of Medium-199 supplemented with 10% foetal calf serum and antibiotics (100 units ml^{-1} penicillin, 100 $\mu g\,ml^{-1}$ streptomycin,

Table 1 Monoamine oxidase A and B activities in cultured human thyroid cells

	Enzyme activity (nmol 30 min^{-1} per 10^6 cells)	
	5-Hydroxytryptamine MAO-A	*2-Phenylethylamine MAO-B*
Control	3.3 ± 0.7	2.1 ± 0.5
Clorygline (10^{-6} M)	0	1.8 ± 1.0
(−)-Deprenyl (10^{-6} M)	2.0 ± 0.3	0

Thyroid cells were preincubated for 20 min at 37°C with the selective MAO inhibitors prior to the addition of 5-HT (200 μM final) and 2-phenylethylamine (20 μM final) as monoamine oxidase substrates. The cells were incubated for a further 30 min and the reaction terminated with tranylcypromine (100 μM final). The deaminated products were isolated as described by Tipton & Youdim (1984). The results are mean ± s.d., $n = 6$ replicates of thyroid cells of a colloid goitre preparation derived from a single individual.

50 μg ml^{-1} neomycin and 2.5 μg ml^{-1} fungizone). After one day at 37°C in an atmosphere of 5% CO_2:95% air in a water-saturated incubator, the medium was removed and the thyroid cells in monolayer culture incubated for 7 days in 0.5 ml Earle's Basal Medium Eagle (BME) supplemented with glutamine (2 mM), HEPES (25 mM), foetal calf serum (10%), KI (1 μM) and antibiotics (as described above) in the presence and absence of TSH (0.5 mu ml^{-1}), the MAO inhibitors clorgyline and l-deprenyl (each at 10^{-7} M and 10^{-6} M) and the MAO substrate tyramine (0.1 and 0.5 mM). At the end of the incubation period, total T_3 secretion was measured in duplicate by radioimmunoassay (RIA) of 50 μl aliquots of medium (intracellular T_3 concentrations were undetectble), using the Amerlex kit from Amersham International (Amersham, England). The antiserum used cross-reacts with L-thyroxine by <0.3%, with 3,5-diiodothyronine by 0.8%, with human thyroglobulin by <0.2%, and 3,3',5'-triiodothyronine (reverse T_3), 3,5-diiodo-L-tyrosine, 3-iodo-L-tyrosine by <0.03%.

All experiments were carried out in triplicate wells and repeated at least twice. Intra assay coefficient of variation (CV) was 7%. Interassay CV was 15% with the thyroid cell preparation used in the experiments shown in Tables 1 and 3, 17% with the thryoid cell preparation used in the experiments shown in Table 2, and 23% with both of these preparations.

Assay of monoamine oxidase activity

Following thawing of cryopreserved human thyroid cells, MAO activity was measured by the luminescence method of Tenne *et al.*, (1985) with octylamine as the substrate. For assaying the different forms of MAO (types A and B), the radiochemical procedure described by Tipton & Youdim (1984) was employed, 5-hydroxytryptamine (5-HT, [2-^{14}C]-5-hydroxytryptamine) as substrate for MAO-type A and [1-^{14}C]-2-phenylethylamine (PEA) as substrate for type B activities.

The assay in a total volume of 500 μl containing 0.05 M sodium phosphate buffer, pH 7.4 and 5-HT (200 μM) or PEA (20 μM) was incubated for 30 min at 37°C. The reaction was terminated by the addition of 100 μl tranylcypromine (10^{-4} M) and the ^{14}C-deaminated metabolites were isolated from an amberlite CG-column and counted in a scintillation counter.

Materials

Materials were obtained from the following sources: culture media from Biological Industries (Beth Haemek, Israel); bovine TSH from Armour (Eastbourne, England); clorgyline and (−)-deprenyl were gifts from May and Baker Ltd. (Dagenham, Essex) and Prof. J. Knoll (Semmelweis University of Medicine, Budapest, Hungary), respectively. [2-^{14}C]-5-hydroxytryptamine creatinine sulphate (50–60 mCi mmol^{-1}) and [1-^{14}C]-phenylethylamine hydrochloride (50–60 mCi mmol^{-1}) were purchased from Amersham International (Amersham, U.K.). All other materials were of the highest purity that could be obtained from Sigma Chemical Co. (St. Louis, MO).

Results

The levels of MAO activity in the different thyroid cell preparations tested (2 colloid goitre, 2 normal and 2 Greaves' thyroid tissues) ranged from 0.1–2.8 nmol h^{-1} per 10^6 cells (octylamine as substrate). Specific type A and B MAO activities were also determined in thyroid cells and the results, shown in Table 1, demonstrate the presence of both MAO-type A (5-HT substrate) and B (2-phenylethylamine substrate) activities. Table 1 also shows the ability of clorgyline to inhibit selectively MAO-type A and

Table 2 Effect of propylthiouracil (PTU) on triiodothyronine (T_3) secretion in cultured human thyrocytes

	T_3 secretion (pg per well)
Cells	99 ± 15
Cells + PTU (0.1 mM)	78 ± 13
Cells + PTU (1 mM)	76 ± 12
Cells + PTU (2 mM)	85 ± 9
Cells + PTU (2 mM) + MAO inhibitors + tyramine	79 ± 14
Cells + TSH	850 ± 25
Cells + TSH + PTU (0.1 mM)	612 ± 18
Cells + TSH + PTU (1 mM)	502 ± 22
Cells + TSH + PTU (2 mM)	331 ± 17
Cells + TSH + PTU (2 mM) + MAO inhibitors + tyramine	325 ± 19

Values shown are the mean ± s.d. T_3 secretion (pg per well) from two experiments, each carried out in triplicate wells, following culture for 7 days of human thyrocytes (10^5 cells per well) in the presence and absence of thyroid stimulating hormone TSH (0.5 mu ml^{-1}), PTU (0.1–2 mM), the MAO inhibitors clorgyline (10^{-6} M) and (−)-deprenyl (10^{-6} M) and the MAO substrate tyramine (0.5 mM). The thyroid cell preparation used in these experiments was obtained from colloid goitre tissue derived from a single individual different from the one used in the experiments shown in Tables 1 and 3.

(−)-deprenyl to inhibit selectively MAO-type B activity. The concentrations of inhibitors chosen were those which previously had been shown to inactivate MAO-A and B selectively (Kalir *et al.*, 1981).

Cultured thyroid cells exhibited maintenance of biochemical competence, as shown by an almost tenfold increase in T_3 secretion following challenge by TSH (Tables 2 and 3). Addition of propylthiouracil (PTU) to the culture system inhibited, in a dose-dependent manner, TSH-stimulated T_3 secretion, up to a 61% reduction in secretion of the thyroid hormone (Table 2), without any toxic effect on cell survival. A similar dose-dependent inhibitory effect was obtained with methimazole, another inhibitor of thyroid hormone synthesis (data not shown). Thus, the bulk of the TSH-stimulated T_3 secretion apparently derives from *de novo* iodothyronine biosynthesis.

Addition of clorgyline and (−)-deprenyl did not significantly alter ($P > 0.05$, Student's t test) basal or TSH-induced T_3 secretion, as shown in Table 3. It is to be noted that the thyroid cell preparation used in these cell culture experiments (i.e. depicted in Table 3) was the one in which the MAO inhibitors were specifically shown to be effective in their ability to

Table 3 Effect of thyroid stimulating hormone (TSH) and monoamine oxidase (MAO) inhibitors on triiodothyronine (T_3) secretion in cultured human thyrocytes

	T_3 secretion (pg per well)
Cells	95 ± 5
Cells + clorgyline (10^{-7} M) + (−)-deprenyl (10^{-7} M)	105 ± 6
Cells + tyramine (0.1 mM)	96 ± 8
Cells + clorgyline (10^{-7} M) + (−)-deprenyl (10^{-7} M) + tyramine (0.1 mM)	99 ± 4
Cells + TSH	1137 ± 18
Cells + TSH + clorgyline (10^{-7} M)	1140 ± 11
Cells + TSH + 1-deprenyl (10^{-7} M)	1102 ± 16
Cells + TSH + clorgyline (10^{-7} M) + (−)-deprenyl (10^{-7} M)	1171 ± 18
Cells + TSH + clorgyline (10^{-6} M) + (−)-deprenyl (10^{-6} M)	1054 ± 48
Cells + TSH + tyramine (0.1 mM)	1103 ± 24
Cells + TSH + tyramine (0.5 mM)	1150 ± 5
Cells + TSH + tyramine (0.1 mM) + clorgyline (10^{-7} M) + 1-deprenyl (10^{-7} M)	1103 ± 24
Cells + TSH + tyramine (0.5 mM) + clorgyline (10^{-7} M) + 1-deprenyl (10^{-7} M)	1088 ± 89

Values shown are the mean ± s.d. T_3 secretion (pg per well) from three experiments, each carried out in triplicate wells, following culture for 7 days of human thyrocytes (10^5 cells per well) in the presence and absence of TSH (0.5 mu ml^{-1}), the MAO inhibitors clorygyline and (−)-deprenyl at the designated concentrations and the MAO substrate tyramine at the indicated concentrations.

The thyroid cell preparation used in these experiments, obtained from goitre tissue derived from a single individual, was the same as that used in the experiments demonstrating specific type A and B MAO activities (i.e. the data shown in Table 1).

inhibit MAO A and B (see data in Table 1). In order to ensure that the results obtained were not due to lack of adequate MAO substrate, tyramine (0.1 or 0.5 mM), a substrate for both enzyme forms (Youdim & Finberg, 1982), was added to the incubation mixture. However, it had no significant effect ($P > 0.05$, Student's t test) on basal or TSH-induced T_3 secretions (Table 3). Moreover, tyramine added to the cultured cells in the presence of a combination of clorgyline and (−)-deprenyl was unable ($P > 0.05$, Student's t test) to alter T_3 secretion (Table 3). Neither did the combination of tyramine plus MAO inhibitors have any significant influence ($P > 0.05$, Student's t test) on the inhibition by PTU of TSH-stimulated T_3 secretion (Table 2).

Discussion

It is now well established that the enzyme MAO exists in at least two major forms, type A and B, which can be distinguished biochemically and pharmacologically on the basis of their substrate specificities and inhibitor sensitivities (Youdim & Finberg, 1982). The present results clearly demonstrate the presence of MAO A and B in human thyroid cells which oxidize 5-HT and 2-phenylethylamine respectively and are selectively inhibited by the MAO inhibitors clorgyline and (−)-deprenyl. Recently, Masini-Repiso *et al.* (1986) reported the presence of MAO A and B activities in bovine thyroid glands. However, these investigators used mitochondria derived from whole bovine thyroid glands which contain different cell types, in contrast to our human thyroid cell preparations which consist of more than 95% follicular epithelial thyroid cells (cytokeratin positive).

A decarboxylase which catalyzes the formation of tyramine from tyrosine has been shown to be present in thyroid tissue (Schulz & Oliner, 1967). This decarboxylase could thus provide an amine substrate for MAO, the oxidation of which could generate H_2O_2. It was therefore postulated (Schulz & Oliner, 1967; Fischer *et al.*, 1968) that this mechanism could serve as a source of H_2O_2 required for thyroid hormone biosynthesis. To substantiate this hypothesis, Huang & Schulz (1972) showed that the MAO substrates tyramine and 2-phenylethylamine stimulated the incorporation of radioactive iodide into bovine thyroid protein. Moreover, the amine-stimulated iodide incorporation was blocked by the presence of high concentrations ($0.1–0.5 \times 10^{-3}$ M) of MAO inhibitors such as isocarboxazid and tranylcypromine (Huang & Schulz, 1972).

In contrast, the present study demonstrates that MAO activity, although present in human thyrocytes, does not seem to play a significant role in the generation of H_2O_2 required for T_3 biosynthesis. The results indeed showed that the MAO substrate tyramine was ineffective in stimulating T_3 secretion and that addition of the selective MAO inhibitors, clorgyline and (−)-deprenyl, in the presence and absence of tyramine, failed to affect TSH-stimulated T_3 secretion in cultured human thyroid cells. Since the bulk of the TSH-stimulated T_3 secretion was shown to originate from new hormone biosynthesis, any effect of MAO on such synthesis would have been reflected in a reduction of T_3 secretion as a result of MAO inhibition.

Possible factors which could account for the discrepancy between our results and those mentioned above could be: (1) the concentration of MAO inhibitors used by Huang & Schulz (1972) were far above those needed to inhibit MAO. Such inhibitor concentrations could also have inactivated thryroid peroxidase and iodide trapping (De Groot & Niepomniszcze, 1977). (2) Species and experimental model used: bovine thyroid slices compared to a thyroid cell culture system using cells of human origin in our study. (3) Measurement of iodide incorporation into general thyroid protein in the latter studies in contrast to the more biologically relevant information provided by measuring thyroid hormone secretion as in our cell cultures.

Another enzyme, xanthine oxidase, has also been claimed to provide a thyroid H_2O_2-generating system necessary for hormone synthesis (Fischer & Lee, 1973). However, tungstate, a potent inhibitor of xanthine oxidase, has been found to lack an inhibitory effect on thyroid hormone synthesis (Kawada *et al.*, 1982).

It would therefore seem that the most likely candidate(s) to serve as the source of H_2O_2 generation necessary for thyroid hormone synthesis is an NAD(P)H oxidase(s). Bjorkman & Ekholm (1984) have suggested that the NAD(P)H oxidase might be located on the apical membrane of thyroid follicles where iodination occurs. Virion and coworkers have characterized an NADPH-dependent H_2O_2 generating system associated with thyroid particulate fraction (Virion *et al.*, 1984; Michot *et al.*, 1985; Dupuy *et al.*, 1986). Both groups of investigators provide evidence that Ca^{2+} seems to be an important factor in the NAD(P)H oxidase regulation of H_2O_2 generation (Bjorkman & Ekholm, 1984; Deme *et al.*, 1985).

In conclusion, we have demonstrated the presence of MAO A and B activities in human isolated thyroid cells. However, it is apparent that these enzyme activities do not seem to play a significant role in T_3 biosynthesis, even though their reaction involves production of H_2O_2.

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Ouabain distinguishes between nicotinic and muscarinic receptor-mediated catecholamine secretions in perfused adrenal glands of cat

Yutaka Yamada, [1]Yoshikazu Nakazato & Akira Ohga

Department of Pharmacology, Faculty of Veterinary Medicine, Hokkaido University, Sapporo, 060, Japan

1 The effect of ouabain on catecholamine (adrenaline and noradrenaline) secretion induced by agents acting on cholinoceptors was studied in perfused cat adrenal glands. Acetylcholine (ACh) (5×10^{-7} to 10^{-3} M), pilocarpine (10^{-5} to 10^{-3} M) and nicotine (10^{-6} to 5×10^{-5} M) caused dose-dependent increases in catecholamine secretion. Both ACh and nicotine released more noradrenaline than adrenaline and the reverse was the case for pilocarpine.

2 Ouabain (10^{-5} M) enhanced catecholamine secretion induced by ACh (10^{-5} M), pilocarpine (10^{-3} M) and nicotine (3×10^{-6} M) during perfusion with Locke solution. The ratio of adrenaline to noradrenaline was not affected by ouabain.

3 In the absence of extracellular Ca^{2+}, ACh and pilocarpine, but not nicotine, still caused a small increase in catecholamine secretions, which were enhanced by treatment with ouabain (10^{-5} M) plus Ca^{2+} (2.2 mM) for 25 min. The effect of ouabain was much more significant on noradrenaline secretion than on adrenaline secretion. The enhanced response was blocked by atropine (10^{-6} M) but not by hexamethonium (5×10^{-4} M).

4 Nifedipine (2×10^{-6} M) inhibited the responses to pilocarpine and nicotine. The treatment with ouabain (10^{-5} M) reversed only the response to pilocarpine and resulted in a significant increase in the proportion of noradrenaline released.

5 It is suggested that ouabain enhances evoked catecholamine secretions by facilitating Ca^{2+} entry through nicotinic receptor-linked Ca^{2+} channels and by increasing the intracellular Ca^{2+} pool linked to muscarinic receptors.

Introduction

It has been reported that cardiac glycosides increase both spontaneous and evoked catecholamine secretions from perfused adrenal glands (Banks, 1967; 1970; García *et al.*, 1981b; Wakade, 1981; Nakazato *et al.*, 1986) and isolated adrenal chromaffin cells (Aunis & García, 1981; Sorimachi *et al.*, 1981; Pocock, 1983a,b). As is the case for the adrenergic nerve terminals (Nakazato *et al.*, 1978; 1980; 1983), these ouabain actions have been mainly explained by an increase in Na^+-dependent Ca^{2+} influx resulting from the inhibition of the Na^+-K^+ pump (García *et al.*, 1980; 1981a; Sorimachi *et al.*, 1981). We also assumed that ouabain increases Ca^{2+} entry through the ACh receptor-linked Ca^{2+} channels and voltage-dependent Ca^{2+} channels by Na^+-Ca^{2+} exchange, of which the rate is accelerated by the reduction of Na^+ electrochemical gradient resulting from the inhibition of the Na^+-K^+ pump, and causes the enhancement in the evoked catecholamine secretions (Nakazato *et al.*, 1986).

We have argued that the mechanism underlying nicotinic and muscarinic receptor-mediated catecholamine secretions is different, as the former is exclusively mediated by voltage-dependent entry of extracellular Ca^{2+} but the latter may be caused by Ca^{2+} mobilized from intracellular storage sites (Nakazato *et al.*, 1984; 1988, Yamada *et al.*, 1988). The positive inotropic action of cardiotonic steroids is interpreted as an increased amount of Ca^{2+} resulting from Na^+-Ca^{2+} exchange after inhibition of the Na^+-K^+ pump by cardiac glycoside. This Ca^{2+} is stored in the sarcoplasmic reticulum, from which more than normal amounts of Ca^{2+} are released when the cell is activated (Blaustein, 1985). It therefore seems possible that ouabain increases intracellularly stored Ca^{2+} and results in the enhancement of catecholamine secretion in response to the activation of muscarinic receptors. If this is

[1] Author for correspondence.

Table 1 Effects of atropine and hexamethonium on acetylcholine (ACh)-induced catecholamine secretion (nmol $5\,min^{-1}$) from perfused cat adrenal glands

	Control			*Atropine*			*Hexamethonium*	
	n	*Ad*	*NA*	n	*Ad*	*NA*	n	*Ad*	*NA*
Resting release	36	1.7 ± 0.2	1.1 ± 0.1 (39.3 ± 0.6%)	4	0.5 ± 0.1**	0.6 ± 0.3 (44.9 ± 9.0%)	6	1.5 ± 0.3	0.5 ± 0.2* (27.4 ± 4.7%)
ACh									
10^{-6} M	5	2.0 ± 0.1	2.2 ± 0.6 (55.9 ± 7.9%)	4	0.3 ± 0.1***	0.4 ± 0.2* (59.8 ± 4.5%)	6	2.7 ± 0.7	1.9 ± 0.7 (42.3 ± 5.1%)
10^{-5} M	17	15.7 ± 1.9	37.6 ± 4.9 (69.5 ± 2.9%)	5	2.6 ± 0.5***	7.0 ± 1.1*** (73.7 ± 2.0%)	6	9.4 ± 2.1*	7.2 ± 1.7*** (43.1 ± 6.4%)
10^{-4} M	4	64.6 ± 11.9	159.5 ± 4.6 (70.0 ± 3.8%)	5	68.1 ± 3.9	113.6 ± 15.5* (61.9 ± 3.2%)	6	15.0 ± 2.9*	21.9 ± 5.0*** (51.5 ± 6.2%)

In this and the following tables, the numbers indicate the mean (±s.e.) of adrenaline (Ad) and noradrenaline (NA) released and the percentage of noradrenaline is given in parentheses under the various conditions indicated. The values of resting output were subtracted from each evoked response. The numbers of cats (*n*) are indicated. Concentrations of atropine and hexamethonium used were 10^{-5} M and 5×10^{-4} M, respectively. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$ when compared with control values.

the case, ouabain is an interesting experimental tool for studying the differences between the mechanisms of nicotinic and muscarinic receptor-mediated catecholamine secretions.

The purpose of the present experiments is to investigate the effect of ouabain on catecholamine secretion evoked by nicotinic and muscarinic agonists in the presence and absence of extracellular Ca^{2+} in isolated and perfused cat adrenal glands.

Methods

Preparations

Cats of either sex, weighing 1.5 to 3.5 kg, were anaesthetized with sodium pentobarbitone ($40\,mg\,kg^{-1}$) intraperitoneally. Both adrenal glands were perfused and isolated following the general procedure described previously (Douglas & Rubin, 1961; Ito *et al.*, 1979). The glands were perfused at a flow rate of 0.6 to $0.8\,ml\,min^{-1}$ and maintained at room temperature (approximately 25°C). The adrenal effluent was collected continuously in 5 min aliquots into glass tubes kept on ice.

The standard perfusion medium was modified Locke solution of the following composition (mM): NaCl 154, KCl 5.6, $CaCl_2$ 2.2, $MgCl_2$ 1.2, Na_2HPO_4-NaH_2PO_4 buffer (pH 7.0) 3 and glucose 10. In the Ca^{2+}-free solutions, $CaCl_2$ was omitted and glycoletherdiaminetetraacetic acid (EGTA) (10^{-5} M) was added. All solutions contained physostigmine (2×10^{-7} M) to prevent hydrolysis of ACh and were bubbled with pure O_2.

Experiments were started 40 to 60 min after isolation of the adrenal glands. Secretagogues were administered for 1 min beginning 1 min before the following 5 min collection periods because of the 1 min dead time of the arterial cannula. Samples collected were acidified with 8 M perchloric acid to a final concentration of 0.4 M and stored on ice until assayed.

Catecholamine assays

Adrenaline, noradrenaline and dopamine were separated by high performance liquid chromatography (h.p.l.c., Jasco) and detected by an electrochemical detector (LC-4B, BAS). The treatment of samples for h.p.l.c. was carried out according to the method described by Salzman & Sellers (1982). Total catecholamines were also assayed by the fluorometric method of Anton & Sayre (1962).

Materials

The following drugs were used: acetylcholine chloride (Ovisort; Daiichi), atropine sulphate (K & K), glycoletherdiaminetetraacetic acid (Wako Pure Chem.), hexamethonium chloride dihydrate (Wako Pure Chem.), nicotine bitartrate (Tokyo Kasei), nifedipine (Bayer), g-strophanthin (Ouabain; Tokyo Kasei), pilocarpine hydrochloride (Tokyo Kasei), physostigmine sulphate (Wako Pure Chem.). All compounds were dissolved in Locke solution, except nifedipine, which was first dissolved in dimethylsulphoxide and then diluted with Locke solution.

Statistics

The data are presented as arithmetic means ±s.e.mean. Significance tests were performed by

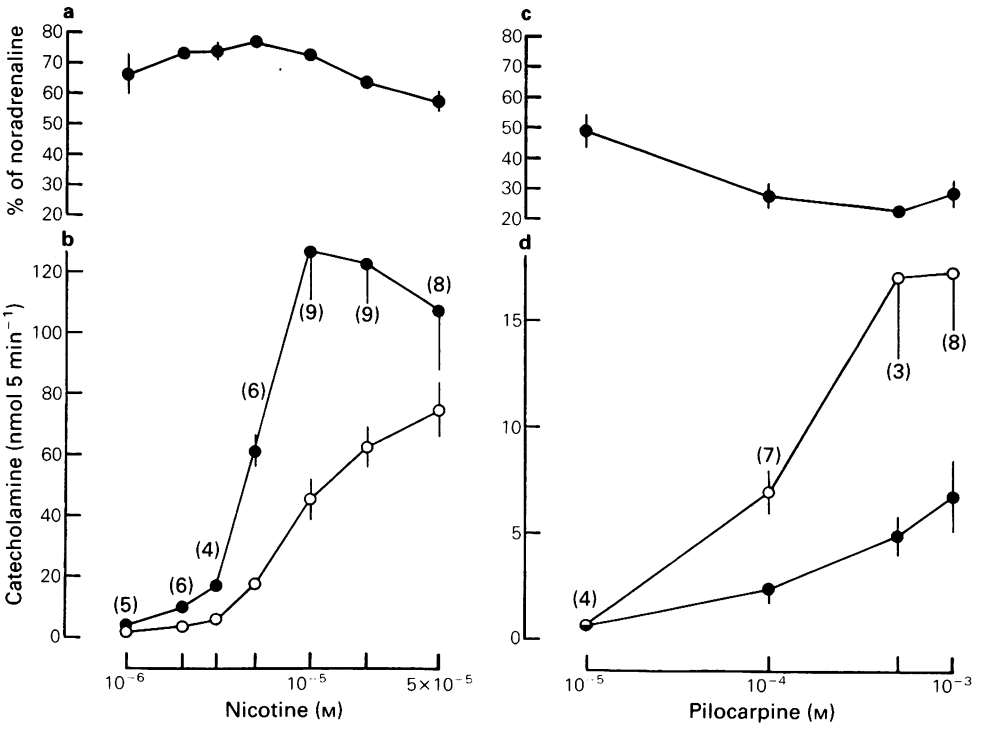

Figure 1 Dose-response curves for catecholamine secretion induced by nicotine (b) and pilocarpine (d) and the percentage of noradrenaline in each evoked response (a,c). Two or three different concentrations of each agonist were infused sequentially for 1 min into single preparations of adrenal glands during perfusion with Locke solution. Means, with s.e.mean indicated by vertical lines (if it exceeds the size of the symbols), of the percentage of noradrenaline (●) in (a) and (c), of the absolute amounts of adrenaline (○) and noradrenaline (●) released in (b) and (d) were plotted against the concentrations of agonists. Values of the resting output were subtracted from the evoked responses. The numbers in parentheses represent the number of experiments. The ordinate scale is the amount of catecholamines released and the abscissa scale the concentration of agonists on a logarithmic scale.

Student's t test. Statistical significance was assumed when $P < 0.05$.

Results

Catecholamine secretions induced by acetylcholine, nicotine and pilocarpine

The resting catecholamine secretion from perfused cat adrenal glands attained a steady level 40 to 60 min after the start of perfusion with Locke solution. The resting secretion consisted of adrenaline and noradrenaline, of which the ratio was approximately 6:4. No dopamine was detectable. When ACh (5×10^{-7} M–10^{-3} M) was infused for 1 min into the adrenal glands, catecholamine secretion was increased in a dose-dependent manner. The ED_{50} values for ACh to release adrenaline and noradrenaline were 2.1×10^{-5} M and 2.6×10^{-5} M, respectively. The dose-response relationships for ACh are partly shown in Table 1. A nicotinic agonist, nicotine (10^{-6} M–5×10^{-5} M), and a muscarinic agonist, pilocarpine (10^{-5} M–10^{-3} M), also caused dose-dependent increases in adrenaline and noradrenaline secretions (Figure 1). The ED_{50} values required for nicotine to release adrenaline and noradrenaline were 8.7×10^{-6} M and 6.7×10^{-6} M, and for pilocarpine they were 9.2×10^{-5} M and 1.5×10^{-4} M, respectively. Dopamine release was also increased by these agonists in parallel with the other two catecholamines, but its percentage of the total catecholamine released was only 0.4 to 2.0%, regardless of either the concentration or the kind of agonist applied.

ACh and nicotine released more noradrenaline than adrenaline and the reverse was the case for the response to pilocarpine. The percentage of noradrenaline released varied a little between different concentrations of each agonist. In the responses to ACh, the percentage of noradrenaline was

Table 2 Effects of ouabain on catecholamine secretions (nmol $5\,min^{-1}$) induced by acetylcholine (ACh), pilocarpine and nicotine from perfused cat adrenal glands

	n	S_1 Ad	S_1 NA	S_2 Ad	S_2 NA	Ratios of S_2/S_1 Ad	Ratios of S_2/S_1 NA
Resting release	7	1.4 ± 0.5	1.2 ± 0.4 (46.7 ± 4.8%)	1.9 ± 0.3	2.0 ± 0.4 (49.8 ± 2.9%)	1.4	1.7
ACh (10^{-5} M)	3	17.2 ± 0.3	30.4 ± 6.1 (64.7 ± 3.4%)	33.6 ± 4.2	54.4 ± 11.7 (60.6 ± 7.0%)	1.9	1.8
Pilocarpine (10^{-3} M)	4	8.1 ± 0.8	5.0 ± 0.7 (38.0 ± 3.3%)	13.8 ± 1.2**	8.9 ± 1.8 (38.1 ± 3.0%)	1.7	1.8
Nicotine (3×10^{-6} M)	4	6.1 ± 1.2	17.0 ± 1.6 (74.1 ± 2.7%)	13.8 ± 2.4*	30.9 ± 4.3* (69.2 ± 2.5%)	2.3	1.8

Secretagogues were applied for 1 min before (S_1) and during (S_2) exposure to ouabain (10^{-5} M). The numbers of cats (n) are indicated. * $P < 0.05$ and ** $P < 0.01$ when compared with S_1 values.

41.3 ± 6.2% at 5×10^{-7} M and 55.9 ± 7.9% at 10^{-6} M. Then it increased to 69.9 ± 5.6% at 2×10^{-6} M and was maintained at a nearly constant level up to 10^{-3} M, the maximum concentration of ACh tested. The value of the noradrenaline percent is partly given in Table 1. In the response to nicotine, the percentage of noradrenaline was 66.5 ± 6.7% at 10^{-6} M. It was increased with increasing concentrations of nicotine until it attained a maximum 77.5 ± 1.7% at 5×10^{-6} M and then declined, attaining a minimum 58.3 ± 3.0% at 5×10^{-5} M. On the other hand, the ratio of noradrenaline to adrenaline in the response to pilocarpine was decreased in a monophasic way as the concentration of pilocarpine was increased. These changes in the percentage of noradrenaline in the responses to nicotine and pilocarpine are plotted in Figures 1a and 1c, respectively.

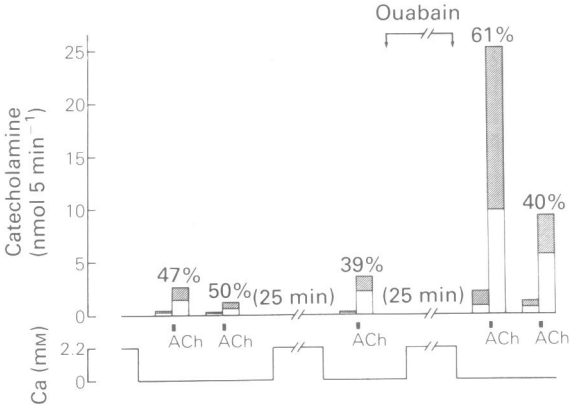

Figure 2 Catecholamine secretion induced by acetylcholine (ACh) during perfusion with Ca^{2+}-free solution before and after readmission of Ca^{2+} with and without ouabain. ACh (10^{-4} M) was applied for 1 min during perfusion with Ca^{2+}-free Locke solution, 10 min after readmission of Ca^{2+} (2.2 mM) for 25 min and 10 min after infusion of ouabain (10^{-5} M) with Ca^{2+} (2.2 mM) for 25 min. In this and the following two figures, the columns represent adrenaline (open) and noradrenaline (hatched) secretion, respectively and the percentage of noradrenaline is given by the numbers above columns.

Effects of cholinoceptor blocking agents on evoked catecholamine secretions

Atropine (10^{-5} M) significantly inhibited the responses to 10^{-6} M and 10^{-5} M ACh by more than 80% of the control regardless of adrenaline or noradrenaline secretion. The response at 10^{-4} M ACh was relatively resistant to atropine, which failed to inhibit adrenaline secretion and reduced noradrenaline secretion by only 30% of the control. In the presence of atropine, noradrenaline was still preferentially released by ACh, though the percentage of noradrenaline was increased by about 4% in the responses to 10^{-6} M and 10^{-5} M ACh and was reduced by about 10% in the response to 10^{-4} M ACh.

Hexamethonium (5×10^{-4} M) did not affect the response to 10^{-6} M ACh, but inhibited adrenaline and noradrenaline secretions evoked by 10^{-5} M ACh by about 40% and 80%, and those induced by 10^{-4} M ACh by 77% and 86%, respectively. The preferential ratios of noradrenaline to adrenaline in the responses to ACh were significantly reduced or actually reversed by hexamethonium. These data are summarized in Table 1. Atropine and hexamethonium in the concentrations mentioned above completely blocked the secretory responses to pilocarpine (10^{-3} M) and nicotine (10^{-5} M), respectively.

Effects of ouabain on evoked catecholamine secretions

The effect of ouabain was studied on the catecholamine secretions induced by ACh, pilocarpine and

Table 3 Acetylcholine (ACh)-induced catecholamine secretion (nmol 5 min^{-1}) from perfused cat adrenal glands in the absence of extracellular Ca^{2+} before and after treatment with ouabain

	n	S_1		S_2		Ratios of S_2/S_1	
		Ad	*NA*	*Ad*	*NA*	*Ad*	*NA*
Resting release	10	0.9 ± 0.2	1.4 ± 0.3 (56.5 ± 4.5%)	1.3 ± 0.3	2.0 ± 0.2 (63.0 ± 3.8%)	1.4	1.4
ACh							
10^{-5} M	4	3.0 ± 1.1	2.5 ± 0.6 (49.7 ± 2.6%)	4.6 ± 1.6	9.2 ± 2.4 (72.0 ± 4.8%)	1.6	3.7
10^{-4} M	3	2.4 ± 0.7	5.4 ± 1.1 (69.4 ± 1.6%)	6.0 ± 0.7*	23.8 ± 5.4* (79.1 ± 2.0%)	2.5	4.4
10^{-3} M	3	2.9 ± 1.2	7.7 ± 1.9 (74.7 ± 3.3%)	7.4 ± 2.1	23.0 ± 3.5* (76.4 ± 3.5%)	2.6	3.0

ACh was applied for 1 min before (S_1) and after (S_2) exposure to ouabain (10^{-5} M) with Ca^{2+} (2.2 mM) for 25 min. The numbers of cats (*n*) are indicated. * $P < 0.05$ when compared with S_1 values.

nicotine during perfusion with Locke solution. ACh (10^{-5} M) was applied repeatedly and pilocarpine (10^{-3} M) and nicotine (3×10^{-6} M) were also infused sequentially for 1 min at 15-min intervals before and 15 min after exposure to ouabain (10^{-5} M) for 25 min. As indicated in Table 2, all evoked catecholamine secretions were enhanced by ouabain by about 2 fold, regardless of adrenaline or noradrenaline secretion. Thus, ouabain did not change the proportion of the two catecholamines released by the three agonists. Ouabain also increased the resting release to an extent equal to or less than the evoked catecholamine secretions (Table 2).

Role of extracellular Ca^{2+} in ouabain action

The results of experiments on the dependency of ouabain action on extracellular Ca^{2+} are described in this and the following sections. ACh (10^{-5} M) was infused repeatedly and nicotine (3×10^{-6} M) and pilocarpine (10^{-3} M) were applied sequentially for 1 min at 15 min-intervals in the absence of extracellular Ca^{2+}. As reported previously (Nakazato *et al.*, 1984; 1988), ACh and pilocarpine, but not nicotine, caused small increases in catecholamine secretion in the absence of Ca^{2+} (plus EGTA 10^{-5} M) (Figures 2 and 3). The magnitude of residual responses in the absence of Ca^{2+} varied from 5 to 25% of the control response in the presence of Ca^{2+} in both ACh- and pilocarpine-induced catecholamine secretion.

As in the previous results (Nakazato *et al.*, 1984), the residual responses declined with repetition of stimulation, but were restored after readmission of Ca^{2+} for 25 min (Figure 2). After exposure to ouabain (10^{-5} M) plus Ca^{2+} (2.2 mM) for 25 min, the responses to ACh (10^{-5} M) and pilocarpine (10^{-3} M) were increased significantly, but nicotine still remained ineffective (Figures 2 and 3). The same results were obtained with differing concentrations of ACh as summarized in Table 3. The resting release was also enhanced after exposure to ouabain with Ca^{2+}, though the enhancement was less than in the case of evoked responses (Table 3). These data indicate that treatment with ouabain plus Ca^{2+}

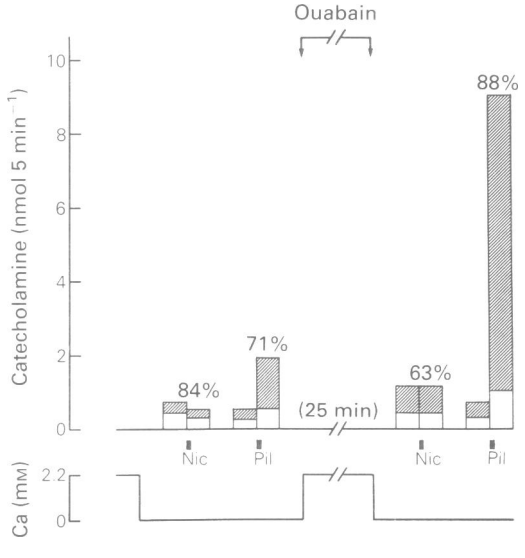

Figure 3 Catecholamine secretion induced by nicotine and pilocarpine during perfusion with Ca^{2+}-free solution before and after exposure to ouabain with Ca^{2+}. Nicotine (Nic, 3×10^{-6} M) and pilocarpine (Pil, 10^{-3} M) were applied sequentially for 1 min at 15 min intervals during perfusion with Ca^{2+}-free Locke solution and 10 min after infusion of ouabain (10^{-5} M) with Ca^{2+} (2.2 mM) for 25 min.

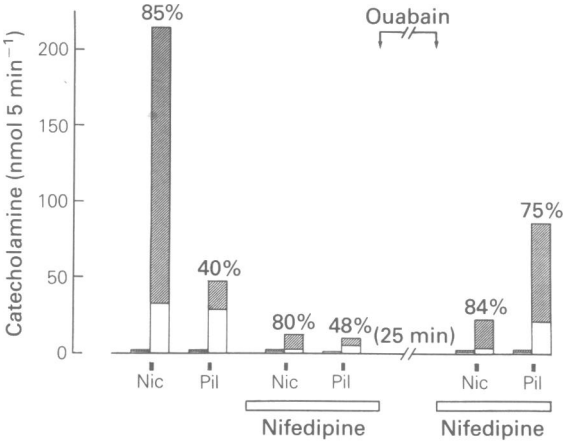

Figure 4 Effect of Ca^{2+} antagonist on catecholamine secretion induced by nicotine and pilocarpine in the presence of Ca^{2+} before and after exposure to ouabain. Nicotine (Nic, 10^{-5} M) and pilocarpine (Pil, 10^{-3} M) were applied sequentially for 1 min at 15 min intervals during perfusion with Locke solution and during infusion of nifedipine (2×10^{-6} M) before and after treatment with ouabain (10^{-5} M) for 25 min.

enhanced noradrenaline secretion more than adrenaline secretion in either the responses to ACh or pilocarpine. Atropine (10^{-6} M) blocked ACh- and pilocarpine-induced responses enhanced by treatment with ouabain, but hexamethonium (5×10^{-4} M) did not.

Next, we determined whether ouabain was effective in enhancing the evoked catecholamine secretion when applied after the removal of extracellular Ca^{2+}. During perfusion with Ca^{2+}-free Locke solution, ACh (10^{-5} M) or pilocarpine (10^{-3} M) was applied for 1 min and then Ca^{2+} (2.2 mM) was reintroduced for 25 min. Five min after the end of readmission of Ca^{2+}, ouabain (10^{-5} M) was added, and another 10 min after the start of ouabain infusion, the adrenal glands were again stimulated with ACh or pilocarpine. There was no enhancement of evoked adrenaline secretions, while noradrenaline secretions were about 1.4 and 2.2 fold of those evoked by ACh and pilocarpine, respectively, before exposure to ouabain (data not shown).

Effects of Ca^{2+} antagonist on evoked catecholamine secretion before and after exposure to ouabain

Nicotine (10^{-5} M) and pilocarpine (10^{-3} M) were infused sequentially for 1 min at 15-min intervals before and during exposure to a dihydropyridine Ca^{2+} antagonist, nifedipine (2×10^{-6} M). Then nifedipine was replaced by ouabain (10^{-5} M) for 25 min, after which this was again replaced by nifedipine, and the glands were stimulated with nicotine and pilocarpine. As shown in Figure 4, nifedipine inhibited the responses to nicotine and pilocarpine before exposure to ouabain. After treatment with ouabain, pilocarpine caused a secretory response almost the same as or greater than that of the control obtained in the absence of the Ca^{2+} antagonist, but nicotine failed to restore the catecholamine secretion to the degree that pilocarpine did. The resting adrenaline and noradrenaline secretions were slightly decreased by nifedipine, the effect of which was practically unaffected by the treatment with ouabain (Table 4). The experiment illustrated in Figure 4 was performed with different concentrations of agonists and the results are plotted in Figure 5a,b for nicotine and 5c,d for pilocarpine. Treatment with ouabain not only reversed the inhibitory effect of nifedipine on adrenaline secretion but also enhanced noradrenaline secretion induced by pilocarpine. Thus, the percentage of noradrenaline was increased significantly in the response to pilocarpine after exposure to ouabain, while that in the response to nicotine remained unchanged. The values for percentage of noradrenaline were calculated from the data indicated in Figure 5 and replotted in Figure 6a,b.

Table 4 Effects of nifedipine on the resting catecholamine release (nmol 5 min^{-1}) before and after treatment with ouabain

Control		*Nifedipine*		*Nifedipine after ouabain*	
Ad	*NA*	*Ad*	*NA*	*Ad*	*NA*
1.3 ± 0.2	0.9 ± 0.2 (40.8 ± 3.1%)	1.1 ± 0.2	0.5 ± 0.1* (35.2 ± 2.7%)	1.1 ± 0.2	0.7 ± 0.1 (40.3 ± 3.3%)

The numbers indicate the mean (±s.e.) of adrenaline (Ad) and noradrenaline (NA) obtained from 12 cats in the presence of nifedipine (2×10^{-6} M) before and after exposure to ouabain (10^{-5} M) for 25 min. * $P < 0.05$ when compared with control values.

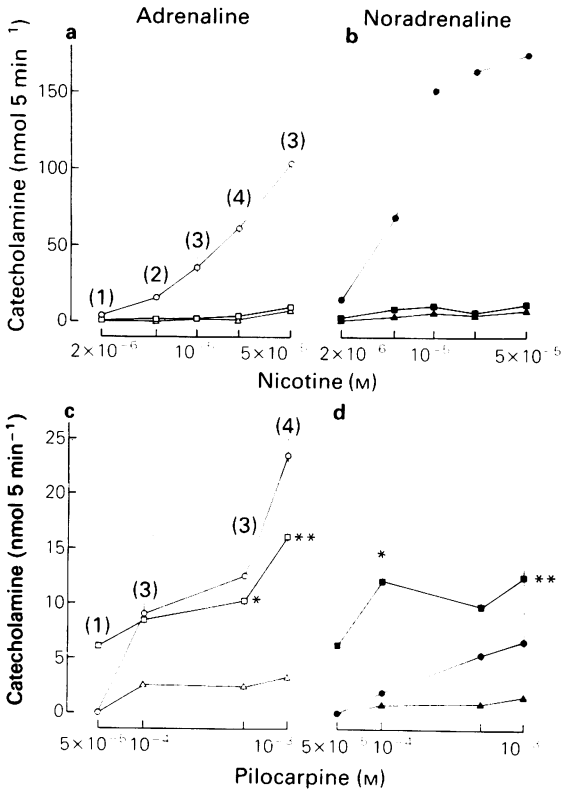

Figure 5 Effect of a Ca^{2+} antagonist (nifedipine) on catecholamine secretion induced by various concentrations of nicotine (a,b) and pilocarpine (c,d) before and after treatment with ouabain. Protocol of the experiment is shown in Figure 4. Means, with s.e.mean indicated by vertical lines if it exceeds the size of the symbols, of adrenaline (a,c) and noradrenaline (b,d) released were plotted against the concentration of agonists. Values of resting output were subtracted from the evoked responses. Symbols indicate the control response (○,●) and the response in the presence of nifedipine (2×10^{-6} M) before (△,▲) and after (□,■) treatment with ouabain (10^{-5} M) for 25 min. The numbers in parentheses represent the number of experiments. * $P < 0.05$, ** $P < 0.005$ when compared with the values obtained before treatment with ouabain (10^{-5} M).

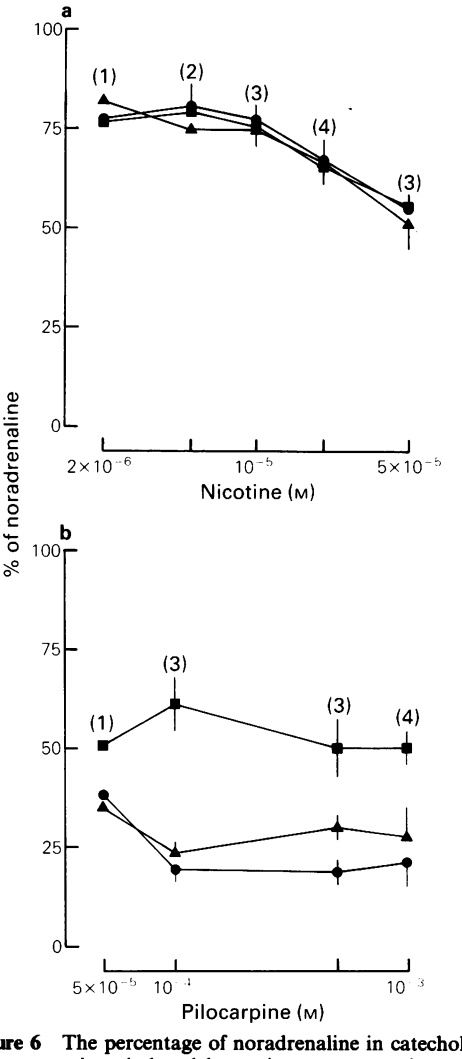

Figure 6 The percentage of noradrenaline in catecholamine secretions induced by various concentrations of nicotine (a) and pilocarpine (b) illustrated in Figure 5. Symbols indicate the percentages of noradrenaline in the control response (●) and in the response in the presence of nifedipine (2×10^{-6} M) before (▲) and after (■) treatment with ouabain (10^{-5} M) for 25 min. The numbers in parentheses represent the number of experiments.

Discussion

The following results were obtained: (1) ACh in lower concentrations increased catecholamine secretion from perfused cat adrenal glands mainly through the activation of muscarinic receptors, and the contribution of nicotinic receptors to ACh-induced response increased with increasing concentrations of ACh, as was reported for guinea-pig adrenal glands (Nakazato *et al.*, 1988). (2) Ouabain increased catecholamine secretions evoked by both nicotinic and muscarinic receptor activation through different mechanisms. (3) Pretreatment with ouabain plus Ca^{2+} enhanced noradrenaline secretion more than adrenaline secretion in response to muscarinic, but not nicotinic, receptor activation in either the

absence of extracellular Ca^{2+} or the presence of the Ca^{2+} antagonist, nifedipine.

Ouabain has been reported to enhance adrenal catecholamine secretion induced by various secretagogues such as Ca^{2+} reintroduced after exposure to Ca^{2+}-free environment (Esquerro *et al.*, 1980; García *et al.*, 1980; 1981a), veratridine (Ito *et al.*, 1979; Wada *et al.*, 1985), nerve stimulation and ACh (Wakade, 1981; Nakazato *et al.*, 1986) and high K^+ (García *et al.*, 1981b; Nakazato *et al.*, 1986). It is known that the activation of nicotinic receptors stimulates catecholamine secretion by increasing Ca^{2+} entry through receptor-linked and/or voltage-dependent Ca^{2+} channels in both perfused rat adrenal glands (Wakade & Wakade, 1983) and bovine isolated adrenal chromaffin cells (Kilpatrick *et al.*, 1981; 1982; Knight & Kesteven, 1983). Thus, we previously suggested that ouabain enhances catecholamine secretion evoked by ACh and high K^+ by increasing the rate of Ca^{2+} influx through the ACh receptor-linked Ca^{2+} channel and/or voltage-dependent Ca^{2+} channels on adrenal chromaffin cells as a result of the inhibition of the Na^+-K^+ pump (Nakazato *et al.*, 1986).

These experiments showed that ouabain was effective in enhancing catecholamine secretion induced by nicotinic receptor activation only in the presence of extracellular Ca^{2+}. One of the most plausible explanations for this ouabain action is the increase in Ca^{2+} entry through either receptor-linked or voltage-dependent Ca^{2+} channels during stimulation with nicotine as suggested previously (Nakazato *et al.*, 1986). However, both resting and muscarinic receptor-mediated catecholamine secretions were increased in either the presence or the absence of extracellular Ca^{2+} if the glands were exposed to ouabain prior to the removal of Ca^{2+}. Furthermore, pretreatment with ouabain enhanced muscarinic receptor-mediated response in the presence of the Ca^{2+} antagonist, nifedipine, leaving the resting secretion unchanged. In addition, even when applied after the removal of Ca^{2+}, ouabain enhanced the muscarinic response, though the enhancement was less than when applied with Ca^{2+}. These results agree with the view that the increase in the resting catecholamine secretion by ouabain may be mediated by the increase in resting Ca^{2+} influx (García *et al.*, 1980; 1981a; Sorimachi *et al.*, 1981) and/or by inhibition of Ca^{2+} efflux (Pocock, 1983b). It has been reported that muscarinic receptor activation causes an increase in adrenal catecholamine secretion independent of extracellular Ca^{2+} in various species (Nakazato *et al.*, 1984; 1988; Wakade *et al.*, 1986; Harish *et al.*, 1987) and in cytosolic free Ca^{2+} in bovine isolated adrenal chromaffin cells without associated catecholamine secretion (Cheek & Burgoyne, 1985; Kao & Schneider, 1985; 1986; Misbahuddin *et al.*, 1985). Furthermore, we recently found that catecholamine secretions induced by ACh as well as caffeine in the absence of extracellular Ca^{2+} were reversibly blocked by the intracellular Ca^{2+} antagonist, TMB-8 (Yamada *et al.*, 1988). It appears, therefore, that ouabain enhances the response to muscarinic stimulation by increasing Ca^{2+} entry, which in turn increases the capacity of the intracellular Ca^{2+} pool linked to muscarinic receptors.

As reported by Douglas & Poisner (1965), nicotine released more noradrenaline than adrenaline, while pilocarpine released more adrenaline than noradrenaline during perfusion with Locke solution. The proportions of these two catecholamines released were not significantly changed over a wide range of concentrations of each agonist. The ratio of noradrenaline to adrenaline released by ACh was basically of the nicotinic type and was not affected by atropine, but was altered to the muscarinic type by hexamethonium. Such dual control of adrenaline and noradrenaline could be peculiar to cats (Ungar & Phillips, 1983). However, Marley & Livett (1987) recently found that in cultured bovine adrenal medullary chromaffin cells, stimulation with nicotine sensitized adrenaline cells, but densenitized noradrenaline cells to subsequent high-K^+ stimulation. Therefore, as they concluded, the differential release of adrenaline and noradrenaline may be generated by different properties of the chromaffin cells themselves. This agrees with the view that adrenaline and noradrenaline cells of cat adrenal medulla possess certain distinguishable characteristics (Rubin & Miele, 1968).

In these experiments, we found that ouabain enhanced adrenaline and noradrenaline secretions evoked by three secretagogues equally (about 2 fold) in the presence of extracellular Ca^{2+}. However, the pretreatment with ouabain plus Ca^{2+} augmented noradrenaline secretion much more than adrenaline secretion in response to subsequent stimulation with pilocarpine in either the absence of extracellular Ca^{2+} or the presence of nifedipine, while nicotine was ineffective. The exact reason for this discrepancy of ouabain action between adrenaline and noradrenaline cells in the absence of Ca^{2+} is not clear at the present time, but the following are possible explanations: (1) Ouabain sensitizes noradrenaline cells much more than adrenaline cells to muscarinic agonists, releasing Ca^{2+} from intracellular pools. (2) Ouabain increases the capacity of the intracellular Ca^{2+} pool much more in noradrenaline cells than in adrenaline cells. (3) The density of membrane Na^+-K^+ ATPase is higher in noradrenaline cells than in adrenaline cells. In any case, ouabain seems to be an interesting agent for the study of the difference in receptor mechanisms and the Ca^{2+} require-

ment for catecholamine secretion in adrenaline and noradrenaline cells.

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The mechanism of action and pharmacological specificity of the anticonvulsant NMDA antagonist MK-801: a voltage clamp study on neuronal cells in culture

Robert F. Halliwell, John A. Peters & [1]Jeremy J. Lambert

Neuroscience Research Group, Department of Pharmacology and Clinical Pharmacology, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY

1 Some possible molecular mechanisms of action of the anxiolytic, anticonvulsant and neuroprotective agent MK-801 have been examined in 'whole-cell' voltage clamp recordings performed on rat hippocampal and cortical neurones, bovine adrenomedullary chromaffin cells and N1E-115 neuroblastoma cells maintained in cell culture.

2 Transmembrane currents recorded from rat hippocampal and cortical neurones in response to locally applied N-methyl-D-aspartate (NMDA) were antagonized by MK-801 (0.1–3.0 μM). Blockade was use-dependent, and little influenced by transmembrane potential. MK-801 (3 μM) had no effect on currents evoked by kainate (100 μM).

3 The antagonism of NMDA-induced currents by MK-801 was only slowly and incompletely reversed when the cell membrane potential was clamped at -60 mV during washout. Prolonged applications of NMDA at $+40$, but not -60 mV during washout, markedly accelerated recovery from block.

4 In contrast to MK-801, ketamine (10 μM) blocked NMDA-induced currents in a voltage-dependent manner. Blockade increased with membrane hyperpolarization and was completely reversible upon washout.

5 MK-801 (1–10 μM) produced a voltage- and concentration-dependent block of membrane currents elicited by ionophoretically applied acetylcholine (ACh) recorded from bovine chromaffin cells. The block was readily reversible upon washout.

6 γ-Aminobutyric acid$_A$ ($GABA_A$) receptor-mediated chloride currents of chromaffin cells were unaffected by MK-801 (1–100 μM). In contrast, such currents were potentiated by diazepam (1 μM). MK-801 (100 μM) had no effect on currents evoked by GABA on hippocampal neurones.

7 MK-801 (10 μM) had little effect on membrane currents recorded from N1E-115 neuroblastoma cells in response to ionophoretically applied 5-hydroxytryptamine (5-HT). Such currents were antagonized by the 5-HT_3 receptor antagonist GR 38032F (1 nM) and also by MK-801 at high concentration (100 μM).

8 Voltage-activated, tetrodotoxin-sensitive, sodium currents of chromaffin cells were unaffected by 10 μM MK-801. However, at a relatively high concentration (100 μM), MK-801 reduced the amplitude of such currents to approximately 77% of control.

9 The relevance of the present results to the central actions of MK-801 is discussed.

Introduction

The excitatory actions of the amino acids L-glutamate and L-aspartate on the central nervous system are mediated through up to four distinct receptor subtypes (Cotman & Iversen, 1987; Watkins & Olverman, 1987). Of these, the receptor selectively activated by N-methyl-D-aspartate (NMDA) has been particularly well characterized. Electrophysiological studies performed on central neurones in cell culture indicate that NMDA activates a cation selective ion channel which conducts

[1] Author for correspondence.

calcium in addition to sodium and potassium ions (Ascher & Nowak, 1987). Membrane currents evoked by NMDA are blocked in a voltage-dependent manner by magnesium ions (Nowak *et al.*, 1984, Mayer & Westbrook, 1985) and are potentiated by glycine, which appears to act through a strychnine-insensitive allosteric regulatory site on the NMDA receptor (Johnson & Ascher, 1987).

Antagonists of the NMDA-ion channel complex are potentially important therapeutic agents; the treatment of certain neurodegenerative disorders being one possible application (Meldrum, 1985). Whilst competitively-acting NMDA receptor antagonists such as D-2-amino-5-phosphonovalerate (APV) have proved useful as *in vitro* research tools, their therapeutic potential is limited since they do not readily cross the blood brain barrier. Recently, the lipophilic compound MK-801 ((+)-5-methyl-10, 11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate) has been shown to be a potent non-competitive antagonist of the NMDA receptor-channel complex (Wong *et al.*, 1986). This compound displays anticonvulsant, sympathomimetic and anxiolytic properties (Clineschmidt *et al.*, 1982) and in some animal models can protect against central neuronal loss induced by NMDA and ischaemic episodes (Gill *et al.*, 1987). Biochemical studies have demonstrated high-affinity stereoselective binding sites for radiolabelled MK-801 in rat brain (Wong *et al.*, 1986) with a topography similar to that described for NMDA receptors (Monaghan & Cotman, 1985; Bowery *et al.*, 1988). The binding of [^{3}H]-MK-801 to rodent brain membranes is enhanced by excitatory amino acids (Foster & Wong, 1987) and by glycine (Wong *et al.*, 1987). Selective non-competitive antagonists of the NMDA receptor channel complex, including the dissociative anaesthetics phencyclidine (PCP) and ketamine (Anis *et al.*, 1983; Harrison & Simmonds, 1985; Martin & Lodge, 1985; Honey *et al.*, 1985), displace [^{3}H]-MK-801 from its binding site with potencies which correlate well with their blocking actions in functional tests (Wong *et al.*, 1986). Such data are consistent with MK-801 binding to a site within the NMDA-gated ion channel rather than the agonist recognition site, a notion supported by the demonstration of agonist-dependent antagonism by MK-801 of NMDA-induced depolarizations in slices of rat cerebral cortex (Kemp *et al.*, 1987). Studies performed on voltage-clamped rodent central neurones have demonstrated that the antagonism of NMDA-induced currents by ketamine is use- and voltage-dependent, suggesting that ketamine also binds to a site within the NMDA-gated ion channel (Honey *et al.*, 1985; MacDonald *et al.*, 1987).

In the present study, the actions of MK-801 and ketamine on NMDA-induced currents recorded from voltage-clamped hippocampal and cortical neurones in cell culture were compared. As ketamine is known to block the nicotinic receptor ion channel (Maleque *et al.*, 1981; Volle *et al.*, 1982), the possibility that MK-801 shares this action was investigated in experiments on voltage-clamped bovine adrenomedullary chromaffin cells. Due to the postulated involvement of γ-aminobutyric acid$_A$ ($GABA_A$) receptors, 5-hydroxytryptamine$_3$ ($5\text{-}HT_3$) receptors and voltage-activated sodium channels in the mode of action of some anxiolytic and anticonvulsant drugs (Willow, 1986; File, 1987; Jones *et al.*, 1988) we additionally evaluated whether or not MK-801 interacts with these sites. A preliminary account of a part of this work has appeared in abstract form (Callachan *et al.*, 1988). Whilst this report was in preparation, a complementary study performed on rodent visual cortical neurones (Heuttner & Bean, 1988) came to our attention.

Methods

Dissociation and culture of hippocampal and cortical neurones

Embryonic rat hippocampal neurones were isolated and cultured essentially as described by Heuttner & Baughman (1986) with minor modifications. E18 embryos, dissected from Sprague-Dawley rats which had been killed by cervical dislocation, were decapitated and the whole brain quickly removed. The cerebral cortices or hippocampi were isolated in Hank's balanced salt solution (HBSS) at ambient temperature (17–21°C), chopped into fragments and incubated for 60 min at 37°C in an enzyme solution containing (in mM): NaCl 116, KCl 5.4, $NaHCO_3$ 26, NaH_2PO_4 1, $CaCl_2$ 1.5, $MgSO_4$ 1, EDTA 0.5, glucose 25, cysteine 1 and papain (20 units ml^{-1}) (pH 7.4). Subsequently, tissue fragments were rinsed in 5 ml of HBSS supplemented with 1 mg ml^{-1} bovine serum albumen (BSA) and 1 mg ml^{-1} ovomucoid. The tissue was transferred into a further 3–4 ml of this solution and dissociated into a cell suspension by gentle trituration with a fire-polished pasteur pipette. Dissociated cells were layered onto 5 ml of HBSS containing BSA (10 mg ml^{-1}) and ovomucoid (10 mg ml^{-1}) and centrifuged at 100 *g* for 10 min. The supernatant was discarded and the cells resuspended in a growth medium composed of Minimal Essential Medium supplemented with 5% (vol/vol) foetal calf serum (FCS), 5% horse serum, streptomycin (50 mg l^{-1}) and penicillin (5×10^4 iu l^{-1}). The final concentrations of glutamine and glucose in the medium were adjusted to 2 and 20 mM respectively. Approximately $3–5 \times 10^5$ cells were plated into

35 mm diameter (Falcon) 'Primaria' culture dishes and incubated in 1.5 ml of growth medium at 37°C in an atmosphere of 95% air, 5% CO_2 at 100% relative humidity. Cultures were fed at 3–4 day intervals by replacing approximately two thirds of the volume of growth medium. The proliferation of non-neuronal cells was suppressed as they approached confluency by the inclusion of cytosine arabinoside (10 μM) in the growth medium for a period of 48 h. In some instances, dissociated hippocampal cells were plated onto confluent monolayers of non-neuronal cells isolated from neonatal rat cerebral cortices by methods similar to those described above. Hippocampal and cortical neurones were used in experiments 10 to 30 days after plating.

Dissociation and culture of chromaffin cells

Bovine adrenomedullary chromaffin cells were isolated and cultured by the method of Fenwick *et al.* (1982) with minor modifications (Cottrell *et al.*, 1987) and used in electrophysiological experiments 1–7 days after plating.

Culture of N1E-115 neuroblastoma cells

N1E-115 cells were cultured according to Peters *et al.* (1988a) and used 2 to 7 days after plating.

Electrical recordings

Agonist- and voltage-activated currents were recorded by the 'whole-cell' clamp mode of the patch-clamp technique (Hamill *et al.*, 1981) with a List Electronics L/M EPC-7 converter headstage and amplifier. Currents were low pass filtered (Bessel characteristic) at the cut-off frequencies indicated in the Figure legends, and recorded onto either magnetic tape with an FM tape recorder (Racal Store 4DS), or onto video tape with a video recorder (Ferguson Videostar) in conjunction with a Sony PCM 701 digital pulse code modulator. In all experiments except those utlizing the agonist NMDA, cells were continuously superfused with a saline containing (in mM): NaCl 140, KCl 2.8, $MgCl_2$ 2, $CaCl_2$ 1 and HEPES-NaOH 10, (pH 7.2). NMDA-induced currents were recorded in a saline nominally free of Mg^{2+} and supplemented with glycine (1 μM) and tetrodotoxin (300 nM–1 μM) to maximize the NMDA-induced response (Nowak *et al.*, 1984; Johnson & Ascher, 1987) and suppress ongoing synaptic activity respectively. Voltage-activated sodium currents were recorded from cells bathed with a nominally Ca^{2+}-free saline containing Co^{2+} (1 mM) to eliminate contamination by voltage-activated Ca-currents. In all experiments K-currents were suppressed by dialysing the cell interior with a Cs-rich pipette solution comprising (in mM): CsCl 140, $MgCl_2$ 2, $CaCl_2$ 0.1, EGTA 1.1 and HEPES-NaOH 10 (pH 7.2). The pipette solution was supplemented with 2 mM Mg-ATP in experiments when GABA-evoked currents were recorded from hippocampal neurones. All recordings were made at room temperature (17°–21°C).

Drug application

Unless specified otherwise, antagonist compounds were introduced to the bath via the superfusion system. In the majority of experiments, agonists were applied locally to cells by pressure ejection (1.4×10^5 Pa) from modified patch pipettes. Alternatively, acetylcholine (ACh), 5-HT and NMDA were applied by ionophoresis from high-resistance (40–180 MΩ) micropipettes by use of a constant-current device based on the design of Dreyer & Peper (1974). Pipettes were back-filled with aqueous solutions containing either ACh bromide (1.0 M), 5-HT creatinine sulphate (20 mM; pH 3.5) or NMDA (20 mM; pH 8.0). ACh and 5-HT were ejected by positive rectangular pulses of current and their diffusional release opposed by retaining currents of −2 to −10 nA. NMDA was applied by negative ejection currents superimposed on a retaining current of 0.6 to 1.6 nA.

Data analysis

Agonist activated currents were analysed either manually from pen recorder traces, or by semi-automated programs (Dempster, 1988) run on a PDP 11-73 minicomputer essentially as described by Peters *et al.* (1988b). Briefly, up to 5 agonist-evoked currents were digitised into 512 or 1024 data points at an appropriate sampling rate, and subsequent to validation, averaged to yield a mean current. For illustration, such averaged currents were then plotted on a Hewlett Packard 7470A plotter. All quantitative data are expressed as the mean ± s.e.mean.

Reagents

All biological and synthetic media employed in cell culture were obtained from Gibco (Paisley, Scotland). Sera were heat-inactivated at 56°C for 30 min before use. Papain, ovomucoid and cysteine were supplied by Sigma. Drugs employed in electrophysiological experiments were obtained from the following sources: acetylcholine bromide, adenosine 5′-triphosphate (Mg salt), γ-aminobutyric acid, DL-2-amino-5-phosphonovaleric acid (APV), glycine,

kainic acid, 5-hydroxytryptamine creatinine sulphate, N-methyl-D-aspartic acid (Sigma); MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine maleate) (Merck, Sharp and Dohme); GR 38032F (1,2,3,9-tetrahydro-9-methyl-3-[(2-methyl-1H-imidazol-1-yl) methyl]-4H-carbazol-4-one hydrochloride dihydrate) (Glaxo); diazepam (Roche); ketamine (Parke Davis). With the exception of diazepam, which was prepared as a concentrate in ethanol, all drugs were freshly dissolved into either recording saline or twice-distilled deionized water. Ethanol, at the final concentration (0.01% vol/vol) used in experiments, had no effect upon the relevant agonist-induced currents.

Results

Inward currents evoked by local applications of NMDA and kainate were recorded under voltage-clamp in neurones from the hippocampus or cerebral cortex. All neurones sampled were sensitive to the agonists and displayed spontaneous synaptic currents if TTX was omitted from the perfusate. There were no obvious differences in characteristics of the agonist-evoked currents recorded from cells of cortical or hippocampal origin. Unless specifically stated otherwise, experiments were conducted at a holding potential of −60 mV.

Figure 1a–c illustrates the influence of the antagonists MK-801 (300 nM), ketamine (10 μM) and APV (10 μM) on inward currents evoked by ionophoretically applied NMDA. Although a quantitative comparison of these three blockers was not attempted, differences in their potency and reversibility were obvious. When applied against responses evoked by pulsatile applications of NMDA (40–80 nA, 1 s, 0.05 Hz), the antagonism produced by low concentrations of MK-801 (100–300 nM) often developed slowly, such that a 'steady-state' block was difficult to establish with certainty. This may, at least in part, have been due to the method of agonist application employed, since subsequent experiments indicated that the antagonist action of MK-801 is largely use-dependent (see below). In 4 cells superfused with solution containing 300 nM MK-801 for at least 15 min, the amplitude of the NMDA-evoked current was estimated to be reduced to 16.8 ± 3.5% of its control value. A more rapid, and essentially complete, blockade of ionophoretically induced responses (NMDA current being reduced to 1.3 ± 1.3% of control, $n = 3$) was observed when MK-801 was bath-applied at a concentration of 3 μM. The blockade produced by MK-801 was very poorly reversed upon washout. In the exemplar cell (Figure 1a) a modest degree of recovery was observed during a 20 min wash with MK-801-free medium, but in other cells no recovery was discernible within this time. Incomplete recovery from blockade by MK-801 has previously been reported (Wong *et al.*, 1986) and will be considered further below. In contrast to MK-801, ketamine (10 μM, Figure 1b) and APV (10 μM, Figure 1c) antagonized responses evoked by ionophoretically applied NMDA in a completely reversible manner. Blockade of NMDA-induced currents by MK-801 and ketamine was selective, as neither compound had any effect upon inward currents evoked by pressure-applied kainate (100 μM) when bath-applied at concentrations of 3 and 10 μM respectively (Figure 1d).

Use-dependent block of NMDA-induced currents by MK-801

It has previously been demonstrated that antagonism of NMDA-evoked currents in hippocampal neurones by ketamine (20 μM) is a use-dependent phenomenon (MacDonald *et al.*, 1987). Figure 2 illustrates the results of experiments designed to test whether or not MK-801 shares this property. On four separate cells, control inward currents to pressure-applied NMDA (100 μM; 0.05 Hz) were recorded at a holding potential of −60 mV. Once a stable response had been observed over several minutes, agonist application was discontinued and the cells superfused with recording medium containing MK-801 (3 μM). If activation of the NMDA-ion channel complex is a prerequisite for blockade by MK-801, it would be anticipated that no antagonism of the response would develop in the absence of agonist. This prediction was approximately borne out by the results of the representative experiment depicted in Figure 2a, where the first of a train of responses of NMDA, recorded following a 10 min pre-exposure to MK-801, was only slightly depressed relative to its control value. Subsequent responses recorded in the presence of MK-801 progressively declined, as would be expected if use-dependent block were occurring. Figure 2b shows the pooled data obtained from the four cells in which the protocol shown in Figure 2a was followed, together with the results of control experiments which rule out the possibility that the 10 min interval during which agonist application was suspended was insufficient to allow the build-up of an effective concentration of MK-801 in the bath. From inspection of the mean data, it is clear that the antagonist action of MK-801 is largely use-dependent. However, the first response in a pulse train initiated after 10 min of pre-exposure to MK-801 was consistently depressed relative to control (by 15.0 ± 5.5%, $n = 4$), and this

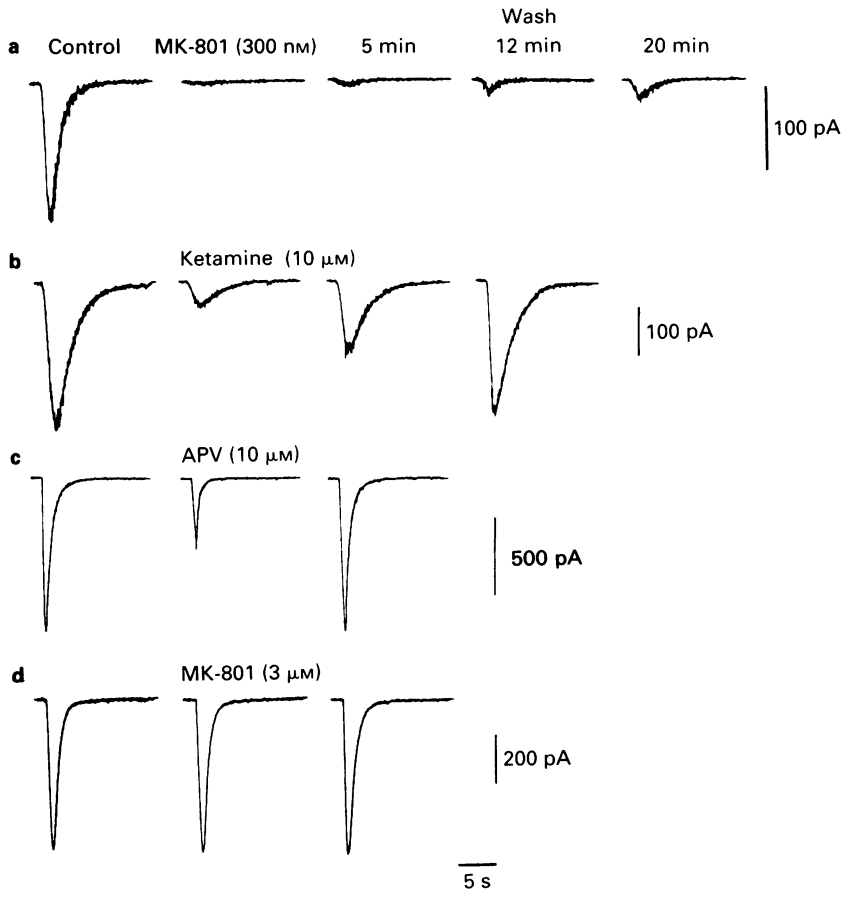

Figure 1 The influence of bath-applied MK-801, ketamine or DL-2-amino-5-phosphonovaleric acid (APV) on membrane currents evoked by N-methyl-D-aspartic acid (NMDA) or kainate. (a) MK-801 (300 nM) antagonizes inward currents elicited by ionophoretically applied NMDA (50 nA, 1 s, 0.05 Hz). Note that blockade was poorly reversed upon washout. (b) Traces illustrating the reversible blockade by ketamine (10 μM) of currents evoked by ionophoretically applied NMDA (40 nA, 1 s, 0.05 Hz). (c) Reversible suppression of NMDA-induced responses by APV (10 μM). NMDA was applied ionophoretically (60 nA, 500 ms, 0.05 Hz). (d) MK-801 (3 μM) has no effect upon inward currents evoked by pressure applied kainate (100 μM, 30 ms, 0.05 Hz). All currents were recorded from hippocampal neurones voltage-clamped at −60 mV. Each trace is the computer generated average of 4 agonist-evoked currents which were in all cases low-pass filtered at 500 Hz.

might suggest that a small component of the block occurs via a use-independent mechanism (see Discussion).

Use-dependent blockade of NMDA-evoked currents by MK-801 was also observed when the agonist was applied by ionophoresis. In contrast, APV (10 μM) antagonized currents induced by ionophoretically applied NMDA in a use-independent manner (data not illustrated), consistent with its proposed role as a competitive antagonist of the NMDA receptor (Harrison & Simmonds, 1985).

Comparison of the influence of membrane potential on blockade of NMDA-induced currents by MK-801 and ketamine

Figure 3 illustrates the results of various experiments in which the influence of membrane potential upon the antagonism of NMDA-evoked currents by MK-801 and ketamine was examined. In initial experiments, MK-801 (300 nM), or ketamine (10 μM), were bath-applied to hippocampal neurones voltage-clamped at a constant holding potential of either

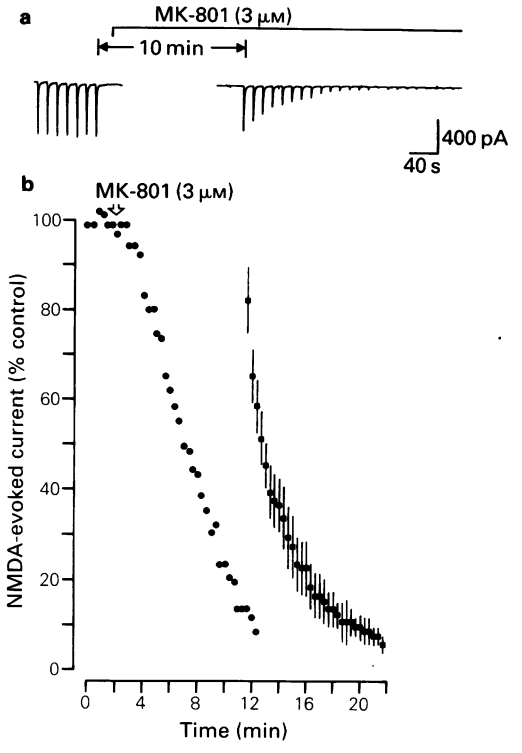

Figure 2 Blockade of N-methyl-D-aspartic acid (NMDA)-evoked currents by MK-801 is use-dependent. (a) Chart recorder trace showing inward currents evoked by pressure applied NMDA (100 μM, 20 ms, 0.05 Hz). The gap in the trace indicates that agonist application was suspended during the introduction of MK-801 (3 μM) to the bath. Following a 10 min equilibration period, agonist application was recommenced. The progressive decline of NMDA-evoked currents in the presence of MK-801 reflects use-dependent block. (b) Plot of NMDA-induced current amplitude, as a percentage of control, against time. The arrow indicates the time at which MK-801 (3 μM) was applied to the bath: (■) represents the mean data obtained from 4 cells in which the protocol described in (a) was followed; (●) show the mean data obtained from 2 cells in which agonist application was continued throughout the application of MK-801. The latter experiment was performed as a control to ensure that a 10 min equilibration period was sufficient to allow an effective concentration of MK-801 to develop in the bath. All experiments were performed at a holding potential of −60 mV and NMDA-evoked currents low-pass filtered at 500 Hz.

−60 or +40 mV. The amplitude of the current evoked by NMDA (100 μM), pressure applied (1.4×10^5 Pa) for 20–30 ms at 20 s intervals, was expressed relative to its control value following a 10 min application of the blocking drug (Table 1). It

Table 1 Influence of holding potential on block by MK-801 and ketamine

	Response amplitude (% control)	
Antagonist (μM)	−60 mV	+40 mV
Unpaired observations		
MK-801 (0.3)	52 ± 4.5 (6)	62 ± 5.0 (5)
Ketamine (10.0)	20 ± 2.5 (4)	89 ± 6.5 (4)
Paired observations		
MK-801 (0.3)	28 ± 4.3 (3)	28.5 ± 7.6 (3)
Ketamine (10.0)	17 ± 2.2 (3)	75 ± 5.5 (3)

should be stressed that whilst blockade by ketamine reached a steady-state within this time, the antagonism occurring to MK-801 did not (see discussion). The results presented in Table 1 suggest that antagonism by MK-801 is little influenced by membrane potential, whereas blockade by ketamine is highly voltage-dependent, the degree of suppression increasing with membrane hyperpolarization.

To examine further the effect of holding potential, experiments were performed in which the influence of MK-801 or ketamine upon the NMDA current-voltage (I–V) relationship was examined over the potential range −60 to +40 mV. In the absence of antagonists, the I–V relationship obtained with either ionophoretic or pressure applications of NMDA to hippocampal or cortical cells was usually linear, or demonstrated a small degree of outward rectification (Figure 3a). In a minority of cells, a complex I–V relationship was observed in which a marked inflection, due to a decrease in slope conductance, was apparent over the potential range −40 to 0 mV. Such 'voltage-dependent inactivation' of NMDA-evoked currents has been described previously by Mayer & Westbrook (1985) and may possibly be linked to an influx of Ca^{2+}. Cells displaying this feature were not studied further.

The results of a representative experiment examining the effect of MK-801 (100 nM) upon the NMDA I–V relationship are illustrated in Figure 3a2. The magnitude of the block produced by MK-801 appeared similar at all holding potentials examined. In contrast, antagonism by ketamine (10 μM) was pronounced only at negative holding potentials as previously observed (MacDonald *et al.*, 1987). Paired observations on the influence of MK-801 and ketamine on the NMDA-evoked response after prolonged application, at holding potentials of −60 and +40 mV, are given in Table 1. The reversal potential of the NMDA-induced current in the presence of either ketamine (10 μM − 8.0 ± 3.0 mV, $n = 3$) or MK-801 (100 nM, −6.0 mV; 300 nM, −4.0 mV) was similar to its control value (−6.0 ± 1.4 mV, $n = 7$).

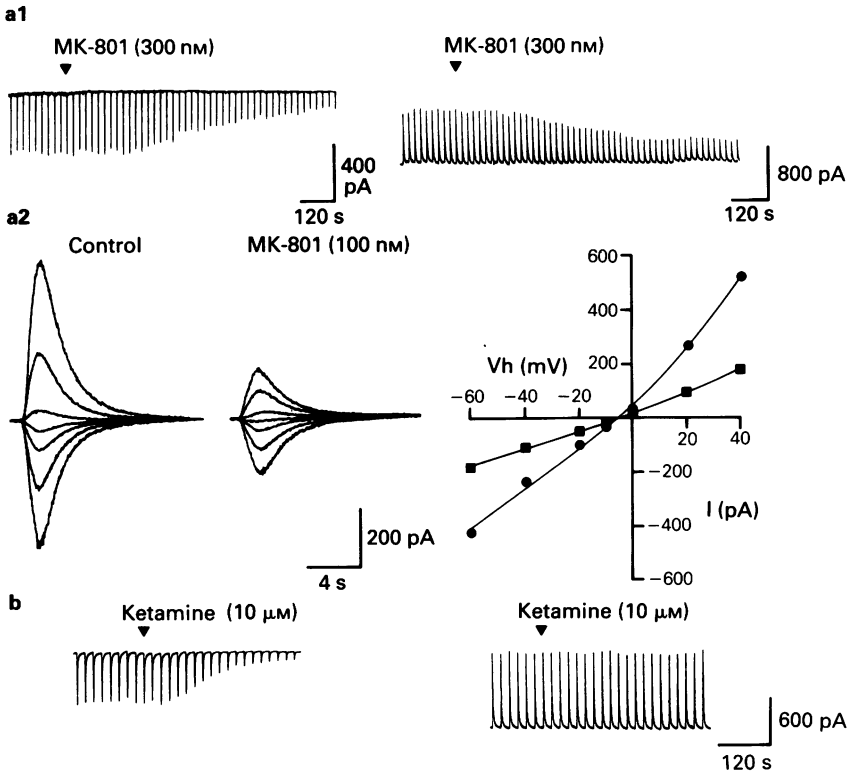

Figure 3 The influence of holding potential on blockade of N-methyl-D-aspartic acid (NMDA)-induced currents by MK-801 and ketamine. (a1) Chart recorder traces illustrating the antagonism of responses to pressure applied NMDA (100 μM, 20 ms, 0.05 Hz) by MK-801 (300 nM) at holding potentials of −60 mV (left) and +40 mV (right). Note that inward and outward currents are similarly depressed. The two recordings shown were performed on different hippocampal neurones. (a2) NMDA-induced currents recorded at holding potentials ranging between −60 and +40 mV from a hippocampal neurone in the absence and presence of MK-801 (100 nM). In order to achieve an approximately steady-state block, MK-801 was applied for 25 min against currents induced by ionophoretically applied NMDA (60 nA, 1 s, 0.05 Hz). Each trace is the average of 4 responses to NMDA and leakage currents have been subtracted by computer programme. The data are shown graphically to the right, where the relationship between NMDA-induced current amplitude and holding potential in the absence (●) and presence (■) of MK-801 is plotted. Note that antagonism appears voltage-independent and is not associated with any change in the reversal potential of the response. (b) Chart recorder traces illustrating the influence of ketamine (10 μM) upon responses to locally applied NMDA (100 μM, 20 ms, 0.05 Hz) recorded at holding potentials of −60 (left) and +40 mV (right). Note that blockade is voltage-dependent. Recordings were obtained from different hippocampal neurones. All currents were low-pass filtered at 0.5 kHz.

Voltage-dependent recovery of NMDA-induced currents from blockade by MK-801

As noted previously, the antagonism of NMDA-induced currents produced by MK-801 (300 nM–3 μM) was very poorly reversed when the membrane potential was held constant at −60 mV and NMDA applied ionophoretically as repetitive 'brief' pulses (1 s duration, 0.05 Hz) during washout (e.g. Figure 1a). Figure 4 illustrates the results of an experiment which demonstrates that recovery from such blockade could be accelerated by prolonged applications of NMDA at a positive holding potential (+40 mV). Briefly, MK-801 (300 nM) was bath-applied against responses induced by brief ionophoretic applications of NMDA. Once substantial antagonism of the response had developed (e.g. Figure 4a), MK-801 was washed out of the bath for at least 10 min. Essentially no recovery occurred during this time. The influence of prolonged (30 or 60 s) ionophoretic application of NMDA upon recovery was then assessed at holding potentials of either −60 or +40 mV. Whilst a prolonged application of agonist at −60 mV had little or no effect upon subsequent

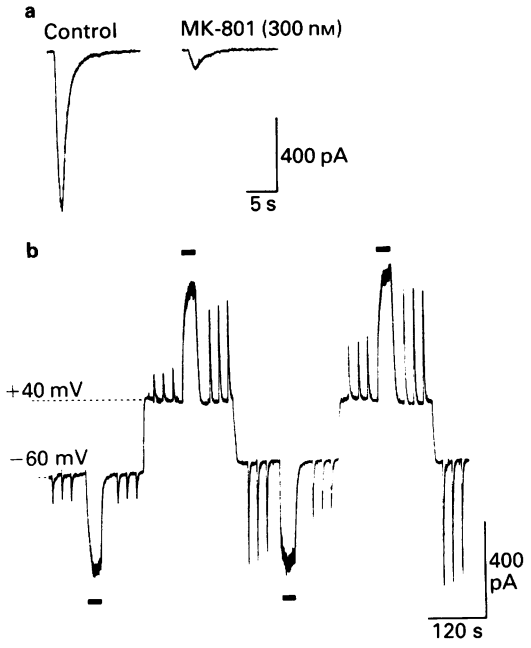

Figure 4 Reversal of MK-801-induced blockade shows voltage-sensitivity. (a) Control responses to ionophoretically applied N-methyl-D-aspartic acid (NMDA) (60 nA, 1 s, 0.05 Hz) and the response recorded following the application of MK-801 (300 nM) for 20 min. Traces are the average of 4 NMDA-induced currents recorded at a holding potential of −60 mV. (b) Chart recorder trace illustrating responses to NMDA during washout of MK-801. The trace starts after 10 min of wash with MK-801-free solution, during which time no recovery of NMDA-evoked response was observed. Prolonged ionophoretic applications of NMDA (60 nA, 30 s) are indicated by the bars adjacent to the trace. Note that the initial prolonged application of agonist at a holding potential of −60 mV has little effect on the amplitude of subsequent responses to brief pulses of NMDA (60 nA, 1 s, 0.05 Hz). In contrast, at a holding potential of +40 mV, a similar prolonged application of NMDA markedly alleviates the block produced by MK-801. Some redevelopment of the block occurs upon returning to a holding potential of −60 mV (see text for further details). Currents were recorded from a hippocampal neurone and low-pass filtered at 0.5 kHz.

responses to brief test pulses of NMDA, a similar treatment at a holding potential of +40 mV enhanced the amplitude of repetitive test responses. Upon returning to a holding potential of −60 mV, a relief of the MK-801-induced blockade was apparent, but this tended to fade with either brief repetitive or prolonged applications of NMDA (Figure 4b). The cycle of relief of blockade by agonist application at +40 mV, and partial redevelopment of block at −60 mV, could be demonstrated many times in an individual cell and was a consistent finding between cells ($n = 5$). In control experiments it was established that: (i) in the absence of MK-801 a prolonged application of NMDA at +40 mV has little effect upon subsequent responses to brief applications recorded at −60 mV, and (ii) depolarization of the cell *per se* is ineffective in relieving blockade evoked by MK-801. Collectively, these observations suggest that recovery from blockade by MK-801 is a use- and voltage-dependent process.

Voltage-dependent blockade of ACh-evoked currents by MK-801

Bovine chromaffin cells possess nicotinic receptors that are pharmacologically similar to those of mammalian autonomic ganglia (Chiapinelli *et al.*, 1988) the activation of which induces the opening of a relatively non-selective cation channel (J.J. Lambert, J.M. Nooney & J.A. Peters, unpublished observations). MK-801 suppressed ACh-evoked currents in a concentration-dependent manner, with an approximate IC_{50} of 1.7 μM at −60 mV (Figure 5a). In contrast to the block of NMDA-evoked currents, the antagonism of ACh-evoked currents by MK-801 was readily reversible at all concentrations tested (1–10 μM) usually within 10 min of starting washout. The block of NMDA-induced currents by MK-801 appeared voltage-independent (Figure 3a). In contrast, the blockade of ACh-induced currents by MK-801 was clearly voltage-dependent, the block increasing with hyperpolarization (Figure 5b).

MK-801 and $GABA_A$ receptors

Bovine chromaffin cells possess $GABA_A$ receptors pharmacologically similar to those of mammalian central neurones (Bormann & Clapham, 1985; Cottrell *et al.*, 1985; 1987). MK-801, at concentrations of 1, 10 and 100 μM had no effect on GABA-evoked whole cell currents in chromaffin cells ($n = 4$ for each concentration examined), whereas the anxiolytic diazepam produced the expected potentiation of GABA-evoked currents (Figure 6a). In acutely isolated CA1 hippocampal neurones, NMDA and the competitive NMDA receptor antagonist APV, have been observed to potentiate membrane currents elicted by GABA (Stelzer & Wong, 1987). However, in experiments on cultured hippocampal neurones, MK-801 (100 μM) had no effect upon GABA-evoked currents ($n = 4$). In common with Stelzer & Wong (1987), we found it necessary to include 4 mM Mg^{2+} and 2 mM ATP in the recording pipette solution in order to avoid a progressive run-down of GABA responses in hippocampal neurones. Interestingly,

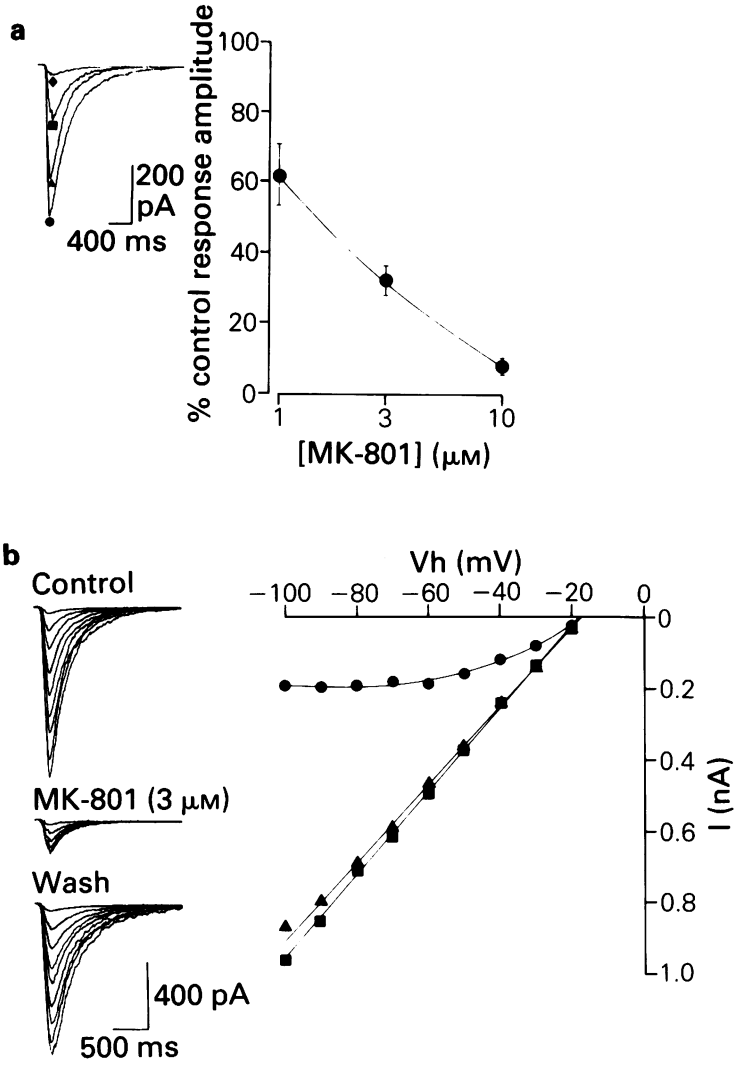

Figure 5 MK-801 antagonizes acetylcholine (ACh)-evoked currents recorded from bovine chromaffin cells. (a) Concentration-dependent blockade of ACh-induced currents by MK-801. Currents-evoked by ionophoretically applied ACh (30 nA, 100 ms, 0.1 Hz) were recorded at a holding potential of −60 mV. Traces, which are the average of 4 ACh-induced currents, illustrate a control response to ACh (●) and responses in the presence of 1 (▲), 3 (■) and 10 (◆) μM MK-801. The blockade produced by all concentrations of MK-801 tested was readily reversible upon washout. (b) Plot of the amplitude of whole cell currents evoked by ionophoretically applied ACh, as a percentage of control, against the log of the concentration of bath-applied MK-801. Data points are the mean of experiments from 4 to 6 cells. The vertical lines indicate s.e.mean. The curve, fitted to the data points by eye, yields an estimated IC_{50} for MK-801 of 1.7 μM. (c) Voltage-dependent blockade of ACh-induced whole-cell currents by MK-801. Membrane currents were evoked by ionophoretically applied ACh (60 nA, 100 ms, 0.1 Hz). The membrane potential was held at −60 mV and 1 s before applying ACh, was transiently stepped for 3 s to the test potential. In each panel the traces illustrate, from uppermost to lowermost, the average of 4 whole-cell currents elicited by ACh at holding potentials of −20 to −100 mV in 10 mV increments. Note that in the presence of MK-801 (3 μM), the ACh-induced current amplitude is essentially constant between holding potentials of −60 and −100 mV, indicating a voltage-dependent block. (d) Graphical representation of the data depicted in (c). The amplitude of the ACh-evoked current is plotted against holding potential before (■) and during (●) the application of MK-801 (3 μM). Virtually complete recovery was apparent upon washout (▲). All currents were low-pass filtered at 500 Hz.

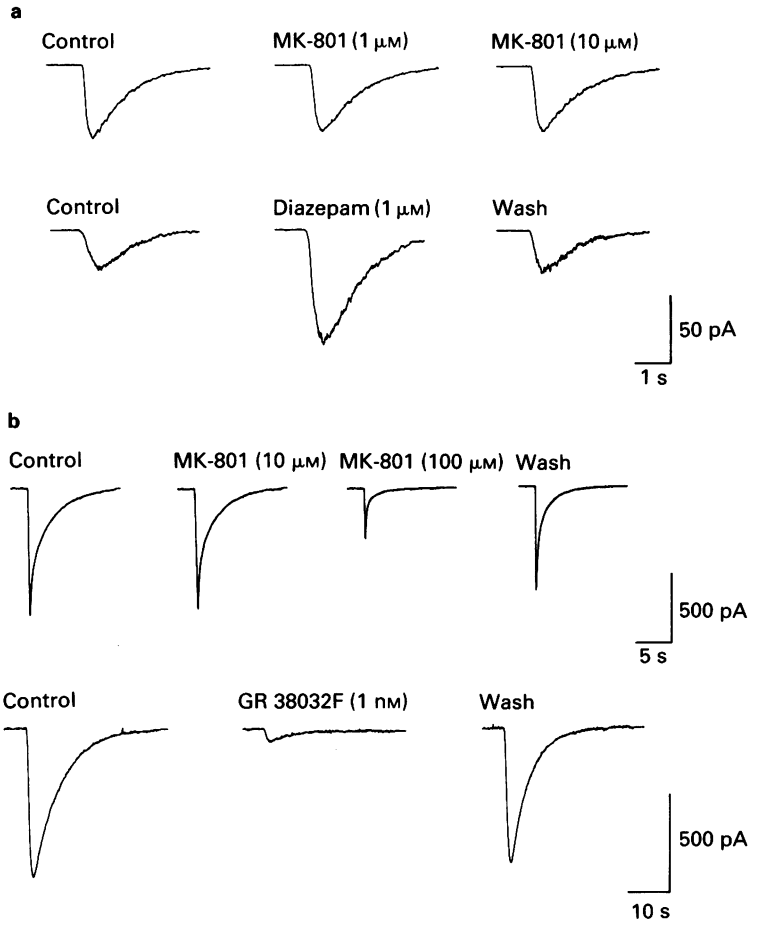

Figure 6 MK-801 does not affect GABA-evoked currents, but at high concentration depresses 5-HT_3 receptor-mediated responses. (a) GABA-induced whole cell currents recorded from bovine chromaffin cells. Membrane currents were evoked by pressure applied GABA (100 μM, 20 ms, 0.05 Hz) and recorded at a holding potential of −60 mV. MK-801 (1 and 10 μM) had no effect on GABA-evoked currents but diazepam (1 μM) reversibly potentiates the response to GABA. (b) 5-HT-induced whole cell currents recorded from N1E-115 cells. Membrane currents were elicited by ionophoretically applied 5-HT (40 nA, 50 ms, 0.02 Hz) and recorded at a holding potential of −60 mV. MK-801 (10 μM) produces only a slight depression of the inward current evoked by 5-HT, but at a concentration of 100 μM, exerts a substantial and reversible antagonism of the response. (c) GR 38032F (1 nM) reversibly antagonizes the 5-HT-induced current in an N1E-115 cell. Membrane currents were evoked by pressure-applied 5-HT (10 μM, 20 ms, 0.011 Hz) and recorded at a holding potential of −60 mV.

ATP-free recording solution appears to support relatively stable GABA responses in bovine chromaffin cells.

MK-801 and 5-HT receptors

Preliminary reports have demonstrated that antagonists of 5-HT_3 receptors such as GR 38032F (Butler *et al.*, 1988) appear to be anxiolytic in some animal models of anxiety (Jones *et al.*, 1988). 5-HT induces currents in N1E-115 neuroblastoma cells which are mediated by 5-HT_3 receptors (Neijt *et al.*, 1988; Peters *et al.*, 1988a). In 3 of 4 cells tested, MK-801 (10 μM) produced a slight suppression of such currents, but was without effect in the remaining cell of this sample. On average, 5-HT-induced currents were reduced to 88.8 ± 2.8% of their control value in the presence of 10 μM MK-801. At a concentration of 100 μM, MK-801 reversibly reduced the amplitude of

the response to 5-HT to 33.8 ± 2.7% ($n = 4$) of control. The 5-HT-induced current was potently antagonized by GR 38032F (1 nM), confirming that such responses are mediated via the 5-HT_3 receptor subtype (Figure 6b).

MK-801 and voltage-activated sodium currents

The anticonvulsants carbamazepine and phenytoin are known to block voltage-activated sodium channels in a use-dependent manner, and such an action may contribute to their anticonvulsant effects (Willow, 1986). In initial experiments on hippocampal neurones, MK-801 (100 μM) reduced the amplitude of inward currents elicited by transiently stepping the membrane potential to 0 mV from a holding potential of −80 mV. Such currents were abolished by tetrodotoxin (0.3 μM), and were therefore due to the opening of voltage-activated sodium channels. No attempt was made to analyse the effect of MK-801 in detail in hippocampal neurones; we simply note that sodium currents were reduced to between 80 and 60% of their control value when the compound was applied at a concentration of 100 μM. Further quantification of this effect in hippocampal neurones was avoided, since we considered the time constant of the voltage-clamp in these cells, relative to the kinetics of the sodium current, insufficient for accurate voltage control. As an alternative, the effect of MK-801 on voltage-activated sodium currents of bovine chromaffin cells was examined, since in such cells adequate voltage control of even rapidly changing currents may be easily attained by use of the whole-cell recording technique (Hamill *et al.*, 1981). Voltage-activated sodium currents of chromaffin cells were completely blocked by tetrodotoxin (0.3 μM, $n = 4$). In contrast, MK-801 (10 μM) had no discernible effect on the sodium current, but at a relatively high concentration (100 μM), rapidly and reversibly reduced it to 77 ± 3.4% ($n = 5$) of control (Figure 7).

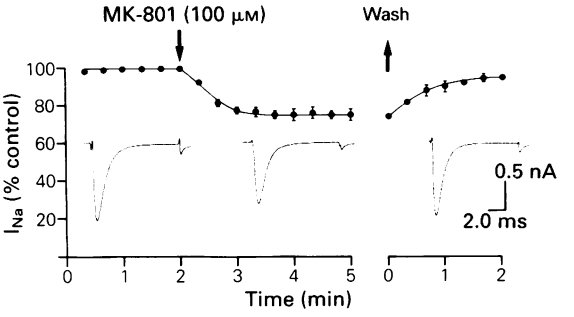

Figure 7 Only relatively high concentrations of MK-801 inhibit voltage-activated sodium currents. Voltage-activated sodium currents were elicited from bovine chromaffin cells by transiently stepping the holding potential from −80 mV to 0 mV (0.5 Hz, 10 ms). Experiments commenced 30 min after forming the whole cell clamp to allow for any change in the sodium current induced by pipette dialysis. Control currents were recorded for 2 min at which time tetrodotoxin (TTX) (0.3 μM) or MK-801 (10–100 μM) was applied to the cell surface by microperfusion for 5 min before removal of the microperfusion pipette and washout of the bath. Such currents were completely blocked by TTX (0.3 μM), unaffected by MK-801 (10 μM) and reduced to 77 ± 3.4% of control by 100 μM MK-801 ($n = 5$). The graph shows the effect of 100 μM MK-801 on voltage-activated sodium current amplitude as a function of time. Each point is the mean of 5 experiments with s.e.mean shown by vertical lines. Computer averaged currents ($n = 10$) are shown before, during, and after washout of 100 μM MK-801. The relatively small capacitative and leakage currents were not subtracted. Currents were low-pass filtered at 2.0 kHz.

Discussion

MK-801 is a potent antagonist of NMDA-induced depolarizations in rat cortical slices (Wong *et al.*, 1986; Kemp *et al.*, 1987; Davies *et al.*, 1988) and spinal cord (Childs *et al.*, 1988). In experiments employing voltage-clamp techniques, MK-801 blocks NMDA-induced currents in rat visual cortical neurones (Huettner & Bean, 1988), and in *Xenopus* oocytes injected with rat brain messenger RNA (Kushner *et al.*, 1988). In agreement with these studies, we now show that MK-801 antagonizes currents evoked by NMDA, but not kainate, in voltage-clamped hippocampal and cortical neurones in cell culture.

It has been suggested that MK-801 might act at the level of the NMDA-activated ion channel, blocking ion conduction (Kemp *et al.*, 1987) in a manner analogous to that proposed for some drugs at the nicotinic receptor of the neuromuscular junction (e.g. Lambert *et al.*, 1983), or autonomic ganglia (e.g. Gurney & Rang, 1984). In support of this suggestion, the block of NMDA-induced currents by MK-801 was largely agonist-dependent. A similar use-dependent block has been obtained in voltage-clamped rat visual cortical neurones (Huettner & Bean, 1988), and in studies employing extracellular recording techniques in rat cortical slices (Wong *et al.*, 1986; Kemp *et al.*, 1987). Consistent with these observations, the binding of radiolabelled MK-801 to rat brain membranes is potentiated by NMDA receptor agonists and by glycine (Foster & Wong, 1987; Wong *et al.*, 1987). However the antagonism by MK-801 of depolarizing responses to NMDA recorded extracellularly from rat spinal cord *in vitro*

does not show agonist-dependency (Childs *et al.*, 1988) and the degree of use-dependency in the cortical slice preparation appears to be temperature-dependent (Davies *et al.*, 1988). In the present work, a small depression of the NMDA-induced current was apparent following a 10 min pre-exposure to MK-801 (3 μM), despite the fact that the application of NMDA was discontinued as MK-801 was introduced to the bath. Such a result might suggest that a small component of the block by MK-801 occurs via a use-independent mechanism. Although the present data do not allow the exclusion of the latter possibility, a more parsimonious explanation is that use-dependent blockade by MK-801 occurs during the rising phase of the initial response, leading to a depression of its amplitude (cf. Lingle, 1983; MacDonald *et al.*, 1987). In this respect it is interesting that the use-dependent nature of blockade by MK-801 was originally demonstrated with comparatively slowly rising agonist responses recorded extracellularly from a cortical slice preparation (Kemp *et al.*, 1987). Incompletely suppressed synaptic activity, and possibly diffusional release of NMDA from the drug application pipette, might in the present experiments, have supplied sufficient agonist for some degree of use-dependent blockade to develop during the 10 min incubation period with MK-801.

In agreement with previous reports (Honey *et al.*, 1985; MacDonald *et al.*, 1987) ketamine blocked NMDA-induced currents in a voltage-dependent manner, the block increasing with hyperpolarization. In contrast, under identical recording conditions, MK-801 appeared equieffective in blocking inward and outward NMDA-induced currents. In this context, it should be noted that the time constant of onset of blockade of NMDA-induced currents by MK-801 is independent of membrane potential (Huettner & Bean, 1988). Like MK-801, the NMDA receptor antagonist DL-2-amino-5-phosphonovaleric acid (APV) blocks NMDA-induced currents in a voltage-independent manner (MacDonald *et al.*, 1987). However, the agonist-dependency of both the blocking action, and binding of MK-801, make it unlikely that the drug binds to the agonist recognition site of the NMDA-receptor-channel complex, as has been suggested for APV. If alternatively, MK-801 blocks the NMDA-activated ion channel, the above results and others (Huettner & Bean, 1988), clearly demonstrate that it does so in a voltage-independent manner.

The recovery from blockade by MK-801 was both use- and voltage-dependent (see also Huettner & Bean, 1988). Similar observations have been made for the block of NMDA ion channels by ketamine (MacDonald *et al.*, 1987), nicotinic ion channels by some methonium compounds (Gurney & Rang, 1984) and ACh-activated channels in lobster muscle fibres by chlorisondamine (Lingle, 1983). To explain these observations, it has been suggested that the drug binds in the lumen of the ion channel and becomes trapped therein when the channel closes (Lingle, 1983; Gurney & Rang, 1984; MacDonald *et al.*, 1987).

In the instances cited above, both blocking and unblocking of the ion channel have been found to be voltage-dependent. An important difference in the present work with MK-801 is the dissimilar influence of membrane potential upon blockade and recovery. MK-801 has a pKa of 8.2 (P. Anderson, unpublished observation) and is therefore mainly in the cationic form at pH 7.2. One interpretation of the voltage-independence of the development of blockade by MK-801 is that it is the minority uncharged form of the drug which blocks the ion channel. The same reasoning suggests that the voltage-dependence of recovery reflects the expulsion of the protonated molecule from the ion channel. One possible explanation of this paradox might be that MK-801 converts to the protonated state once bound to its site within the channel. Alternatively, it may be speculated that the NMDA ion channel undergoes some voltage-dependent conformational change which specifically facilitates unblocking, or that it is the net direction of ion flow through the channel, rather than membrane potential *per se*, which influences recovery.

Both ketamine and PCP are known to block the nicotinic receptor-channel complex in a non-competitive manner. The mechanism of blockade is not well understood, but is thought to involve a blockade of both open and closed nicotinic ion channels by the drug (e.g. Maleque *et al.*, 1981; Volle *et al.*, 1981; Aguayo & Albuquerque, 1986; but see Changeux *et al.*, 1986). The present study demonstrates that MK-801 is a potent antagonist of currents evoked by nicotinic receptor activation in bovine chromaffin cells. Unlike the antagonism of NMDA-induced responses, blockade of ACh-evoked currents by MK-801 reached a steady-state relatively quickly (within approximately 2–3 min), was fully reversible upon washout, and was at least partly voltage-dependent with an IC_{50} of 1.7 μM (at -60 mV). The latter of these characteristics implicates the protonated form of MK-801 in the blockade of ACh-induced currents. Whether or not the phenomena of use-dependent blocking and recovery observed with MK-801 at the NMDA-ion channel complex occur at the nicotinic channel also, remains to be determined. Although a high concentration (100 μM) of MK-801 was observed to antagonize 5-HT_3 receptor-mediated currents in N1E-115 cells, this system and kainate-induced currents were minimally affected by doses of MK-801 (1–10 μM) which markedly suppress ACh-evoked currents in chromaf-

fin cells and NMDA-evoked currents in central neurones. Thus the blockade of ACh-induced responses by MK-801 is at least partially selective, and could be due to an association of the drug with a specific site on the nicotinic-channel complex. The primary amino acid sequence of the nicotinic receptor-ion channel complex of the *Torpedo* electroplaque organ is known and progress in identifying the amino acids to which PCP binds has been reported (Changeux & Revah, 1987). When the amino acid sequence of the NMDA ion channel is determined, it will be of interest to compare the PCP/MK-801 binding site with that of its counterpart on the nicotinic receptor.

MK-801 is a potent anticonvulsant, anxiolytic and neuroprotective agent (Clineschmidt, 1982; Clineschmidt *et al.*, 1982; Gill *et al.*, 1987). This behavioural profile may be due entirely to the known antagonism of the NMDA-receptor-channel complex by MK-801. However, the possibility exists that other receptors or ion channels may contribute to its effects. Anxiolytics of the benzodiazepine type are thought to act primarily by binding to a site associated with the $GABA_A$ receptor (e.g. Martin, 1987). A recent report has demonstrated that the NMDA receptor antagonist carboxypiperazine phosphonic acid (CPP) is a potent displacer of flunitrazepam binding (White *et al.*, 1988), although MK-801 binding is unaffected by clonazepam (Wong *et al.*, 1986). In the present study, MK-801 had no effect on GABA-induced currents which were, however, potentiated by the anxiolytic diazepam. This result is consistent with radioligand binding studies (Wong *et al.*, 1986) and the insensitivity of the anticonvulsant action of MK-801 to the benzodiazepine antagonist Ro15-1788 (Clineschmidt, 1982), an agent which reverses the flunitrazepam-induced potentiation of GABA-induced currents in bovine chromaffin cells (Cottrell *et al.*, 1987). These results suggest that the central effects of MK-801 are unlikely to be mediated directly by $GABA_A$ receptors.

In preliminary studies, antagonists of the $5\text{-}HT_3$ subclass of receptor have been reported to be active in animal tests predictive of anxiolytic effects in man (Jones *et al.*, 1988). In the present study, $5\text{-}HT_3$ receptor-mediated currents recorded from N1E-115 cells were minimally affected by MK-801 at a concentration of 10 μM, but substantial antagonism was observed with a ten fold higher concentration of the drug. To place this action in perspective, it should be noted that the $5\text{-}HT_3$ receptor antagonist and putative anxiolytic GR 38032F virtually abolished the 5-HT-induced current at a concentration of 1 nM. In contrast to the present results, MK-801 has recently been shown to potentiate 5-HT-induced depolarizations in a rat spinal cord preparation, possibly due to an effect of MK-801 on 5-HT uptake (Childs *et al.*, 1988).

Carbamezepine and phenytoin are known to block voltage-activated sodium channels in a use-dependent manner, a molecular mechanism thought to contribute to their anticonvulsant actions (Willow, 1986). In the present study, although high doses of MK-801 did block voltage-activated sodium channels, it seems unlikely that such an action is important in its anticonvulsant effect when compared with the potent blockade of NMDA-induced currents produced by the drug.

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MEETINGS CALENDAR

21–23 March 1989

British Opioid Colloquium, Reading, U.K. (Dr. J.R. Traynor, Department of Chemistry, Loughborough University, Leics LE11 3TU, U.K.)

29 March–1 April 1989

International Symposium on Serotonin from Cell Biology to Pharmacology and Therapeutics, Florence, Italy (Secretariat, Fondazione Giovanni Lorenzini, Via Monte Napoleone 23, 20121 Milan, Italy).

2–5 April 1989

Seventh National Meeting of the Brain Research Association, Sheffield, U.K. (Dr. N. Payne, Dept. of Anatomy, Sheffield University, Sheffield, U.K.)

3–4 April 1989

British Neuroendocrine Group Meeting, Bristol, U.K. (Dr. C.D. Ingram, Department of Anatomy, The Medical School, University Walk, Bristol BS8 1TD).

5–7 April 1989

British Pharmacological Society Spring Meeting, Bristol, U.K. (Closed meeting for members and guests.)

6–7 April 1989

International Symposium on Psychiatric Neuropathology and Neurochemistry, London, U.K. (Dr. R.W. Kerwin, Department of Neuropathology, Institute of Psychiatry, De Crespigny Park, London SE5).

7 April 1989

British Pharmacological Society. Symposium on the NMDA Receptor, Bristol, U.K. (Dr. G.L. Collingridge, Department of Pharmacology, The Medical School, University of Bristol, Bristol, BS8 1TD, U.K.)

21–22 April 1989

Satellite Symposium of Int. Soc. for Neurochemistry Congress, Adenosine Receptors, Agarne, Portugal (Professor J.A. Ribeiro, Laboratory of Pharmacology, Gulbenkian Institute of Science, 2781 Oeiras, Portugal).

30 April–5 May 1989

Fifth Int. Symposium on the Adrenal Chromaffin Cell as a Neuroendocrine model; from Basic to Clinical Aspects, Jerusalem, Israel (Dr. K. Rosenheck, Dept. of Membrane Research, Weizmann Institute of Science, Rehovot 76100, Israel).

22–26 May 1989

Second Meeting of the International Neurotoxicology Association, Barcelona, Spain. (Dr. E. Rodriquez Farre, Dept. of Pharmacology & Toxicology, CSIC, Jorge Girona Salgado, 18–26, E-08034, Spain).

25–27 May 1989

4th International Symposium on Calcium Antagonists, Florence, Italy. (Secretariat, Fondazione Giovanni Lorenzini, Via Monte Napoleone 23, 20121 Milan, Italy).

1–2 June 1989

International Workshop on Sensory Neuropeptides, Florence, Italy. (Miss A. Giannini, Istituto Farmacobiologico Malesci, S.p.A., Dept. Pharmacology, Via Porpora 22, 50144 Florence, Italy).

1–3 June 1989

International Symposium on Kidney, Proteins & Drugs, Montecatani Terme, Italy. (Dr. Claudio Bianchi, Centro Nefrologico, Clinica Medica Generale 2, University of Pisa, 56100 Pisa, Italy).

28 June–2 July 1989

Ethnicity & Cardiovascular Diseases Conference, Nairobi, Kenya. (Ms. C. Cate, Int. Society on Hypertension in Blacks, 69 Butler Street, S. E., Atlanta, GA 30303, USA).

12–14 July 1989

British Pharmacological Society Summer Meeting. Leeds, U.K. (Closed meeting for members and guests)

16–21 July 1989

Vth International Congress of Toxicology, Brighton, U.K. (IUTOX '89, Congress House, 65 West Drive, Sutton, Surrey SH2 7NB, U.K.)

1–3 September 1989

Second International FIP Symposium on Disposition & Delivery of Peptide Drugs, Leiden, The Netherlands. (Dr. J. Verhoef, Centre for Bio-Pharmaceutical Sciences, PO Box 9502, 2300 RA Leiden, The Netherlands).

17–20 September 1989

1st International $GABA_B$ Symposium, Cambridge, U.K. (Professor N.G. Bowery. Department of Pharmacology, School of Pharmacy, 29/39 Brunswick Square, London WC1N 1AX.)

13–15 September 1989

British Pharmacological Society Autumn Meeting. Manchester, U.K. (Closed meeting for members and guests)

3–5 January 1990

British Pharmacological Society Winter Meeting. London, U.K. (Closed meeting for members and guests)

18–20 April 1990

British Pharmacological Society Spring Meeting. Sheffield, U.K. (Closed meeting for members and guests)

29 May–1 June 1990

Fondazione Giovanni Lorenzini Conference on Prostaglandins and Related Compounds, Florence, Italy (Fondazione Giovanni Lorenzini, Via Monte Napoleone, 23, 20121 Milan, Italy.)

1–6 July 1990

IUPHAR 11th International Congress of Pharmacology, Amsterdam, Netherlands (Contact to be advised.)

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PAPERS

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