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Training and employment in pharmacology

Fourth updating survey on the organisation of pharmacology in Great Britain: Summary of findings

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This report presents the results of a study of training and employment in pharmacology in Great Britain, carried out by the Institute of Manpower Studies on behalf of the British Pharmacological Society. It updates material collected by the Society in three earlier studies published in 1966, 1974 & 1986. The present survey was completed in October 1990.

The report focuses on the results of a review of existing official data sources, new surveys of academic departments, and of students graduating in 1983 and 1987.

Over the last 25 years the numbers employed in academic pharmacology departments have grown; most rapidly in the 1960s and at much slower rates in the 1980s. In 1989 the surveyed departments had over 400 academic staff and almost 1500 students. The average size of departments has grown from 5.1 academic staff per department in 1964 to 9.1 academic staff in 1989. Between 1983–89, the sizes of individual departments have, however, changed dramatically, with one quarter contracting and one quarter expanding their number of academic staff by almost 50%. The number of research staff has grown rather faster, especially in the 1980s, as has the number of undergraduate and postgraduate students. In the 1980s the growth has been concentrated in the university sector.

Since 1964 the proportion of staff with degrees in pharmacology has risen from a small minority to over one third; the proportion with PhDs has risen to 86 percent. The age structure became slightly older in the 1980s.

The funding of research staff and students has continued on its long term trend towards industrial and foundation sources, who now provide over half of this type of research funding.

The main source of recruitment to academic and research posts continues to be from within higher education, although fewer recruits have been coming straight after qualification. There were no recruits reported from industry. The departments had little problem filling the expanding number of posts in the 1980s. The disciplines of those recruited have become more diverse, although about a half have backgrounds in pharmacology.

The numbers leaving for early retirement have fallen significantly since the early 1980s; the proportions leaving for industry (under 20 per cent) and overseas (under 20 per cent) have not changed significantly.

Students' reasons for undertaking pharmacology courses have changed little in the 1980s and still focus on the intrinsic interest of the subject. Nearly a third thought that their studies were a great advantage in their subsequent employment and half thought positively about their preparation; these proportions had changed little in the 1980s. Little change was apparent in their subsequent destinations; they entered a wide spread of activities, including R & D and academic research, while further degrees were an important destination for first degree graduates.

Overall, respondents expected the growth patterns of the 1980s to continue in the 1990s, especially in the case of the universities. There was a view that there was a need for more postgraduates and research posts in the next decade, although this report is not able to provide an industrial perspective or comment on the likely availability of research funding in the 1990s. In terms of course content, recent graduates thought that there should be an increase in work experience and business-related topics in undergraduate courses.

Copies of the survey report, which includes full data, questionnaires and interpretations, can be obtained (price £10) from the office of the General Secretary of the British Pharmacological Society.

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The non-peptide NK_1 receptor antagonist, (±)-CP-96,345, produces antinociceptive and anti-oedema effects in the rat

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The non-peptide NK_1 receptor antagonist, (±)-CP-96,345, has been evaluated for antinociceptive activity in two well-characterized inflammatory pain models in the rat. (±)-CP-96,345 abolished carrageenin-induced mechanical hyperalgesia, significantly reduced carrageenin-induced paw oedema and attenuated the second phase of the formalin response. The results suggest that NK_1 receptor activation occurs during the induction of inflammatory pain states in the rat.

Keywords: (±)-CP-96,345; non-peptide NK_1 receptor antagonist; antinociception; anti-oedema

Introduction The pharmacological actions of the tachykinin, substance P, are thought to be mediated predominantly through a distinct neurokinin receptor subtype which has been termed NK_1 (see Guard & Watson, 1991). Although it has been suggested that NK_1 receptors are involved in a variety of physiological and pathophysiological processes in both the central and peripheral nervous systems, definitive evidence has awaited the discovery of potent, selective antagonists that effectively block NK_1 receptor-mediated responses. In this respect, Snider *et al.* (1991) described recently the pharmacology of the first non-peptide NK_1 receptor antagonist, CP-96,345 ((2S,3S)*cis*-2-(diphenylmethyl)-*N*-[(2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]octan-3-amine).

One of the key areas in which substance P is thought to play an important role is in nociception. Experimental evidence suggests that substance P can be released from the peripheral nerve terminals of primary afferent C-fibres to produce inflammation or from the central terminals of these fibres to modulate nociceptive transmission at the level of the spinal cord dorsal horn (Otsuka & Yanagisawa, 1987). The aim of the present study was to assess the ability of CP-96,345 to produce antinociceptive and anti-oedema effects in two well-established models of inflammatory pain in the rat. As a number of studies have indicated that CP-96,345 has lower affinity for the rat NK_1 receptor than in other species (Beresford *et al.*, 1991a; Gitter *et al.*, 1991; Watling *et al.*, 1991), doses of CP-96,345 that block peripherally-mediated NK_1 responses in the rat were also determined. Throughout this study the racemic version of this compound, (±)-CP-96,345, has been used.

Methods *NK_1 receptor-mediated paw oedema in the rat* Male RH, Glaxo-bred rats (75-120 g) were injected by the intraplantar route (dose volume 100 µl) with a range of doses of the selective NK_1 agonist GR73632 (Hagan *et al.*, 1991; 0.03 to 1 nmol; $n = 5$ per group) in the presence of a subthreshold dose of calcitonin-gene-related peptide (CGRP, 0.1 nmol), and the resulting paw oedema measured 30 min later by use of a plethysmometer. Vehicle or (±)-CP-96,345 (24 µmol kg^{-1}) were administered subcutaneously (dose volume 4 ml kg^{-1}) at varying times before induction of oedema. ED_{50} values (95% confidence limits) for producing oedema (dose causing 50% of maximum oedema) were determined in the absence and presence of (±)-CP-96,345 and dose-ratios were calculated.

Carrageenin-induced hyperalgesia and oedema in the rat Hyperalgesia and oedema were induced in male RH, Glaxo-bred rats (75–120 g) by intraplantar injection with 100 µl of 2% carrageenin in saline. At various time points before and after carrageenin administration, nociceptive pressure thresholds were measured with an analgesymeter (Ugo Basile) and paw volumes were measured with a plethysmometer (Ugo Basile). Hyperalgesia was assessed as the decrease in the pressure threshold required to evoke a paw withdrawal response and oedema was measured as the percentage increase in paw volume determined before carrageenin injection. Vehicle or (±)-CP-96,345 (8, 24 or 73 µmol kg^{-1}; $n = 8$ per group) were administered subcutaneously (dose volume 4 ml kg^{-1}) 30 min before and at 60 and 150 min after carrageenin. This dosing regimen was chosen on the basis of data obtained from experiments performed in the NK_1 receptor-mediated paw oedema model (see above).

Formalin-induced paw licking in the rat Formalin-induced hindpaw licking was induced in male RH, Glaxo-bred rats (40–70 g) by intraplantar injection with 50 µl of 5% phosphate-buffered formalin (Dubuisson & Dennis, 1977). Immediately following injection rats were placed in perspex observation chambers (25 × 40 × 20 cm) and the duration of licking of the injected paw was scored for 0–5 min (first phase) and 15–30 min (second phase). Vehicle or (±)-CP-96,345 (8, 24 or 73 µmol kg^{-1}; $n = 6$ per group) were administered subcutaneously (dose volume 4 ml kg^{-1}) 30 min before formalin. ED_{50} values (95% confidence limits) for blockade of either phase of the licking response (dose causing 50% inhibition) were calculated as appropriate.

Drugs (±)-CP-96,345 (*cis*-2-(diphenylmethyl)-*N*-[(2-methoxyphenyl)-methyl]-1-azabicyclo [2.2.2]octan-3-amine) and GR73632 (δAva[L-Pro^9, N-MeLeu^{10}]SP(7-11)) were synthesized in the Department of Medicinal Chemistry, Glaxo Group Research Ltd. Calcitonin-gene-related peptide (CGRP) and carrageenin were purchased from the Sigma Chemical Company.

Results *NK_1 receptor-mediated paw oedema in the rat* GR73632 (0.03 to 1 nmol), with CGRP (0.1 nmol), produced a dose-related oedematous response; ED_{50} 0.06 (0.04–0.1) nmol. Pretreatment (30 min) with (±)-CP-96,345 (24 µmol kg^{-1}, s.c.) produced a parallel rightward shift in the dose-response curve to GR73632 with CGRP; dose-ratio 22.3

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(7-64). Increasing the pretreatment time to 60 or 90 min produced dose-ratios of 26.0 (10–77) and 6.5 (2–19), respectively.

Carrageenin-induced hyperalgesia and oedema in the rat Intraplantar injection of 2% carrageenin produced a reduction in the nociceptive pressure threshold (hyperalgesia) and an increase in the paw volume (oedema) in the injected paw. Both effects were maximal at 3 h after injection. Repeated subcutaneous administration of (±)-CP-96,345 (8, 24 or 73 μmol kg^{-1}) produced a dose-dependent blockade of the carrageenin-induced hyperalgesia (Figure 1a); complete blockade of the hyperalgesia was obtained with (±)-CP-96,345 at 73 μmol kg^{-1}, s.c. A significant blockade of paw oedema was obtained with (±)-CP-96,345 at 24 and 73 μmol kg^{-1}, s.c. (Figure 1b). At the 3 h time-point, (±)-CP-96,345 (73 μmol kg^{-1}, s.c.) produced a 60% reduction in oedema. No overt behavioural effects were observed with (±)-CP-96,345 at the doses tested and this antagonist had no effect on the nociceptive pressure threshold or paw volume of the non-injected paw.

Formalin-induced paw licking in the rat Intraplantar injection of 5% formalin evoked a characteristic biphasic licking response. The duration of licking (mean ± s.e.mean) for the first phase (0–5 min) was 72.3 ± 8.5 s and for the second phase (15–30 min) was 160.3 ± 16.7 s. Pretreatment (30 min) with (±)-CP-96,345 (8, 24 or 73 μmol kg^{-1}, s.c.) had no significant effect against the first phase of the formalin response (Figure 2a), but produced a dose-dependent blockade of the second phase; ED_{50} 33.9 (19.4–81.3) μmol kg^{-1}, s.c. (Figure 2b).

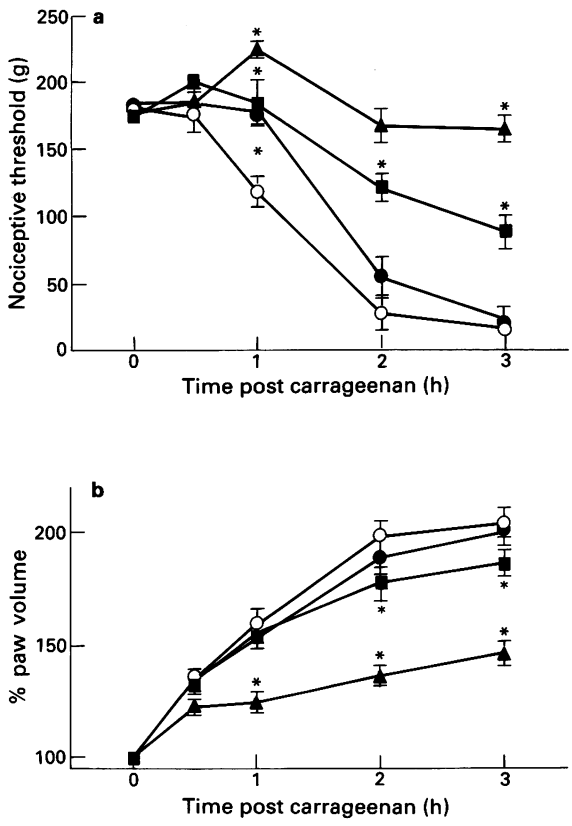

Figure 1 Effect of the non-peptide NK_1 receptor antagonist, (±)-CP-96,345, against (a) carrageenin-induced hyperalgesia and (b) carrageenin-induced paw oedema in the rat. Symbols represent animals treated with vehicle (○) or (±)-CP-96,345 at 8 (●), 24 (■) or (▲) 73 μmol kg^{-1}, s.c.; see methods for details. Only data for the carrageenin-injected paw is shown for clarity. Each point represents mean data from 8 animals; vertical bars show s.e.mean. * $P < 0.05$ significantly different from vehicle-injected animals (Mann-Whitney test).

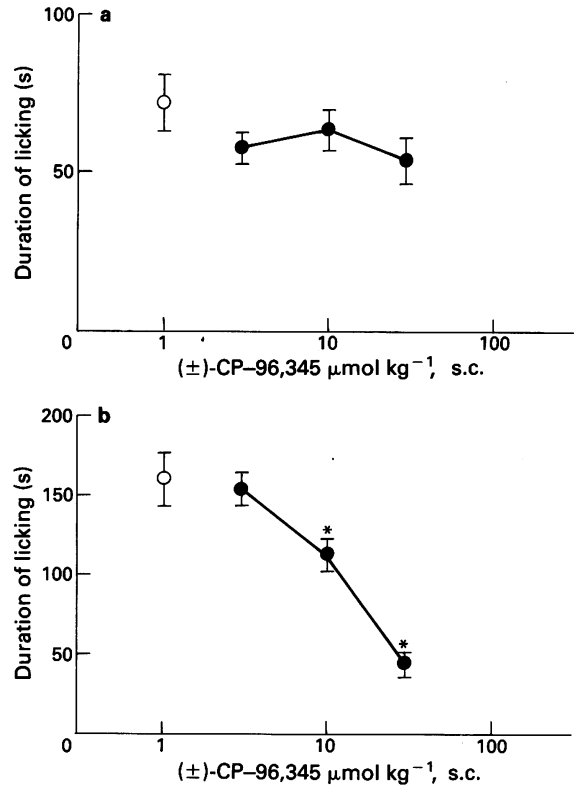

Figure 2 Effect of the non-peptide NK_1 receptor antagonist, (±)-CP-96,345, against (a) the first phase and (b) the second phase of the formalin-induced paw licking response in the rat. Symbols represent animals treated with vehicle (○) or (±)-CP-96,345 (●); see Methods for details. Each point represents mean data from 6 animals; vertical bars show s.e.mean. * $P < 0.05$ significantly different from vehicle-injected animal (Mann-Whitney test).

Discussion The results indicate that the non-peptide substance P antagonist, (±)-CP-96,345, has antinociceptive and anti-oedema activity in two well-characterized models of inflammatory pain in the rat. These effects occur at doses that effectively and specifically (Birch *et al.*, 1991) block NK_1 receptor-mediated paw oedema. As these doses do not block centrally-mediated NK_1 responses in the rat (Birch *et al.*, 1991), this action of (±)-CP-96,345 is possibly mediated at a peripheral site.

The selective blockade by (±)-CP-96,345 of the second phase of the formalin response is indicative of an action against inflammatory pain as this phase involves an inflammatory component (Dubuisson & Dennis, 1977). Although, carrageenin injection leads to the generation of a range of inflammatory mediators (including 5-hydroxytryptamine (5-HT) and bradykinin), the action of (±)-CP-96,345 in this model is not mediated via an action of 5-HT or bradykinin receptors as paw oedema induced by these agents is not blocked by this antagonist (Birch *et al.*, 1991). Interestingly, (±)-CP-96,345 caused complete blockade of carrageenin-induced hyperalgesia but only a 60% decrease in oedema. This may indicate that NK_1 receptors play a pivotal role in the induction of hyperalgesia but are only one of a number of receptor systems involved in the oedematous response.

In order to confirm that the antinociceptive and anti-oedema effects of (±)-CP-96,345 are mediated at NK_1 receptors, similar studies are underway with the selective, peptide NK_1 receptor antagonist GR82334, which is also an effective blocker of NK_1 responses in the rat (Beresford *et al.*, 1991b; Birch *et al.*, 1991).

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Heterogeneity of endothelin-sarafotoxin receptors mediating contraction of pig coronary artery

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The contractile effects of endothelin-1, endothelin-3, sarafotoxin 6b and sarafotoxin 6c were studied in endothelium-denuded rings of pig coronary artery. Endothelin-1, sarafotoxin 6b and sarafotoxin 6c produced monophasic concentration-response curves (mean EC_{50} values 6.7, 14.8 and 1.6 nM), whereas the concentration-response curve to endothelin-3 was biphasic (mean EC_{50} values 9.6 nM and 0.32 μM). The maximal effect of sarafotoxin 6c was about one third of that reached by the other peptides. The higher sensitivity component of the curve to endothelin-3 was abolished in the presence of sarafotoxin 6c (0.3 μM), while the EC_{50} value for the other component remained unchanged. Sarafotoxin 6c (0.3 μM) failed to alter the EC_{50} values of endothelin-1 and sarafotoxin 6b. These data strongly suggest the presence of at least two endothelin-sarafotoxin receptors mediating contraction of pig coronary artery, one with the profile of the endothelin ET_A receptor subtype, the other recognizing sarafotoxin 6c and endothelin-3, but not endothelin-1 and sarafotoxin 6b, being thus different from the ET_B receptor subtype.

Keywords: Endothelin-1; endothelin-3; sarafotoxin 6b; sarafotoxin 6c; endothelin receptor subtypes; pig coronary artery; vascular smooth muscle contraction

Introduction Endothelins and sarafotoxins constitute a family of peptides with 21 amino-acids which have potent vasoconstrictor properties (Inoue *et al.*, 1989; Kloog & Sokolovsky, 1989). Recently, the complementary DNAs encoding two distinct endothelin receptors have been cloned. The first receptor, now referred to as the ET_A receptor, was characterized by the rank order of binding affinities: endothelin-1 > sarafotoxin 6b > endothelin-3 (Arai *et al.*, 1990). The other receptor (ET_B) was non-isopeptide-selective (Sakurai *et al.*, 1990). In addition, the isopeptide sarafotoxin 6c appears to be quite useful for discriminating between ET_A and ET_B receptors, since it has been reported as a selective ligand for the ET_B receptor with very weak affinity for the ET_A receptor (Williams *et al.*, 1991). Endothelin-sarafotoxin receptors mediating vascular contraction are generally believed to be of the ET_A receptor subtype. This is mostly based on the rank order of potency: endothelin-1 > sarafotoxin 6b > endothelin-3, although the potency ratio between endothelin-1 and endothelin-3 is often much less than expected (see Randall, 1991).

In the present work, evidence is presented that multiple endothelin-sarafotoxin receptors mediate contraction of pig isolated coronary artery. Apart from a receptor fulfilling the current criteria for defining the ET_A receptor, another one recognizes particularly sarafotoxin 6c and endothelin-3, but not endothelin-1 or sarafotoxin 6b, thus being different from the ET_B receptor.

Methods Pig hearts were obtained from a local abbattoir (within 15 min of death) and transferred to the laboratory in ice-cold Krebs bicarbonate solution (composition in mM: NaCl 122, KCl 5, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2PO_4 1, $CaCl_2$ 1.25 and glucose 11.5). The first 3 cm of the left circumflex coronary artery were dissected out and freed of surrounding adipose tissue. Four adjacent rings (2–3 mm long) were cut and mounted between parallel hooks under a tension of 4 g in 10 ml organ chambers containing Krebs solution maintained at 37°C and bubbled with a gas mixture of 95% O_2:5% CO_2. Endothelium was removed from tissues by rubbing the interior of the ring with a wooden matchstick. This procedure abolished the endothelium-dependent relaxation to substance P (10 nM). Changes in tension were recorded isometrically. Tissues were allowed a period of equilibration of 2 h, during which the solution was periodically replaced and the tension repeatedly readjusted to 4 g. They were first contracted by a maximally effective concentration of prostaglandin $F_{2\alpha}$ (30 μM). When the contraction had reached a constant value, substance P (10 nM) was added as a test of efficiency of endothelium removal. After a further 90 min period of washout and resting, during which the tension was readjusted to 4 g, a cumulative concentration-response curve was constructed for one of the indicated peptides. Only one such curve was obtained from one ring. When studied as an antagonist, sarafotoxin 6c (0.3 μM) was present in the bath for at least 30 min before the concentration-response curve to one of the other peptides was started and control tissues were run in parallel. Peptides (Bachem, Bubendorf, Switzerland or Novabiochem, Läufelfingen, Switzerland) were stored as stock solutions in water at −20°C and used within one month. Prostaglandin $F_{2\alpha}$ (Biokema, Crissier-Lausanne, Switzerland) was prepared daily. Concentration-response curves were analyzed by a non-linear regression computer programme (De Lean *et al.*, 1980) according to the equation:

$$E = \sum_{i=1}^{n} \frac{E_{max_i}}{1 + (EC_{50_i}/[A]^{m_i})}$$

where E is the effect and [A] is the agonist concentration; E_{max_i}, EC_{50_i} and m_i are respectively the maximal effect, the half-maximal effective concentration and the slope factor for the receptor site i. Statistical analysis was performed by the F-test according to Snedecor & Cochran (1967). The best fit with a one or multiple receptor model was found by increasing the number of receptor sites until the F-value reached a maximum. Contractile effects are given in g with s.e.mean. EC_{50} values are given as geometric means with 95% confidence limits in parentheses.

Results Endothelin-1 (0.1 nM–0.3 μM), endothelin-3 (1 nM–3 μM), sarafotoxin 6b (0.1 nM–0.3 μM) and sarafotoxin 6c (0.1 nM–30 nM) produced concentration-dependent contractions of pig coronary artery (Figure 1). The maximal effect elicited by sarafotoxin 6c (4.0 ± 0.7 g, $n = 14$) was about one third of that reached by endothelin-1 (11.5 ± 0.7 g, $n = 29$),

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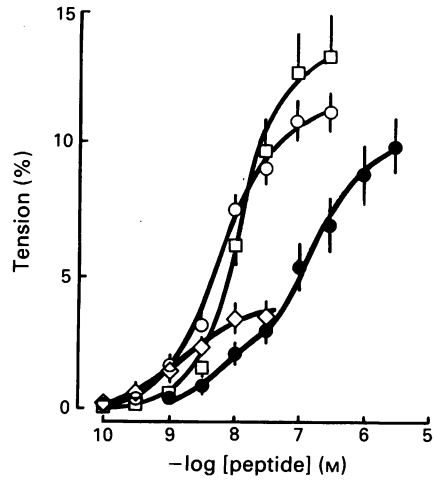

Figure 1 Concentration-response curves to endothelin-1 (○), endothelin-3 (●), sarafotoxin 6b (□) and sarafotoxin 6c (◇) for contraction of pig coronary artery. Data are means, with s.e.mean represented by vertical bars, of 29, 19, 15 and 14 values, respectively.

whereas those elicited by sarafotoxin 6b (14.5 ± 1.7 g, $n = 15$) and endothelin-3 (9.8 ± 1.0 g, $n = 19$) were not significantly different from the maximal effect of endothelin-1 ($P > 0.05$, Student's t test). Concentration-response curves to endothelin-1, sarafotoxin 6b and sarafotoxin 6c appeared to be monophasic and fitted best with a single receptor model (slope factors 1.04, 1.23 and 0.95, respectively). EC_{50} values were 6.7 nM (5.0–8.7) for endothelin-1, 14.8 nM (11.0–20.0) for sarafotoxin 6b and 1.6 nM (0.8–3.2) for sarafotoxin 6c. However, the curve to endothelin-3 was shallow (slope factor 0.64 in a one-receptor model) and fitted best with a two-receptor model. It was resolved into a higher sensitivity component representing 37% of the total effect (3.9 ± 0.7 g, slope factor 0.91), with an EC_{50} value of 9.6 nM (4.7–19.5) and a lower sensitivity component representing 63% of the total effect (6.7 ± 0.9 g, slope factor 1.00), with an EC_{50} value of 0.32 μM (0.20–0.54).

Concentration-response curves to endothelin-1, sarafotoxin 6b and endothelin-3 were performed in the presence of sarafotoxin 6c (0.3 μM; Figure 2). Sarafotoxin 6c (0.3 μM) itself produced a peak contraction followed by a stable plateau (1.3 ± 0.2 g, $n = 26$) after 30 min to 1 h. Subsequent curves to endothelin-1 (Figure 2a) or sarafotoxin 6b (Figure 2b) were not significantly shifted with respect to control curves. Thus, EC_{50} values for endothelin-1 were respectively 7.2 nM (4.0–13.2) and 9.1 nM (5.4–15.5) in paired control and sarafotoxin 6c-treated arteries ($n = 10$). E_{max} values (9.2 ± 1.1 g versus 9.7 ± 1.3 g) and slope factors (0.86 in both cases) were also unchanged. EC_{50} values for sarafotoxin 6b were 15.1 nM (8.1–28.2) and 12.6 nM (8.1–19.5) in paired control and sarafotoxin 6c-treated arteries ($n = 7$). E_{max} values for sarafotoxin 6b were 14.9 ± 3.0 g and 16.0 ± 2.5 g, slope factors 1.14 and 1.38, in paired control and sarafotoxin 6c-treated arteries.

In the presence of sarafotoxin 6c (0.3 μM), the higher sensitivity component of the endothelin-3 curve was abolished, whereas the lower sensitivity component remained unchanged compared to the control curve (Figure 2c). In control arteries ($n = 9$) the best fit was to a two-receptor model. The parameters of the higher sensitivity component were: E_{max} value 3.7 ± 0.9 g, slope factor 1.00, EC_{50} value 6.0 nM (2.5–14.8). Those of the lower sensitivity component were: E_{max} value 8.5 ± 1.4 g, slope factor 1.00, EC_{50} value 0.26 μM (0.14–0.47). By contrast, in paired sarafotoxin 6c-treated arteries, the curve to endothelin-3 now fitted best with a one-receptor model, yielding an EC_{50} value of 0.25 μM (0.13–0.47), a slope factor of 1.31 and an E_{max} value of 10.3 ± 1.6 g ($n = 9$).

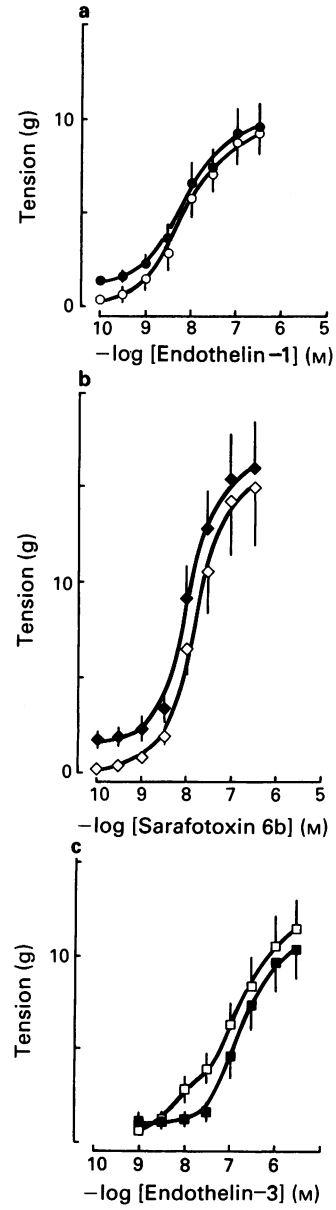

Figure 2 Concentration-response curves to (a) endothelin-1, (b) sarafotoxin 6b and (c) endothelin-3 in the absence (open symbols) and in the presence (closed symbols) of sarafotoxin 6c (0.3 μM). Data are means, with s.e.mean represented by vertical bars, of 10, 7 and 9 values, respectively. The proper effect of sarafotoxin 6c is taken into account.

Discussion Heterogeneity of functional endothelin receptors has been suggested in guinea-pig pulmonary artery, based on the observation that endothelin-3 did not exhibit cross-tachyphylaxis towards endothelin-1 (Cardell *et al.*, 1991). The present study provides good evidence that endothelin-3 stimulates two receptors to produce contraction of pig coronary artery. The concentration-response curve to endothelin-3 was clearly biphasic, showing a higher and a lower sensitivity component, at variance with those to endothelin-1, sarafotoxin 6b or sarafotoxin 6c. Furthermore, the higher sensitivity component disappeared in the presence of sarafotoxin 6c. By contrast, the lower sensitivity component of the endothelin-3 curve remained unchanged under these conditions, as did the EC_{50} values of endothelin-1 and sarafotoxin 6b.

These findings strongly suggest that the receptor involved in the higher sensitivity component of the endothelin-3 curve recognizes sarafotoxin 6c, but not endothelin-1 or sarafotoxin 6b under the present conditions. It follows that this receptor cannot be the ET_B receptor, which has roughly equal affinity for all four isopeptides (Sakurai *et al.*, 1990; Williams *et al.*, 1991). On the other hand, it is very unlikely that, at the concentration used (0.3 μM) sarafotoxin 6c acted on the receptor(s) stimulated by endothelin-1, sarafotoxin 6b and higher concen-

trations of endothelin-3. This, in addition to the rank order of potency endothelin-1 > sarafotoxin 6b > endothelin-3 (lower sensitivity component), is compatible with an action at the ET_A receptor (Arai *et al.*, 1990; Lin *et al.*, 1991). It remains, however, to be proven by the use of a selective antagonist.

In conclusion, there is evidence for at least two endothelin-sarafotoxin receptors mediating contraction of pig isolated coronary artery. One has the pharmacology of the ET_A receptor. The other recognizes sarafotoxin 6c and endothelin-3, but not endothelin-1 and sarafotoxin 6b. Hence it is different from the non-selective ET_B receptor. The relatively high potency of sarafotoxin 6c described in the present study might help explain why this peptide increases blood pressure *in vivo* (Williams *et al.*, 1991).

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MK801 attenuates behavioural adaptation to chronic nicotine administration in rats

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The effect of administering MK801 (0.3 mg kg^{-1}) during chronic nicotine (0.4 mg kg^{-1}) treatment was studied on locomotor activity in rats. Sensitization of the locomotor activity response to nicotine was seen with chronic nicotine treatment. This sensitization was attenuated in rats that had received MK801 30 min before the daily nicotine injections during chronic treatment. These results suggest that the NMDA receptor may be involved in the adaptive processes seen with chronic nicotine administration.

Keywords: Nicotine; locomotor activity; MK801; NMDA receptor

Introduction The N-methyl-D-aspartate (NMDA) antagonist MK801 [(+)-5-methyl-10,11-dihydro-5H-dibenzo(a,d)cyclohepten-5,10-imine] was developed initially for its ability in preventing neurological disorders such as epileptogenesis and neurodegeneration. However with the increasing number of behavioural studies, it is becoming evident that excitatory amino acid systems have a more central role in other behaviours and in learning and memory (Ward *et al.*, 1990).

Many responses of rats to morphine, amphetamines and cocaine are known to exhibit behavioural plasticity, and thus may show either tolerance or sensitization (reverse tolerance) with repeated exposures to the drugs (Goudie & Emmett-Oglesby, 1989). A recent series of reports has shown that MK801 blocks the development of sensitization or tolerance to several psychoactive substances. Both amphetamine- and cocaine-induced sensitization of locomotor stimulation in mice have been blocked (Karler *et al.*, 1989), as well as sensitization to cocaine-induced convulsions and stereotypy in rats (Karler *et al.*, 1989). Tolerance to the analgesic effects of morphine in rats can be attenuated by MK801 (Trujillo & Akil, 1991). In addition, MK801 attenuated the development of morphine-dependence as assessed by naloxone-precipitated withdrawal (Trujillo & Akil, 1991). Such findings suggest that diverse drugs can trigger a common mechanism of adaptation that is sensitive to MK801.

It was our aim to extend these findings by investigating whether MK801 could also attenuate an adaptive response seen with chronic administration of nicotine. Repeated exposures of rats to small doses of nicotine can produce sensitization to the locomotor stimulant effect (Clarke & Kumar, 1983; Ksir *et al.*, 1987). Larger doses of nicotine produce a strong depressant effect on locomotor activity to which tolerance develops rapidly with repeated exposures, thus unmasking the underlying locomotor stimulation (Clarke & Kumar, 1983; Ksir *et al.*, 1987).

Methods The animals used in this experiment were 32 male hooded Lister rats (Harlan Olac, Bicester) weighing between 250–300 g. Rats were individually housed with free access to food and water, in rooms maintained at 20–22°C with a regular light dark cycle (light from 08 h 00 min to 20 h 00 min).

Nicotine bitartrate (BDH, Dorset) and MK801 (donated by Merck, Sharpe and Dohme, Harlow) were each dissolved in saline and pH adjusted with NaOH to 7. Nicotine was administered subcutaneously (1 ml kg^{-1}) and MK801 was given intraperitoneally (2 ml kg^{-1}). Saline was injected as vehicle control.

Rats were randomly assigned to 4 groups. The regimen of chronic treatment for two groups was MK801 (0.3 mg kg^{-1}, i.p.) followed 30 min later by nicotine (0.4 mg kg^{-1}, s.c.; MK-N) or saline (MK-S). The other two groups received saline injection (i.p.) followed by nicotine (0.4 mg kg^{-1}, s.c.; S-N) or saline (SC; S-S). Rats were chronically treated daily as described above for 14 days, receiving half of their treatments before they were placed in locomotor activity cages for 60 min immediately after injection of nicotine or saline; on alternate days, they were returned to their home cages immediately after injection.

Following chronic treatment, each rat was tested with a randomised sequence of doses of nicotine (0.1, 0.2, 0.4, 0.8 mg kg^{-1}) and saline. Successive treatments were tested on alternate days, with only the usual chronic treatments given on intervening days. Rats were injected with nicotine or saline and were immediately placed into photocell activity cages (Clarke & Kumar, 1983). The number of cage crosses (movements from one beam of infra-red light to another) was recorded over 60 min. Results were analysed by one- and two-factor analyses of variance for repeated measures. Dunnett's *t* test was used as a test of significance at each dose utilised.

Results Illustrated in Figure 1 are activity scores for doses of nicotine in the four chronically treated groups. An initial two-factor analysis of variance for repeated measures showed a significant interaction of the chronic treatment regimen with

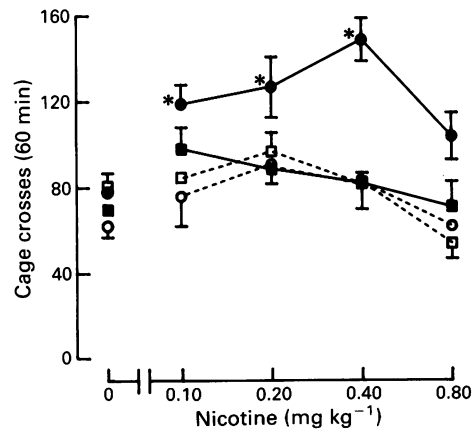

Figure 1 The effects of nicotine on mean locomotor activity in four groups of rats ($n = 8$) (s.e.mean shown by vertical bars). Rats were initially given daily chronic treatment consisting of two injections 30 min apart; saline-saline (S-S, ○), saline-nicotine (S-N, ●), MK801-saline (MK-S, □) and MK801-nicotine (MK-N, ■). Following 14 days of chronic treatment, each rat was tested with a randomized series of nicotine doses (0.1, 0.2, 0.4 and 0.8 mg kg^{-1}) and saline, and locomotor activity was measured. For each group of rats, asterisks denote significant increases above saline control scores as determined by Dunnett's *t* test (* $P < 0.01$).

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dose of nicotine [$F(12,112) = 2.47$, $P < 0.01$]. Separate single-factor analyses of variance were then calculated for each group.

No increases in activity were observed with graded doses of nicotine in S-S treated rats [$F(4,28) = 2.09$, NS]. However with chronic nicotine (S-N) treatment, dose-related increases in activity were apparent [$F(4,28) = 12.56$, $P < 0.001$]. Significant increases above control (saline) activity were noted with $0.1\,mg\,kg^{-1}$ ($P < 0.01$), $0.2\,mg\,kg^{-1}$ ($P < 0.01$) and $0.4\,mg\,kg^{-1}$ ($P < 0.01$) of nicotine (Figure 1). Analysis of the time-course of effects in these chronically-treated animals indicated prominent increases in activity during the second 30 min of the testing sessions: for instance with $0.4\,mg\,kg^{-1}$ nicotine, locomotor activity increased by 37% in the first 30 min, followed by an increase of 233% during the second 30 min. This behavioural sensitization was not seen when MK801 was administered before nicotine during chronic treatments. In such animals (M-N), no dose-related increases were observed [$F(4,28) = 2.7$, NS] with nicotine injections. Activity following $0.4\,mg\,kg^{-1}$ nicotine showed no increase in the first half of test sessions (0%), and an 88% increase during the second 30 min of the tests. Chronic treatment with MK801 alone (M-S), produced an overall significant change in locomotor activity with nicotine dose [$F(4,28) = 4.48$, $P < 0.01$], but Dunnett's t test showed that no individual dose of nicotine differed significantly from saline (Figure 1).

Discussion The present data show that when administered 30 min before daily doses of nicotine, MK801 can attenuate the locomotor stimulation seen with chronic nicotine treatment in rats. The vertical shift of the nicotine dose-response curve observed as a result of chronic nicotine treatment (Figure 1) was similar to that reported by Ksir *et al.* (1987). In accordance with observations of Clarke & Kumar (1983) the sensitization response was most prominent during the latter half of the test. MK801 administered before chronic nicotine treatment completely blocked this sensitization, whereas it did not influence locomotor activity during tests with rats that did not receive chronic treatment with nicotine. Nicotine may therefore be included amongst the other psychoactive drugs that are sensitive to MK801. A similarity in the mechanism of action of these psychoactive drugs suggests a mechanism for the effect of MK801. Morphine, amphetamine, cocaine and nicotine can all increase extracellular levels of dopamine at terminals of the mesolimbic dopamine system (Di Chiara & Imperato, 1988). MK801 may therefore be affecting the functioning of these dopamine terminals. Support for this linkage to dopamine comes from recent studies showing that MK801 can block (1) the priming effect of apomorphine on turning behaviour produced by the dopamine agonist SKF 38393 (Morelli & Di Chiara, 1990) and (2) long term sensitization of dopamine receptors with SKF 38393 (Criswell *et al.*, 1990).

An alternative explanation could be that MK801 may be acting in similar manner to mecamylamine, a centrally acting nicotinic antagonist which can block nicotine-induced locomotor activity (Clarke & Kumar, 1983). Recent *in vitro* experiments have provided evidence which support this hypothesis. MK801 blocked the ability of the potent nicotinic agonist (+)-anatoxin to open the ion channels in chronically denervated rat soleus muscles (Ramoa *et al.*, 1990). Furthermore MK801 also depressed nicotine-evoked γ-aminobutyric acid (GABA) release from rat hippocampal synaptosomes (Ramoa *et al.*, 1990). However these effects were obtained when MK801 was applied acutely before evoking these responses with nicotine or its analogues and were not examined in relation to adaptive responses to nicotine. It is also known that MK801 can impair learning in the radial arm maze (Ward *et al.*, 1990) and thus, it is conceivable that MK801 may block learning processes that might be involved in sensitization to nicotine. Sensitization to some drug effects may involve learning (Goudie & Emmett-Oglesby, 1989), but such evidence has not been forthcoming for nicotine.

The present study suggests that adaptive changes seen with chronic nicotine treatment may involve the NMDA receptor because the range of drugs known to be sensitive to MK801 can be extended to include nicotine. Tests with additional NMDA antagonists will be necessary to verify this interpretation. These adaptive changes are thought to play a contributory role in the initiation of the smoking habit and, therefore, excitatory amino antagonists may provide a novel approach for modifying adaptive processes in tobacco addiction.

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ATP modulates the efferent function of capsaicin-sensitive neurones in guinea-pig isolated atria

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1 The effect of adenosine triphosphate (ATP) and its stable analogues, α,β-methylene-ATP and β,γ-methylene-ATP, on the efferent function of capsaicin-sensitive non-adrenergic, non-cholinergic (NANC) nerves was tested in guinea-pig isolated atria.

2 Transmural nerve stimulation of atria isolated from reserpine-pretreated guinea-pigs, in the presence of 1 μM atropine and 0.3 μM CGP 20712A, induced a transient positive inotropic effect attributable to calcitonin gene-related peptide (CGRP) release from NANC nerve endings.

3 ATP (1–30 μM) concentration-dependently reduced the cardiac response to transmural nerve stimulation, without affecting the inotropic response to 10 nM exogenous CGRP. The inhibitory effect of ATP was competitively antagonized by the P_1-purinoceptor antagonist, 8-phenyltheophylline (8-PT, 1 μM), but was unaffected by the P_2-purinoceptor antagonist, suramin (100 μM).

4 β,γ-Methylene-ATP in the same concentration range as ATP, inhibited the cardiac response to transmural nerve stimulation. The inhibitory effect of β,γ-methylene ATP was antagonized by 1 μM 8-PT. The desensitizing agonist for P_2-purinoceptors, α,β-methylene ATP did not induce any inhibitory effect either on the cardiac response to transmural nerve stimulation or on the inhibitory effect curve for ATP.

5 The inhibitory effect of ATP on the NANC neurotransmission was inconsistently modified in the presence of 10 μM α,β-methylene-adenosine diphosphate, an inhibitor of the 5'-ectonucleotidases.

6 These results demonstrate that ATP modulates the efferent function of cardiac NANC nerve endings through prejunctional inhibitory receptors belonging to the P_1 type. The metabolic conversion of ATP to adenosine does not seem to be a pre-requisite for the ATP agonist activity.

Keywords: ATP; purinoceptors; NANC neurotransmission; capsaicin-sensitive neurones; guinea-pig atria

Introduction

Primary sensory neurones can have both an afferent and an efferent function due to the release of their transmitter content at the peripheral level (Maggi & Meli, 1988; Burnstock, 1990). In the cardiac tissue the presence of capsaicin-sensitive non-adrenergic, non-cholinergic (NANC) nerves has been demonstrated by immunohistochemical (Hougland *et al.*, 1986; Wharton *et al.*, 1986) and pharmacological studies (Saito *et al.*, 1986). The efferent function of the capsaicin-sensitive primary sensory nerves can be activated by different stimuli, including electrical field stimulation (Maggi & Meli, 1988). The antidromic invasion of capsaicin-sensitive neurones in cardiac tissue induces a positive inotropic effect, mainly due to calcitonin gene-related peptide (CGRP) release (Miyauchi *et al.*, 1987; Saito *et al.*, 1987; Maggi *et al.*, 1991). It has been reported that several substances, including opioid peptides (Mantelli *et al.*, 1989; 1990a; Giuliani *et al.*, 1990), α-adrenoceptor agonists (Mantelli *et al.*, 1990b), neuropeptide Y (Giuliani *et al.*, 1989a) and the peptide galanin (Giuliani *et al.*, 1989b) modulate the efferent function of capsaicin-sensitive neurones in cardiac tissue. Moreover it has been recently demonstrated that endogenous adenosine, released during the transmural nerve stimulation of guinea-pig isolated atria, exerts an inhibitory control of NANC neurotransmission through prejunctional A_1 receptors (Rubino *et al.*, 1990; 1991).

Adenosine shares with adenine nucleotides, including adenosine 5'-triphosphate (ATP), its inhibitory control of the neurotransmission process both in the central nervous system and in peripheral organs by reducing the transmitter release and altering the responses of the target organ to the transmitter released (Fredholm & Hedqvist, 1980; Williams, 1987). In the heart, adenosine and ATP, released during nerve activity and during the contractile response of the muscle cells to nerve stimulation or other stimuli, play a crucial role in the regulation of sympathetic neurotransmission by acting on specific cell membrane receptors (Hedqvist & Fredholm, 1979; Fredholm & Hedqvist, 1980).

A regulatory role for adenosine has been demonstrated on the cardiac NANC neurotransmission (Rubino *et al.*, 1990); however no information is available on the role of ATP in the modulation of NANC neurotransmission in the heart. Therefore in the present study we have investigated the effect of ATP on the efferent function of primary sensory neurones in guinea-pig isolated atria and have tested whether ATP was active *per se* or by its breakdown to adenosine. Moreover, since the purinoceptors have been classified into the P_1 and P_2 subtypes (Burnstock, 1978), we have also characterized the kind of purinoceptors involved in the inhibitory action of ATP.

Methods

Experimental procedure

The experimental model used for this study has been previously described in more detail (Mantelli *et al.*, 1989). Atrial preparations were isolated from male guinea-pigs (200–300 g) pretreated with reserpine (5 mg kg^{-1}, i.p.) 24 h before the experiment. Preparations were mounted vertically in an organ bath containing Tyrode solution of the following composition (mM): NaCl 115, KCl 4.7, $CaCl_2$ 1.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 10, oxygenated with 95% O_2 and 5% CO_2 and kept at the constant temperature of 30°C. The atria were stretched until the maximum force of contraction was reached and then maintained at this length throughout the experiment. The isometric contraction was recorded by an isometric transducer and a d.c. preamplifer on a pen recorder (Battaglia Rangoni KV 135) and on a dual beam oscilloscope (Tektronix D 13).

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Preparations were driven electrically at a constant rate (4 Hz) by punctate electrodes connected with a pulse generator (Tektronix type 161 pulse generator). Transmural nerve stimulation was performed by applying trains of field pulses (50–100 mA intensity; 1 ms duration) through two platinum plates, parallel to the preparations, connected with a second pulse generator (MARB 82/2/200). Trains of field pulses, consisting of two pulses for each of 40 consecutive contractions, were applied during the absolute refractory period in order to avoid interference with the normal rhythm of contractility. The parasympathetic component of the response to field stimulation was eliminated by the addition of 1 μM atropine to the Tyrode solution. Moreover 0.3 μM CGP 20712A (a β_1-adrenoceptor selective antagonist) was added to the solution in order to eliminate the residual adrenergic component of the response. As already described, in these experimental conditions the cardiac response to transmural nerve stimulation was abolished by the treatment with the peptidergic toxin capsaicin (Mantelli *et al.*, 1989) and was concentration-dependently reduced by the CGRP receptor antagonist, hCGRP (8–37), used at micromolar concentrations (Rubino *et al.*, 1991), thus confirming the hypothesis that NANC neurotransmission was being evaluated. After a period of equilibration of at least 60 min, trains of field pulses were applied at 15 min intervals. The response to transmural nerve stimulation was evaluated as the difference between the maximum force of contraction developed after transmural nerve stimulation and the basal contractility (ΔFc). Since the response to each train remained reproducible for many consecutive tests, it was possible to obtain cumulative concentration-effect curves for the agonists. Increasing concentrations of the agonists were added to the bathing solution after a control train; transmural nerve stimulation was then applied again after 5 min contact with all of the agonists tested. Only one concentration-effect curve was usually obtained in each preparation. The control response to field stimulation was taken as 100% and the effect of the agonist tested, alone and in the presence of antagonist, was evaluated as percentage inhibition of the control response. The incubation periods for suramin and 8-phenyltheophylline (8-PT) were 10 and 20 min of contact, respectively, before treating the atrial preparation with the agonist.

Drugs

Adenosine triphosphate (ATP), α,β-methylene-ATP, β,γ-methylene-ATP, α,β-methylene-ADP, suramin, rat calcitonin gene-related peptide (CGRP) and reserpine were purchased from Sigma Chemical Co., St. Louis, U.S.A. Atropine sulphate was obtained from BDH, Poole, England. 8-Phenyltheophylline (8-PT) was furnished by Calbiochem, San Diego, California, U.S.A.; 2-hydroxy-5-{2-[hydroxy-3-(4-[(1-methyl-4-trifluoromethyl)1H-imidazol-2-yl-]-phenoxy)-propyl]aminoethoxyl}-benzamide (CGP 20712A) was kindly supplied by Ciba Geigy, Basel, Switzerland.

A stock solution of 10 mM 8-PT was made up in 80% ethanol containing 0.2 M NaOH; further dilutions were made in distilled water.

Data evaluation

The data shown in the test and in the figures are reported as mean values $\pm$ s.e.mean. Statistical analysis was performed by means of Student's *t* test for unpaired data.

Results

Effect of ATP alone and in the presence of 8-phenyltheophylline (8-PT) or suramin

Transmural nerve stimulation of guinea-pig atria, isolated from reserpine-pretreated animals, in the presence of 1 μM atropine and 0.3 μM CGP 20712A, increased cardiac contractility from 341.8 $\pm$ 18.6 to 520.9 $\pm$ 26.0 mg (n = 36). The maximum inotropic response to transmural nerve stimulation was reached within about 40 s and cardiac contractility declined to basal values in about 5 min. A recording taken from a typical experiment showing the cardiac response to transmural nerve stimulation in control conditions and in the presence of 3 and 30 μM ATP is shown in Figure 1. It is evident that the addition of 1 μM 8-PT to the bathing solution reversed the inhibitory effect of 30 μM ATP. ATP (1–30 μM) reduced the cardiac response to transmural nerve stimulation in a concentration-dependent manner (Figure 2). The maximum effect consisted of 52.7 $\pm$ 3.4% inhibition of control response to transmural nerve stimulation and was almost

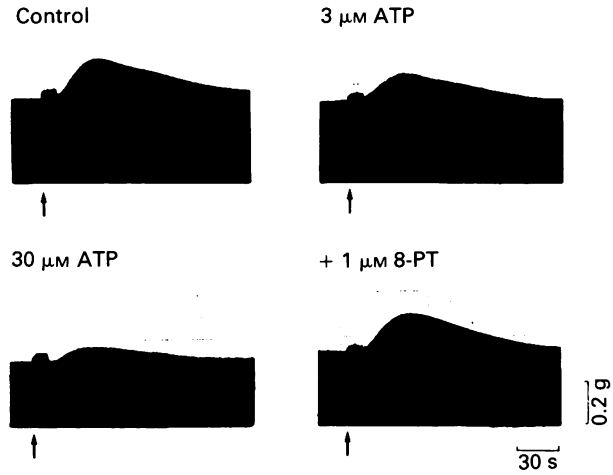

Figure 1 Records taken from a typical experiment showing the response to transmural nerve stimulation on atria obtained from reserpine-pretreated guinea-pig, in the presence of 1 μM atropine and 0.3 μM CGP 20712A. The cardiac response to transmural nerve stimulation before and after treatment with increasing concentrations (3 and 30 μM) of ATP is shown. The addition to the bathing solution of 1 μM 8-phenyltheophylline (8-PT) reversed the inhibitory effect of 30 μM ATP. The arrows indicate the beginning of transmural nerve stimulation.

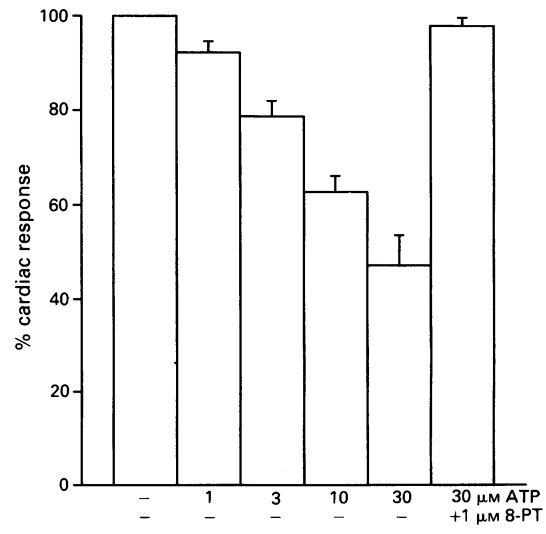

Figure 2 Concentration-dependent inhibitory effect of ATP on the cardiac response to transmural nerve stimulation. The increase in cardiac contractility induced by transmural nerve stimulation was taken as 100%. 8-Phenyltheophylline (8-PT) was added to the bathing solution in the presence of the highest ATP concentration. Means of 6 experiments with s.e.mean shown by vertical bars.

completely abolished by 1 μM 8-PT (Figure 2). At concentrations of 10 and 30 μM, ATP had a slight direct negative inotropic effect; the basal contractility was reduced from 341.6 ± 56.9 to 309.6 ± 54.4 mg in the presence of 10 μM ATP ($n = 6$) and to 266.6 ± 50.7 mg, in the presence of 30 μM ATP ($n = 6$). Although a 100% inhibition of the cardiac response to transmural nerve stimulation was never reached, higher concentrations of ATP were not tested to prevent the direct negative inotropic effect from interfering with the evaluation of the ATP reduction in cardiac response to electrical stimulation.

The concentration-inhibitory effect curve for ATP was not affected by a concentration of suramin (100 μM) (Figure 3) that fully antagonizes P_2-purinoceptor activation. On the other hand, the control curve for ATP was shifted to the right in a parallel fashion by 1 μM 8-PT (Figure 3). The basal cardiac contractility was affected neither by suramin not by 8-PT. Whereas 1 μM 8-PT increased the control response to transmural nerve stimulation by about 30% (ΔFc was 193 ± 16 and 243 ± 22.3 mg in the absence and presence of 8-PT, respectively) as already reported (Rubino *et al.*, 1990), suramin did not affect the cardiac response to transmural nerve stimulation.

Effect of ATP on the cardiac response to exogenous calcitonin gene-related peptide

At the highest concentration tested, ATP did not modify the positive inotropic effect of exogenous CGRP, thus suggesting a prejunctional inhibitory effect of ATP. A concentration of CGRP (10 μM) was selected that produced a positive inotropic response superimposable on that obtained with transmural nerve stimulation of atrial preparations. When 10 nM CGRP was added to the bathing solution in the absence and in the presence of 30 μM ATP the cardiac contractility increased from 329.2 ± 20.4 to 516.0 ± 31.6 mg ($n = 10$) and from 263.5 ± 34.3 to 441.7 ± 36.7 mg ($n = 8$), respectively. As reported above, 30 μM ATP reduced the basal contractility by about 20%, but the preparations were still able to respond to the exogenously applied CGRP, the ΔFc being 187.4 ± 19.7 and 178.3 ± 27.1 mg in the presence and absence of ATP, respectively.

Effects of stable analogues of ATP, α,β-methylene-ATP and β,γ-methylene-ATP

The effect of slowly degradable ATP analogues on the cardiac response to transmural nerve stimulation was tested. α,β-Methylene-ATP, which first activates and then desensitizes selectively the P_2-purinoceptor at micromolar concentrations did not show any effect either on the cardiac response to transmural nerve stimulation or on the basal contractility (data not shown). Furthermore, in preparations exposed to a desensitizing concentration of α,β-methylene-ATP (50 μM) for at least 20 min the concentration-inhibitory effect curve for ATP was not statistically different from that for ATP alone (Figure 3). These observations argue against the involvement of P_2-purinoceptors in mediating the effect of ATP.

β, γ-Methylene-ATP, in the same concentration range as ATP (1–30 μM), concentration-dependently reduced the cardiac response to transmural nerve stimulation (Figure 4), without affecting basal cardiac contractility in a significant way. The maximum inhibitory effect reached with 30 μM β,γ-methylene-ATP consisted of 58.8 ± 4.5% inhibition of the control response to transmural nerve stimulation, and was not statistically different from the maximum inhibitory effect of ATP. The inhibitory effect of β,γ-methylene-ATP at the highest concentration tested (30 μM) was completely antagonized by 1 μM 8-PT (Figure 4).

Effect of ATP in the presence of α,β-methylene-ADP

The effect of ATP in the presence of the inhibitor of 5'-ectonucleotidases, α,β-methylene-ADP, used at a concentration fully active in inhibiting the enzyme in the cardiac tissue was evaluated. The inhibitory effect of increasing concentrations of ATP in the presence of 10 μM α,β-methylene-ADP, compared to the effect of ATP in control conditions, is shown in Figure 5. ATP partially lost its inhibitory effect on the cardiac response to transmural nerve stimulation only at the lower concentrations used (1 and 3 μM). However it was still able to reduce the response to transmural nerve stimulation by 34.9 ± 7.2 and 56.0 ± 9.1% at 10 and 30 μM, respectively. The effect of 30 μM ATP alone and in the presence of α,β-methylene-ADP was reversed by 1 μM 8-PT (not shown). α,β-Methylene-ADP did not have any effect either on basal con-

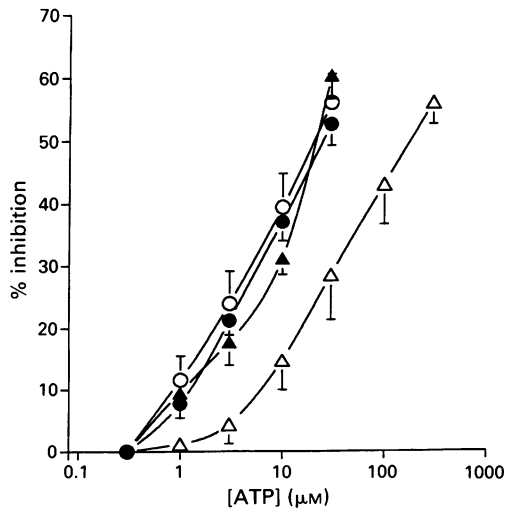

Figure 3 Concentration-inhibitory effect curves for ATP alone (●; $n = 6$) and in the presence of 100 μM suramin (○; $n = 10$), 1 μM 8-phenyltheophylline (8-PT; △; $n = 6$) and 50 μM α,β-methylene-ATP (▲; $n = 6$) on the cardiac response to transmural nerve stimulation. The response to transmural nerve stimulation obtained before drug addition was considered as 100% and the effect of ATP, alone and in the presence of suramin, 8-PT or α,β-methylene ATP, was evaluated as percentage inhibition of the control response.

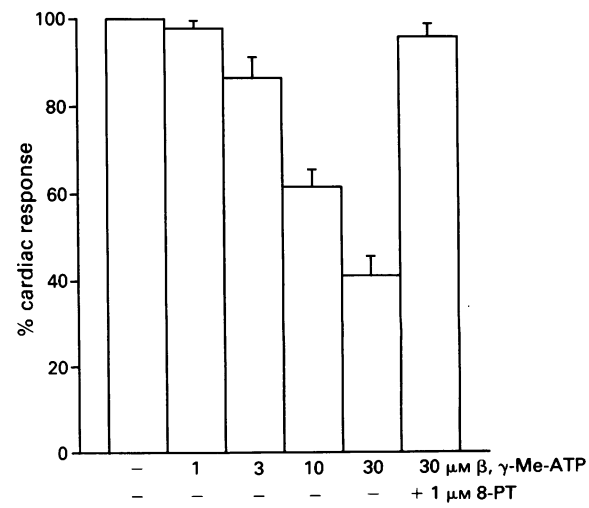

Figure 4 Concentration-dependent inhibitory effect of β,γ-methylene-ATP (β,γ-Me-ATP) on the cardiac response to transmural nerve stimulation. The increase in cardiac contractility induced by transmural nerve stimulation was taken as 100%. 8-Phenyltheophylline (8-PT) was added to the bathing solution in the presence of the highest β,γ-methylene-ATP concentration. Means of 4 experiments with s.e.mean shown by vertical bars.

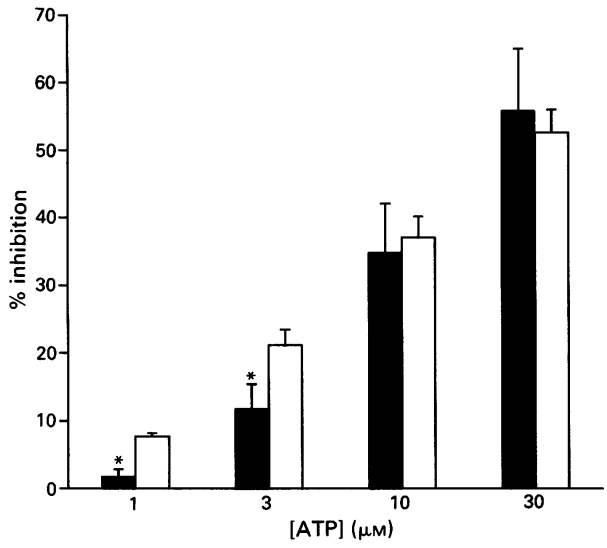

Figure 5 Inhibitory effect of increasing concentrations of ATP alone ($n = 6$, open columns) and in the presence of 10 μM α,β-methylene-ADP ($n = 6$, solid columns). The response to transmural nerve stimulation obtained before drug addition was taken as 100% and the effect of ATP alone and in the presence of α,β-methylene-ADP was evaluated as percentage inhibition of the control response. * for at least $P < 0.05$.

tractility or on the control response to transmural nerve stimulation.

Discussion

Evidence has been produced indicating that purines are released as cotransmitters or neuromodulators following nerve stimulation in various peripheral organs, including the heart (Fredholm & Hedqvist, 1980). Besides the neuronal origin for adenosine and ATP, purine release has been demonstrated to occur in cardiac muscle cells, in response to nerve stimulation or other noxious stimuli, such as hypoxia or ischaemia (Forrester & Williams, 1977; Rubio & Berne, 1985). This last condition has been reported to activate the efferent function of capsaicin-sensitive NANC cardiac neurones (Franco-Cereceda *et al.*, 1989). In addition, the presence of quinacrine-positive fibres containing ATP has been demonstrated in guinea-pig atria (Crowe & Burnstock, 1982). Recent evidence indicates the presence of receptors for ATP on intracardiac neurones in culture, supporting the hypothesis that both adenosine and adenine nucleotides play a role in the regulation of cardiac function (Allen & Burnstock, 1990). Actually adenosine and ATP regulate adrenergic neurotransmission in the heart through pre- and postsynaptic effects mediated by specific receptors (Hedqvist & Fredholm, 1979; Fredholm & Hedqvist, 1980; Burnstock, 1980). Adenyl compounds may also have a physiological modulatory action on acetylcholine release from cholinergic nerve terminals in the heart (Burnstock, 1980).

In addition to being controlled by sympathetic adrenergic and parasympathetic cholinergic nerves, cardiac functions may be controlled by NANC nerves, present in the heart of several species including man (Franco-Cereceda, 1988). We have recently demonstrated that endogenous adenosine regulates cardiac NANC neurotransmission through prejunctional inhibitory receptors belonging to the A_1 subtype (Rubino *et al.*, 1990; 1991). In the present study we demonstrate that the adenosine nucleotide ATP exerts an inhibitory control of cardiac NANC neurotransmission in guinea-pig isolated atria, which seems to be mediated by prejunctional receptors. ATP, at the highest concentrations tested, induced a slight negative inotropic effect, as previously observed by other authors (Burnstock & Meghji, 1981). However, the maximum inhibitory effect of cardiac response to transmural nerve stimulation induced by 30 μM ATP was more than two times greater than the direct negative inotropic effect induced by the same concentration of ATP. Moreover ATP, at the highest concentration tested, did not modify the inotropic effect of exogenous CGRP, thus indicating a prejunctional activity. The inhibitory effect of ATP on cardiac NANC neurotransmission appeared to be mediated by receptors sensitive to methylxanthines, since 8-PT, a potent P_1-purinoceptor antagonist (Griffith *et al.*, 1981), competitively antagonized the inhibitory effect of ATP. On the other hand, suramin, a reversible antagonist at the P_2-purinoceptor (Dunn & Blakeley, 1988; Hoyle *et al.*, 1990), did not affect ATP inhibitory activity in our experimental model. Thus our results indicate that the inhibitory effect of ATP on cardiac NANC neurotransmission is mediated by prejunctional P_1-purinoceptors. This hypothesis is further supported by results obtained with the selective P_2-purinoceptor agonist, α,β-methylene-ATP, which was used as a tool for revealing the presence of P_2 purinoceptors, because of its ability to activate and then desensitize this kind of receptor (Kasakov & Burnstock, 1983). If P_2-purinoceptors were involved in ATP activity it would not be possible to observe the inhibitory effect of ATP, after challenging the atrial preparations with the desensitizing agonist, α,β-methylene-ATP. However in our experimental model α,β-methylene ATP did not show either any direct effect or reduction of ATP inhibitory activity, thus excluding the involvement of a P_2-purinoceptor in the ATP effects.

In the heart, ATP is rapidly hydrolyzed to form 5'-AMP and adenosine (Hopkins, 1973), the conversion of 5'-AMP by 5'-ectonucleotidases being the final step in the hydrolysis of ATP to adenosine (Pearson *et al.*, 1980). This enzyme is inhibited by α,β-methylene-ADP (Bruns, 1980). It could be suggested that ATP must be converted to adenosine in order to cause its presynaptic action on the cardiac NANC neurotransmission. However, our observation that in the presence of the enzyme inhibitor, α,β-methylene-ADP, only the lowest concentrations of ATP tested partly lost their inhibitory effect, indicates that conversion to adenosine is not a prerequisite for the ATP agonist activity at the P_1-purinoceptor. These findings suggest that ATP can be active by itself at this receptor, as already shown in the guinea-pig atria (Burnstock & Meghji, 1981; Collis & Pettinger, 1982). Further evidence that the breakdown of ATP is not necessary for activation of the P_1-purinoceptor is provided by the observation that the hydrolysis resistant β,γ-methylene isostere of ATP showed potent P_1-purinoceptor agonist activity. Actually, β,γ-methylene-ATP was active at the same concentrations as ATP in inhibiting NANC neurotransmission and its inhibitory activity was reversed by 8-PT. Moreover, at the concentrations tested, β,γ-methylene-ATP was less active than ATP in exerting the direct negative inotropic effect, as previously reported by Burnstock & Meghji (1981). The agonist activity of ATP analogues, including β,γ-methylene-ATP, at P_1-purinoceptors, antagonized by xanthines has already been demonstrated by other authors (Burnstock & Meghji, 1981; Wiklund & Gustaffson, 1986).

In conclusion, our findings provide the first evidence that ATP inhibits the efferent function of capsaicin-sensitive NANC neurones in cardiac tissue. Although it was not possible to support this evidence with measurements of CGRP release, the findings clearly point out a prejunctional site of action, in which P_1 purinoceptors are involved.

Similar results have been obtained in another peripheral preparation, the guinea-pig bronchi by Kamikawa & Shimo (1989). In this preparation, electrical field stimulation induced a noncholinergic functional response due to substance P release, which was inhibited by both ATP and adenosine through prejunctional P_1-purinoceptors.

Furthermore, we have also demonstrated that the degradation of ATP to adenosine by ectoenzymes does not seem to be a prerequisite for activation of the P_1-purinoceptors, although

a part of the ATP effect might be attributable to its conversion to adenosine. Thus endogenous adenosine, as already reported (Rubino *et al.*, 1990), and ATP, as demonstrated in the present study, regulate the peripheral function of capsaicin-sensitive cardiac neurones. The potential interplay between the endogenous purine nucleosides and nucleotides seems to represent a complex and ductile mechanism by which the efferent function of NANC neurotransmission can be regulated.

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Cardiovascular actions of the κ-agonist, U-50,488H, in the absence and presence of opioid receptor blockade

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1 The cardiovascular actions of U-50,488H, a κ-receptor agonist, were studied in rat isolated perfused hearts, and in anaesthetized rats, over concentrations or doses generally above those required to produce κ-receptor-mediated effects.

2 U-50,488H dose-dependently decreased left-ventricular peak systolic pressure and beating rate *in vitro* and reduced blood pressure and heart rate *in vivo*.

3 Over the concentration range of 1–30 μM *in vitro*, and the dose-range of 0.5–32 μmol kg^{-1} *in vivo*, U-50,488H prolonged the P–R, QRS and Q–T intervals of the ECG.

4 The effects of U-50,488H were not antagonized by an opioid receptor antagonist, naloxone (1 μM or 8 μmol kg^{-1}). Similarly, the opioid receptor antagonist, MR 2266, at 8 μmol kg^{-1} did not significantly reduce the cardiovascular actions of U-50,488H *in vivo*.

5 The actions of U-50,488H on responses to electrical stimulation were also studied. Over the dose range of 0.5–32 μmol kg^{-1}, U-50,488H altered thresholds and effective refractory period. It had a biphasic action on thresholds for induction of ventricular fibrillation. Thresholds were decreased at lower doses (0.5–4 μmol kg^{-1}) but increased at higher doses (8–32 μmol kg^{-1}). The effects of lower doses were blocked by naloxone. Effective refractory period and threshold pulse width only increased with dose.

6 In conclusion, U-50,488H at high concentration, had direct depressant actions on cardiac contractility, electrical excitability and the ECG. These depressant effects were not antagonized by the opioid receptor antagonists, naloxone and MR 2266, and probably do not involve opioid receptors. Furthermore, some of the observed effects were those expected to result from sodium channel blockade.

Keywords: U-50,488H; ECG; electrical stimulation; sodium channel blockade; naloxone; MR 2266; rat hearts

Introduction

U-50,488H, a selective κ-receptor agonist (Lahti *et al.*, 1982), has been shown to induce ventricular arrhythmias in the isolated perfused heart of the rat when given as a bolus of 44 or 132 nmol (Wong *et al.*, 1990). Further, the incidence and severity of ischaemia and reperfusion-induced arrhythmias are reduced by the opioid receptor antagonists naloxone (Sarne *et al.*, 1991) and MR 2266 (Wong *et al.*, 1990). Other studies suggest that κ-receptor blockade may be antiarrhythmic (Sitsapesan & Parratt, 1989) whereas buprenorphine, an agent with mixed actions on μ- and κ-receptors, may exert antiarrhythmic effects directly, independent of opioid receptors (Boachie-Ansah *et al.*, 1989). In view of the possibility that U-50,488H has direct cardiac effects, as well as the fact that the bolus doses used by Wong *et al.* (1990) may have produced transient high concentrations, we decided to extend that study. We used a wide range of steady state concentrations and compared effects *in vitro* with those *in vivo* to determine whether the former actions could be observed in intact animals.

U-50,488H has recently been shown to exert sodium channel blocking actions in neuronal tissue; actions which are not reversed by opioid receptor antagonists (Alzheimer & Ten Bruggencate, 1990). It thus appeared useful to examine U-50,488H for cardiovascular actions which could relate to sodium channel blocking actions in cardiac tissue and to use naloxone to block possible opioid-receptor-mediated effects so revealing direct cardiac effects.

Known sodium channel blockers *in vivo* depress blood pressure and heart rate, prolong P–R and QRS intervals and depress electrical excitability. Concentrations *in vitro* corresponding to effective plasma concentrations *in vivo* depress contractility, lower heart rate and widen P–R and QRS intervals in rat isolated hearts (Walker & Beatch, 1988; Abraham *et al.*, 1989; Howard & Walker, 1990). Such concentrations also depress the rise rate of phase 0 of intracellular action potentials.

We have systematically investigated the actions of U-50,488H in rat isolated hearts and in pentobarbitone-anaesthetized rats. We assessed the effects of U-50,488H on left-ventricular peak systolic pressure, heart rate and the ECG *in vitro*. *In vivo*, blood pressure, heart rate and the ECG effects were examined together with sensitivity to electrical stimulation of the left ventricle. Results suggested that, at concentrations or doses greater than those required to produce agonism at κ-receptors, U-50,488H blocked sodium channels in rat heart.

Methods

Male Sprague-Dawley rats (150–350 g) were used in accordance with the guidelines of the University of British Columbia's Animal Care Committee. Intact rats were anaesthetized with pentobarbitone (60 mg kg^{-1}, i.p.). When required, the trachea was cannulated for artificial ventilation at a stroke volume of 10 ml kg^{-1}, 60 strokes min^{-1}. Body temperature was monitored by rectal thermometer and maintained between 36–37°C with a heating lamp.

Isolated hearts

The procedure described by Curtis *et al.* (1986) was used. Rats were killed and their hearts excised for immediate perfusion with cold Krebs-Henseleit solution. Thereafter they were mounted on a modified Langendorff apparatus (Curtis *et al.*, 1986) and perfused at a constant pressure of 100 mmHg with oxygenated Krebs-Henseleit solution (pH 7.4), and at a temperature of 37°C.

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The left atrium was removed and a compliant balloon inserted into the left ventricle. The end diastolic pressure in the balloon was adjusted to 10 mmHg with approximately 0.5 ml of saline. Pressures within the balloon were monitored on a Grass Polygraph (model 7D) while the maximum rate of intraventricular pressure development ($+dp.dt^{-1}_{max}$) was obtained with a Grass differentiator (model 7P20C).

The ECG was recorded via two atraumatic silver-ball electrodes placed on the epicardial surface of the right atrium and left ventricle, respectively. The ECG was recorded on a Grass Polygraph at a bandwidth of 0.1–40 Hz. All hearts were of similar weight (1.2–1.6 g).

A random block design was used to assign hearts to the following groups: saline, and U-50,488H (at 1, 3, 10 and 30 μM for 10 min each) in the presence or absence of 1 μM naloxone, a concentration at least 100× the pA_2 (Martin, 1983). Hearts were left to stabilize for 10 min before addition of saline vehicle, or naloxone, 10 min prior to dosing with U-50,488H.

In vivo *effects of U-50,488H*

In pentobarbitone-anaesthetized, and artificially ventilated rats ($n = 6$ per group), the left carotid artery and right jugular vein were cannulated. Dose-response curves were constructed for U-50,488H with cumulative doses of 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 and 32.0 μmol kg^{-1} i.v. Each dose was infused over 2 min and the blood pressure, heart rate and ECG were recorded 10 min later, just before addition of the next dose.

In a separate experiment, and according to a random and blind design, either saline, naloxone (8 μmol kg^{-1}) or MR 2266 (8 μmol kg^{-1}) was administered before giving U-50,488H at 16 μmol kg^{-1}. All rats were allowed to stabilize for 10 min before receiving the first injection of saline, or either of the two opioid antagonists. The dose of U-50,488H was chosen from the previous dose-response study as one that produced significant, but not maximal, responses which could be expected to be blocked completely by the high doses of antagonists used. We performed an initial *in vivo* trial study with naloxone and the dose we decided to use was the highest dose without effect on the ECG, blood pressure or heart rate and much higher than the pA_2 (Martin, 1983). This chosen dose could be expected to block effectively any opioid receptor-dependent effects of U-50,488H, even at the highest doses.

Electrical stimulation studies

In pentobarbitone-anaesthetized rats the left-carotid artery and right jugular vein were cannulated. Two Teflon-coated silver wire stimulating-electrodes were inserted through the chest wall and implanted in the left ventricle (Walker & Beatch, 1988). The inter-electrode distance was approximately 1 mm and square wave stimulation was used to determine: threshold current for capture (iT–μA), threshold pulse width for capture (tT–ms), maximum following frequency (MMF–Hz), ventricular fibrillation threshold (VFt–μA) and effective refractory period (ERP–ms) according to previously described methods (Howard & Walker, 1990).

The variables iT and tT approximate to the rheobase and chronaxie, respectively, of the i vs t curve and are a measure of sodium channel availability. Unfortunately, since iT and tT cannot be measured under rigidly controlled conditions they are imperfect measures. ERP and MFF are related such that it might be expected that MFF(Hz) = 1000/ERP(ms). However, although the two are similar they are sufficiently different to warrant reporting both. Thus while ERP, as measured, is a reasonable measure of effective refractive period, MFF is more a measure of relative refractory period, and ventricular functional refractory period, and thus can exhibit a different sensitivity to drugs from ERP. The process of determination of MFF, namely a steadily increasing frequency of stimulation, can be associated with accumulation of extracellular K^+ thereby adding an extra component to what would otherwise be another measure of ERP. MFF and ERP are not equally sensitive to frequency-dependent sodium channel blockers (Walker & Beatch, 1988). Stimulation studies were performed only *in vivo* since results are more predictable in the intact animal than in isolated hearts. The full profile of U50,488H on the various stimulation variables is given here to allow for comparison with the profiles obtained for other Class 1 anti-arrhythmic drugs (e.g. as in Abraham *et al.*, 1989).

The stimulation variables were determined 5 min after each dose of saline, or U-50,488H, in the absence or presence of a single previous dose (8 μmol kg^{-1}) of naloxone. U-50,488H was given cumulatively at 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 and 32.0 μmol kg^{-1}.

Drugs

The drugs used were: U-50,488H (trans-(±)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]-benzene-acetamide methane sulphonate), naloxone and MR 2266 ((−)-5,9-alpha-diethyl)-2-(3-furylmethyl-2′-hydroxy-6,7-benzomorphan). U-50,488H and MR 2266 were generous gifts from the Upjohn Company and Boehringer Ingelheim (Canada) Ltd., respectively. Naloxone hydrochloride was purchased from DuPont Pharmaceutical Co. All drugs were prepared as stock solutions dissolved in a 0.9% NaCl solution for *in vivo* studies. Drugs were dissolved in Krebs-Henseleit for *in vitro* studies.

Statistical analysis

Statistical analyses were performed with the NCSS statistical package (Hintze, 1987) and values are presented as mean ± s.e.mean. General Linear Model ANOVA was used for balanced studies and multiple comparisons made by Duncan's test. A difference at $P < 0.05$ was considered significant. The effects of U-50,488H are expressed as changes from the pre-U-50,488H value in the absence, or presence, of naloxone. Two types of statistical comparisons were made. With 'raw' data, simple comparisons of means were made. However, in dose-response studies, the mean percentage changes induced by different doses of U50,488H were compared with zero in a manner analogous to that used in t tests for differences.

Results

Figure 1 shows the effects of U-50,488H on peak systolic left ventricular pressure and heart rate in terms of changes from the pre-U-50,488H values in isolated hearts. Corresponding changes in ECG intervals recorded from rat isolated perfused hearts are shown in Figure 2.

U-50,488H dose-dependently reduced peak-systolic left ventricular pressure and heart rate, while the maximum rate of intra-ventricular pressure development ($+dp.dt^{-1}_{max}$) was also reduced (data not shown). It also dose-dependently prolonged both P–R interval and QRS duration. All of the above effects were still present in the presence of 1 μM naloxone. The only notable difference between the dose-response curves in the presence or absence of naloxone was for a tendency for higher peak systolic pressure in the presence of naloxone. The effects of naloxone on isolated hearts were not statistically different from those of saline alone.

In intact pentobarbitone-anaesthetized rats, U-50,488H dose-dependently lowered both blood pressure and heart rate (Figure 3a). In addition, it prolonged the P–R and Q–T intervals and widened the QRS complex in a dose-dependent manner (Figure 3b). In both Figure 3a and b changes from pre-drug values are shown. Statistically significant depression of blood pressure and heart rate occurred after 0.5 μmol kg^{-1} whereas larger doses (2–4 μmol kg^{-1}) had to be given before statistically significant changes were seen in P–R interval and QRS width. In the separate experiment summarized in Table 1, blood pressure, heart rate and ECG changes induced by 16 μmol kg^{-1} U-50,488H were not prevented by pretreatment

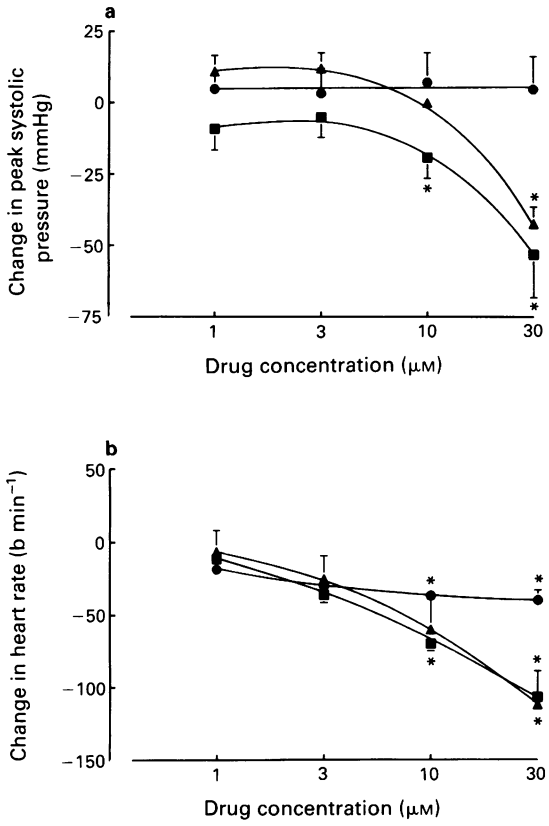

Figure 1 Dose-related effects of U-50,488H on peak systolic ventricular pressure and heart rate in rat isolated perfused hearts: (a) shows changes, from pre-drug values, in peak systolic pressure (mmHg) and (b) heart rate (beats min^{-1}) from pre U-50,488H values. The symbol (●) is for data from control animals treated with saline alone, (■) for U-50,488H treatment, and (▲) for U-50,488H in the presence of naloxone (1.0 μM). Each point is mean with s.e.mean shown by vertical bars, $n = 5$. * $P < 0.05$ for difference from zero change. The pre U-50,488H, or saline, means ranged from 259 ± 11 to 269 ± 12 beats min^{-1} for heart rate and 129 ± 5 to 145 ± 8 mmHg for peak systolic left ventricular pressure.

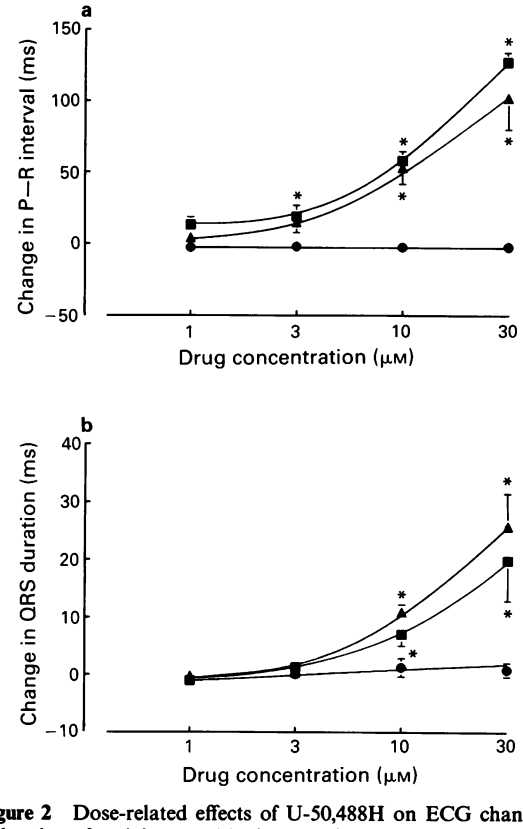

Figure 2 Dose-related effects of U-50,488H on ECG changes in rat isolated perfused hearts: (a) changes, from pre-drug values, in P-R interval (ms) and (b) QRS width (ms). The symbol (●) is for saline control alone, (■) for U-50,488H and (▲) for U-50,488H in the presence of naloxone (1.0 μM). Values are mean with s.e.mean shown by vertical bars, $n = 5$. * $P < 0.05$ for difference from zero change. The pre U-50,488H, or saline, means ranged from 45 ± 2 to 47 ± 3 ms, for P–R, and 33 ± 1 to 35 ± 1 ms for QRS.

with high doses of either MR 2266 or naloxone. The antagonist drugs alone had limited effects; naloxone did not change any of the variables whereas MR 2266 induced slight bradycardia and P–R prolongation (Table 1).

Table 1 The effects of U-50,488H at a dose of 16 μmol kg^{-1} on blood pressure, heart rate and ECG in pentobarbitone-anaesthetized rats in the absence or presence of the opioid antagonists, naloxone and MR 2266 (both at 8 μmol kg^{-1})

Treatment	*Pretreatment*	*Post-treatment*	*Post U-50,488H*	%
		BP (mmHg)		
Saline	132 ± 4	136 ± 6	107 ± 5†	(−20 ± 6)†
Naloxone	137 ± 6	143 ± 5	120 ± 8†	(−16 ± 7)†
MR 2266	136 ± 7	142 ± 9	118 ± 11†	(−18 ± 4)†
		HR (beats min^{-1})		
Saline	365 ± 13	364 ± 6	261 ± 14†	(−25 ± 2)†
Naloxone	405 ± 20	403 ± 25	323 ± 24†	(−20 ± 3)†
MR 2266	389 ± 1	337 ± 20*	271 ± 12†	(−22 ± 3)†
		P–R (ms)		
Saline	49 ± 1	48 ± 1	58 ± 2†	(+18 ± 4)†
Naloxone	47 ± 2	48 ± 2	57 ± 2†	(+20 ± 3)†
MR 2266	48 ± 1*	54 ± 2*	58 ± 2†	(+8 ± 3)†
		QRS (ms)		
Vehicle	29.6 ± 0.7	30.0 ± 0.7	32.8 ± 0.4†	(+10 ± 2)†
Naloxone	28.6 ± 0.4	29.4 ± 0.2	31.8 ± 0.2†	(+8 ± 2)†
MR 2266	30.0 ± 0.8	31.1 ± 0.5	33.8 ± 0.2†	(+9 ± 2)†

Values are mean ± s.e.mean, $n = 5$. BP = mean blood pressure; HR = heart rate. Values in parentheses are the percentage changes between post-treatment and post U-50,488H values. After administration of U-50,488H all values were statistically significantly (†) different ($P < 0.05$) from the pre-U-50,488H, i.e. post-saline, naloxone or MR 2266 treatment values. * $P < 0.05$ for means after treatment with either saline, naloxone or MR 2266 versus pretreatment means.

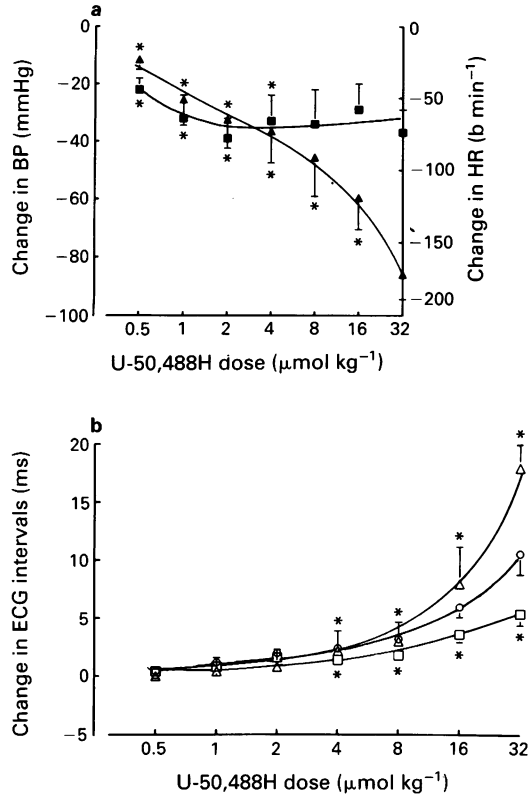

Figure 3 Dose-related effects of U-50,488H on blood pressure, heart rate and the ECG in pentobarbitone-anaesthetized rats: (a) changes, from pre-drug values, in blood pressure (mmHg) and heart rate (beats min^{-1}) while (b) shows the corresponding changes in P–R, QRS and Q–T (ms). The symbol (■) is for blood pressure, (▲) for heart rate, (△) for P–R interval, (□) for QRS and (○) for Q–T. Values are mean with s.e.mean shown by vertical bars, $n = 5$–10. Significant difference from zero change at $^*P < 0.05$. The pretreatment means were 123 ± 8 mmHg for blood pressure, 381 ± 26 for heart rate, 47 ± 1 ms for P–R, 29 ± 1 for QRS and 42 ± 1 for Q–T.

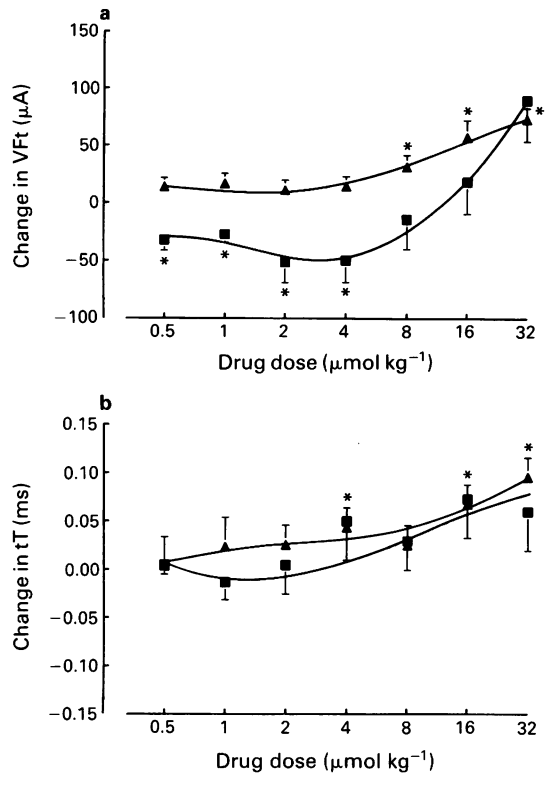

Figure 4 Effects of U-50,488H on ventricular fibrillation threshold (VFt) (a) and threshold pulse width (tT) (b) in pentobarbitone-anaesthetized rats subject to electrical stimulation. The symbol (■) indicates changes from pre-drug values with U-50,488H alone, or in the presence of naloxone pretreatment (▲). Saline control values are not shown but are indicated in the text. Values are mean with s.e.mean shown by vertical bars, $n = 5$–12. $^*P < 0.05$ for difference from zero change. Control (pre-drug) values ranged from 208 ± 15 to $265 \pm 25\,\mu$A for VFt and 53 ± 3 to 54 ± 4 ms for tT.

Figures 4 and 5 summarize the effects of U-50,488H on responses to electrical stimulation in intact rats expressed as changes from values just prior to administration of U-50,488H. Data for saline control rats showed no changes with time over the experimental period. Thus values at the beginning and end of the experimental period in the saline-treated group were 240 ± 25 and $294 \pm 36\,\mu$A for VFt, 0.32 ± 0.04 and 0.27 ± 0.03 ms for tT, 53 ± 3 and 48 ± 4 ms for ERP and 13.9 ± 0.6 and 14.7 ± 0.7 Hz for MFF, respectively.

The dose-dependent changes in VFt produced by U-50,488H (Figure 4) were biphasic in nature. Reductions occurred over the dose range of 0.5–4 μmol kg^{-1} while VFt increased with dose after attaining a minimum value at 4 μmol kg^{-1}. The initial phase of reduced values was greatly attenuated by pretreatment with naloxone. In Figure 4 it can be seen that tT was prolonged in a dose-related manner with only a suggestion of an initial fall and this was not seen after naloxone pretreatment. Similar findings were made with iT. In the absence of naloxone, iT initially fell by $-30 \pm 10\,\mu$A ($P < 0.05$) after a total dose of 4 μmol kg^{-1} and then increased with dose to reach a value of $+79 \pm 34\,\mu$A ($P < 0.05$). With naloxone pretreatment no significant initial fall was seen and iT rose in a monotonic manner with increasing dose.

ERP lengthened dose-dependently at all dose levels (Figure 5a) in a monotonic dose-dependent manner and this was not influenced by naloxone pretreatment. In keeping with the prolongation of ERP, the closely related but different variable, MFF, was dose-dependently reduced. This change was accentuated rather than reduced by pretreatment with 8 μmol kg^{-1} naloxone.

Naloxone, *in vivo*, had no statistically significant effects on electrical stimulation. Thus differences before and after naloxone were $+19 \pm 12\,\mu$A for iT, $+32 \pm 18\,\mu$A for VFt, -0.01 ± 0.03 ms for tT, -0.1 ± 0.8 Hz for MFF and $+2 \pm 4$ ms for ERP. None of these changes was statistically significant.

Discussion

The present study revealed some interesting actions of U-50,488H on rat hearts. These actions occurred at doses and concentrations above those required for κ-agonism and for the most part were not abolished by naloxone. In isolated hearts a naloxone-resistant depression of contractility, rate and the ECG were seen. Similar depression *in vivo* would account at least in part, for reductions in blood pressure and heart rate seen in intact rats.

In both isolated and intact hearts, QRS width and P–R interval were prolonged by U-50,488H in a manner unaffected by naloxone. It is generally accepted that Class I antiarrhythmics, i.e. myocardial sodium channel blockers, can produce increases in QRS width. Prolongation of the P–R interval can be produced by a variety of different drugs including Class I antiarrhythmics.

U-50,488H, at the relatively high doses and concentrations used in this study produced significant actions on responses to electrical stimulation. These included increases in iT, tT and VFt. These responses were characteristic of those produced by blockade of sodium channels in rat heart (Abraham *et al.*, 1989). However, it was noticeable that lower concentrations of U-50,488H, i.e. those most likely to be associated with selective effects on opioid receptors, reduced VFt in a manner that was attenuated by naloxone. The same pattern, but to a less marked extent, was seen with iT and tT. Thus, especially in

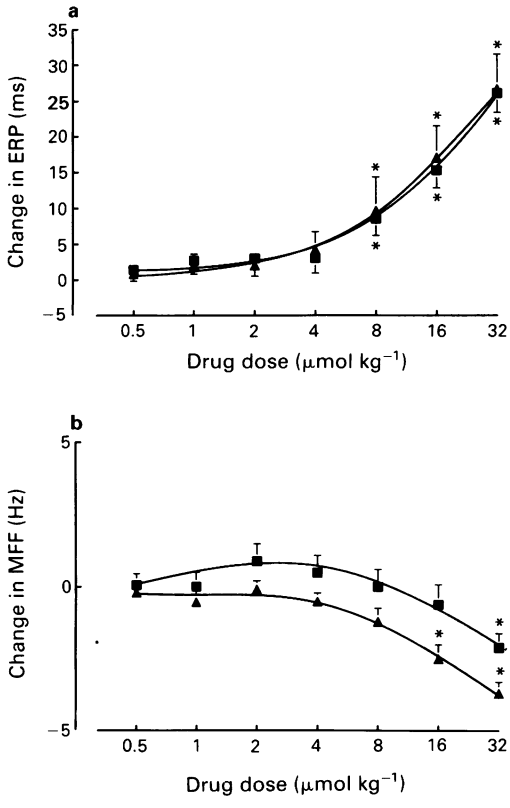

Figure 5 Effects of U-50,488H on effective refractory period (ms) (a) and maximum following frequency (Hz) (b) in pentobarbitone-anaesthetized rats subject to electrical stimulation. The symbol (■) indicates changes from pre-drug values with U-50,488H alone, or in the presence of naloxone pretreatment (▲). Saline control values are not shown but are indicated in the text. Values are mean with s.e.mean shown by vertical bars, $n = 5$–12. * $P < 0.05$ for difference from zero change. Control values ranged from 53 ± 3 to 58 ± 3 ms for ERP and 13.4 ± 0.4 to 15.3 ± 1.0 Hz for MFF.

the case of VFt, U-50,488H appeared to have two actions; a lower dose and possibly arrhythmogenic effect mediated through opioid receptors, and a higher dose and possible anti-arrhythmic effect independent of opioid receptors.

ERP was increased and MFF decreased in a dose-dependent manner by U-50,488H. These are characteristic actions of drugs which block sodium channels and/or prolong action potential duration. U-50,488H prolonged Q–T in the manner typical of a Class Ia antiarrhythmic drug.

In summary the profile of action of U-50,488H, especially at higher doses and concentrations, was consistent with sodium channel blockade. Depression of blood pressure, or contractility, and heart rate, prolongation of P–R and QRS, elevation of thresholds for induction of ventricular fibrillation or capture, are all produced by sodium channel blockade as has been illustrated previously with tetrodotoxin (Abraham *et al.*, 1989). Prolongation of ERP can be expected to occur with Class Ia sodium channel blockers and Class III anti-arrhythmics. It should be noted that for the majority of the measured variables there was a clear dose-response relationship. Even in the case of VFt, where such a dose-response relationship was not so obvious, because of the initial fall, blockade with naloxone clearly revealed the same underlying trend as seen with other variables. In concert with this, preliminary studies (unpublished observations) in rat isolated cardiac cells show that U-50,488H blocks sodium currents in these cells at concentrations found effective in rat isolated hearts.

The relationship between the above findings and potential arrhythmogenic or antiarrhythmic actions of U-50,488H are complex. Thus reductions in VFt seen at lower doses can be interpreted as being arrhythmogenic whereas sodium channel blockade can be either antiarrhythmic or arrhythmogenic in a manner that depends both upon dose and prevailing conditions. Thus U-50,488H may be biphasic in action: arrhythmogenic or pro-arrhythmic, at lower doses, and antiarrhythmic at higher doses. Such actions agree with previous findings in another study in which U-50,488H induced arrhythmias in rat isolated hearts when given in bolus doses (Wong *et al.*, 1990). They also are in agreement with the suggestion that opioid agonists and antagonists have anti-arrhythmic actions independent of opioid receptors (Sarne *et al.*, 1991).

At lower doses the possible arrhythmogenic action of U-50,488H on VFt was blocked by naloxone, indicating an opioid receptor-mediated effect, a finding in agreement with the suggestions of Parratt & Sitsapesan, (1986) and Wong & Lee (1987). Thus the present study helps explain discrepancies as to the effects of opioid agonists and antagonists on arrhythmias (see Sarne *et al.*, 1990).

In a separate study (unpublished observations), we found that U-50,488H, given at a single dose of 8 μmol kg^{-1} had antiarrhythmic actions against arrhythmias induced by occlusion of a coronary artery in anaesthetized rats. These antiarrhythmic actions were not blocked by pretreatment with naloxone. In the present study the same dose, albeit given cumulatively rather than as a single dose, gave incomplete indications of sodium channel blockade. Thus P–R and QRS were both increased by this dose. On the other hand Q–T was increased as was ERP, findings associated with Class Ia actions and/or potassium channel blockade. The 8 μmol kg^{-1} (cumulative) dose was associated with an increased VFT in presence of naloxone but not in its absence, an indication, perhaps, of the presence of sodium blockade masked by an equal but opposite action mediated via κ-agonism. It remains to be tested whether with lower doses, where κ-agonism would be expected to predominate, proarrhythmic actions occur with coronary occlusion in the rat.

In conclusion, the present study provides evidence that U-50,488H, a κ-receptor agonist, can have complex cardiovascular actions. Some of the effects may be mediated via opioid receptors while others, especially at higher doses, are independent of opioid receptors and may involve sodium channel blockade. The exact relationship between the findings in this study and effects on arrhythmias have still to be fully explained.

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The non-peptide tachykinin antagonist, CP-96,345, is a potent inhibitor of neurogenic inflammation

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1 Release of the tachykinin, substance P, from the peripheral terminals of polymodal afferent C-fibres is thought to be largely responsible for the vasodilatation and plasma protein extravasation described as neurogenic inflammation. The effects of CP-96,345, a non-peptide antagonist at the substance P (NK_1) receptor, on these vascular reactions were investigated in the rat.

2 Intravenously (i.v.) injected CP-96,345 (0.4–3.0 μmol kg^{-1}) prevented the drop in blood pressure, a measure of the peripheral vasodilatation, evoked by substance P and neurokinin A in a dose- and time-dependent manner, but did not affect that elicited by the non-tachykinin peptides calcitonin gene-related peptide and vasoactive intestinal polypeptide.

3 Plasma protein extravasation evoked by i.a. infusion of substance P, antidromic stimulation of the saphenous or the vagus nerve, and stimulation of cutaneous afferent nerves with mustard oil, were each significantly inhibited by CP-96,345 (3.0–9.0 μmol kg^{-1}, i.v.). Furthermore, CP-96,345 was orally active in blocking mustard oil-induced plasma extravasation with an ED_{50} of 10 μmol kg^{-1}.

4 The inhibition of substance P-induced vasodilatation and of neurogenic plasma extravasation by CP-96,345 was stereospecific as the inactive isomer CP-96,344 (**2R**, **3R** enantiomer of CP-96,345) had no effect.

5 Thus CP-96,345 is a specific, highly potent, long-acting and orally active inhibitor of tachykinin-mediated neurogenic inflammation.

Keywords: Neurogenic plasma extravasation; vasodilatation; substance P; neurokinin A; calcitonin gene-related peptide; vasoactive intestinal polypeptide; substance P antagonist; oral activity

Introduction

Polymodal afferent C-fibres contain the vasoactive tachykinins, substance P and neurokinin A, as well as calcitonin gene-related peptide (CGRP), peptides which are released following nerve stimulation at the central and peripheral terminals of these neurones. Release of these peptides from their central terminals induces autonomic reflexes, neuroendocrine regulations and the sensation of pain (for reviews see Lembeck, 1987; 1988). When the peripheral terminals of these fibres are stimulated by chemical, thermal or mechanical stimuli, the release of neuropeptides from these terminals induces vasodilatation and plasma protein extravasation in the skin (Lembeck & Holzer, 1979) and in the gastric mucosa (Holzer *et al.*, 1991), as well as oedema and bronchoconstriction in the airways (Lundberg & Saria, 1987; Barnes *et al.*, 1988). Since it has been proposed that substance P and neurokinin A, among these peptides, play a pathophysiological role in neurogenic inflammation in the skin and airways (Holzer, 1988), there seems to be a definite therapeutic potential for the use of metabolically stable tachykinin antagonists in these acute inflammatory conditions.

Recently the non-peptide compound CP-96,345, or (2S,3S)-*cis*-2-(diphenylmethyl)-N-((2-methoxyphenyl)methyl)-1-azabicyclo [2.2.2]octan-3-amine, has been shown to displace substance P and substance P analogues from binding to cell membranes in the CNS and has been classified as a selective NK_1 receptor antagonist (Snider *et al.*, 1991; McLean *et al.*, 1991; Rouissi *et al.*, 1991). The compound has been shown to inhibit selectively the effects of substance P on smooth muscle preparations and on locus coeruleus neurones *in vitro* and to block the salivary secretion evoked by substance P *in vivo* (Snider *et al.*, 1991; McLean *et al.*, 1991).

The present study was designed to examine whether CP-96,345 would also inhibit the effects of endogenous substance P released from peripheral terminals of afferent nerve fibres *in vivo*, and, since the compound does not have a peptide structure, to assess its oral activity.

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Methods

Female Sprague-Dawley rats (body weight 200–250 g) were used for all the experiments. All animals were anaesthetized with an i.p. injection of sodium pentobarbitone (50 mg kg^{-1}). One jugular vein was cannulated for the i.v. injection of drugs, and in experiments in which arterial blood pressure and heart rate were recorded, one carotid artery was cannulated.

To evoke peptide-induced hypotension, a dose of 30 pmol kg^{-1} substance P corresponding to its ED_{50} (Maggi *et al.*, 1987) was injected i.v. at 5 min intervals to give a reproducible brief fall in mean arterial blood pressure of about 20 mmHg. A similar reproducible fall in blood pressure was also induced by neurokinin A (150 pmol kg^{-1}), CGRP (45 pmol kg^{-1}) and vasoactive intestinal polypeptide (VIP, 90 pmol kg^{-1}) injected at 5 min intervals. Each peptide dose was injected in 0.1 ml of saline during a time period of 20 s.

The tachykinin antagonist CP-96,345 or its inactive isomer CP-96,345 (**2R**, **3R** enantiomer of CP-96,345) was slowly injected i.v. at the appropriate concentration in 1 ml of saline over a time period of 1 min, 5 min before the third injection of the peptide agonists.

To evoke plasma protein extravasation into the skin of the hind paw, the superficial epigastric artery was cannulated in one set of experiments for retrograde i.a. infusion of substance P (3.7 pmol min^{-1} during 5 min) into the femoral artery. In another set of experiments the saphenous nerve was cut in the thigh, and its distal part placed on bipolar platinum electrodes and stimulated for 5 min (10 V, 1 ms, 5 Hz). In a third set of experiments the dorsal skin of the hind paw was painted with 5% mustard oil in paraffin oil (w/w) twice during a time period of 10 min (for details of these methods see Lembeck & Holzer, 1979). To induce plasma protein extravasation in the trachea, the right cervical vagus nerve was exposed and cut

and its distal end was electrically stimulated for 5 min (10 V, 0.2 ms, 10 Hz; Lundberg & Saria, 1982).

Evans Blue (50 mg kg^{-1}), used as a marker for extravasated proteins, was injected i.v. 7 min before the stimulation period whereas CP-96,345, CP-96,344 or saline were injected i.v., as described above, 5 min before the stimulation period. After the end of the stimulation experiments in the paw, the rats were killed by bleeding and the dorsal paw skin was removed. Immediately after stimulation of the vagus nerve, the thorax was opened and the animals were perfused with 50 ml saline during 30 s via the aorta. A portion of the trachea (1 cm) adjacent to the stem bronchi was excised. All the tissues were weighed, Evans Blue was extracted with formamide and quantitated photometrically (Lembeck & Holzer, 1979).

To determine oral activity of CP-96,345 or CP-96,344 against mustard oil-induced plasma protein extravasation, the compounds were dissolved in 0.1% methyl cellulose/water and dosed orally 1 h before the mustard oil challenge. In these experiments a slightly different protocol was employed. The whole foot below the ankle was painted with 5% mustard oil in paraffin oil and 20 min later the foot was amputated, frozen in liquid nitrogen, pulverized and the Evans Blue dye extracted. For this set of experiments the basal dye content in the foot painted only with the vehicle paraffin oil is given for comparison. Basal dye content accounts for the blood volume left in larger vessels and an increase above basal levels represents mustard oil-induced dye extravasation and increased intravascular volume.

Substances

Compound CP-96,345, or (2S,3S)-*cis*-2-(diphenylmethyl)-N-((2-methoxyphenyl)methyl)-1-azabicyclo[2.2.2]octan-3-amine, and CP-96,344 (2**R**,3**R** enantiomer of CP-96,345) were generously provided by Dr Michael Snider from Pfizer Central Research Division (U.S.A.); substance P, neurokinin A, CGRP and VIP were obtained from Peninsula (U.S.A.), and Evans Blue was obtained from Fluka (Switzerland).

Results

To assess the potency and duration of action of CP-96,345 we chose to calibrate its effect against the hypotensive action of substance P. The i.v. injection of CP-96,345 (0.4–3.0 μmol kg^{-1}) inhibited the substance P-induced fall in blood pressure in a dose-dependent fashion (Figure 1). The largest dose of CP-96,345 used in this assay (3.0 μmol kg^{-1}) caused an almost complete inhibition of the hypotensive effect of substance P for up to 30 min. The inactive isomer CP-96,344 (2**R**, 3**R** enantiomer of CP-96,345) had no effect when tested also at a dose of 3.0 μmol kg^{-1} ($n = 3$, results not shown). Whereas CP-96,345, injected i.v. in a dose of 3.0 μmol kg^{-1}, caused also almost complete inhibition of the hypotensive action of another tachykinin, neurokinin A, for up to 30 min, it did not affect the hypotensive actions of the unrelated peptides CGRP and VIP in the time period 5 min to 30 min after its i.v. injection. As an example, the hypotensive responses of the four vasoactive peptide agonists investigated 10 min after 3 μmol kg^{-1} CP-96,345 are given for comparison in Figure 2. The i.v. injection of 3.0 μmol kg^{-1} of CP-96,345 alone had a slight depressant effect on blood pressure (-10 ± 1 mmHg below the pre-injection level which was 136 ± 3 mmHg, $n = 20$) and on heart rate, which fell from 400 ± 10 b.p.m. to 360 ± 10 b.p.m. ($n = 20$), effects not related to any substance P-antagonistic action because they were shared by the inactive isomer CP-96,344 ($n = 5$).

The effect of CP-96,345 on the plasma protein extravasation evoked by three different means of sensory nerve stimulation (mustard oil, antidromic saphenous or efferent vagus nerve stimulation) was compared with that observed on substance P-induced plasma extravasation (close i.a. infusion into the femoral artery). A 3.0 μmol kg^{-1} dose of CP-96,345 was sufficient to inhibit significantly the effect of mustard oil whereas a 9.0 μmol kg^{-1} dose of the substance P antagonist was required for a complete blockade (Figure 3). Plasma protein extravasation induced by substance P or by saphenous or vagus nerve stimulation was also markedly inhibited by 3.0 μmol kg^{-1} of CP-96,345 (Figure 3). The inactive isomer CP-96,344 was tested for its action against plasma protein extravasation following an antidromic saphenous nerve stimulation and was found to have no antagonistic action ($n = 3$, results not shown).

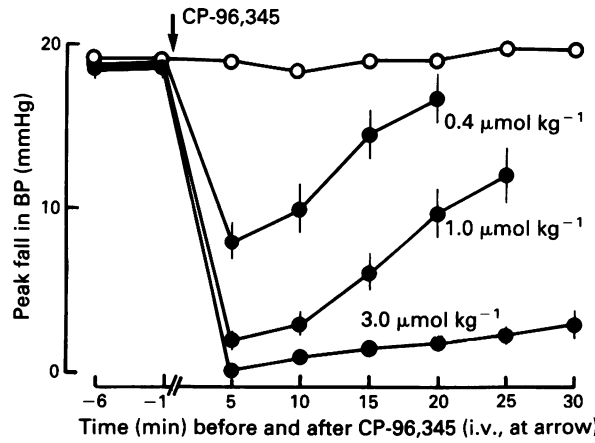

Figure 1 Inhibition of the fall in blood pressure (BP) induced by substance P (30 pmol kg^{-1}, i.v.) in pentobarbitone-anaesthetized rats. Substance P caused a short fall in BP which was of constant magnitude when the peptide was injected at 5 min intervals (○). Following the i.v. injection of CP-96,345 (0.4–3.0 μmol kg^{-1}, ●), but not that of saline used as a control (○), the fall in BP was reduced; both the magnitude and duration of the effect of CP-96,345 were dose-dependent. Whereas a dose of 0.4 μmol kg^{-1} of CP-96,345 blocked only the next two applications of substance P, 3.0 μmol kg^{-1} of CP-96,345 abolished almost completely the substance P responses for the next 30 min ($P < 0.05$, Quade test). Means of 4–5 experiments with s.e.mean shown by vertical lines.

The efficiency of the oral administration of CP-96,345 was examined in the mustard oil-induced plasma extravasation test in the rat foot. CP-96,345 inhibited this reaction in a dose-dependent manner with an oral ED_{50} of 10 μmol kg^{-1} (Figure 4). The inhibition was stereospecific and the inactive isomer

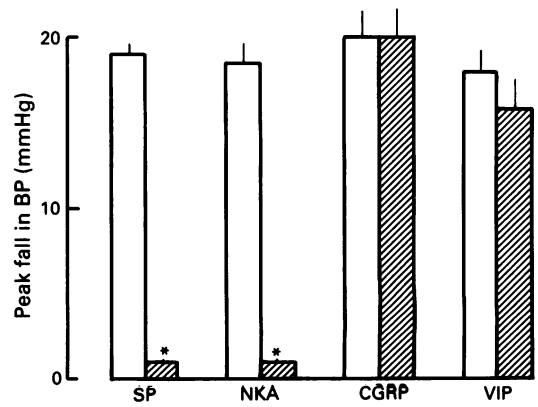

Figure 2 Fall in blood pressure (BP) in pentobarbitone-anaesthetized rats following the i.v. injections of substance P (SP, 30 pmol kg^{-1}), neurokinin A (NKA, 150 pmol kg^{-1}), calcitonin gene-related peptide (CGRP, 45 pmol kg^{-1}) and vasoactive intestinal polypeptide (VIP, 90 pmol kg^{-1}). The fall in blood pressure observed with all four peptides was of similar size before the injection of CP-96,345 (open columns). Ten min after CP-96,345 injection (3.0 μmol kg^{-1}, i.v., hatched columns) the effects of substance P and neurokinin A were almost abolished (* $P < 0.05$, t test), those of CGRP and VIP remained unchanged. Means of 4–5 experiments; vertical lines show s.e.mean.

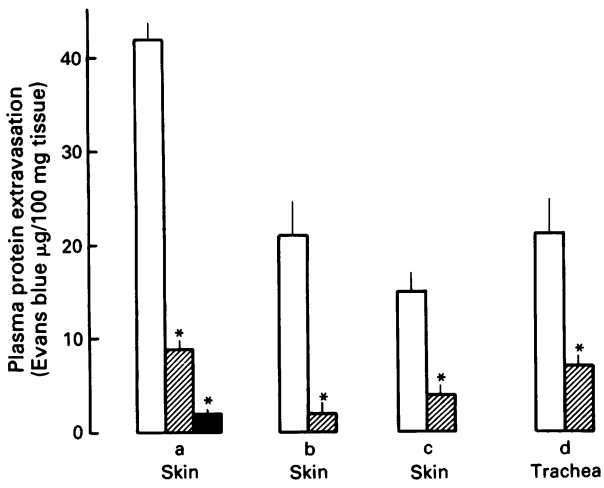

Figure 3 Plasma protein extravasation (measured by Evans blue content of tissues) in pentobarbitone-anaesthetized rats. Controls = open columns. CP-96,345 (3.0 μmol kg^{-1} = hatched columns, 9.0 μmol kg^{-1} = solid column) was injected i.v. 5 min before the following treatments: (a) Plasma protein extravasation in the skin of the hind paw following painting the paw with 5% mustard oil in paraffin oil. (b) Plasma protein extravasation in the skin of the hind paw following antidromic stimulation of the saphenous nerve (10 V, 1 ms, 5 Hz for 5 min). (c) Plasma protein extravasation in the skin of the hind paw following close i.a. infusion of substance P (3.7 pmol min^{-1}) into the femoral artery. (d) Plasma protein extravasation in the trachea following stimulation (10 V, 0.2 ms, 10 Hz for 5 min) of the centrally divided right vagus nerve. Means of $n = 4$–6 for each group; vertical lines show s.e.mean. * $P < 0.05$ compared to the appropriate control by t test or ANOVA.

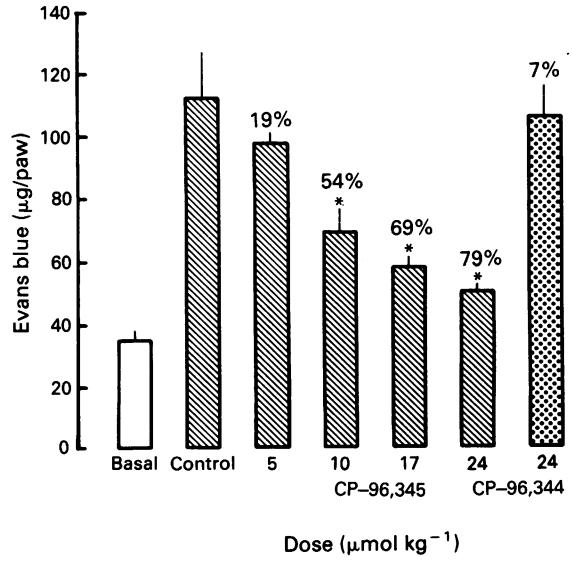

Figure 4 Dose-dependent inhibition of mustard oil-induced Evans blue accumulation in the paw of anaesthetized rats. CP-96,345 (hatched columns) and the inactive enantiomer CP-96,344 (stippled column) were dissolved in 0.1% methyl cellulose-water and given orally 1 h before the mustard oil challenge as in Figure 3. Each column represents the mean of 5–6 rats (s.e.mean shown by vertical lines) and the number above indicates % inhibition after subtracting the basal (open column, without mustard oil, see methods). * indicates $P < 0.01$ compared with control by 1 way ANOVA.

CP-96,344 had no effect up to 24 μmol kg^{-1}. Basal dye content of the foot (see methods) was not changed by the highest dose of CP-96,345 (24 μmol kg^{-1}) employed ($n = 5$, results not shown).

Discussion

CP-96,345 exhibited stereospecific antagonistic effects against tachykinin-induced hypotension as well as against substance P-induced, or several types of neurogenic plasma protein extravasation. Earlier investigations have demonstrated that the hypotensive responses to the i.v. injection of substance P (Burcher *et al.*, 1977), neurokinin A (Hua *et al.*, 1984; Maggi *et al.*, 1987), CGRP (Lappe *et al.*, 1987) and VIP (Said & Mutt, 1970) can be regarded as a correlate of peripheral vasodilatation. The tachykinin-induced hypotension (vasodilatation) has been characterized as a tachykinin effect mediated by the NK_1 receptor (Maggi *et al.*, 1987). Although the compound CP-96,345 has been classified as selective NK_1 receptor (substance P receptor) antagonist, this does not exclude its antagonistic action against neurokinin A which can also interact with the NK_1 receptor. However, the antagonistic effects of CP-96,345 were observed only against tachykinins and not against unrelated peptides such as CGRP or VIP (as demonstrated here with their hypotensive action) nor against bradykinin-, leukotriene D_4-, histamine-, or PAF-induced plasma protein extravasation (Nagahisa, personal communication).

The specific substance P-antagonistic effects of CP-96,345 were clearly separable from its unspecific, slightly depressant, effects on blood pressure and heart rate, because the latter were also observed after administration of the inactive isomer, CP-96,344.

The almost complete inhibition of the various types of neurogenic plasma protein extravasation in skin and trachea in the rat would indicate that such phenomenon predominantly resulted from the release of substance P and neurokinin A. However, vasodilatation induced by the coreleased CGRP, itself unaffected by CP-96,345, could certainly have remained. The present findings also do not eliminate a synergy of oedema formation in the skin between CGRP and tachykinins (Brain & Williams, 1985; Gamse & Saria, 1985), since inhibition of either component may significantly modulate oedema formation.

The main advantages of CP-96,345 are its oral activity and long duration of action. The peptide-antagonists of the tachykinins developed so far were metabolically unstable and have thus, apart from a few exceptions (Lundberg *et al.*, 1983; Maggi *et al.*, 1991), only been applied locally in the tissue (Lembeck *et al.*, 1982; Xu *et al.*, 1991). Although the effects of CP-96,345 have still to be explored on the different functions of substance P-containing neurones in the intestinal intrinsic nerve plexus and in the CNS under *in vivo* conditions, the potent effects on afferent neurones characterizes the compound as a useful and practical tool for the study of tachykinin-mediated neurogenic inflammation.

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Adenine nucleotide-induced inhibition of binding of sulphonylureas to their receptor in pancreatic islets

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1 The effects of the Mg complex of adenosine 5'-triphosphate (MgATP) on binding of sulphonylureas to microsomes obtained from mouse pancreatic islets were examined.

2 MgATP inhibited the binding of both glibenclamide and tolbutamide to microsomes.

3 Binding of [^{3}H]-glibenclamide inhibited by MgATP was not further diminished by Mg^{2+}-bound adenosine 5'-(β,γ-imidotriphosphate) (AMP-PNP) or free adenosine 5'-diphosphate (ADP). Higher concentrations of MgAMP-PNP induced a partial reversal of the inhibitory effect of MgATP on [^{3}H]-glibenclamide binding.

4 The apparent dissociation constant (K'_D) for binding of [^{3}H]-glibenclamide remained constant when the MgATP concentration was increased from 100 to 500 μM.

5 Extracellular ADP did not markedly stimulate insulin release from mouse pancreatic islets.

6 It is concluded that sulphonylureas and cytosolic nucleotides exert their inhibitory effects on the K-ATP-channels of β-cells by binding to different sites. The binding properties of the sulphonylurea receptor seem to be modulated by protein phosphorylation.

Keywords: Adenine nucleotides; glibenclamide; sulphonylurea receptor; pancreatic islets

Introduction

Initiation of insulin release requires increase in the cytosolic Ca^{2+}-concentration of the pancreatic β-cell. Enhancement of Ca^{2+}-influx across the plasma membrane is induced by depolarization of the β-cell to the threshold potential at which voltage-dependent Ca^{2+}-channels are activated (for reviews see Hedeskov, 1980; Henquin & Meissner, 1984; Matthews, 1985). Glucose depolarizes the β-cell by inhibiting an adenosine 5'-triphosphate-dependent K^+-channel (K-ATP-channel) in the plasma membrane, probably via increasing the cytosolic ratio of adenosine 5'-triphosphate (ATP) to adenosine 5'-diphosphate (ADP), and sulphonylureas depolarize the β-cell by inhibiting the same channel via direct interaction with the plasma membrane (Ashcroft *et al.*, 1984; Cook & Hales, 1984; Sturgess *et al.*, 1985; for reviews see Ashcroft & Rorsman, 1989; Dunne & Petersen, 1991).

In microsomal membranes from insulin-secreting tumour cells or pancreatic islets, a binding site was identified which represents the sulphonylurea receptor (Geisen *et al.*, 1985; Schmid-Antomarchi *et al.*, 1987; Gaines *et al.*, 1988; Panten *et al.*, 1989; Niki *et al.*, 1989). In microsomes from mouse pancreatic islets, the Mg complex of ATP, ADP or some other phosphate and thiophosphate group donating adenine and guanine nucleotides inhibited glibenclamide binding (Schwanstecher *et al.*, 1991). Glibenclamide binding was not inhibited by free nucleotides (in the absence of Mg^{2+} ions) or nonhydrolyzable analogues, e.g. adenosine 5'-(β,γ-imidotriphosphate) (AMP-PNP). The data suggested that protein phosphorylation modulates the affinity of the sulphonylurea receptor. However, the possibility was not ruled out that MgATP and the Mg complexes of related nucleotides competed with glibenclamide for the same binding site. Moreover, it was not examined whether adenine nucleotides can compete with glibenclamide for the same binding site when the conformation of the sulphonylurea receptor is altered by the Mg complex of ATP or other phosphate and thiophosphate group donating nucleotides.

In insulin-secreting tumour cells (hamster pancreatic β-cell line HIT T15) extracellular ADP stimulated insulin secretion and decreased glibenclamide binding to intact cells (Niki *et al.*, 1990). These effects were specific for ADP which inhibited glibenclamide binding also in HIT T15 cell microsomes incubated in the absence of Mg^{2+}. It was suggested that extracellular ADP and glibenclamide interact with the same binding site at the sulphonylurea receptor.

The aim of the present study was to test the hypothesis that adenine nucleotides and glibenclamide do not compete for the same binding site at the sulphonylurea receptor of pancreatic islet cells. The results are in favour of this hypothesis.

Methods

Measurement of [^{3}H]-glibenclamide binding to microsomes

Preparation of microsomes from pancreatic islets of obese-hyperglycaemic mice which contain more than 90% β-cells (Gepts *et al.*, 1960), measurement of [^{3}H]-glibenclamide binding (at 22°C, in 50 mM Tris, adjusted with HCl to pH 7.4) and data analysis were performed as previously described (Schwanstecher *et al.*, 1991). With the exception of the experiments in Figure 1, nonspecific binding was determined by incubations in the presence of additional unlabelled glibenclamide (0.1 μM). Concentrations of free nucleotides, free Mg^{2+} ions and Mg complexes of nucleotides were calculated as described below.

Measurement of insulin secretion

Male albino mice (NMRI, 11–15 weeks old, fed *ad libitum*) were used. Insulin secretion from batches of 30 pancreatic islets (isolated by collagenase digestion, perifused at 0.9 ml min^{-1} and 37°C) was measured as described previously (Panten *et al.*, 1990). The islets were perifused for 90 min in the presence of 3 or 10 mM D-glucose and then for 30 min in the presence of 40 mM D-glucose. From min 61 to min 90 the media were supplemented with ADP (2 mM) and additional $MgCl_2$ to hold the free Mg^{2+} concentration constant.

Treatment of results

Values are presented as mean value ± s.e. mean for independent experiments using separate preparations of isolated islets or microsomal membranes. Significances were calculated by the two-tailed matched pairs signed rank test of Wilcoxon or

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by the two-tailed U-test of Wilcoxon and of Mann and Whitney with the Bonferroni correction for multiple comparisons. $P < 0.05$ was considered significant.

The theoretical curve for a competitive interaction of glibenclamide and the Mg complex of ATP was calculated from the following equation: $K'_D = K_D \times (1 + [MgATP]/K_{MgATP})$ (Ehlert, 1986). K'_D represents the apparent dissociation constant for binding of glibenclamide to the sulphonylurea binding site in the presence of MgATP, K_D the dissociation constant for binding of glibenclamide in the absence of MgATP, K_{MgATP} the assumed dissociation constant for binding of MgATP to the sulphonylurea binding site.

K_D and K'_D for binding of tolbutamide to the sulphonylurea receptor were calculated according to Cheng & Prusoff (1973) assuming $K_D = 0.3$ nM or $K'_D = 1.8$ nM for binding of glibenclamide in the absence or presence of 100 μM MgATP, respectively (Schwanstecher *et al.*, 1991).

Compounds and solutions

Na_2ATP, KADP and Li_4AMP-PNP were from Boehringer (Mannheim, Germany). Glibenclamide and tolbutamide were from Hoechst (Frankfurt, Germany); [^{3}H]-glibenclamide (51 Ci mmol^{-1}, 96% radiochemical purity) was from NEN (Dreieich, Germany). All other chemicals were obtained from the sources described elsewhere (Schwanstecher *et al.*, 1991).

The free Mg^{2+} concentration in the incubation media was held close to 1 mM by adding appropriate amounts of $MgCl_2$ to the nucleotide containing solutions. The concentration of free ATP, AMP-PNP or ADP was 1.9, 1.5 or 27% of total concentrations of ATP, AMP-PNP or ADP, respectively. The required amounts of $MgCl_2$ and the composition of the solutions were calculated for pH 7.4 with a computer programme (Fabiato, 1988). The logarithms of the absolute stability constants (log K) for binding of H^+ and Mg^{2+} to ATP or ADP were taken from Sigel *et al.* (1987) or Taqui Khan *et al.* (1962), respectively. The corresponding log K values for AMP-PNP were calculated from the values for ATP and the differences between the values for ATP and AMP-PNP (Yount *et al.*, 1971; Pettit & Siddiqui, 1976). All log K values were corrected for the ionic strength of the incubation buffer (0.04 M) (Harrison & Bers, 1987). The log K values for all proton-ligand complexes were corrected by 0.08 in order to include the proton activity coefficient appropriate for the bath solution (Harrison & Bers, 1989).

Results

We have previously observed that the Mg complex of ATP or some other related nucleotides inhibits glibenclamide binding to microsomal membranes from pancreatic islets (Schwanstecher *et al.*, 1991). Competition or saturation experiments revealed that MgATP increased the apparent dissociation constant for glibenclamide binding (K'_D) by 3.6 or 6 fold, respectively. This different extent of the response to MgATP was probably due to the differences in experimental design and evaluation of the data when measuring K'_D in competition or saturation experiments. In the present study we wanted to know whether MgATP also inhibits binding of other sulphonylureas. Figure 1 shows that in the absence or presence of 100 μM of MgATP, unlabelled tolbutamide inhibited specific binding of [^{3}H]-glibenclamide half-maximally at 19.8 $\pm$ 1.6 μM or 35.8 $\pm$ 5.6 μM, respectively. These half-maximally inhibitory concentrations (IC_{50}) were significantly different ($P < 0.03$) and corresponded to K_D or K'_D values for binding of tolbutamide of 11.9 or 32.3 μM, respectively. The slope parameter (n) was -0.94 or -0.93 in the absence or presence of MgATP, respectively, suggesting that there is only one class of high affinity binding sites for tolbutamide both in the absence and presence of MgATP. These results resemble the effect of MgATP on the inhibition of [^{3}H]-glibenclamide binding by unlabelled glibenclamide and thus indicate that MgATP inhibits binding of tolbutamide.

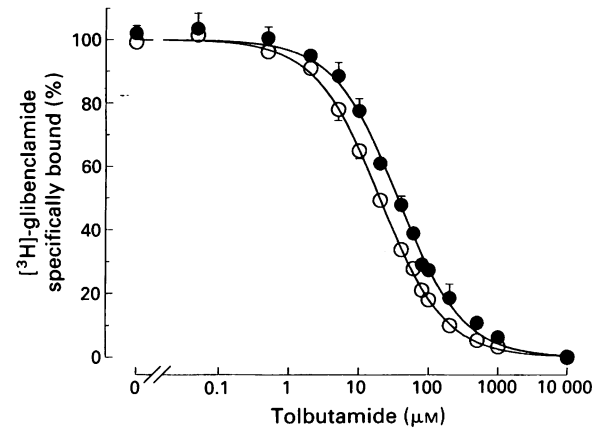

Figure 1 Effect of tolbutamide on binding of [^{3}H]-glibenclamide to microsomes from mouse pancreatic islets. Inhibition of [^{3}H]-glibenclamide binding by tolbutamide in the absence (○) or presence (●) of 0.1 mM MgATP was measured in 1 ml of buffer containing [^{3}H]-glibenclamide (0.2 nM), free Mg^{2+} (1 mM) and the indicated tolbutamide concentrations (logarithmic scale). Incubations were started by addition of microsomal protein (15 μg ml^{-1}) and terminated after 60 min by filtration. Binding in the presence of 10 mM tolbutamide was taken as nonspecific binding. Results are presented as percentages of specific binding of [^{3}H]-glibenclamide in the absence of tolbutamide and in the absence (○) or presence (●) of MgATP. MgATP inhibited binding of [^{3}H]-glibenclamide (0.2 nM) in the absence of tolbutamide by 71 $\pm$ 4%. Values are given as mean values (with s.e.mean shown by vertical bars when larger than symbols) for results from 4 separate experiments using separate membrane preparations.

Previous saturation experiments revealed that 100 μM MgATP increased the apparent dissociation constant (K'_D) for binding of [^{3}H]-glibenclamide from 0.3 to 1.8 nM (Schwanstecher *et al.*, 1991). As shown in Figure 2, there was no further increase in the K'_D value at higher MgATP concentrations. This approach of the K'_D value to a limiting value is not in accordance with the theoretical curve for a competitive interaction of glibenclamide and MgATP (Figure 2).

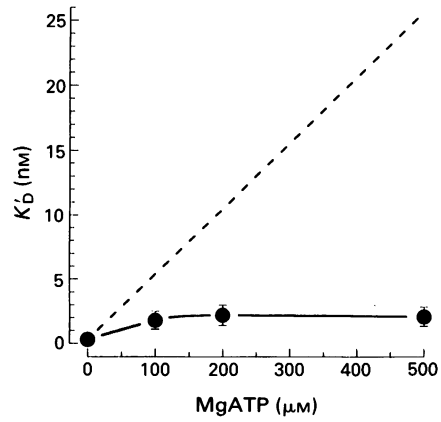

Figure 2 Effect of the Mg complex of ATP (MgATP) on the apparent dissociation constant (K'_D) for glibenclamide in microsomes from mouse pancreatic islets. Binding of [^{3}H]-glibenclamide was measured in 1 ml of buffer containing [^{3}H]-glibenclamide (0.05–12 nM), free Mg^{2+} (1 mM) and the indicated concentrations of MgATP. Incubations were started by addition of microsomal protein (15 μg ml^{-1}) and terminated after 60 min by filtration. Values are given as mean values (with s.e.mean shown by vertical bars) for results from 3–4 separate experiments using separate membrane preparations. The dashed line shows the relationship between K'_D and MgATP concentration expected for a competitive interaction of glibenclamide and MgATP at a common binding site. The dissociation constant for binding of MgATP to the sulphonylurea binding site (K_{MgATP}) was assumed to be 12 μM (Schwanstecher *et al.*, 1991).

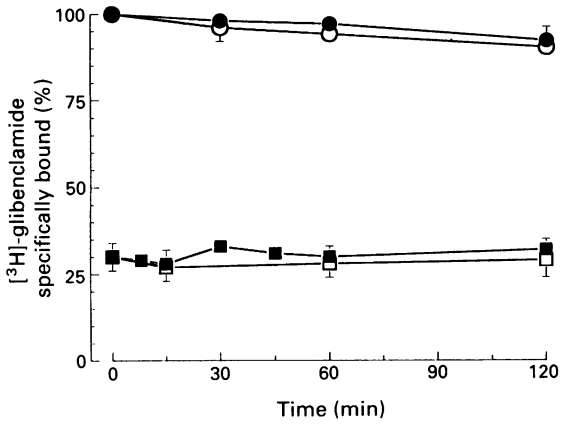

Figure 3 Kinetics of effects of MgAMP-PNP on binding of [^{3}H]-glibenclamide to microsomes from mouse pancreatic islets. Microsomes (15 μg ml^{-1}) were preincubated for 30 min in buffer containing 0.3 nM [^{3}H]-glibenclamide and 1 mM free Mg^{2+} (circles) or 1 mM free Mg^{2+} + 0.2 mM MgATP (squares). At zero time an aliquot of buffer without (open symbols) or with (filled symbols) adenosine 5'-(β,γ-imidotriphosphate) (AMP-PNP) was added. The final concentrations were 0.2, 0.3 or 1 mM for MgATP, MgAMP-PNP or free Mg^{2+}, respectively. Aliquots were filtered at the indicated time. Nonspecific binding was determined using preincubations and incubations in the presence of additional 0.1 μM unlabelled glibenclamide and did not vary significantly up to min 120. Results are presented as percentages of specific binding of [^{3}H]-glibenclamide in the absence of nucleotides at zero time. Values are given as mean values (with s.e.mean shown by vertical bars when larger than symbols) for results from 4 separate experiments using separate membrane preparations.

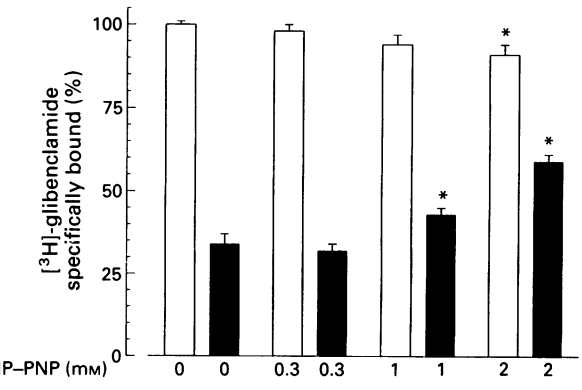

Figure 4 Concentration-dependent effects of adenosine 5'-(β,γ-imidotriphosphate) (AMP-PNP) on binding of [^{3}H]-glibenclamide to microsomes from mouse pancreatic islets. After preincubation (30 min) in buffer containing 1 mM free Mg^{2+} (open columns) or 1 mM free Mg^{2+} + 0.1 mM MgATP (filled columns), buffer without or with AMP-PNP was added to give the indicated final concentrations of MgAMP-PNP. Incubations were terminated 60 min later by filtration. Values are given as mean values (with s.e.mean shown by vertical bars) for results from 6 separate experiments using separate membrane preparations. Asterisks indicate significant differences ($P < 0.02$) from the corresponding incubation without MgAMP-PNP. For further details see legend to Figure 3.

The opening activity of the K-ATP-channel seems to require that the channel is phosphorylated by a membrane-bound protein kinase which uses MgATP as a substrate (Ashcroft & Rorsman, 1989). However, the channel openings can be inhibited by sulphonylureas or nucleotides (e.g. ATP, ADP, AMP-PNP) both in the absence or presence of Mg^{2+} ions (Ashcroft & Rorsman, 1989). Binding experiments in a previous (Schwanstecher *et al.*, 1991) and in the present study show that [^{3}H]-glibenclamide binding in the presence of MgATP is further diminished by increasing concentrations of glibenclamide, diazoxide and tolbutamide. Therefore, we examined whether adenine nucleotides might compete with sulphonylureas for binding to the microsomal binding site, the state of which was altered by preincubation with MgATP. Binding of [^{3}H]-glibenclamide inhibited by MgATP was not further diminished within 2 h after addition of MgAMP-PNP (300 μM) (Figure 3). At higher concentrations of MgAMP-PNP (1 or 2 mM) a partial reversal of the inhibitory effect of MgATP on [^{3}H]-glibenclamide binding was observed (Figure 4). However, it is unclear whether Mg^{2+}-bound AMP-PNP or free AMP-PNP caused this reversal since the concentrations of both were increased simultaneously. In the absence of MgATP, 2 mM MgAMP-PNP slightly inhibited [^{3}H]-glibenclamide binding (Figure 4). Free ADP (0.1–0.5 mM) did not alter [^{3}H]-glibenclamide binding inhibited by 100 μM MgATP (results not shown).

Extracellular ADP (1–2 mM) has been reported to stimulate markedly insulin release from tumour cells (hamster pancreatic β-cells) incubated in the presence of 1.2 mM Mg^{2+} (Niki *et al.*, 1990). Therefore, we examined the effects of ADP on insulin secretion from perifused mouse pancreatic islets. In conjunction with 3 mM D-glucose, ADP (2 mM) did not stimulate insulin secretion ($n = 6$, results not shown). In the presence of 10 mM D-glucose, ADP (2 mM) induced only a very small increase of insulin secretion which lasted for 1 min ($n = 6$, results not shown). The maximum secretory response to ADP (2 mM) was 9% of the first peak of insulin release observed 4 min after transition to 40 mM D-glucose.

Discussion

The results indicate that MgATP inhibits microsomal binding of both glibenclamide and tolbutamide. Therefore, it seems likely that these effects of MgATP reflect a general phenomenon being valid for all sulphonylureas.

Evidence was previously presented that in islet microsomes the high affinity binding site for sulphonylureas represents the sulphonylurea receptor (Panten *et al.*, 1989). The present study shows that the inhibitory effect of MgATP on binding of sulphonylureas to their receptor cannot be explained by an interaction of MgATP and sulphonylureas at the same high affinity site. The identity of the site by which MgATP regulates binding of sulphonylureas to their receptor is unknown. However, the data described in the present and in a previous study are consistent with the view that MgATP acts by serving as a substrate for one or several kinases which regulate the binding properties of the sulphonylurea receptor by phosphorylating the receptor protein or a closely associated protein (Schwanstecher *et al.*, 1991).

Phosphorylation of the K-ATP-channel in pancreatic β-cells is probably required to maintain channel function (Ashcroft & Rorsman, 1989). Patch-clamp experiments indicated that half-maximal inhibition of the K-ATP-channels of pancreatic β-cells is induced by 0.4 nM, 50 μM or 60 μM of free concentration of glibenclamide, ADP or AMP-PNP, respectively (Zünkler *et al.*, 1988; Ashcroft & Kakei, 1989; Schwanstecher, Dickel & Panten, unpublished observations). The Mg complex of AMP-PNP, too, is inhibitory on the K-ATP-channels in β-cells ($EC_{50} = 25\ \mu$M; Schwanstecher, Dickel & Panten, unpublished observations; Ashcroft & Kakei, 1989). As MgAMP-PNP and free ADP did not inhibit [^{3}H]-glibenclamide binding in the presence of MgATP, sulphonylureas and nucleotides seem to close the K-ATP-channel via different binding sites, both under phosphorylating (this study) and dephosphorylating conditions (Schwanstecher *et al.*, 1991).

Millimolar concentrations of AMP-PNP antagonized the inhibitory effect of MgATP on glibenclamide binding to islet microsomes. This finding could reflect displacement of MgATP from the binding site mediating the inhibitory effect of MgATP. Since AMP-PNP is no substrate for protein kinases (Yount *et al.*, 1971), it may be speculated that a displacement takes place at the active site of a protein kinase which inhibits binding of sulphonylureas by phosphorylating the sulphonylurea receptor.

Experiments with HIT-cells suggested that the β-cell sulphonylurea receptor is an ADP-binding protein (Niki *et al.*, 1990). This interpretation mainly resulted from two observations: (1) in the absence of Mg^{2+}, ADP inhibited [^{3}H]-glibenclamide binding to HIT-cell microsomes; (2) in the presence of Mg^{2+}, extracellular ADP strongly stimulated insulin secretion from HIT-cells. In a previous study we have shown that the first observation is not seen in microsomes from pancreatic islets (Schwanstecher *et al.*, 1991). The present study demonstrates that the second observation with HIT-cells, is also not seen in β-cells from pancreatic islets. Thus, the phenomenon described by Niki *et al.* (1990) has nothing to do with the inhibitory effect of MgATP on sulphonylurea binding in β-cell microsomes.

In conclusion, the present study supports the view that sulphonylureas and cytosolic nucleotides (e.g. free ATP) exert their inhibitory effects on the K-ATP-channels of β-cells by binding to different sites. It is suggested that the proteins organizing the K^+ channel comprise the receptors for sulphonylureas and channel-blocking nucleotides. A protein kinase tightly associated with the K-ATP-channel appears to exert additional regulation via phosphorylation of one or several channel proteins. MgATP serves as substrate for this phosphorylation which inhibits the binding of sulphonylureas to their receptor.

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Sympathoinhibitory effects of rilmenidine may be mediated by sites located below the brainstem

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1 To determine the site of action of rilmenidine, we examined its effects on arterial blood pressure (BP), heart rate (HR) and postganglionic renal sympathetic nerve activity (RSNA) after intracerebroventricular (i.c.v.) administration (300 $\mu g\,kg^{-1}$), in groups (all $n = 6$) of conscious and freely moving, pentobarbitone-anaesthetized and pentobarbitone-anaesthetized and spinally transected, fifteen week-old male spontaneously hypertensive rats (SHRs).

2 In conscious SHRs, which exhibited a low sympathetic nerve activity (RSNA: $3.4 \pm 0.9\,\mu V$), rilmenidine was inactive on systolic BP (SBP), diastolic BP (DBP), HR and RSNA.

3 In intact pentobarbitone-anaesthetized SHRs, which exhibited an elevated sympathetic nerve activity (RSNA: $10.6 \pm 0.9\,\mu V$), rilmenidine exerted potent antihypertensive (ΔSBP: $-37 \pm 4\%$; ΔDBP: $-43 \pm 6\%$), bradycardic (ΔHR: $-32 \pm 3\%$) and sympathoinhibitory (ΔRSNA: $-68 \pm 9\%$) activities.

4 In pentobarbitone-anaesthetized SHRs with cervical spinal cord transection, BP was markedly decreased and sympathetic nerve activity (RSNA: $10.3 \pm 3.1\,\mu V$) returned to the level observed in conscious SHRs (RSNA: $3.6 \pm 0.5\,\mu V$). In these conditions, rilmenidine remained sympathoinhibitory (ΔRSNA: $-74 \pm 5\%$).

5 In conclusion, we have shown that pentobarbitone-anaesthesia enhances the peripheral sympathetic tone by a central action, as the spinal cord transection allows RSNA to return to normal levels and that, spinal or ganglionic structures could be a major site of the sympathoinhibitory action of rilmenidine.

Keywords: α_2-Adrenoceptors; blood pressure; intracerebroventricular; renal sympathetic nerve activity; rilmenidine; spinal transection; spontaneously hypertensive rats; sympathetic nervous system

Introduction

For clonidine and related drugs like rilmenidine, several sites of action (for review see Jarrott *et al.*, 1987; Jarrott, 1988) have been proposed: brainstem (Bousquet & Guertzenstein, 1973; Bousquet *et al.*, 1981; Punnen *et al.*, 1987), spinal cord (Connor *et al.*, 1981; Solomon *et al.*, 1989) and/or peripheral presynaptic α_2-adrenoceptors (Timmermans *et al.*, 1981). Moreover, although one mechanism proposed for the antihypertensive action of rilmenidine in spontaneously hypertensive rats (SHRs) has been a reduction in peripheral sympathetic tone (Van Zwieten, 1988), to our knowledge, no studies have been able to demonstrate the direct action of rilmenidine on the peripheral sympathetic nervous system activity in anaesthetized or conscious SHRs. Otherwise, previous studies in conscious unrestrained (Sannajust *et al.*, 1989; 1992) and pentobarbitone-anaesthetized (Sannajust *et al.*, 1992) SHRs suggested that rilmenidine did not act primarily and exclusively on the central nervous system (forebrain/brainstem) to lower arterial blood pressure (BP) and heart rate (HR).

Therefore, the aim of the present study was to determine whether or not these drugs could exert their sympathoinhibitory effect by acting on sites located outside the brain. Consequently, we measured the arterial BP and the renal sympathetic nerve activity (RSNA) after i.c.v. injections of rilmenidine in conscious, pentobarbitone-anaesthetized and, pentobarbitone-anaesthetized and spinal cord transected SHRs.

Methods

Eighteen 15 week-old male SHRs (IFFA-Credo, Les Onçins, France) weighing 290 to 337 g, were used in these experiments.

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Preparation of the animals (for details see Sannajust et al., *1992)*

In all animals, one week before experimentation, a stainless-steel guide cannula was implanted. After a 5-day period of recovery, two polyethylene catheters were inserted into the left femoral artery and vein for BP measurement and drug administrations, respectively. On the day of the experiment, the arterial line of conscious and pentobarbitone-anaesthetized SHRs was connected to a pressure transducer coupled to a recorder. The body temperature of anaesthetized animals was maintained at 37 ± 1°C by means of a heating pad and the trachea was cannulated for artificial ventilation with room air ($10\,ml\,kg^{-1} \times 65\,min^{-1}$).

Renal sympathetic nerve activity measurement

Efferent renal sympathetic nerve activity was measured by the technique described by Thoren & Ricksten (1979), with minor modifications. Briefly, under methohexitone-anaesthesia (i.v. injection of a 3% solution of methohexitone, adapted for each animal when required) for conscious SHRs or under pentobarbitone-anaesthesia ($60\,mg\,kg^{-1}$, i.p.) for anaesthetized SHRs, the renal sympathetic nerve was approached dorsolaterally through an incision in the left flank extending from the latissimus dorsalis muscle to the level of the sternum. Ventral retraction of the spleen and the kidney allowed for the dissection of nerves located along the left renal artery. The left renal nerve was isolated and carefully freed from surrounding tissue with the aid of a stereoscopic dissecting microscope. A thin platinum-iridium bipolar electrode was placed around the nerve and insulated with silicone rubber (Wacker Silgel 604, Adrian, U.S.A.). The electrode cable was sutured to the surrounding muscles, and the free end was passed subcutaneously to the back of the neck, exteriorized, and sutured in place. The rats were allowed a 2 h period to recover from anaesthesia before starting the experiment. The nerve signals were amplified ($\times$ 50,000) by a Grass P511.J. amplifier (Grass Instr. Co., Quincy, U.S.A.) with a low- and high-frequency

Table 1 Effects of pentobarbitone-anaesthesia on the baseline values of the parameters recorded in fifteen week-old male SHRs

	Conscious (n = 6)	Pentobarbitone-anaesthetized: Intact (n = 6)	Pentobarbitone-anaesthetized: Before transection (n = 6)
SBP (mmHg)	178 ± 8	155 ± 11†	121 ± 14*††
DBP (mmHg)	118 ± 10	114 ± 11	87 ± 13*††
HR (b.p.m.)	375 ± 15	393 ± 11	325 ± 12**††
RSNA (μV)	3.4 ± 0.9	10.6 ± 0.9††	10.3 ± 3.1††

Data are mean ± s.e.mean
* $P < 0.05$; ** $P < 0.01$; vs intact-anaesthetized SHRs. (Mann-Whitney-U-test).
† $P < 0.05$; †† $P < 0.01$; vs conscious SHRs (Mann-Whitney-U-test).

cut-off set at 100 and 1,000 Hz, respectively. The amplified nerve activity was displayed on a Gould oscilloscope DSO-1602 (Gould Instr. S.A.F., Paris, France). For the integration of discharges the nerve signals appearing at the oscilloscope outputs were rectified and displayed along with arterial BP and HR on a computer MOTOROLA SYS-3304/NY/082 (Motorola Co., Tempe, U.S.A.) (Gustin *et al.*, 1990). The background non neural activity was taken as the activity recorded after injection of a ganglionic blocker (trimethaphan camsilate: 10 mg kg^{-1}, i.v.). This background activity was determined at the end of the experiment and substracted from the total integrated activity to allow the quantitative estimation of sympathetic nerve activity.

Spinal cord transection

Six pentobarbitone-anaesthetized animals were subjected to spinal transection at the level of the fifth cervical (C5) segment. The spinal cord was exposed via a dorsal laminectomy from cervical level C1 to C6, then the vertebral disk between C4 and C5 segments was removed to allow visualization of the dorsal surface of the cord. Once the dura was perforated, the exposed spinal cord was kept moist with warm mineral oil. The spinal cord was completely transected at C5 level, first, with a scalpel blade and second, in order to ensure the completeness of the transection with a dental hand-drill. Immediately, a disk of gel foam (Spongel*, Lab. Houde, Paris, France), approximately 1 mm in width, was placed into the sectional spinal cord space, the muscles were sutured and the incision was closed.

Blood pressure, heart rate and renal sympathetic nerve activity signals processing

Systolic BP (SBP), diastolic BP (DBP), heart rate (HR) and rectified nerve signals of RSNA were continuously recorded in freely moving and anaesthetized groups of animals by a computerized technique (Gustin *et al.*, 1990).

Experimental protocols

In all groups of SHRs, i.c.v. cannula and, arterial and venous catheters were implanted one week and 48 h, respectively, before the start of the recording sessions. Injection volumes were 2 μl per animal for i.c.v. and 0.5 ml kg^{-1} for i.v. injections. In conscious and unrestrained animals, the i.c.v. and i.v. polyethylene tubes were extended outside the registration cage to avoid stressing the animals. Finally, at the end of each experiment, i.v. injections of the ganglionic blocker trimethaphan camsilate (10 mg kg^{-1}) were used to estimate the background noise level of the nerve activity.

Conscious SHRs ($n = 6$) Each awake rat was placed in a cylindrical cage and connected to the BP recording system for a 0.5 to 1.0 h control period. Then, under methohexitone-anaesthesia, the renal nerve bipolar electrode was implanted and spike activity was monitored. After a 2 h period which allowed the animal to recover from anaesthesia and the cardiovascular parameters to stabilize, BP, HR and RSNA were continuously recorded for 5 to 6 h. After a 1 h control period, each animal received one i.c.v. injection of artificial cerebrospinal fluid (CSF) followed by one i.c.v. injection of rilmenidine (300 μg kg^{-1}).

Pentobarbitone-anaesthetized SHRs ($n = 6$) After connection to the BP recording system, implantation of the renal nerve bipolar electrode and cannulation of the trachea, each animal was placed in a stereotaxic frame, for an equilibration period which allowed the cardiovascular parameters, blood gases and pH to stabilize. Then, BP, HR and RSNA were recorded continuously for a 1 h control period and a 3–4 h period during which, each animal received one i.c.v. injection of artificial CSF followed by one of rilmenidine (300 μg kg^{-1}).

Pentobarbitone-anaesthetized and spinally-transected SHRs ($n = 6$) The animals were prepared as described above for anaesthetized SHRs. Then, they were mounted on a stereotaxic frame in order to hold the head steady whilst the spinal cord was dissected and BP, HR and RSNA were continuously recorded. When the cardiovascular parameters returned to control values, the spinal cord was completely transected at C5 level and they were monitored for a 3–4 h period. Twenty to thirty min after transection, when all the cardiovascular parameters were stabilized, each animal received one i.c.v. injection of artificial CSF followed by one of rilmenidine (300 μg kg^{-1}).

Assessment of the guide-cannula implantation and of spinal transection

At the end of each experiment, the guide-cannula position was assessed as previously described in Sannajust *et al.* (1992). The spinal transection was confirmed *post-mortem* by dissection of the site of lesion.

Statistical evaluation

Data are expressed as mean ± standard error of the mean (s.e.mean). At any given time of the recording, the significance of the differences was assessed by means of a Mann-Whitney-U-test. Wilcoxon's signed rank-test for paired differences was used when appropriate. The interpretation of multiunit nerve recordings has several problems, as it is function of the number of active fibres, their firing frequency and of the contact between the nerve bundle and the electrode. However, since great care was taken to standardize the mechanical preparation of nerve bundles only slight differences were observed among the various SHRs and we, therefore, compared RSNA between groups.

Drugs and solutions

Rilmenidine [N-(dicyclopropylmethyl)-amino-2Δ2-oxazoline] phosphate (I.R.I. Servier, France) was dissolved in artificial CSF (Palaic *et al.*, 1967) and maintained in sealed vials at 37°C and adjusted to pH 7.3 just before injection. [Val^5]-angiotensin II acetate and trimethaphan camsilate were obtained from Sigma Chemical Company (St-Louis, U.S.A.) and Hoffmann-La-Roche Lab., (Basle, Switzerland) respectively.

Results

Effects of i.c.v. administration of rilmenidine in conscious SHRs

In freely moving, quietly resting SHRs, basal SBP, DBP, HR and RSNA (average of the 20 min of control period preceding i.c.v. administrations) are indicated in Table 1. As shown in Figure 1, artificial CSF as well as rilmenidine (300 μg kg^{-1},

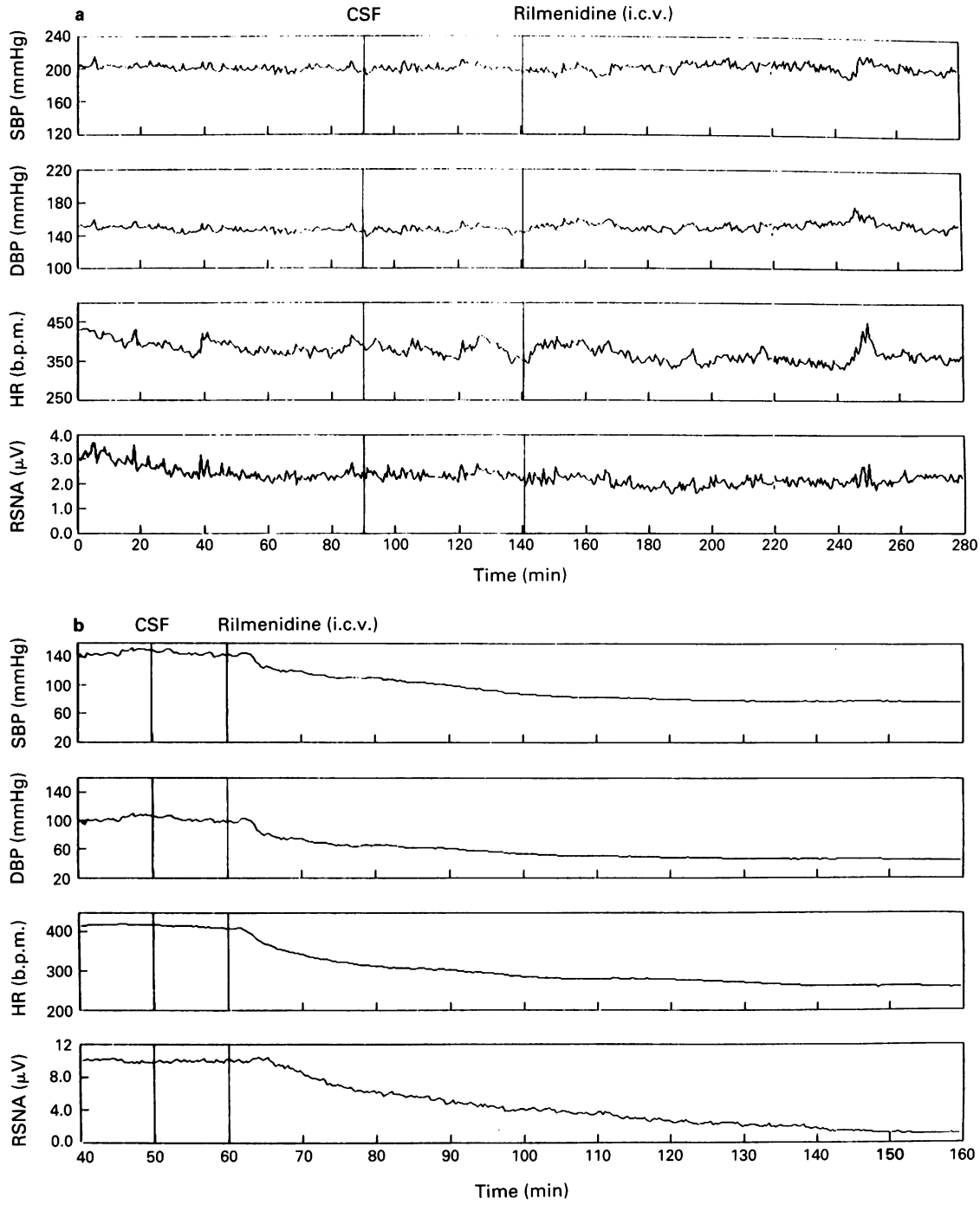

Figure 1 Systolic (SBP), diastolic (DBP) blood pressure, heart rate (HR) and renal sympathetic nerve activity (RSNA) before and after i.c.v. injection of cerebrospinal fluid (CSF) and rilmenidine ($300\,\mu g\,kg^{-1}$) in one conscious (a) and one pentobarbitone-anaesthetized spontaneously hypertensive rat (b). Each point of the graph is the mean of the values recorded during 60 s periods. The background noise of RSNA has been subtracted.

i.c.v.) did not affect these parameters. On the other hand, the ganglionic blocker given at the end of experiment, markedly reduced SBP, DBP, HR and RSNA.

Effects of pentobarbitone-anaesthesia (see Table 1)

Pentobarbitone-anaesthesia significantly ($P < 0.05$) lowered SBP and markedly (3 fold) increased RSNA. The surgical preparation required for the spinal cord transection slightly reduced BP and HR, but did not affect the RSNA level. Figure 2 indicates that the bursts of spikes of the renal nerve were more marked and exhibited a more clear-cut rhythmicity in pentobarbitone-anaesthetized (b) than in conscious (a) SHRs.

Effects of i.c.v. administration of rilmenidine in pentobarbitone-anaesthetized SHRs

In anaesthetized intact SHRs, rilmenidine ($300\,\mu g\,kg^{-1}$, i.c.v.) induced rapid, marked and long-lasting decreases in SBP, DBP, HR and RSNA (Figure 1). The maximum effects appeared around 90 min after i.c.v. injection, and were as follows: SBP ($-37 \pm 4\%$), DBP ($-43 \pm 6\%$), HR ($-32 \pm 3\%$) and RSNA ($-68 \pm 9\%$). As shown in Figure 1, 5 min after injection, HR, SBP and DBP were significantly ($P < 0.05$) decreased ($-11 \pm 2\%$, $-8 \pm 2\%$ and $-9 \pm 4\%$ respectively) while RSNA remained stable. Then SBP, DBP, HR and RSNA decreased in parallel to reach the maximal effect within 90 min.

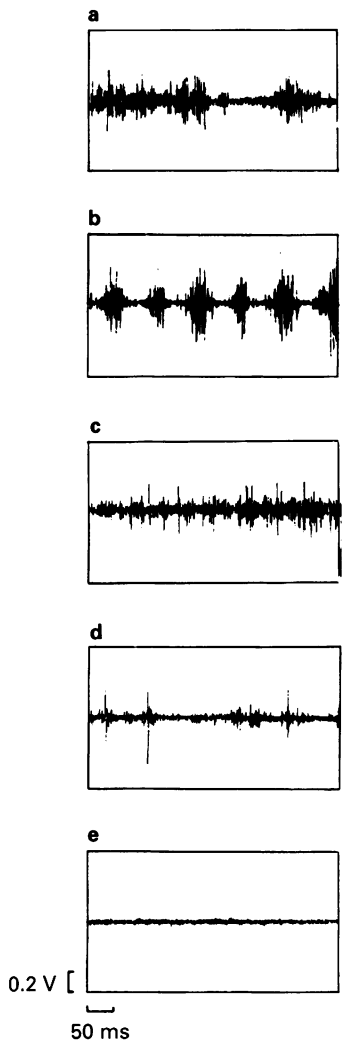

Figure 2 Typical oscilloscope recordings of the signals of renal sympathetic nerve activity (RSNA) in (a) one conscious, (b) one pentobarbitone-anaesthetized spontaneously hypertensive rat, (c) during baseline after spinal cord transection, (d) after i.c.v. injection of rilmenidine (300 $\mu g\,kg^{-1}$) and (e) after ganglionic blockade.

Effects of spinal cord transection

As indicated in Table 1 and Figure 3, the spinal cord dissection prior to the transection induced significant decreases in SBP and HR, but did not affect RSNA which was identical to that of pentobarbitone-anaesthetized intact SHRs. Acute spinal cord transection at C5 level, markedly ($P < 0.05$) and irreversibly reduced SBP, DBP, HR and RSNA ($-48 \pm 4\%$; $-52 \pm 4\%$; $-18 \pm 6\%$ and $-56 \pm 8\%$ respectively) (see Figures 2 and 3). Only 2 out of 6 animals in this experiment responded to the transection with a rapid and very short-lasting initial increase in BP, HR and RSNA, but, immediately after the end of transection, SBP, DBP, HR and RSNA decreased to levels significantly below control period. HR was less markedly reduced than BP. Interestingly, the spinal cord transection did not completely abolish RSNA as (see Figure 4) its level ($3.6 \pm 0.5\,\mu V$) returned to that of conscious SHR ($3.4 \pm 0.9\,\mu V$), but as shown in Figure 2b suppressed its rhythmicity.

Effects of i.c.v. administration of rilmenidine in pentobarbitone-anaesthetized and spinally transected SHRs

After spinal transection, average SBP, DBP, HR and RSNA were 61 ± 4 mmHg; 40 ± 4 mmHg; 267 ± 24 b.p.m. and $3.6 \pm 0.5\,\mu V$ respectively. In these new basal conditions (Figure 3), artificial CSF was devoid of effect and rilmenidine ($300\,\mu g\,kg^{-1}$, i.c.v.), did not alter BP but, significantly ($P < 0.05$) decreased HR ($-6 \pm 1\%$) and RSNA ($-22 \pm 4\%$) 5 min after the administration. The maximum effects (HR: $-16 \pm 3\%$; RSNA: $-74 \pm 5\%$) were obtained 50 min after the injection. Interestingly as indicated in Figure 4, the rilmenidine-induced relative decrease in RSNA was identical in intact and in spinal cord-transected SHRs.

Discussion

The major finding of this study is that rilmenidine acts to lower BP by reducing peripheral sympathetic nervous system (SNS) activity and that the site of this sympathoinhibitory effect is not only located in the brainstem but also in the spinal or ganglionic structures.

Rilmenidine is an amino-oxazoline, structurally related to imidazolines such as clonidine, which exhibits α_2-adrenoceptor agonist activities (Laubie *et al.*, 1985) and antihypertensive properties. Although the mechanism proposed for the antihypertensive action of rilmenidine in SHRs is a reduction in peripheral sympathetic tone (Van Zwieten, 1988), to our knowledge, no studies have demonstrated its direct action on the peripheral SNS activity in anaesthetized or conscious SHRs.

Previous studies in conscious, unrestrained (Sannajust *et al.*, 1989) and pentobarbitone-anaesthetized (Sannajust *et al.*, 1992) SHRs have shown that the ability of rilmenidine and clonidine to lower BP could be related to the level of the sympathetic drive and conversely to the integrity of the cardiac baroreflex. The absence of antihypertensive effects of rilmenidine or clonidine administered by the i.c.v. route in conscious SHRs, suggests that these drugs could interact with central structures like forebrain (Klevans *et al.*, 1973; Van den Buuse *et al.*, 1990) and brainstem (Gomez *et al.*, 1990), which play an activator or inhibitor role of sympathetic tone, respectively (Head, 1991). Therefore, in this study we examined in conscious unrestrained, pentobarbitone-anaesthetized and pentobarbitone-anaesthetized and spinally transected SHRs, the effects of rilmenidine administered by i.c.v. route, on BP, HR and postganglionic efferent RSNA. We have chosen to record the nerve traffic in the renal nerve because it represents a major sympathetic nerve implicated in the BP regulation. The renal nerve is well known to influence cardiovascular resistance, Na^+ and water reabsorption and renin secretion (DiBona, 1982). Under these conditions, in conscious SHRs, the absence of RSNA change after i.c.v. administered rilmenidine ($300\,\mu g\,kg^{-1}$ chosen from the dose-response curves obtained in previous experiments, Sannajust *et al.*, 1992) rules out a possible compensation, by other pressor systems, of the BP effects of rilmenidine. On the other hand, it seems unlikely that the lack of effect of rilmenidine on RSNA could depend on a balance of the two components (pressor and depressor) and that the vasomotor influence of the forebrain structures would be quantitatively opposed to the vasoinhibitory influence of the brainstem. This is in accordance with the recent demonstration by Gomez *et al.* (1990) that midcollicular decerebration did not affect the hypotensive effect of rilmenidine microinjected into the vasomotor centre (C1 area) of the rostral ventrolateral medulla; therefore, forebrain structures are not implicated in the mechanism of the antihypertensive action of rilmenidine.

In a second set of experiments, we administered rilmenidine by the i.c.v. route in pentobarbitone-anaesthetized SHRs. These experiments were designed to confirm the increase in SNS activity previously hypothesized (Sannajust *et al.*, 1992) on the basis of indirect evidence such as the great hypotensive effect of the ganglionic blocker, trimethaphan and to demonstrate whether the ability of rilmenidine to lower BP could be related to the degree of central sympathetic drive. It is generally accepted (Zimpfer *et al.*, 1982; Murthy *et al.*, 1982; Matsukawa & Ninomiya, 1989) that barbiturate-anaesthesia

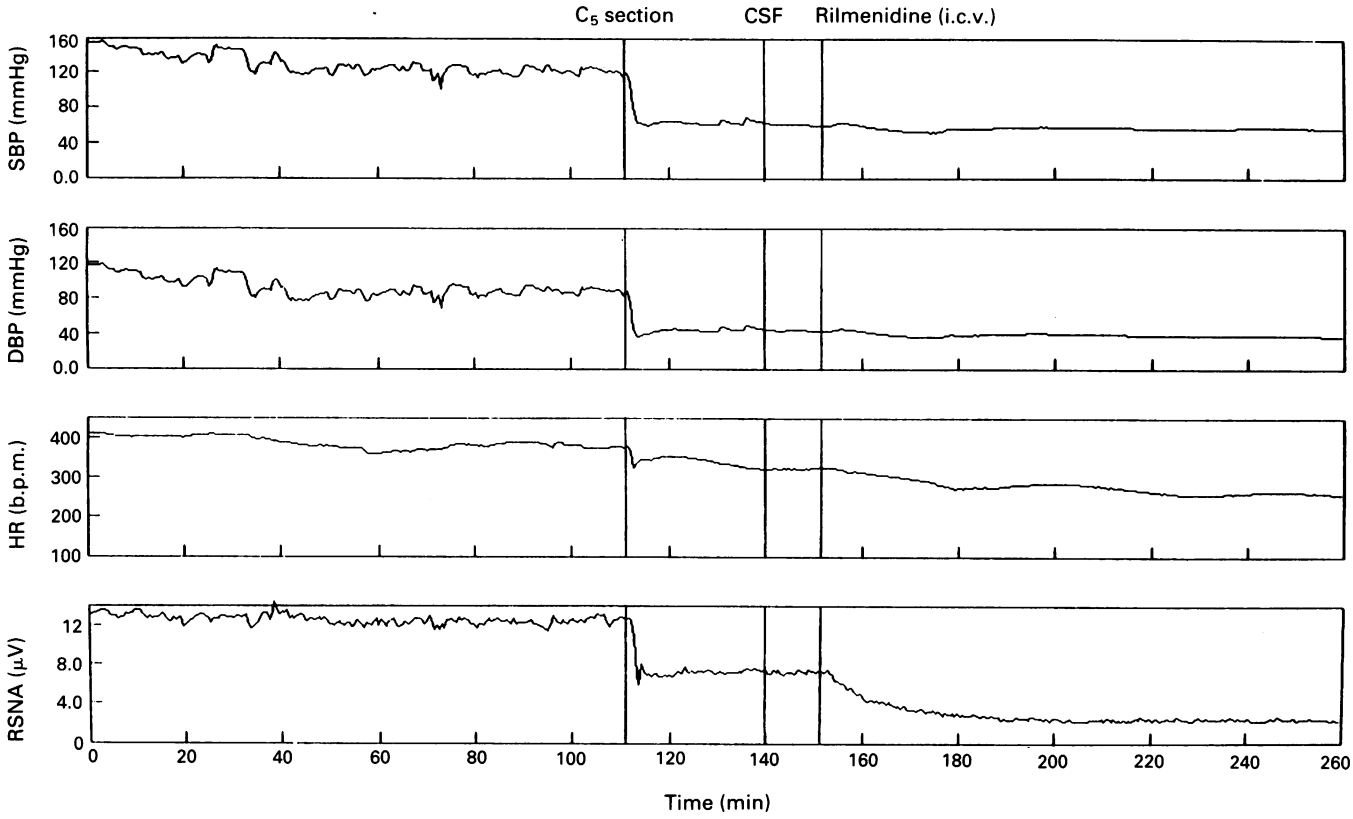

Figure 3 Systolic (SBP), diastolic (DBP) blood pressure, heart rate (HR) and renal sympathetic nerve activity (RSNA) before and after spinal cord transection then, after i.c.v. injection of cerebrospinal fluid (CSF) and of rilmenidine ($300\,\mu g\,kg^{-1}$) in one pentobarbitone-anaesthetized spontaneously hypertensive rat. Each point of the trace is the mean of the values recorded during 60 s periods. The background noise of RNSA has been subtracted.

increases the SNS activity and we have observed that the anaesthetized SHRs present a 3 fold higher RSNA level than the conscious SHRs. Interestingly, by using our technique of continuous recording of the different cardiovascular parameters we observed that RSNA decreased after the onset of the BP and HR decreases. This delay (2–3 min) demonstrates that the decrease in BP could result firstly from a sympathetic-independent decrease in HR, possibly mediated by vagal stimulation, and secondly from a reduction in sympathetic nerve activity which would induce an additional decrease in HR and a reduction in the cardiac output. Therefore, we have to consider that, like clonidine (Bonham *et al.*, 1984), rilmenidine may act at least, on two different sites, one controlling HR and the other controlling RSNA. Moreover, these results demonstrate that rilmenidine diminishes the electrical activity of postganglionic sympathetic fibres and provides direct evidence of a preganglionic or ganglionic site of action. Nevertheless, although we had quantified the sympathoinhibitory effect of i.c.v. rilmenidine we have yet to determine whether the effect is of central or peripheral origin.

A final set of experiments was designed to determine whether or not the sympathoinhibitory effect of rilmenidine was due to an action within the brainstem or could be the result of a peripheral action. I.c.v. administered rilmenidine may reach peripheral sites either by leaking from the cerebral ventricles into the systemic circulation or by diffusing through the CSF to spinal sites (e.g. the intermediolateralis tractus: IML) controlling the peripheral SNS tone. For this reason, we performed an acute spinal cord transection at the C_5 cervical level in pentobarbitone-anaesthetized rats. As previously reported by Schramm & Livingstone (1987) and Taylor & Schramm (1987), the spinal cord transection profoundly and irreversibly decreased SBP and DBP but less markedly reduced HR. Interestingly, the spinal cord transection did not completely abolish the RSNA which returned to the level observed in conscious SHRs, demonstrating that pentobarbitone-anaesthesia enhances the peripheral SNS tone by a central effect. Moreover, these observations suggest that the SNS activity in intact rats is generated not only by spinal or medullary centres, but also by interaction, at spinal levels, of excitatory and inhibitory information descending via multiple pathways. Spinal or ganglionic systems also contribute to the SNS tone (Schramm & Chornoboy, 1982). In these conditions, despite the normal RSNA level, i.c.v. rilmenidine was still able to decrease RSNA markedly to a degree similar to that observed in intact pentobarbitone-anaesthetized SHRs. This clearly demonstrates that this sympathoinhibition after spinal cord transection cannot be explained by a central effect of rilmenidine, but by action at sites situated below the C_5 level. As the measured RSNA is postganglionic, these sites may be located in the IML of the thoracolumbar spinal cord or in ganglionic structures. In addition, as rilmenidine has no effect in conscious SHRs with similar RSNA level, it must be emphasized that spinal cord transection has altered the mechanisms of control of the sympathetic tone. In that respect, Elghozi *et al.* (1989) have shown that the IML column receives noradrenergic fibres which have an important tonic inhibitory action on spinal preganglionic sympathetic motoneurones and non-adrenergic fibres which have an excitatory action on these same motoneurones; furthermore, these two kinds of fibres could, via stimulation of α_2-adrenoceptors, influence the vasomotor tone and HR.

Taken together, the results of the present study lead to the hypothesis that, in conscious SHRs, the tonic influence of the inhibitory inputs from the arterial baroreceptors and the noradrenergic fibres could counteract the effects of rilmenidine which are mediated via stimulation of α_2-adrenoceptors. Pentobarbitone-anaesthesia may only suppress the influence

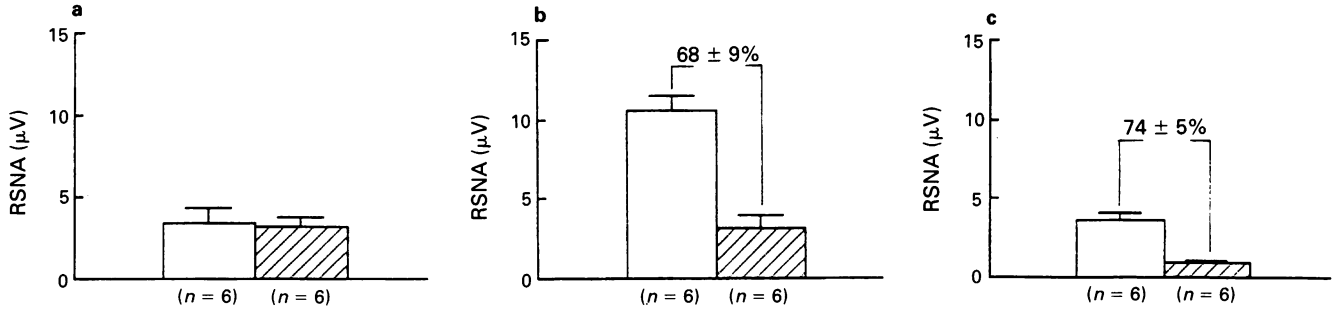

Figure 4 Effects of i.c.v. injections of rilmenidine ($300\,\mu g\,kg^{-1}$) on the renal sympathetic nerve activity (RSNA) in conscious (a), intact anaesthetized (b) or anaesthetized and spinal cord transected (c) spontaneously hypertensive rats. (Open columns: before injection; hatched columns: after injection). n = number of animals.

of inhibitory noradrenergic fibres, inducing an increase of the SNS activity and, as the cardiac baroreflex is abolished by pentobarbitone, anaesthesia could favour the action of rilmenidine. Similarly, in pentobarbitone-anaesthetized and spinally transected SHRs, the influences of both noradrenergic and non-adrenergic fibres may be blocked, thereby explaining the identical RSNA basal level observed in conscious animals and, thus unmasking the ability of rilmenidine to exert its action by stimulation of unoccupied α_2-adrenoceptors.

Such spinal or ganglionic sites of action obviously require further investigations of the location and nature of the receptors involved. However, the present work demonstrates that pentobarbitone-anaesthesia enhances the peripheral sympathetic tone by a central action. The antihypertensive effects of rilmenidine are closely related to the pre-existing level of the sympathetic drive and the mechanism of the sympatho-inhibitory action of rilmenidine seems to involve sites located below the brainstem.

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Influence of anaesthesia on the cardiovascular effects of rilmenidine and clonidine in spontaneously hypertensive rats

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1 The acute cardiovascular effects of two α_2-adrenoceptor agonists, rilmenidine and clonidine, were studied in 15-week-old male spontaneously hypertensive rats (SHRs). The effects of these drugs were compared with intravenous (i.v.) and intracerebroventricular (i.c.v.) administration in conscious and pentobarbitone-anaesthetized SHRs, in which aortic blood pressure (BP) was continuously recorded.

2 In conscious SHRs, i.v. doses of either rilmenidine (30, 100, 300 $\mu g\,kg^{-1}$) or clonidine (3, 10, 30 $\mu g\,kg^{-1}$) induced dose-dependent short-lasting increases in BP followed by moderate decreases associated with bradycardia, while the same three doses of both drugs given i.c.v. were devoid of BP and heart rate (HR) effects.

3 Pentobarbitone-anaesthesia increased the sympathetic control of BP and suppressed the cardiac baroreflex sensitivity.

4 In anaesthetized SHRs, i.v. injections of the same 3 doses of rilmenidine and clonidine induced a slight increase in BP, rapidly followed by profound and long-lasting BP and HR decreases. Surprisingly, when given i.c.v., these 3 doses lowered BP and HR to the same extent but in a more progressive manner.

5 The lack of efficacy of both drugs in conscious SHRs after the i.c.v. administration of i.v. active doses and the lack of more marked and rapid effects in anaesthetized SHRs, after i.c.v. than after i.v. injections, question the involvement of a major central site of action for these antihypertensive α_2-adrenoceptor agonists. Moreover, these results show that the cardiovascular effects of these drugs are profoundly influenced by baseline sympathetic nervous system activity which is enhanced by pentobarbitone-anaesthesia.

Keywords: α_2-Adrenoceptors; blood pressure; conscious rats; intracerebroventricular; intravenous; pentobarbitone-anaesthetized rats; clonidine; rilmenidine; spontaneously hypertensive rats

Introduction

Rilmenidine [(N-dicyclopropylmethyl)-amino-2-oxazoline] is an oxazoline analogue of clonidine with similar antihypertensive properties (Laubie *et al.*, 1985; Van Zwieten *et al.*, 1986; Van Zwieten, 1988). While initially rilmenidine and clonidine were considered as potent α_2-adrenoceptor agonists, actually, both belong to the new class of mixed α_2-adrenoceptor/imidazoline-preferring-receptor agonists. The pharmacological profile of rilmenidine differs markedly from that of clonidine. Rilmenidine is more selective for α_2- than for α_1-adrenoceptors (Guicheney *et al.*, 1981; Verbeuren *et al.*, 1990) and has a 2.5 times greater selectivity for the imidazoline-preferring-receptors over cortical α_2-adrenoceptors than clonidine (Gomez *et al.*, 1991). In addition, the main difference observed in both animals and man, is that rilmenidine induces a lower incidence of central nervous system side effects than clonidine (Laubie *et al.*, 1985; Fillastre *et al.*, 1988), lower sedative effects (Laubie *et al.*, 1985; Mahieux, 1989) and lower rebound effects during withdrawal (Sannajust *et al.*, 1989; Galley *et al.*, 1988). The mechanism of the antihypertensive action of rilmenidine is, like clonidine, believed to rely upon the stimulation of α_2-adrenoceptors (Laubie *et al.*, 1985) or imidazoline-preferring-receptors (Tibirica *et al.*, 1988; Ernsberger *et al.*, 1990) in the lateralis reticularis nucleus (Bousquet & Feldman, 1987) which induces a decrease in the sympathetic nervous system (SNS) activity (Van Zwieten *et al.*, 1983; Punnen *et al.*, 1987). The assumption of such a central action is based upon several experiments showing that intracerebroventricular (i.c.v.) or intravertebral injections of clonidine (Schmitt, 1977) or of rilmenidine (Laubie *et al.*, 1985) decrease blood pressure (BP) and heart rate (HR) at doses that are ineffective by the intravenous (i.v.) route. However, it must be emphasized that, the exact site(s) of action of these drugs within the brainstem (Van Zwieten *et al.*, 1983) as well as the receptors mediating their effects—α_2-adrenoceptors versus imidazoline-preferring receptors—remains unclear. Furthermore, most of the above cited experiments were conducted in anaesthetized animals. Since anaesthetics usually induce sympathetic activation (Matsukawa & Ninomiya, 1989), this could have influenced the cardiovascular effects of clonidine and related drugs. Indeed, the rare studies of these drugs in conscious animals produced conflicting results. When given i.c.v., clonidine did not affect (Bonham *et al.*, 1984) or decrease BP (Kawasaki *et al.*, 1986) in normotensive conscious rats, while it did not consistently modify BP of conscious spontaneously hypertensive rats (SHRs) (Baum & Becker, 1982). In previous experiments, we observed that in conscious freely moving SHRs, rilmenidine and clonidine, administered i.v., exhibited moderate antihypertensive activity and that this effect was more marked in active rats, i.e. with an activated SNS, than in resting animals (Sannajust *et al.*, 1989). Therefore, the purpose of the present work was to evaluate the effects of anaesthesia on the cardiovascular effects of rilmenidine and clonidine given by i.v. or i.c.v. routes in adult SHRs.

Methods

Animals

Ninety 15 week-old male SHRs (IFFA-Credo, Les Onçins, France), weighing 291 to 339 g, were used. Prior to experiments, the animals were kept for a two week habituation period in our animal house under controlled conditions (temperature: 21 ± 1°C, humidity: 60 ± 10% and lighting:

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0800 h 00 min–20 h 00 min). They received a standard rat chow (Entretien UAR, Villemoisson s/Orge, France) containing less than 0.3% sodium, with tap water *ad libitum*.

Preparation of conscious SHRs

One week before experimentation, a stainless-steel guide-cannula (0.8 mm outer diameter, 11.0 mm length) was implanted into the right lateral cerebroventricle, under chloral hydrate anaesthesia (300 mg kg^{-1}, i.p.), according to coordinates given by De Groot (1959). Briefly, the head of the animal was placed in a small-animal stereotaxic apparatus (La Précision Cinématographique Française, Asnières, France), the incisor bar of which was positioned 3.3 mm above horizontal zero passing through the plane of the interaural line. A 1.0 mm hole was drilled perpendicular to the skull, 5.4 mm anterior and 1.7 mm lateral to the horizontal zero. The tip of the guide-cannula was positioned 7.5 mm vertically to the horizontal zero. Then, the guide-cannula was anchored to the skull with dental cement and a stylet was locked inside. After surgery, the animals received a subcutaneous (s.c.) injection of gentamycin sulphate (10 mg kg^{-1}) (Unicet Lab., Levallois-Perret, France), and were allowed to recover for 5 days before catheter implantation. This latter was performed as previously described (Cerutti *et al.*, 1985) in halothane (2% in O_2) anaesthetized animals. A polyethylene (PE) tubing composed of PE 20 (0.40 mm internal diameter (I.D.)) and PE 60 (0.76 mm I.D.) was inserted into the lower abdominal aorta and passed under the skin to emerge through an aluminum tube fixed on the skull of the animal. Another catheter (PE 50: 0.58 mm I.D.) was inserted into a jugular vein for i.v. injections. After a 2-day recovery period, the animals were placed into a cylindrical cage maintained in controlled conditions of temperature (21 ± 1°C) and lighting (08 h 00 min–20 h 00 min). The arterial line was connected to a BP transducer (Statham P 23ID, Gould Inc., Cleveland, U.S.A.) through a rotating swivel (Ets R.P., Lyon, France) which allowed the rats to move freely. An overnight habituation period was allowed before starting the recordings.

Preparation of anaesthetized SHRs

The day of the experiment, SHRs were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and two catheters composed of PE 10 (0.28 mm I.D.) and PE 50 (0.58 mm I.D.) were inserted into the left femoral artery and vein, respectively. The arterial catheter was connected to the BP transducer through a three-way stopcock to allow a continuous infusion (0.4 ml h^{-1}) of isotonic glucose containing sodium pentobarbitone (6 mg kg^{-1} h^{-1}). Body temperature was maintained at 37 ± 1°C by means of a heating pad (Harvard Blanket Homeothermic System, Millis, U.S.A.). The trachea was cannulated for artificial ventilation (Harvard apparatus, model 683, Millis, U.S.A.) with room air (10 ml kg^{-1} × 65 min^{-1}). The animal was then placed in the stereotaxic apparatus and a guide-cannula was implanted into the right lateral cerebroventricle as previously described. At the beginning and at the end of each experiment, arterial blood gases and pH were measured with a blood gas and pH analyser (Instrumentation Laboratory, model 213, Milano, Italy).

Data collection

Arterial BP and HR were continuously recorded in freely moving and pentobarbitone-anaesthetized animals by a computerized technique (Gustin *et al.*, 1990). Briefly, the BP signal was transmitted to a BP analyser (Gould Processor Amplifier 134615-52, Cleveland, U.S.A.), then digitized and processed by a computer (SYS-3304/NY/082, Motorola Microsystems, Tempe, U.S.A.) which allows real-time, beat-by-beat determination of systolic (SBP) and diastolic (DBP) BP, mean arterial pressure (MAP) and HR, graphical monitoring and data storage on cartridge tapes (DC 600A, 3M, Germany). Off-line subsequent data analysis such as graphical and statistical analysis was performed on a workstation (SUN 3/50M, Sun Microsystems, Mountain View, U.S.A.).

Sympathetic control of arterial pressure

Sympathetic control was assessed at the beginning of each experiment as the maximum BP decrease induced by the short acting ganglion blocker, trimethaphan camsilate (10 mg kg^{-1}, i.v.). Previous experiments, using direct measurement of the renal sympathetic nerve activity in conscious and anaesthetized SHRs, have demonstrated that this dose of trimethaphan totally abolishes, for a few minutes, the electrical activity of the postganglionic renal fibers (unpublished observation).

Cardiac baroreflex sensitivity

Cardiac baroreflex sensitivity was determined by the beat-by-beat study of the changes in SBP and heart period (HP) [60 000/HR: ms] induced by i.v. injections of phenylephrine (Phen, 5 μg kg^{-1}) and nitroglycerin (NG, 100 μg kg^{-1}) (Smyth *et al.*, 1969). As previously described (Su *et al.*, 1986), for each injection the computer calculated the delay between the drug-induced SBP and HP changes that gave the best linear relationship between these two parameters. When the correlation coefficient reached the $P < 0.001$ significance level, the slope (ms mmHg^{-1}) of this relationship was used as an index of the baroreflex sensitivity. For each rat, the baroreflex sensitivity was expressed as the mean of the slopes obtained after three injections of Phen and NG.

Assessment of the guide-cannula implantation

Two criteria were used to assess guide-cannula implantation. Firstly, it was checked that i.c.v. injections of angiotensin II (300 ng kg^{-1}) induced both a drinking behaviour, within 1–2 min, and a pressor response in conscious animals (Hoffman *et al.*, 1977) and a pressor response in anaesthetized animals. Secondly, at the end of each experiment 2 μl of methylene blue dye [5% in artificial cerebrospinal fluid (CSF)] was injected and the brain was removed for histological examination. Correct implantation was assumed when heavy staining of the ventricles, including the fourth ventricle, could be observed.

Experimental protocols

In all the experiments, the injected volumes were 0.5 ml kg^{-1}, i.v. and 2 μl, i.c.v. per animal. The ascertainment of the stability of the preparations during the course of the experiments was previously described (Sannajust *et al.*, 1989); therefore two series of experiments were designed.

Conscious SHRs BP and HR were recorded for 0.5 to 1 h (control period). Then each animal successively received one i.v. injection of trimethaphan followed, 1 h later by three i.v. bolus injections of Phen and NG. When all the cardiovascular parameters had returned to control values, SHRs were randomly allocated to i.v. or i.c.v. injections and to rilmenidine (i.v.: $n = 7$; i.c.v.: $n = 6$) or clonidine (i.v.: $n = 6$; i.c.v.: $n = 6$) groups. Each rat received three successive increasing doses of rilmenidine (30–100–300 μg kg^{-1}) or of clonidine (3–10–30 μg kg^{-1}) given at 3 h intervals.

Pentobarbitone-anaesthetized SHRs After an equilibration period which allowed the cardiovascular parameters, blood gases and pH to stabilize, BP and HR were recorded for a 0.5 h control period. Then each animal received trimethaphan and 1 h later, 3 injections of Phen and of NG. When all the parameters had returned to control values the rat received, either i.v. or i.c.v., one dose of rilmenidine (30 or 100 or 300 μg^{-1}) or clonidine (3 or 10 or 30 μg kg^{-1}), which was preceded by the injection of corresponding vehicle. Therefore, 12

groups of rats (all $n = 5$–6) were studied and the route of administration, drug and dose were randomized.

Statistical evaluation

Data are expressed as mean ± s.e.mean. Wilcoxon's signed rank-test for paired differences or Wilcoxon's unpaired test for comparison of two independent groups were used when appropriate.

Drugs and solutions

Rilmenidine [N-(dicyclopropylmethyl)-amino-2Δ2-oxazoline] phosphate (I.R.I. Servier, France), clonidine [2-(2-6, dichlorophenyl)-amino-2 imidazoline] hydrochloride (Boehringer-Ingelheim, Germany) were dissolved either in artificial CSF and maintained in sealed vials at 37°C or in sterile saline, for i.c.v. or i.v. administrations respectively. The artificial CSF (Palaic *et al.*, 1967) contained (millimolar): NaCl 126.5, KCl 2.4, $CaCl_2$ 1.30, $MgCl_2$ 0.83, $NaHCO_3$ 27.5, KH_2PO_4 0.50, urea 2.7 and glucose 3.9. It was stored at −4°C, pre-heated to 37°C and adjusted to pH 7.3 immediately before starting the i.c.v. injections. [Val^5]-angiotensin II acetate was obtained from Sigma Chemical Company (St Louis, U.S.A.). Trimethaphan camsilate (Hoffmann-La-Roche Lab., Basel, Switzerland), phenylephrine chlorhydrate (Sigma Lab., St Louis, U.S.A.) and nitroglycerin (Besins-Iscovesco Lab., Paris, France) were dissolved in sterile saline.

Results

Studies in conscious SHRs

As presented in Figure 1, i.v. injections of three doses of rilmenidine induced a sharp dose-dependent BP increase which lasted for 5 to 10 min and was followed by a progressive and long-lasting dose-dependent decrease. The maximal effect reached: 18 ± 3, −26 ± 3 and −34 ± 2 mmHg for SBP and −13 ± 3, −16 ± 4 and −20 ± 3 mmHg for DBP at the doses of 30, 100 and 300 $\mu g\,kg^{-1}$, respectively. These BP changes were significant at the $P < 0.05$ level for the two lower doses and at the $P < 0.01$ level for the 300 $\mu g\,kg^{-1}$ dose and were associated with a slight bradycardia which did not reach the level of statistical significance (Figure 3). Clonidine (Figure 2) induced similar decreases in BP (SBP: −20 ± 2, −28 ± 3 and −34 ± 4 mmHg; DBP: −11 ± 1, −18 ± 3 and −22 ± 4 mmHg for the doses of 3, 10 and 30 $\mu g\,kg^{-1}$, respectively). These decreases were statistically significant at the $P < 0.01$ level for SBP and DBP only for the 30 $\mu g\,kg^{-1}$ dose and at the $P < 0.05$ level for DBP at the lower doses. Therefore, in conscious SHRs, the equipotent rilmenidine/clonidine ratio is approximately 10. However, the effects of clonidine differed from those of rilmenidine in that the hypertensive peak was more marked and there was a more marked and long-lasting bradycardia which reached statistical significance (−130 ± 5 b.p.m., $P < 0.05$) only for the 30 $\mu g\,kg^{-1}$ dose. As indicated in Figure 3, clonidine 30 $\mu g\,kg^{-1}$, i.v.

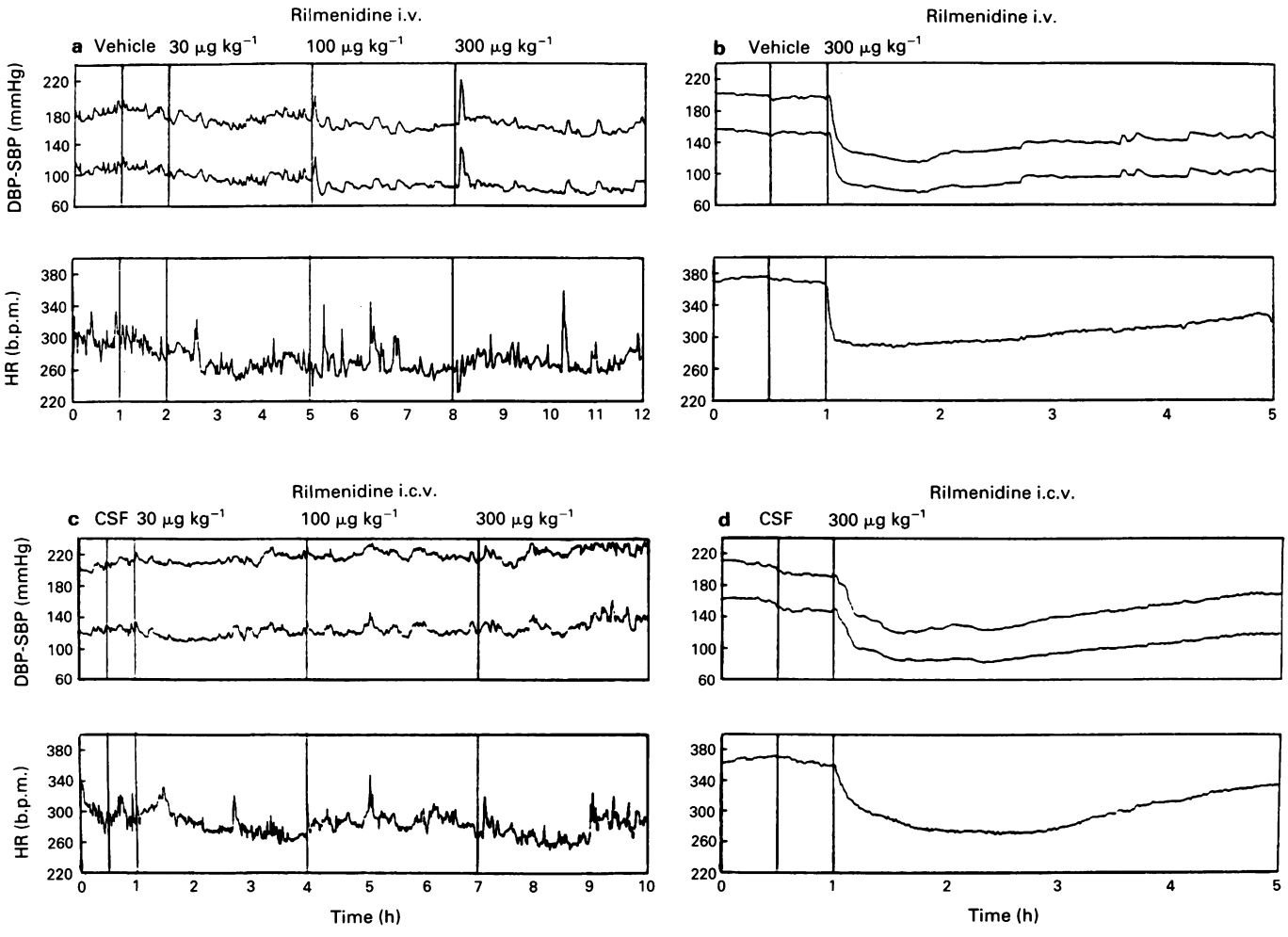

Figure 1 Changes in systolic (SBP), diastolic (DBP) blood pressure and heart rate (HR) before and after i.v. (a, b) or i.c.v. injection (c, d) of rilmenidine in, one conscious (a, c) and one anaesthetized (b, d) 15 week-old male spontaneously hypertensive rat. Each point of the graph is the mean of the values recorded during an 80 s period.

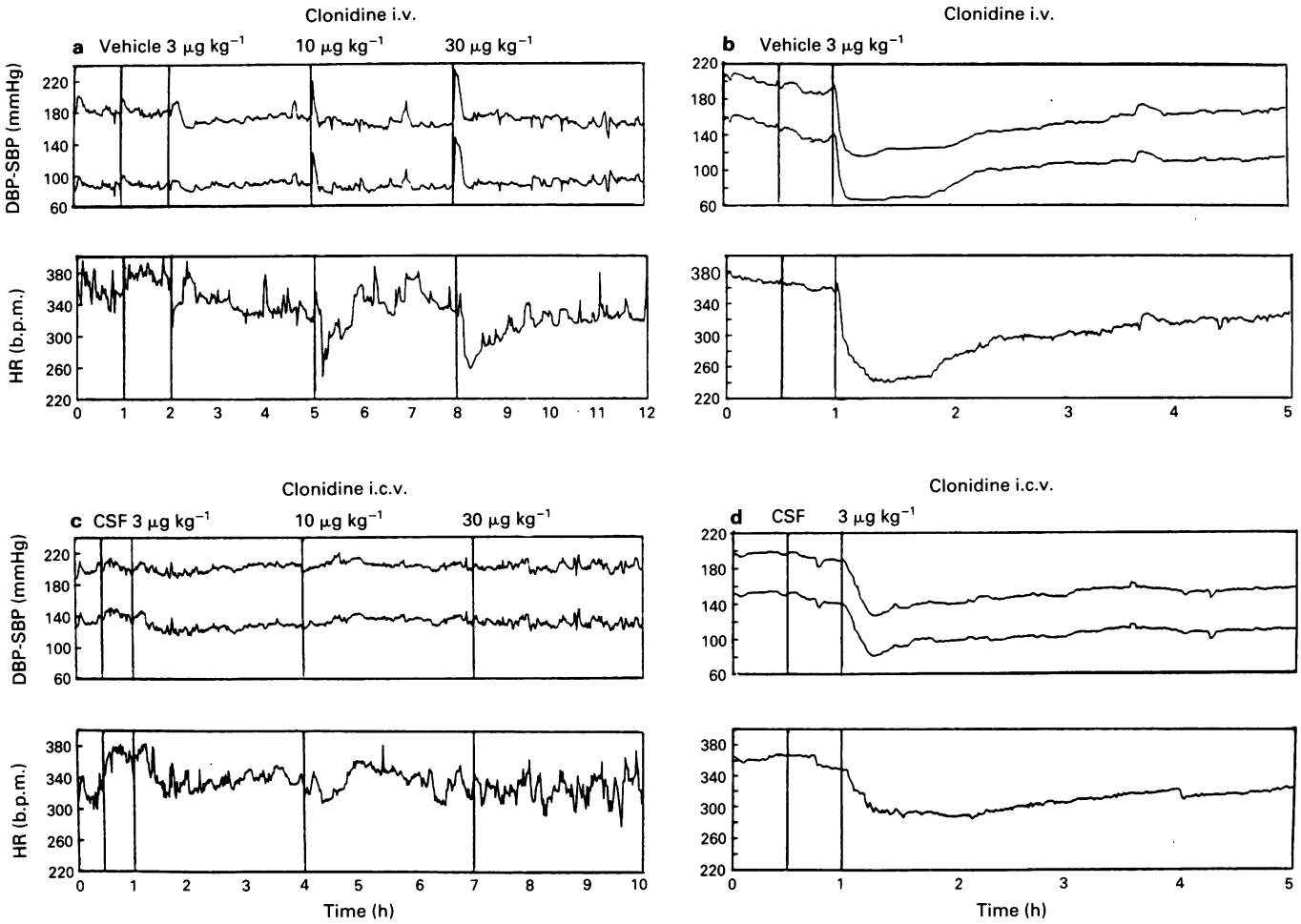

Figure 2 Changes in systolic (SBP), diastolic (DBP) blood pressure and heart rate (HR) before and after i.v. (a, b) or i.c.v. injection (c, d) of clonidine in, one conscious (a, c) and one anaesthetized (b, d) 15 week-old male spontaneously hypertensive rat. Each point of the graph is the mean of the values recorded during an 80 s period.

decreased HR to the level observed after ganglionic blockade with trimethaphan.

I.c.v. injections of angiotensin II (300 ng kg^{-1}) induced an almost immediate drinking behaviour which lasted for 10 to 15 min and was associated with a SBP increase of 28 ± 3 mmHg (Table 1). As indicated in Figures 1, 2 and 3, neither rilmenidine nor clonidine when given i.c.v., induced significant changes in BP and HR.

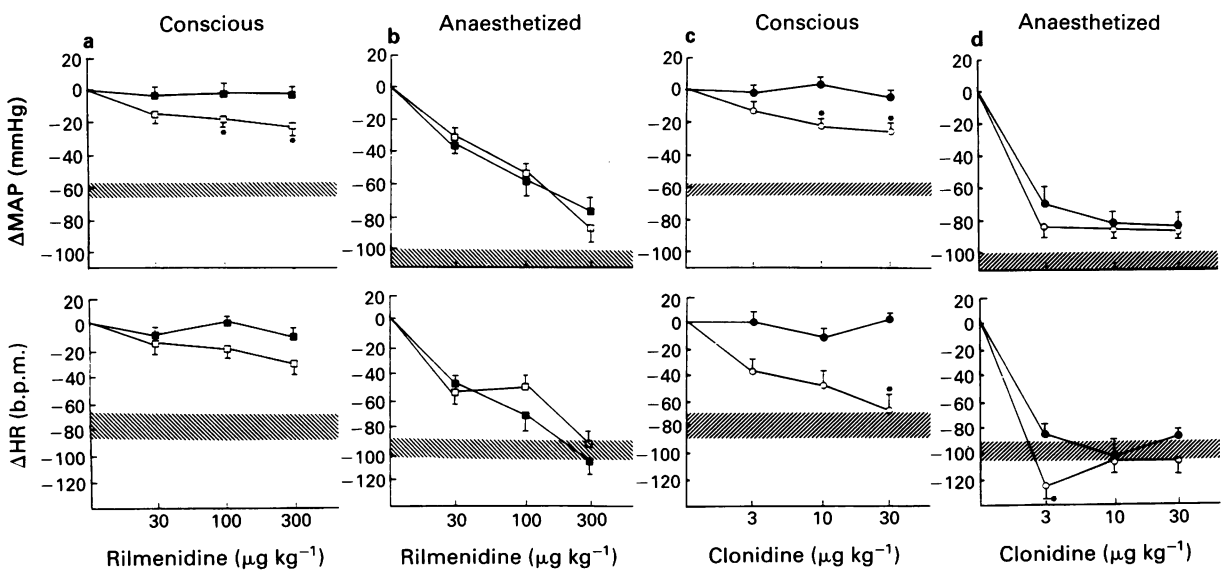

Figure 3 Maximum decreases in mean arterial pressure (ΔMAP) and heart rate (ΔHR) after administration of rilmenidine (a, b) by i.v. (□) or i.c.v. (■) routes, or of clonidine (c, d) by i.v. (○) or i.c.v. (●) routes in groups of 6 conscious or pentobarbitone-anaesthetized 15 week-old male spontaneously hypertensive rats. The hatched area corresponds to the mean (± s.e.mean) values observed after an i.v. injection of trimethaphan (10 mg kg^{-1}).
* $P < 0.05$ vs i.c.v.

Table 1 Effects of pentobarbitone-anaesthesia on the cardiovascular parameters of 15 week-old male SHRs

	Conscious ($n = 13$)	*Pentobarbitone* ($n = 65$)
Baseline		
SBP (mmHg)	198 ± 3	193 ± 2
DBP (mmHg)	120 ± 3	147 ± 2**
MAP (mmHg)	146 ± 3	163 ± 2**
HR (b.p.m.)	324 ± 14	364 ± 3**
Effects of trimethaphan ($10\,mg\,kg^{-1}$, i.v.)		
ΔSBP (mmHg)	−70 ± 5	−105 ± 3**
ΔDBP (mmHg)	−58 ± 4	−101 ± 3**
ΔMAP (mmHg)	−62 ± 3	−103 ± 3**
ΔHR (b.p.m.)	−78 ± 10	−97 ± 6
Cardiac baroreflex sensitivity (ms $mmHg^{-1}$)		
Phen	0.62 ± 0.11	0.13 ± 0.02**
NG	0.86 ± 0.11	0.06 ± 0.01**
Angiotensin II ($300\,ng\,kg^{-1}$, i.c.v.)		
ΔSBP (mmHg)	28 ± 3	15 ± 1**
ΔDBP (mmHg)	26 ± 2	11 ± 1**
ΔMAP (mmHg)	27 ± 2	12 ± 1**
ΔHR (b.p.m.)	16 ± 4	6 ± 2*

Values are means ± s.e.mean. For abbreviations see text.
* $P < 0.05$; ** $P < 0.01$ vs conscious.

Effects of pentobarbitone-anaesthesia

As indicated in Table 1, pentobarbitone slightly increased baseline HR and significantly enhanced DBP. The influence of SNS on BP was markedly increased by pentobarbitone anaesthesia as demonstrated by the greater hypotensive response to the ganglionic blocker, trimethaphan, and the cardiac baroreflex sensitivity was almost abolished, when measured after Phen and NG injections. Finally, the pressor effects of i.c.v. angiotensin II were lower in anaesthetized animals than in conscious rats. Blood gases and pH were stable throughout the experiment and were as follows: before Po_2: 86 ± 0.2 mmHg, Pco_2: 35 ± 0.6 mmHg and pH: 7.47 ± 0.007, and at the end of the experiments, Po_2: 84 ± 0.7 mmHg, Pco_2: 37 ± 0.7 mmHg and pH: 7.47 ± 0.009.

Studies in pentobarbitone-anaesthetized SHRs

The effects of i.v. injection of increasing doses of rilmenidine and clonidine in pentobarbitone-anaesthetized SHRs were strikingly different from those observed in conscious conditions. As shown by Figures 1 and 2, the immediate pressor effect was almost abolished. Most significantly, both rilmenidine and clonidine evoked a rapid and profound decrease in BP and in HR. Both parameters tended to return to baseline more rapidly after rilmenidine than after clonidine, but remained significantly lowered for the 4 h of observation. Figure 3 indicates that the peak effects of rilmenidine were dose-dependent, while the lowest dose used of clonidine ($3\,\mu g\,kg^{-1}$) evoked the maximum antihypertensive ($P < 0.001$) and bradycardic ($P < 0.001$) effects. Therefore, in anaesthetized SHRs, the equipotent rilmenidine/clonidine ratio was at least 100.

When considering the i.c.v. injections, both drugs (Figures 1 and 2) evoked a profound and long-lasting decrease in BP and HR, the magnitude of which was similar to that observed after the i.v. injection of the same doses (Figure 3). However, the effects of i.c.v. injections differed from those of i.v. administration by the absence of a short lasting preliminary pressor response and by a more progressive decrease in BP. Finally, the maximum BP and HR effects of rilmenidine and clonidine did not differ from those of trimethaphan, except that the maximum bradycardic response obtained after i.c.v. administration of the lowest dose of clonidine was greater than that of trimethaphan.

Discussion

The mechanisms whereby rilmenidine (an original antihypertensive compound with an oxazoline ring) and clonidine (the prototype of central antihypertensive agents with an imidazoline structure) lower BP and HR have been related to a decrease in the peripheral SNS activity mediated by the stimulation of postsynaptic α_2-adrenoceptors located within the central nervous system (for review see Schmitt, 1977; Van Zwieten *et al.*, 1983). However, the precise site(s) of action of clonidine remains unclear (Jarrott *et al.*, 1987) and it has recently been suggested that the receptors involved differ from α_2-adrenoceptors and are more specific for the imidazole structure (Meeley *et al.*, 1986; Lachaud *et al.*, 1988; Ernsberger *et al.*, 1990). Furthermore, in previous experiments (Sannajust *et al.*, 1989), we observed that in conscious SHRs, rilmenidine like clonidine induced significant but mild decreases in BP and HR, associated with a slight reduction in the urinary excretion of catecholamines taken as an index of the peripheral SNS activity. The discrepancy between these results and most of those of the literature prompted us to assess the importance of a central site of action and the effects of anaesthesia on the cardiovascular effects of rilmenidine and clonidine.

For that purpose, drugs were given by the i.v. and i.c.v. routes. In conscious freely moving SHRs, the results obtained after i.v. injections of rilmenidine or clonidine suggest that, besides a reduction in the peripheral SNS activity, clonidine more than rilmenidine lowers HR by another mechanism, which has been proposed to be a central activation of the vagal tone (Schmitt & Laubie, 1983). However, the most striking result was that the doses of rilmenidine and clonidine, which were active when given i.v., were devoid of cardiovascular effects when administered i.c.v.

As we have postulated previously (Sannajust *et al.*, 1989) that the importance of the effects of rilmenidine were related to the level of the sympathetic drive of the animals. The experiments were repeated in rats anaesthetized with pentobarbitone, an agent that enhanced the SNS tone (Touw *et al.*, 1980) and almost abolished the cardiac baroreflex sensitivity. Under these conditions, when given i.v., we observed consistent differences in the BP effects of the two drugs. The absence of initial pressor effect of i.c.v. injections of rilmenidine and clonidine in anaesthetized SHRs is likely to be due to the lack of stimulation of α_1/α_2-vascular adrenoceptors (Kobinger & Pichler, 1975; Timmermans & Van Zwieten, 1980) by drugs leaking from the cerebral ventricle to the systemic circulation. Moreover, the slower onset of antihypertensive action after i.c.v. than after i.v. administration, may represent the time needed for the small i.c.v. injected volume ($2\,\mu l$) to reach peripheral sites of action, such as those in the medulla oblongata (Sun & Guyenet, 1986; Punnen *et al.*, 1987) or the thoracolumbar spinal cord (Connor & Finch, 1981; Solomon *et al.*, 1989). This finding, which is especially clear with rilmenidine, argues against the hypothesis of a preferential central action, as it is difficult to conceive that the same amount of drug would more rapidly reach the medulla oblongata when given i.v. than when given i.c.v. Several studies have examined the disposition of clonidine in rats after i.v. administration. Conway & Jarrott (1980) found that the administration of clonidine ($20\,\mu g\,kg^{-1}$, i.v.) led to a rapid distribution, with the highest levels being obtained in all the tissues 2 min after the injection. At the peak of the antihypertensive response, less than 2% of the injected dose was present in the brain. These results emphasize that a considerable amount of clonidine does not enter the central nervous system and that a peripheral action of the antihypertensive effect of clonidine must be considered. Therefore as: (1) in anaesthetized SHRs i.v. doses were found as potent and more rapidly active than i.c.v. doses

and, (2) in conscious SHRs the doses which were active i.v. were devoid of effect when given i.c.v., it may be proposed that both drugs do not act primarily on the central nervous system to lower BP and HR and that they perhaps do not work through identical mechanisms. Moreover, the lack of effect of i.c.v. rilmenidine or clonidine in conscious SHRs raises several hypotheses. The use of insufficient doses or the injection of a volume too small to reach the ventral part of the medulla oblongata can be ruled out on the basis of the efficacy of the same doses i.v. in conscious SHRs and of the same doses given i.c.v. in anaesthetized animals. Another possibility is that clonidine and related drugs exert different cardiovascular effects by acting on different nuclei of the brain. Kawasaki & Takasaki (1986) observed that i.c.v. BHT-920, another α_2-adrenoceptor agonist, exhibited pressor effects in conscious rats while it was depressor in pentobarbitone-anaesthetized animals. They speculated that BHT-920 could be pressor by acting on suprabulbar structures and depressor on bulbar sites, and that anaesthesia had specifically suppressed the suprabulbar action. Such an hypothesis which suggests that the pressor and depressor effects of rilmenidine or clonidine act in balanced opposition in conscious SHRs, has been recently ruled out by Gomez *et al.* (1991), who demonstrated that decerebration did not change the cardiovascular effects of rilmenidine.

Therefore, the most likely explanation for the marked differences observed between the effects of i.c.v. rilmenidine and clonidine in conscious and anaesthetized animals may involve the pentobarbitone-induced changes in SNS activity and in the cardiac baroreflex sensitivity. In anaesthetized SHRs with an elevated sympathetic drive, the sympathoinhibitory action of rilmenidine and clonidine can be fully expressed. In addition, it is not opposed by a baroreflex activation. In freely moving and unstressed SHRs, with a low SNS activity, clonidine or rilmenidine can exert only small sympathoinhibitory effect. This slight effect is opposed by the baroreflex activation; thus Bonham *et al.* (1984) demonstrated that, when given i.c.v. clonidine was devoid of effects in conscious normotensive rats but became hypotensive after sinoaortic denervation.

In conclusion, this study does not allow the exclusion of a central action of rilmenidine and clonidine. However, it clearly demonstrates that, if such an action occurs, it may not be of major importance in the cardiovascular effects of these drugs when they are given in conscious and unstressed SHRs with a normal baroreflex sensitivity. In addition, it shows that the ability of these two drugs to lower BP could be related to the level of sympathetic drive. Finally, the experiments described in the paper show the importance of conscious animals for the study of antihypertensive agents which interfere with the autonomic nervous system.

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Effects of BRL 38227 on potassium currents in smooth muscle cells isolated from rabbit portal vein and human mesenteric artery

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1 Single smooth muscle cells were isolated from the rabbit portal vein and the human mesenteric artery and whole cell currents recorded at room temperature from either cell type by the whole cell voltage clamp technique.

2 In the rabbit portal vein cells addition of 10 μM BRL 38227 induced a quasi-instantaneous, voltage-insensitive and time-independent current which had a reversal potential of −75 mV under experimental conditions where the calculated E_K was −83 mV.

3 Cells were held at 0 mV and BRL 38227 was added cumulatively to construct a dose-response relationship. BRL 38227 (0.03–10 μM) caused a dose-dependent outward shift in the holding current with an EC_{50} of 1.3 μM.

4 BRL 38227 (10 μM) had no effect on the delayed rectifier K^+ current measured in the presence of 5 mM tetraethylammonium and no effect on the Ca^{2+}-activated K^+ current measured in the presence of 5 mM 4-aminopyridine. Similarly BRL 38227 had no effect on the Ca^{2+} current.

5 The BRL 38227-induced current was blocked by glibenclamide (10 μM) and phentolamine (100 μM), specific blockers of the ATP-sensitive K^+ current in single cells.

6 In human isolated mesenteric artery cells, BRL 38227 (10 μM) induced a glibenclamide-sensitive current similar to, but smaller than, that observed in the rabbit portal vein.

7 We conclude that in these cells, BRL 38227 activates a potassium conductance which has the electrophysiological and pharmacological characteristics of ATP-sensitive K^+ channels.

Keywords: Smooth muscle; rabbit portal vein; human mesenteric artery; BRL 38227; potassium currents

Introduction

BRL 38227 [(−)-6-cyano-3,4-dihydro-2,2-dimethyl-*trans*-4-(2-oxo-1-pyrrolidyl)-2H-1-benzopyran-3-ol)] is one of a novel class of compounds, termed potassium (K^+) channel openers, that have been shown to produce relaxation of various visceral and vascular smooth muscles (Hamilton & Weston, 1989). Their main mode of action is the activation of a potassium conductance resulting in hyperpolarization of the resting membrane potential. At present, several different potassium channels have been proposed as sites of action of these drugs. Beech & Bolton (1989c) suggested that the mode of action of cromakalim, the mixture of BRL 38227 and its (+)-enantiomer, was a pharmacological alteration of the time-dependent voltage-sensitive delayed rectifier current. Others have identified an ATP-sensitive K^+ conductance which is activated by cromakalim (Standen *et al.*, 1989) while both small (Okabe *et al.*, 1990) and large (Gelband *et al.*, 1989) conductance Ca^{2+}-activated K^+ channels have been proposed as the possible site of action for the potassium channel openers.

The whole cell K^+ currents present in the rabbit portal vein have been well characterized by Beech & Bolton (1989a,b). They identified a tetraethylammonium (TEA)-sensitive Ca^{2+}-activated K^+ current responsible for both spontaneous transient outward currents and a noisy sustained outward current elicited by depolarization positive of 0 mV. They also found a delayed rectifier K^+ current activated at potentials positive of −40 mV and a voltage-dependent K^+ current with fast kinetics. In the same cell Hume & Leblanc (1989), under different conditions, identified four currents including a background K^+ current, a delayed rectifier K^+ current and two distinct Ca^{2+}-activated K^+ currents as suggested by earlier single channel studies (Inoue *et al.*, 1985; 1986).

The objective of the present study was to identify the BRL 38227-sensitive currents in cells isolated from both the rabbit portal vein and human mesenteric artery. BRL 38227 was found to elicit a time-independent voltage-insensitive K^+ conductance. The blockade of this conductance by glibenclamide and phentolamine suggested the site of action of BRL 38227 was the ATP-sensitive K^+ channel.

Methods

Cell preparation

Rabbit portal vein Cells were isolated by the technique of Beech & Bolton (1989a). Adult rabbits of either sex weighing 2–3 kg were killed by i.v. injection of phenobarbitone. The main branch of the portal mesenteric vein was removed, placed in physiological saline solution (PSS) and dissected free of fat and connective tissue. It was then cut into four small equal sized pieces which were then placed into a low-Ca^{2+} PSS for 10–20 min. The tissue was subsequently incubated for 30 min at 37°C in low-Ca^{2+}-PSS containing papain (1 mg ml^{-1}), dithiothreitol (1 mM) and bovine serum albumin (1–2 mg ml^{-1}). The tissue was placed in more enzyme-free low-Ca^{2+} PSS and triturated with a wide-bore Pasteur pipette to dislodge the cells. Calcium in the medium was then increased to about 0.7 mM, and the cells were stored at 4°C for use within 9 h.

Human mesenteric artery Fat attached to sections of stomach or small intestine removed during routine surgery at St

[1] Author for correspondence.

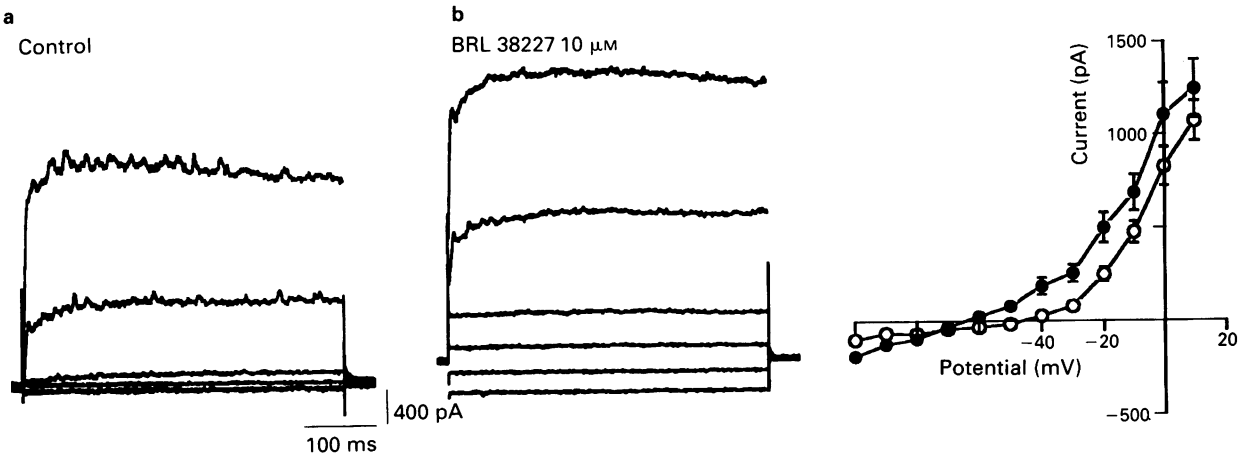

Figure 1 Effects of BRL 38227 on whole cell K^+ currents in cells isolated from the rabbit portal vein. (a) Representative current traces from a single cell in normal PSS held at −60 mV and stepped from −90 mV to 10 mV with 20 mV increments in the absence and presence of 10 μM BRL 38227 (bath applied). (b) The *I–V* relationship of the K^+ current, measured at the peak of the current, in normal PSS for six cells (mean with s.e.mean shown by vertical bars) under control conditions (○) and in the presence of 10 μM BRL 38227 (●). The current augmented by BRL 38227 had an apparent reversal potential of −75 mV.

Thomas's Hospital was immediately cut away, placed into cold physiological saline solution (PSS), and small arteries (0.5–3 mm, outer diameter) were dissected out. Arteries were then opened longitudinally and cut transversely into small equally sized sections. These sections were used immediately or were stored for up to two days in PSS at 4°C for further isolation. To obtain isolated cells, four sections of tissue were incubated in Ca^{2+}-free PSS for 30 min at 37°C. The tissue was then placed in 2 ml low-Ca^{2+} PSS containing papain (1.5–2.5 mg ml^{-1}), collagenase (1–1.7 mg ml^{-1}, Sigma Type XI), dithiothreitol (1 mM) and bovine serum albumin (2.5–3.5 mg ml^{-1}) for 50–60 min at 37°C. Following digestion the tissue was then placed in enzyme-free Ca^{2+}-free PSS, gently triturated and stored in a similar fashion to the rabbit portal vein cells.

Electrophysiology

Voltage clamp and cell current clamp experiments were carried out at room temperature (23°–27°C) by use of an Axopatch 1A patch clamp amplifier (Axon Instruments, Foster City, CA, U.S.A.). Patch pipettes, which were made from borosilicate glass, had resistances of 2–6 MΩ when filled with normal intracellular solution. Corrections for cell capacitance

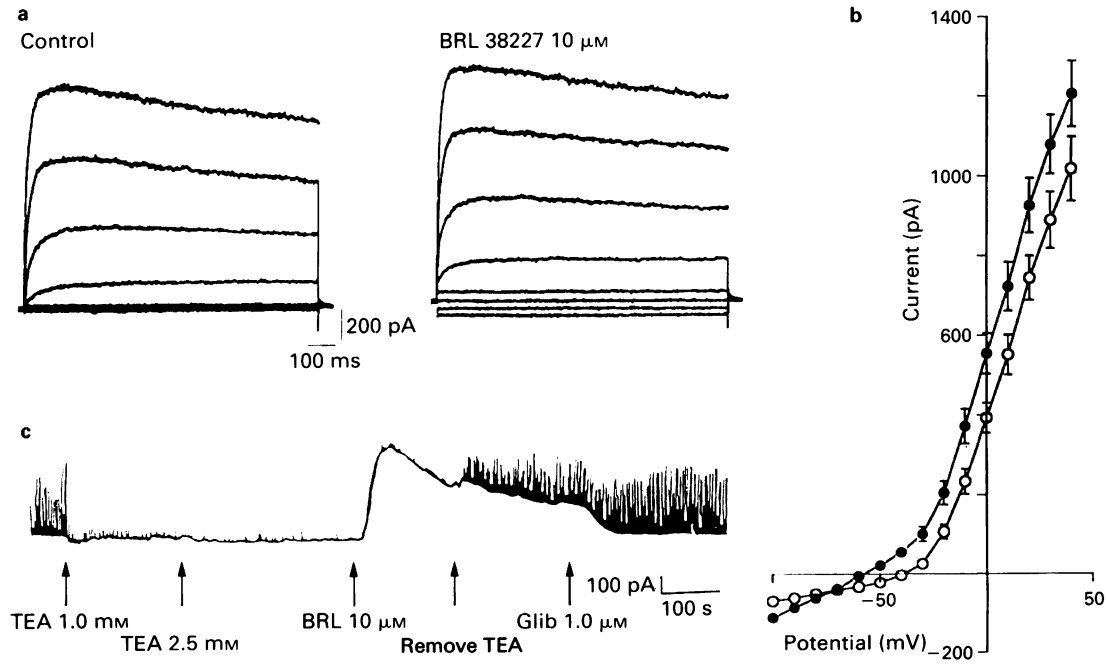

Figure 2 Action of BRL 38227 on the whole cell K^+ currents in the presence of 5 mM tetraethylammonium (TEA) to block the Ca^{2+}-activated K^+ current. (a) Traces from a typical cell in the presence of 5 mM TEA held at −60 mV and stepped over a range of potentials from −100 mV to +40 mV with 20 mV increments in the absence (left) and presence of BRL 38227. In (b) the current-voltage relationship of the TEA (5 mM)-insensitive potassium current before (○) and after (●) the addition of BRL 38227 (10 μM) is shown for 13 cells (mean with s.e.mean shown by vertical bars). The apparent reversal potential of the BRL 38227-stimulated current was again −75 mV. (c) Spontaneous transient outward currents (STOCs) were elicited from a cell held at 0 mV. TEA dose-dependently inhibited the STOCs; however, addition of BRL 38227 (BRL) still induced a large outward current in the presence of TEA. On removal of TEA, STOCs returned and there was a slight increase in the BRL 38227-induced current. Addition of glibenclamide (Glib) completely inhibited the BRL 38227-induced current with no effects on STOCs.

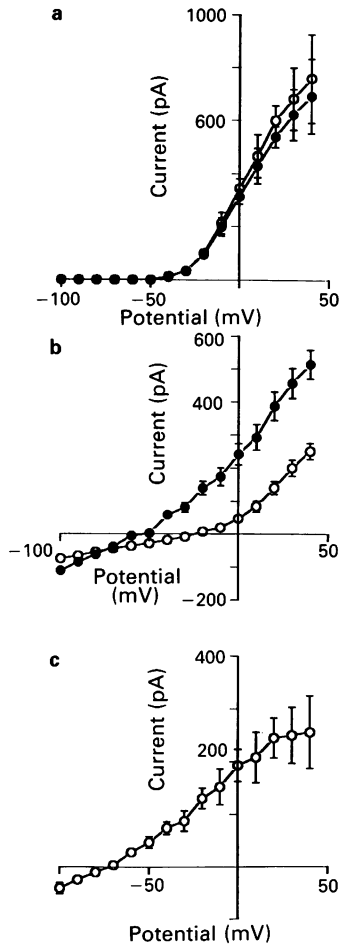

Figure 3 The effects of BRL 38227 on the time-dependent delayed rectifier current (a) and on the quasi-instantaneous current (b, c) in the presence of tetraethylammonium 5 mM. Panel (a) shows the time-dependent current, calculated by subtracting the quasi-instantaneous current measured at the end of the downstroke of the capacitance artifact at the beginning of the voltage step from the peak current. This time-dependent current was plotted before (○) and after (●) the addition of BRL 38227. (b) The plot of the quasi-instantaneous current as measured previously in the presence (●) and absence (○) of BRL 38227. (c) The current induced by BRL 38227 was calculated by subtracting the currents shown in (b).

and series resistance were not deemed necessary for the experiments carried out. Data were stored on computer hard disc and DAT tape, digitized with a Labmaster DMA TL-1-125 interface, and analysed by the pClamp 5.0 programme (Axon Instruments). Since BRL 38227 affected a non-inactivated current, 'leak' currents were not normally calculated and subtracted except where stated.

Solutions

Physiological saline solution (PSS) contained (in mM): NaCl 130, KCL 5, $MgCl_2$ 1.2, $CaCl_2$ 1.5, glucose 10 and HEPES 10. This and all other solutions were buffered to pH 7.2 with NaOH. Low-Ca^{2+} PSS was identical, except that only 10 μM $CaCl_2$ was added to the solution.

The external solution for measuring K currents was PSS, while the pipette solution contained (in mM): KCl 140, HEPES 10, $MgCl_2$, 1, EGTA 1, and Na_2ATP 1. For measurement of the Ca^{2+} channel current, cells were incubated in a solution containing (in mM): NaCl 120, KCl 5, $MgCl_2$ 1.2, $BaCl_2$ 10, glucose 10, and HEPES 10. The internal solution for measuring the Ba^{2+} current contained (in mM): CsCl 130, $MgCl_2$ 2.5, HEPES 10, EGTA 10 and Na_2ATP 1. Stock solutions of BRL 38227 (1 mM) were made up in a 10% dimethylsulphoxide (DMSO) solution whereas glibenclamide (10 mM) stock was made up daily in 100% DMSO.

Papain, dithiothreitol (DTT), bovine serum albumin (BSA), 4-aminopyridine (4-AP), tetraethylammonium chloride (TEA), adenosine 5'-triphosphate (ATP), ethylene glycol *bis*-(b-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) and glibenclamide were all obtained from Sigma. BRL 38227 was kindly provided by Dr T. Hamilton at SmithKline Beecham Pharmaceuticals Ltd, Harlow, Essex.

Results

Effects of BRL 38227 on the whole cell K^+ currents of the rabbit portal vein

The currents elicited from cells dialysed with high potassium intracellular solution, and held at −60 mV, were recorded over a range of test potentials from −100 mV to +40 mV. Time-dependent K^+ currents were detected at all test potentials positive of −40 mV (Figure 1); these increased in a voltage-dependent manner as described by Beech & Bolton (1989b) and Hume & Leblanc (1989). Under normal conditions in physiological saline the resting potential measured in current clamp was −32 mV ± 8 (mean ± s.d., n = 19). Application of BRL 38227 (10 μM) produced the onset of an outward shift in the holding current at −60 mV which reached a sustained maximum after 30 s–1 min, at which time the resting membrane potential was seen to have hyperpolarized to −53 mV ± 14 (n = 10). Concurrent with the outward shift in the holding current was an increase in the slope of the current voltage (I–V) relationship over the whole range of test potentials. The current voltage relationships in the presence and absence of BRL 38227 intersected at −75 mV, indicating that current elicited by the drug reversed at this potential. The calculated E_K under these conditions was −83 mV.

To study the specific K^+ conductances being augmented by BRL 38227, 5 mM TEA, externally applied, was used to block the Ca^{2+} activated K^+ current (Figure 2a). Addition of 5 mM TEA to the extracellular solution had no significant effect on the resting membrane potential measured in current clamp. As described by Beech & Bolton (1989b), the TEA-insensitive current appeared to consist predominantly of a delayed rectifier. By use of the same protocol as above, currents were elicited over a range of test potentials from −100 mV to +40 mV stepping from a holding potential of −60 mV (Figure 2a and b). Addition of BRL 38227 (10 μM) produced a similar response to that already described in Figure 1, with an increase in the slope of the I–V plot and reversal of the BRL 38227 stimulated current near −75 mV. Similarly the resting membrane potential was raised to −53 mV ± 11 (n = 25).

Further evidence that the K^+ conductance stimulated by BRL 38227 was not the Ca^{2+}-activated K^+ current is illustrated in Figure 2c. Spontaneous transient outward currents (STOCs), which are the result of the opening of calcium-dependent K^+ channels stimulated by the spontaneous cyclical release of calcium from an intracellular store (Benham & Bolton, 1986), were evoked by holding a cell at 0 mV (Figure 2c). Addition of 2.5 mM TEA completely inhibited the STOCs, confirming block of the Ca^{2+}-dependent K^+ current. Subsequent application of BRL 38227 (10 μM) resulted in a large outward current. The STOCs returned upon removal of TEA with very little apparent effect on the BRL 38227 response. The response to BRL 38227 was then completely inhibited by glibenclamide (1 μM), a selective inhibitor of the ATP-sensitive K^+ current. Neither BRL 38227 nor glibenclamide appeared to affect STOCs.

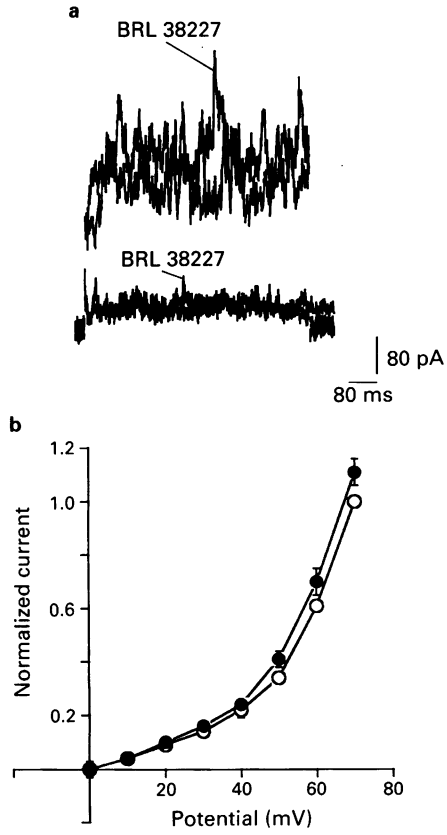

Figure 4 Effects of BRL 38227 on the Ca^{2+}-activated K^+ current in cells held at 0 mV in the presence of 4-aminopyridine 5 mM. (a) Representative traces from a cell held at 0 mV and stepped to +10 mV and +70 mV in the presence and absence of BRL 38227. (b) The I-V relationship of the Ca^{2+}-activated K^+ current for 4 cells (mean with s.e.mean shown by vertical bars) before (○) and after (●) addition of BRL 38227.

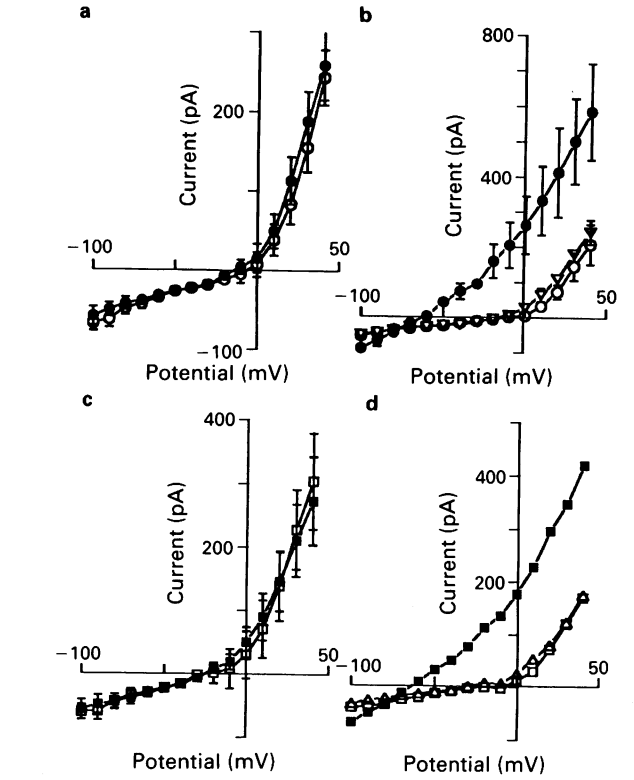

Figure 6 The effects of glibenclamide 10 μM (a, b) and phentolamine 100 μM (c, d) on the control instantaneous current (a, c) and the BRL 38227-induced instantaneous current (b, d). Glibenclamide (a, $n = 7$, mean ●, with s.e.mean shown by vertical bars) and phentolamine (c, $n = 3$, ■) had no effect on the control (○, □) instantaneous current. Plot (b) shows the current-voltage relationship of the quasi-instantaneous current ($n = 3$) under control conditions (○) after the addition of BRL 38227 (●) and finally in the presence of BRL 38227 and glibenclamide (▽). Plot (d) shows similar results for the quasi-instantaneous current in a cell under control conditions (□) in the presence of BRL 38227 (■) and after addition of BRL 38227 and phentolamine (△).

On closer examination of the TEA-insensitive K^+ conductance (Figure 2a) it was possible to separate out two distinct components which were differentially affected by BRL 38227. The first component was a time-independent quasi-instantaneous current which could be estimated at the beginning of the current trace, at the end of the down-stroke of the capacitance artifact. Superimposed upon this was a time-dependent current which rose to a peak and then decayed slowly. Subtraction of the instantaneous current from the total peak current in the presence of 5 mM TEA (Figure 3a) resulted in a time-dependent voltage-sensitive current which activated at potentials positive of −40 mV. This current was equivalent to delayed rectifier as described by Hume & Leblanc (1989), and was unaffected by BRL 38227. The application of BRL 38227 in the presence of 5 mM TEA (Figure 2a) did, however, increase markedly the instantaneous component of the TEA-insensitive current.

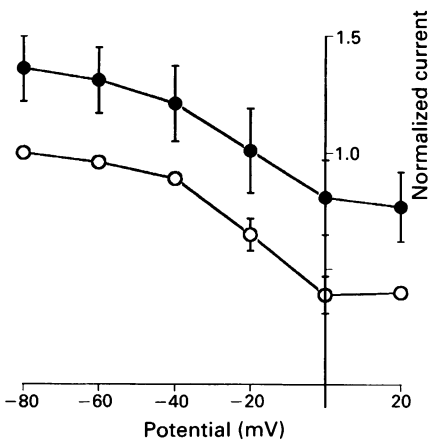

Figure 5 The availability of the tetraethylammonium (TEA)-insensitive K^+ current in the absence (○) and presence (●) of BRL 38227 in five cells (mean with s.e.mean shown by vertical bars). Cells were stepped for 10 s to a conditioning potential between −80 mV to +20 mV with 20 mV increments and then stepped to a test potential of +40 mV. The plot shows the current elicited from the test potential from each conditioning step; the current magnitude is expressed as a fraction of the test potential current elicited for each cell after the −80 mV conditioning pulse under control conditions.

Analysis of the quasi-instantaneous current (Figure 3b) showed that BRL 38227 increased the current at all potentials. The BRL 38227-induced current had a reversal potential of −75 mV. This is similar to the calculated K^+ conductance reversal potential, under the experimental conditions used, of −83 mV. Subtraction of the quasi-instantaneous current in the presence and absence of BRL 38227 gave a measure of the BRL 38227-induced current (Figure 3c). The current-voltage relationship of the induced current was linear between −100 mV to +10 mV and showed increasing inward rectification at potentials positive of this. The linear nature of the current-voltage relationship indicated the BRL 38227-induced current showed little voltage-dependence.

The action of BRL 38227 (10 μM) on the Ca^{2+}-activated K^+ current was investigated, under conditions designed to minimize other currents. Cells were held at a potential of 0 mV to

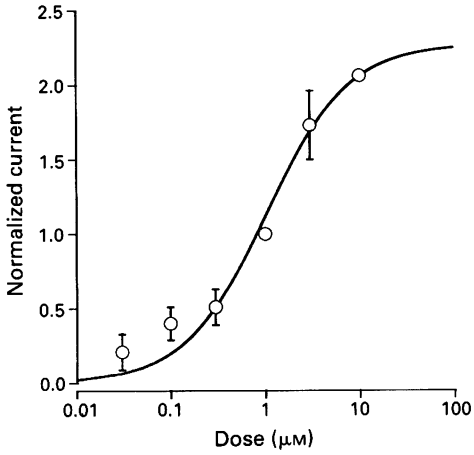

Figure 7 The concentration-response curve to BRL 38227 (10 μM). Cells were held at 0 mV and the outward current elicited by increasing doses of BRL 38227 was measured and expressed as a fraction of the current stimulated by a 1 μM dose (mean with s.e.mean shown by vertical bars, $n = 5$).

inactivate maximally the delayed rectifier K^+ current (Beech & Bolton, 1989b, see also Figure 5). 4-Aminopyridine (4-AP, 5 mM) was also present in order to block any residual delayed rectifier. When cells were then stepped over a range of test potentials from 0 mV to +70 mV, a noisy, non-inactivating current, which appeared to be comprised predominantly of a Ca^{2+}-activated K^+ current, was elicited. Application of BRL 38227 (10 μM), as shown in Figure 4, had no effect on this outward current under these conditions.

The voltage-dependence of the BRL 38227-induced conductance was further investigated by studying the availability of TEA-insensitive current (Figure 5). Cells were held for 10 s at

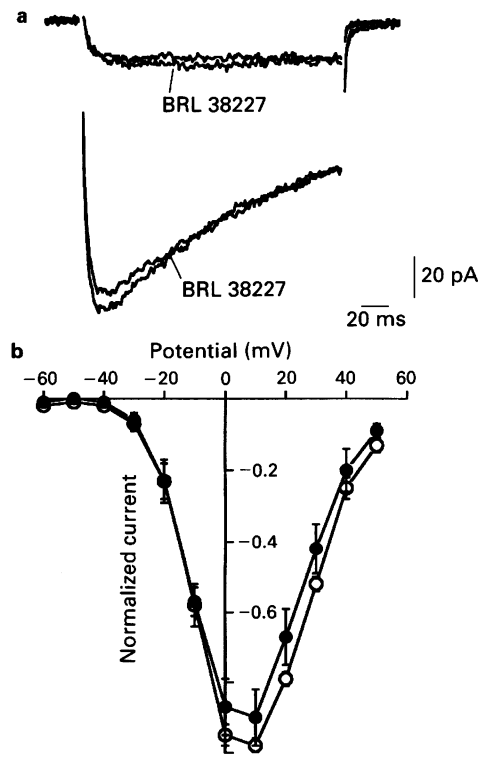

Figure 8 Effects of BRL 38227 on the whole cell calcium current: (a) shows representative traces from a cell held at −60 mV and stepped to −20 mV and 0 mV before and after the addition of BRL 38227. Plot (b) shows the current-voltage relationship of the peak calcium current (mean with s.e.mean shown by vertical bars, $n = 4$) in the absence (○) and presence (●) of BRL 38227.

potentials between −80 mV and +20 mV, and then stepped to a test potential of +40 mV for 100 ms. The peak of the current at the test voltage for each prepulse potential was measured and normalized to the current elicited by the −80 mV prepulse step for each cell. This procedure was carried out in 5 cells in the presence and absence of BRL 38227 (10 μM). BRL 38227 caused an upward parallel shift in the availability of the current, indicating that the induced current did not share the sensitivity of the delayed rectifier current to depolarization of the conditional potential. It is noteworthy that the quasi-instantaneous current in control cells also did not inactivate as the holding potential was depolarized.

To identify the current induced by BRL 38227, the effects of glibenclamide and phentolamine, both blockers of the ATP-sensitive K^+ conductance (Standen *et al.*, 1989; Plant & Henquin, 1990), were investigated. In the absence of BRL 38227, neither glibenclamide (10 μM) nor phentolamine (100 μM) had an effect on the instantaneous current evoked from cells held at −60 mV and stepped over the range of potentials from −100 mV to +40 mV (Figure 6a,c). However, after pretreatment with BRL 38227, application of glibenclamide (10 μM) completely blocked any previously induced current (Figure 6b) leaving only the control instantaneous conductance. Under identical conditions, phentolamine (100 μM) had similar effects, completely reversing any stimulation of the current by BRL 38227 (Figure 6d).

The dose-response relationship to BRL 38227 was constructed over a range of concentrations from 30 nM to 10 μM. Cells were held at a potential of 0 mV, and the outward shift in the holding potential induced by several sequentially added concentrations of drug measured. Data in each cell were normalised to the effect observed in 1 μM BRL 38227, as illustrated in Figure 7. The dose-response relationship was fitted to a sigmoid curve using a Hill Coefficient of 1 (i.e. assuming no cooperativity) and an EC_{50} of 1.3 μM was calculated.

Previous work has suggested that cromakalim, the racemic mixture of BRL 38227 and its enantiomer, caused an inhibition of the inward current carried by Ca^{2+} channels in vascular cells (Okabe *et al.*, 1990; Post *et al.*, 1991). We therefore examined the effects of BRL 38227 on the whole cell current through Ca^{2+} channels, using a caesium based pipette solution and an external solution containing 10 mM Ba^{2+}. The Ba^{2+} current evoked from a holding potential of −60 mV was apparent positive of −30 mV and peaked at around +10 mV. As shown in Figure 8, BRL 38227 (10 μM) had no significant effect on the *I-V* plot or kinetics of the Ba^{2+} current. BRL 38227 50 μM also had no effect on the Ba^{2+} current in the one cell in which it was tested.

Effect of BRL 38227 on the potassium current in human mesenteric artery

The action of BRL 38227 on the K^+ current in cells isolated from the human mesenteric artery was studied (Figure 9) under conditions similar to those previously used in the experiments with the rabbit portal vein. Cells were held at a potential of −60 mV and stepped over a range of test potentials from −100 mV to +20 mV. K^+ currents were elicited at test potentials positive of −30 mV and appeared to have very little time-dependence. Application of BRL 38227 (10 μM) induced a time-independent current, particularly apparent at more negative test potentials, with a reversal potential of −75 mV. A further increase in the BRL 38227 concentration to 50 μM did not appear to increase the current augmented by 10 μM BRL 38227 suggesting full activation of the current in these cells was achieved by a maximum dose of 10 μM. The current activated by BRL 38227 was then inhibited by the blocker of the ATP-sensitive K^+ channel, glibenclamide (10 μM).

It is noteworthy that the dominant current at positive potentials is a noisy, TEA- and Ca-sensitive K^+ current which

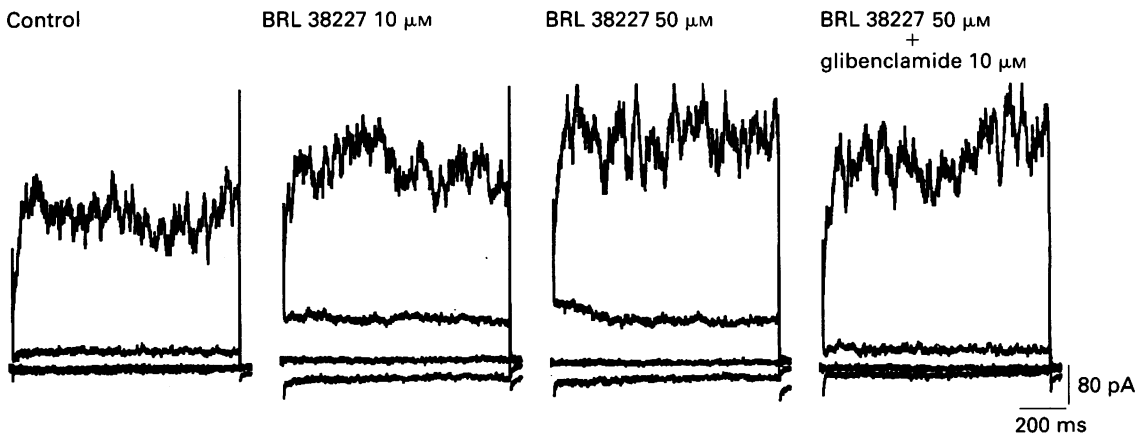

Figure 9 Effects of BRL 38227 (10 and 50 μM) on whole cell K^+ currents in a cell isolated from the human mesenteric artery. The cell was held at −60 mV and stepped to test potentials of −100 mV, −60 mV, −20 mV and +20 mV under control conditions, in the presence of 10 μM and 50 μM BRL 38227 and after the addition of 50 μM BRL 38227 and 10 μM glibenclamide.

is insensitive to both BRL 38227 and glibenclamide (Figure 9, see Aaronson & Smirnov, 1991, for description of outward current in these cells). Figure 10 illustrates that the blockade of this current with 5 mM TEA unmasks the effect of 10 μM BRL 38227 at positive potentials. As in the rabbit portal vein, the BRL 38227-stimulated current shows no evidence for voltage- or time-dependence, and demonstrates a reversal potential between −70 and −80 mV.

It is of interest that even though these cells demonstrate a substantial outward current at positive potentials, an inward tail current is often present when cells are stepped back to −60 mV (e.g. Figure 9). This tail current must therefore represent a current which is either partially or entirely carried by an ion other than K^+. The tail current is especially prominent in cells buffered with a low (0.2 mM) EGTA concentration, and is also most prominent at potentials at which the voltage-gated Ca^{2+} current is maximal, suggesting that it may be Ca^{2+}-activated (Smirnov & Aaronson, unpublished). It is also markedly stimulated by BRL 38227, especially at 50 μM, and this stimulation is reversed by glibenclamide (Figure 9).

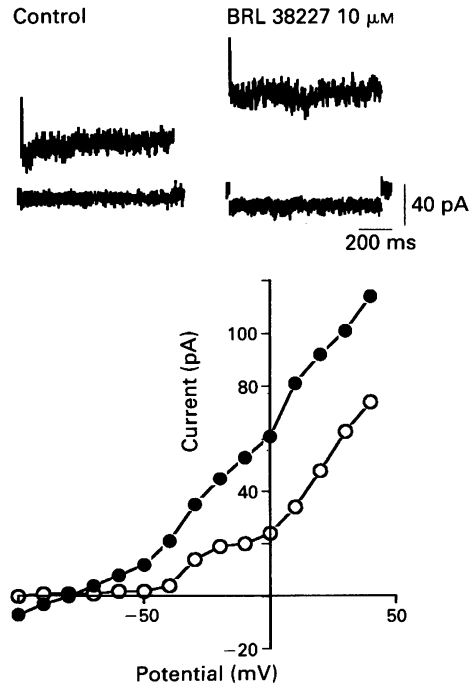

Figure 10 Effects of BRL 38227 on the whole cell K^+ current in human mesenteric artery cells in the presence of 5 mM tetraethylammonium (TEA). (a) The effect of BRL 38227 in a cell held at −60 mV and stepped to −100 mV and +20 mV. (b) Currents elicited from 1 cell in the presence of 5 mM TEA, and the presence (●) and absence (○) of BRL 38227 (10 μM).

Discussion

The results of these experiments suggest that in the rabbit portal vein and human mesenteric artery, BRL 38227 activates a K^+ conductance which is voltage-insensitive and time-independent. The activation of this conductance results in a hyperpolarization of the resting cell membrane potential. The similarity of apparent reversal potential of this current (−75 mV) to the calculated reversal potential for a pure K^+ current of −83 mV suggests the conductance elicited is carried by K^+. This is in agreement with other investigators using K^+ channel openers (Weir & Weston, 1986; Cook, 1988; Quast & Cook, 1989).

Previous publications have suggested the presence of at least three K^+ currents in single cells isolated from the rabbit portal vein, as discussed in the introduction, which could be considered as the site of action for BRL 38227. Work by Beech & Bolton (1989c) has shown that cromakalim does not act upon the voltage-sensitive K^+ current described as I_{fo} (Beech & Bolton, 1989a) a transient, rapidly inactivating current present at only hyperpolarized holding potentials.

In the same paper (Beech & Bolton, 1989c) postulated that cromakalim (the racemic mixture of BRL 38227 and its enantiomer) acted upon the delayed rectifier K^+ current to short-circuit the voltage gate of the channels and render the current voltage-insensitive and time-independent. Their conclusions were based on the similarity in potency of the channel blockers phencyclidine, quinidine and 4-AP for the delayed rectifier and the cromakalim-induced currents. Our experiments in the presence of TEA (5 mM) to block the Ca^{2+}-activated K^+ current present in both visceral (Benham & Bolton, 1986; Bolton & Lim, 1989) and vascular (Beech & Bolton, 1989b) smooth muscle cells do not suggest any action of BRL 38227 on the delayed rectifier K^+ current. Rather it appears from our work that the current induced by BRL 38227 is superimposed upon the delayed rectifier K^+ current. Evidence for this is two fold. Firstly, subtraction of the quasi-instantaneous current from the total current elicited in the presence of 5 mM TEA gave a measure of the time-dependent current referred to previously as the delayed rectifier (Beech & Bolton, 1989; Hume & Leblanc, 1989). The magnitude of the time-dependent current was unaffected by 10 μM BRL 38227 (Figure 3). Secondly, BRL 38227 caused a parallel upward shift in the availability of the TEA-insensitive current, indicating the activation of a holding potential-insensitive conduc-

tance that was independent of the voltage-sensitive delayed rectifier current, which retained its characteristic potential sensitivity.

Several researchers have suggested the site of action of the K^+ channel openers to be one of the Ca^{2+}-activated K^+ channels. Gelband *et al.* (1989) suggested cromakalim increased the P_{open} of a large conductance Ca^{2+}-activated K^+ channel with similar properties to channels found in a variety of visceral and vascular smooth muscle cells. Others have described nicorandil activating a Ca^{2+}-activated K^+ channel with a conductance of 30 pS (Inoue *et al.*, 1989). More recently, Kajioka *et al.* (1990) presented evidence of nicorandil acting upon a low conductance Ca^{2+}-dependent and ATP-sensitive K^+ in the rat portal vein. Our observations in both rabbit portal vein and human mesenteric artery cells, however, do not indicate a significant action of BRL 38227 on the TEA- and Ca^{2+}-sensitive current. In the rabbit portal vein, currents elicited by stepping positive from a holding potential of 0 mV in the presence of 4-AP (5 mM) should reflect primarily the activation of Ca^{2+}-activated K^+ current. BRL 38227 had no significant effect on the current-voltage relationship under these conditions. We observed spontaneous transient outward currents (STOCs), which have been shown to be caused by Ca^{2+} activated K^+ channels opened by Ca^{2+} released from intracellular stores (Benham & Bolton, 1986). These were blocked almost completely by 2.5 mM TEA, a concentration that had only a minor effect on the response to BRL 38227.

Human mesenteric cells also exhibit prominent STOCs and a noisy K^+ current which is markedly diminished by high concentrations of intracellular EGTA or BAPTA and which is blocked by TEA with an IC_{50} of 0.8 mM (Aaronson & Smirnov, 1991). This current, which is very pronounced at positive potentials (Figure 9, step to +20 mV), is also unaffected by BRL 38227, even at high concentrations.

Hume & Leblanc (1989) described a time-independent K^+ current in rabbit portal vein cells that was inhibited by the removal of extracellular Ca^{2+}; this current may be equivalent to the quasi-instantaneous current we observed in the absence of BRL 38227. It is important to note, however, that this current was not affected by concentrations of glibenclamide and phentolamine that completely blocked the effect of BRL 38227 on the quasi-instantaneous current. Therefore, we do not feel that BRL 38227 increased the existing quasi-instantaneous current; rather it induced a new time-independent conductance.

It has been postulated that part of the relaxant action of cromakalim is an inhibition of the voltage-operated Ca^{2+} current (Okabe *et al.*, 1990). Post *et al.* (1991) showed, however, that BRL 38227 did not inhibit I_{Ca} in colonic smooth muscle. Our observation that BRL 38227 did not reduce I_{Ca} in the rabbit portal vein is therefore consistent with this latter report.

The final class of potassium channels to be considered as a possible site of action for 'K^+ channel openers' are the ATP-sensitive K^+ channels. Since their initial discovery in cardaic cells (Noma, 1983), ATP-sensitive channels have been identified in a variety of tissues including pancreatic β-cells (Cook & Hales, 1984), skeletal muscle (Spruce *et al.*, 1985) and more recently in vascular (Standen *et al.*, 1989) smooth muscle. In other tissues this channel has been shown to have a high selectivity for K^+ ions, to exhibit little voltage sensitivity and to inwardly rectify at potentials positive of +20 mV (for review see Ashcroft & Ashcroft, 1990). Our observation that BRL 38227 induced a current with a reversal potential near the K^+ equilibrium potential and a linear current-voltage relationship which begins to inwardly rectify at positive potentials are in complete agreement with these observations. Further evidence of the action of BRL 38227 being on ATP-sensitive K^+ channels is the blocking of the BRL 38227-induced current by phentolamine and by the sulphonylurea compound, glibenclamide. Both drugs have previously been shown to inhibit selectively the relaxant actions of K^+ channel openers in a variety of smooth muscles (McPherson & Angus, 1989; Murray *et al.*, 1989; Nielsen-Kudsk *et al.*, 1990). In other tissues they have been shown to be selective inhibitors of the ATP-sensitive K^+ current (glibenclamide, Standen *et al.*, 1989; Belles *et al.*, 1987; phentolamine, Plant & Henquin, 1990). Thus under our experimental conditions the current induced by BRL 38227 appears to have the characteristics of the ATP-sensitive K^+ current, as described in other tissues. Final confirmation of the nature of this current must, however, await single channel recordings, which we have not yet carried out.

In the cells isolated from the human mesenteric artery, BRL 38227 produced similar results to those obtained in the rabbit portal vein cells. BRL 38227 caused the appearance of a time-independent current even at very negative test potentials, which was specifically blocked by glibenclamide, and which had a reversal potential between −70 and −80 mV. It appears therefore BRL 38227 induced a K^+ conductance in the human mesenteric artery cells which has similar characteristics to that present in the rabbit portal vein. It is interesting, however, that in human mesenteric artery cells, BRL 38227 also increased the magnitude of an inward tail current which could not have been caused by this time-dependent K^+ current. Although we have not studied this tail current in detail, its enhancement by low $EGTA_i$ and by I_{Ca} suggests that it is Ca^{2+}-activated. We speculate that, as in other types of smooth muscle cell (Chopra *et al.*, 1990), BRL 38227 may inhibit Ca^{2+} uptake into the sarcoplasmic reticulum. Thus, Ca^{2+} entering through voltage gated Ca^{2+} channels during depolarizing steps might be sequestered to a smaller extent in the presence of BRL 38227, leading to a rise in subplasmalemmal $[Ca^{2+}]_i$ and the stimulation of a Ca^{2+}-dependent tail current. Confirmation of this possibility must, however, await additional study.

In conclusion, it appears that BRL 38227 induces a current through channels which have the pharmacological and physiological characteristics of an ATP-sensitive K^+ conductance. The existence of this current in both cell models studied, taken in conjunction with the work of other investigators, suggests this conductance may be ubiquitous in vascular smooth muscle cells.

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Impaired endothelium-dependent relaxation of dog coronary arteries after myocardial ischaemia and reperfusion: prevention by amlodipine, propranolol and allopurinol

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1 Anaesthetized, open-chest dogs were subjected to 60 min of left circumflex coronary artery occlusion followed by 90 min of reperfusion. Endothelium-dependent and -independent relaxant responses of the isolated coronary arterial rings were then investigated.

2 The endothelium-dependent, acetylcholine-induced relaxation of ischaemic/reperfused arterial rings was significantly attenuated in comparison to control rings (1.9 fold rightward shift, ischaemic/reperfused maximum relaxation = 57 ± 13% of control maximum relaxation; $P < 0.05$). In contrast, glyceryl trinitrate produced similar relaxant responses in control and ischaemic rings.

3 Pretreatment of dogs with either amlodipine ($3\,\mu g\,kg^{-1}\,min^{-1}$, i.v.) or propranolol ($1\,mg\,kg^{-1}$, i.v.) completely prevented the postischaemic impairment of endothelium-dependent relaxant responses (100 ± 3% and 90 ± 5% of control maximum relaxation, respectively).

4 Allopurinol pretreatment ($25\,mg\,kg^{-1}$, p.o. 24 h previously, plus $50\,mg\,kg^{-1}$ i.v. 5 min before arterial occlusion) partially protected against endothelial dysfunction by preventing the ischaemia-induced rightward shift of the acetylcholine relaxation curve and increasing the maximum relaxation response (83 ± 7% of control rings).

5 These results confirm that endothelium-dependent coronary vascular relaxation is impaired by ischaemia and reperfusion, and that the ischaemia-induced impairment is reduced by pretreatment with amlodipine, propranolol or allopurinol.

Keywords: Acetylcholine; allopurinol; amlodipine; coronary artery; endothelium; ischaemia; glyceryl trinitrate; propranolol; reperfusion; vasodilatation

Introduction

Regional myocardial infarction and reduced cardiac function caused by an acute period of coronary artery occlusion and reperfusion is accompanied by vascular endothelial cell injury (Armiger & Gavin, 1975; Sobey *et al.*, 1990a), large coronary artery constriction (Sobey *et al.*, 1990a) and an impairment of endothelium-dependent coronary vasodilatation both *in vivo* and *in vitro* (Ku, 1982; VanBenthuysen *et al.*, 1987; Mehta *et al.*, 1989a,b; Sobey *et al.*, 1990a).

Coronary artery endothelial damage, with consequent alterations in artery reactivity, has been proposed to contribute to the clinical occurrence of coronary vasospasm during reperfusion of ischaemic myocardium (Ku, 1982). However, there is little information regarding the mechanisms of the endothelial damage or the potential for its prevention by pharmacological manipulation.

An excessive influx of calcium into cardiac myocytes, generation of cytotoxic oxygen-derived free radicals and myocardial cell membrane instability are all processes that are considered to contribute to the myocardial damage caused by ischaemia and reperfusion. Evidence supporting these proposals is that postischaemic cardiac function is improved by administration of calcium entry inhibitors (Kloner & Przylenk, 1990), scavengers of oxygen-derived free radicals (Jolly *et al.*, 1984; Ambrosio *et al.*, 1986; Werns *et al.*, 1988; Tamura *et al.*, 1988) or inhibitors of free radical generation (Werns *et al.*, 1986; Chambers *et al.*, 1987), and membrane stabilizing compounds such as propranolol (Nayler *et al.*, 1980; Ogawa *et al.*, 1989).

There have been relatively few studies of the capacity of these cardioprotective agents to protect against ischaemia-induced damage to coronary arteries. Mehta *et al.* (1989a) reported that administration of superoxide dismutase or the thromboxane synthetase inhibitor, U63557A, to anaesthetized dogs before coronary reperfusion protected against loss of endothelium-dependent coronary vasorelaxation *in vitro*. The authors attributed the protective action of U63557A to an increased platelet prostacyclin production and consequent inhibition of superoxide anion generation by neutrophils (Mehta *et al.*, 1989a). In addition, these authors found that superoxide dismutase (Mehta *et al.*, 1989b), but not the calcium antagonist, verapamil (Mehta *et al.*, 1989c), prevented the attenuation of endothelium-dependent coronary flow responses *in vivo* after ischaemia and reperfusion. VanBenthuysen *et al.* (1987) similarly found no protective action of verapamil against postischaemic coronary endothelial dysfunction. Thus, there is some evidence that superoxide anions, but not calcium entry, contribute to postischaemic endothelial dysfunction. However, more studies are warranted to assess the efficacy of other agents on postischaemic coronary vascular function. Therefore, in the present study we have investigated further the mechanisms of the impaired endothelium-dependent coronary vasorelaxation in a canine model of acute ischaemia and reperfusion. In these experiments, separate groups of dogs were pretreated with amlodipine (a dihydropyridine-type calcium entry inhibitor), allopurinol (a xanthine oxidase inhibitor) and propranolol (a β-adrenoceptor antagonist and membrane stabilizer), and the ability of these agents to protect the endothelium from ischaemic damage was evaluated.

Methods

Experimental preparation

Twenty-five mongrel dogs of either sex (10–33 kg) were sedated with an intramuscular injection of xylazine ($1\,mg\,kg^{-1}$; Bayer, Australia), and anaesthetized by intravenous injection of sodium thiopentone ($25–30\,mg\,kg^{-1}$; May & Baker, Australia), followed by α-chloralose ($70\,mg\,kg^{-1}$; BDH Chemicals, England) which was supplemented as necessary. The dogs were ventilated artificially with room air plus

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additional oxygen as necessary to maintain arterial blood gas and pH levels within the physiological range (Po_2: 85–110 mmHg; Pco_2: 30–35 mmHg; pH: 7.25–7.35). Systemic arterial blood pressure was measured by inserting a catheter into the right femoral artery, and connecting it to a Druck pressure transducer. Heart rate was monitored with a cardiotachometer coupler which was triggered by the electrocardiogram (ECG). A left thoracotomy was performed and the heart was suspended in a pericardial cradle. A proximal segment (approximately 1 cm in length) of the left circumflex coronary artery was dissected clear of connective tissue and inserted into an electromagnetic flow probe connected to a flowmeter (Gould Statham, U.S.A.) for coronary blood flow measurement.

In vivo *experimental protocol*

The vascular effects of coronary ischaemia and reperfusion were studied in four groups of dogs: (1) dogs receiving no pretreatment ($n = 8$); (2) dogs pretreated with amlodipine ($3\,\mu g\,kg^{-1}\,min^{-1}$, i.v., starting 90 min before arterial occlusion and continuing throughout the period of ischaemia and reperfusion; $n = 5$); (3) dogs pretreated with allopurinol ($25\,mg\,kg^{-1}$, p.o. 24 h previously, plus $50\,mg\,kg^{-1}$, i.v. administered 5 min before arterial occlusion; $n = 6$); and (4) dogs pretreated with propranolol ($1\,mg\,kg^{-1}$ i.v., 30 min before arterial occlusion, and supplemented with $0.5\,mg\,kg^{-1}\,h^{-1}$, i.v.; $n = 6$). In all dogs, left circumflex coronary artery blood flow, systemic arterial blood pressure and heart rate were recorded simultaneously on a Grass model 7D Polygraph. Total coronary vascular resistance was calculated as the quotient of arterial pressure and coronary blood flow. In Groups 1, 2 and 3 (4 of 6 dogs in group 3) left ventricular pressure was measured with a pressure transducer-tipped catheter (Millar) passed through the left atrium into the left ventricle. Peak positive LV *dp/dt*, an index of ventricular contractility, was obtained by electronic differentiation of the left ventricular pressure pulse. A stabilization period of approximately 20 min was allowed following surgery. Each dog was included in only one experimental group.

Five minutes before occlusion of the left circumflex coronary artery, lignocaine hydrochloride (50 mg, i.v.; Astra Pharmaceuticals, Australia) was administered to reduce arrhythmias. Total occlusion of the left circumflex coronary artery, distal to the first main branch, was obtained with a small bulldog clip. After 60 min of arterial occlusion the clip was removed, allowing reperfusion of the ischaemic myocardium. Left circumflex coronary artery blood flow was restricted to the pre-occlusion level for approximately 10 min by partially occluding the artery with a 10 cm length of cotton tape placed around the artery. This procedure limits reperfusion hyperaemia, thus reducing the severity of reperfusion arrhythmias and the incidence of haemorrhagic myocardial infarction (Lucchesi *et al.*, 1976). Recordings of the ECG (leads I, II and III) were made throughout each experiment. After 90 min of reperfusion the dogs were killed with an intravenous injection of sodium thiopentone (400–800 mg), and the hearts were removed and stored in Krebs-bicarbonate solution at 4°C overnight.

Isolated coronary arterial rings

The left circumflex coronary artery, proximal (control) and distal (ischaemic and reperfused) to the site of occlusion, was dissected and cleared of superficial connective tissue with care being taken to avoid damage to the endothelial surface. In some experiments the left anterior descending coronary artery was also used to provide control arterial rings. Rings, 4–5 mm in width, were cut from each artery at least 5 mm from the point of occlusion. The arterial rings were mounted on stainless steel hooks in 10 ml organ baths, and bathed in Krebs-bicarbonate solution of the following composition (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, (+)-glucose 5.0, $NaHCO_3$ 25.0 and $CaCl_2 \cdot 2H_2O$ 2.54. The arterial rings were maintained at 37°C and gassed with 5% CO_2 in O_2, and were allowed to equilibrate for 40 min during which time resting passive tension was adjusted to 4 g. The arterial rings were then briefly contracted with 25 mM KCl to demonstrate their viability, and the KCl was quickly rinsed from the baths. After a further equilibration period of 40 min, the arterial rings were contracted with the thromboxane-mimetic U46619 (0.03 μM; 50–70% of maximum U46619 contraction) to produce a stable increase in tension. Increasing concentrations of either acetylcholine or glyceryl trinitrate were then added to the bath in a cumulative manner until maximal relaxation was obtained. Relaxation responses are expressed as a percentage inhibition of the U46619 contraction. To verify that proximal and distal left circumflex coronary artery segments normally exhibit similar relaxant responses to acetylcholine and glyceryl trinitrate, similar experiments were carried out in coronary arteries from a further seven dogs not subjected to coronary ischaemia.

Drugs

Acetylcholine perchlorate (BDH Chemicals, Poole, Dorset, U.K.) and (±)-propranolol hydrochloride (Sigma Chemical Co., St. Louis, MO, U.S.A.) were dissolved and diluted in 0.9% saline. Glyceryl trinitrate (Tridil, Boots Co., Australia) was diluted in 0.9% saline. U46619 (9,11-dideoxymethanoepoxy-9α,11α-prostaglandin $F_{2\alpha}$, Upjohn Co., Kalamazoo, MI, U.S.A.) was dissolved in absolute ethanol and diluted in 0.2 M $NaHCO_3$. Amlodipine maleate (Pfizer, Sandwich, Kent, U.K.) was dissolved and diluted in water for injection. Allopurinol (Sigma) was dissolved in 50 ml 0.9% saline (high pH with 2 M NaOH).

Calculations and statistics

All data presented are the mean ± s.e.mean of n experiments. A one-way analysis of variance and Duncan's test for multiple comparisons was used to analyse the effect of drug treatment and ischaemia/reperfusion on *in vivo* baseline levels. Concentration-relaxation curves generated by acetylcholine in each experiment were fitted to a sigmoidal curve by a computer which then calculated the EC_{50} and maximum relaxation values. Student's paired t test was then used to compare the mean maximum and mean EC_{50} values for control and ischaemic/reperfused arterial rings in each group, and also the magnitude of precontraction of these rings by U46619. To evaluate the effectiveness of each pretreatment on endothelium-dependent responses, the calculated maximum acetylcholine-induced vasorelaxation of the ischaemic arterial rings from each dog was expressed as a percentage of the calculated control maximum response, and the overall group means were compared by a one-way analysis of variance and Duncan's test for multiple comparisons. As concentration-relaxation curves generated by glyceryl trinitrate were not sigmoidal, statistical comparisons of control and ischaemic/reperfused curves were carried out with a one-way analysis of variance and Duncan's test for multiple comparisons. Maximum relaxations by glyceryl trinitrate were compared by Student's t test for paired data. A P value of less than 0.05 was considered to be statistically significant in each analysis.

Results

Effects of pharmacological pretreatments on baseline cardiovascular variables before coronary occlusion

Allopurinol treatment caused an increase in heart rate (Table 1, $P < 0.05$), whilst propranolol decreased heart rate (Table 1,

Table 1 Effect of left circumflex coronary artery occlusion and reperfusion on baseline variables

Group	*Control*	*Treated*	*Post-Isch*
(*a*) *AP* (mmHg)			
1 No pretreatment	108 ± 6	NA	98 ± 4
2 Amlodipine	104 ± 8	102 ± 10	94 ± 11
3 Allopurinol	108 ± 8	98 ± 6	104 ± 7
4 Propranolol	126 ± 4	121 ± 4	105 ± 6**
(*b*) *CBF* (ml min^{-1})			
1 No pretreatment	23 ± 6	NA	20 ± 5
2 Amlodipine	18 ± 3	22 ± 5	31 ± 2
3 Allopurinol	19 ± 4	37 ± 9	18 ± 5
4 Propranolol	32 ± 3	30 ± 3	19 ± 1**
(*c*) *HR* (b min^{-1})			
1 No pretreatment	135 ± 15	NA	125 ± 12
2 Amlodipine	123 ± 13	116 ± 10	126 ± 19
3 Allopurinol	121 ± 9	164 ± 11*	127 ± 5
4 Propranolol	153 ± 9	122 ± 6*	115 ± 6*
(*d*) *LVP* (mmHg)			
1 No pretreatment	164 ± 7	NA	153 ± 5
2 Amlodipine	159 ± 9	158 ± 9	153 ± 22
3 Allopurinol	158 ± 10	161 ± 15	164 ± 7
(*e*) *LV* dP/dt (mmHg s^{-1})			
1 No pretreatment	2536 ± 188	NA	1857 ± 122*
2 Amlodipine	2333 ± 663	2750 ± 641	2713 ± 689
3 Allopurinol	2850 ± 203	2637 ± 424	2475 ± 403
(*f*) *CVR* (mmHg min ml^{-1})			
1 No pretreatment	5.2 ± 0.6	NA	6.7 ± 1.5
2 Amlodipine	6.3 ± 1.1	5.8 ± 1.8	3.1 ± 0.3
3 Allopurinol	6.4 ± 1.4	3.3 ± 0.9	9.2 ± 4.9
4 Propranolol	3.9 ± 1.6	4.3 ± 0.5	5.9 ± 2.4

Post-isch: post-ischaemia; AP: arterial pressure; CBF: coronary blood flow; HR: heart rate; LVP: left ventricular pressure; CVR: coronary vascular resistance; NA: not applicable.
* Significantly different from control.
** Significantly different from control and treated; $P < 0.05$.

$P < 0.05$). No other variable was significantly altered by the pretreatment protocol in any group.

In vivo *effects of coronary occlusion and reperfusion*

In comparison to pre-occlusion levels, 60 min of left circumflex coronary artery occlusion and 90 min of reperfusion resulted in reductions of LV *dP/dt* in non-treated dogs (Group 1; Table 1; $P < 0.05$), and of systemic arterial pressure and left circumflex coronary artery blood flow in propranolol-treated dogs (Group 4, Table 1; $P < 0.05$). All other haemodynamic variables in all groups were unaltered by the ischaemia/reperfusion protocol (Table 1). Alterations in the ECG indicative of myocardial ischaemia were observed following coronary artery occlusion in all dogs.

Isolated coronary arterial rings

Concentration-relaxation curves were obtained for acetylcholine and glyceryl trinitrate in non-ischaemic (control) and ischaemic/reperfused coronary arterial rings which were precontracted with U46619 (0.03 μM).

Ischaemia and reperfusion significantly attenuated the maximum acetylcholine-induced relaxations in coronary arteries from dogs that were not pretreated with any pharmacological agent (Group 1: ischaemic = 57 ± 13% of control, $P < 0.05$; Figure 1, Table 2). In addition, ischaemia and reperfusion resulted in a significant (1.9 fold) rightward shift of the acetylcholine relaxation curve in rings from Group 1 dogs ($P < 0.05$, Table 2).

Acetylcholine-induced maximum relaxations were also attenuated in arteries from allopurinol-treated dogs (Group 3: ischaemic = 83 ± 7% of control, $P < 0.05$; Figure 2, Table 2). In contrast, both amlodipine (Group 2, ischaemic = 100 ± 3% of control; Figure 3, Table 2) and propranolol (Group 4, ischaemic = 90 ± 5% of control; Figure 4, Table 2) pretreatments prevented the attenuation of maximum acetylcholine-induced vasorelaxation by ischaemia and reperfusion. However, in each of the three treatment groups EC_{50} values for acetylcholine concentration-relaxation responses were similar in control and ischaemic/reperfused arterial rings (Table 2).

Glyceryl trinitrate-induced relaxant responses of control and ischaemic/reperfused arterial rings did not differ within any group (data from Group 1 shown in Figure 5).

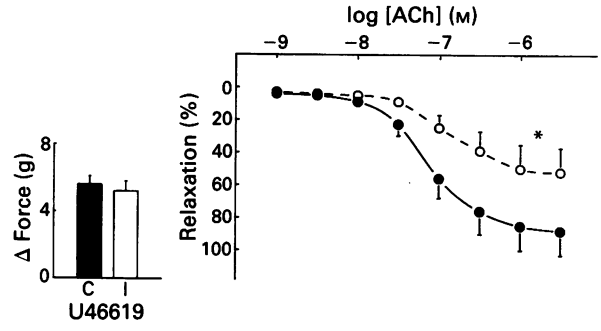

Figure 1 Acetylcholine (ACh) concentration-response curve in control (●) and ischaemic/reperfused (○) coronary arterial rings from dogs that received no pharmacological pretreatment prior to coronary occlusion and reperfusion (Group 1, $n = 8$ dogs). Ischaemia and reperfusion caused a 1.9 fold rightward shift in the curve ($P < 0.05$; Student's *t* test), and significantly reduced the magnitude of maximum relaxation (*$P < 0.05$; Student's *t* test). The bar graph shows that control (C) and ischaemic/reperfused (I) arterial rings were precontracted with U46619 (0.03 μM) to a similar level.

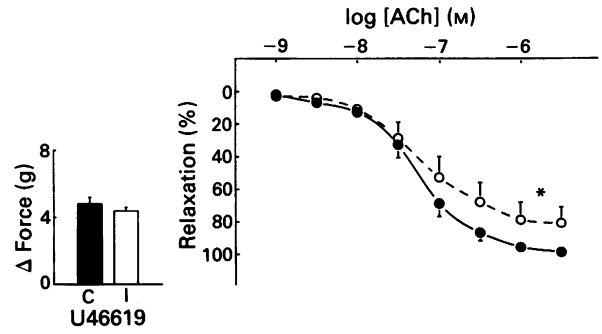

Figure 2 Acetylcholine (ACh) concentration-response curve in control (●) and ischaemic/reperfused (○) coronary arterial rings from dogs that were pretreated with allopurinol prior to coronary occlusion and reperfusion (25 mg kg^{-1} ingested 24 h previously, plus 50 mg kg^{-1} i.v. administered 5 min before arterial occlusion; Group 3; $n = 6$ dogs). Allopurinol treatment prevented the rightward shift in the curve caused by ischaemia and reperfusion, but the magnitude of maximum relaxation was still reduced in these arteries (* $P < 0.05$).

Table 2 Effect of ischaemia and reperfusion on EC_{50} (−log M) and maximum (%) values for acetylcholine concentration-relaxation curves

Group	*Cont EC_{50}*	*Isch EC_{50}*	*Cont max*	*Isch max*
1	7.005 ± 0.113	6.730 ± 0.118*	88 ± 15	53 ± 15**
2	7.160 ± 0.102	7.043 ± 0.088	107 ± 11	107 ± 8
3	7.247 ± 0.107	7.093 ± 0.160	99 ± 3	82 ± 9**
4	7.261 ± 0.138	7.124 ± 0.159	89 ± 7	81 ± 8

Cont: control; Isch: ischaemic/reperfused; Group 1: no pretreatment; Group 2: amlodipine-treated; Group 3: allopurinol-treated; Group 4: propranolol-treated.
* Significantly different from control EC_{50}.
** Significantly different from control maximum; $P < 0.05$.

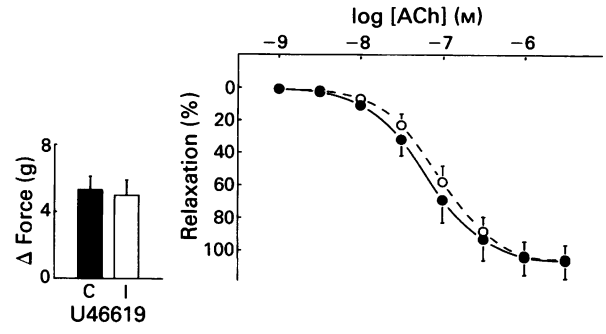

Figure 3 Acetylcholine (ACh) concentration-response curve in control (●) and ischaemic/reperfused (○) coronary arterial rings from dogs that were pretreated with amlodipine prior to coronary occlusion and reperfusion ($3\,\mu g\,kg^{-1}\,min^{-1}$, i.v., commencing 30 min before occlusion; Group 2; $n = 5$ dogs). Amlodipine treatment prevented the postischaemic attenuation of endothelium-dependent coronary relaxation.

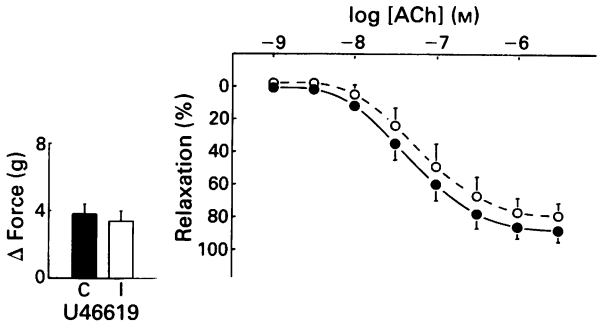

Figure 4 Acetylcholine (ACh) concentration-response curve in control (●) and ischaemic/reperfused (○) coronary arterial rings from dogs that were pretreated with propranolol before coronary occlusion and reperfusion ($1\,mg\,kg^{-1}$ i.v. initially, and supplemented with $0.5\,mg\,kg^{-1}\,h^{-1}$, i.v.; Group 4; $n = 6$ dogs). Propranolol treatment prevented the postischaemic attenuation of endothelium-dependent coronary relaxation.

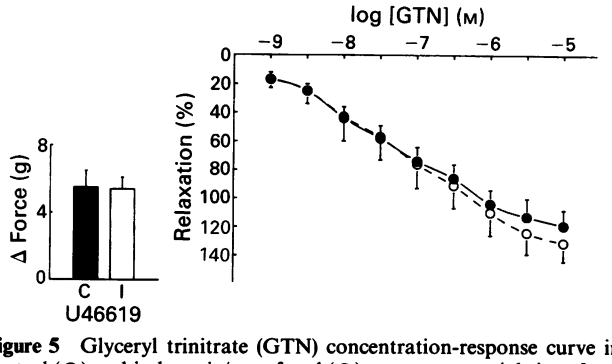

Figure 5 Glyceryl trinitrate (GTN) concentration-response curve in control (●) and ischaemic/reperfused (○) coronary arterial rings from dogs that received no pharmacological pretreatment before coronary occlusion and reperfusion (Group 1, $n = 8$ dogs). There was no difference between the relaxant responses of control and ischaemic/reperfused rings.

Proximal and distal segments of non-ischaemic left circumflex coronary arteries were found to respond in an identical manner to either acetylcholine or glyceryl trinitrate, thus validating the proximal circumflex artery as a non-ischaemic control segment (data not shown). We have shown previously that the left anterior descending and left circumflex coronary arteries respond similarly to these substances (Sobey *et al.*, 1990a).

Discussion

In this study we investigated the effects of a number of pharmacological agents on endothelium-dependent relaxation of dog coronary arteries *in vitro*, following 60 min of coronary artery occlusion and 90 min of reperfusion *in vivo*. These experiments confirmed previous findings (Ku, 1982; VanBenthuysen *et al.*, 1987; Sobey *et al.*, 1990a) that endothelium-dependent but not endothelium-independent vasorelaxation is impaired *in vitro* following an acute period of coronary ischaemia and reperfusion. We now show that three apparently unrelated pharmacological agents which are known to inhibit ischaemia-induced cardiac damage, amlodipine (a calcium antagonist), propranolol (a β-adrenoceptor antagonist) and allopurinol (a xanthine oxidase inhibitor), all offer significant protection against the impairment of endothelium-dependent vasorelaxation by ischaemia and reperfusion.

Continuous intravenous infusion of amlodipine started before coronary occlusion completely preserved acetylcholine-induced endothelium-dependent relaxation of the isolated ischaemic/reperfused arteries. Amlodipine is a dihydropyridine calcium entry blocking agent, related structurally to nifedipine. Like nifedipine, amlodipine reduces ultimate myocardial calcium accumulation and infarct size in anaesthetized dogs when administered prior to an acute period of coronary artery occlusion and reperfusion (Hoff *et al.*, 1989). In our study, amlodipine indeed prevented the postischaemic reduction in cardiac function. The calcium-blocking action of the dihydropyridines is known to be relatively selective for vascular smooth muscle compared to cardiac muscle (Singh, 1986). However, as demonstrated in the present study and previously (VanBenthuysen *et al.*, 1987; Sobey *et al.*, 1990a), the direct nitrovasodilator-induced relaxation is unaffected by acute ischaemia and reperfusion, indicating that vascular smooth muscle function is not impaired in this model. Furthermore, relaxation of non-ischaemic arterial rings from dogs pretreated with amlodipine, propranolol or allopurinol was normal in response to acetylcholine. These pharmacological agents were administered at a dose that did not affect baseline cardiac contractility or coronary blood flow. Therefore it appears that amlodipine, as well as propranolol and allopurinol, protected the coronary vascular endothelium from ischaemic damage and dysfunction. It is possible that all three compounds protected the endothelium indirectly by reducing the severity of ischaemia, thus improving cellular integrity. Inhibition by amlodipine of both calcium entry and the loss of calcium homeostasis in vascular endothelial cells during ischaemia and reperfusion may account for its preservation of endothelial function.

It is believed that a major mechanism of endothelial cell damage associated with ischaemia and reperfusion is via the generation of cytotoxic oxygen-derived free radicals following the reintroduction of oxygen to the tissue during reperfusion. Free radicals, such as the superoxide anion, are capable of directly damaging cell membranes through the peroxidation of membrane lipids, and damage to the coronary endothelium has been proposed as the primary site of reperfusion injury in the ischaemic heart (Schrader, 1985). Indeed, administration of free-radical scavengers has been reported to attenuate the impairment of coronary vascular injury and regional coronary blood flow in anaesthetized dogs subjected to acute myocardial ischaemia and reperfusion (Przylenk & Kloner, 1989; Mehta *et al.*, 1989a,b). Allopurinol is known to reduce myocardial infarct size in anaesthetized dogs (Werns *et al.*, 1986; Chambers *et al.*, 1987). In the present study, allopurinol treatment did prevent the reduction in cardiac contractility observed after ischaemia and reperfusion in dogs receiving no pretreatment. Furthermore, the ischaemia-induced shift in the acetylcholine concentration-relaxation curve was prevented, and the maximum relaxant response to acetylcholine was increased in arterial rings from these dogs, indicating partial protection of endothelial function by allopurinol. Inhibition of superoxide anion production by xanthine oxidase, an enzyme localized in the vascular endothelium (Ferrari *et al.*, 1990), is the mechanism most likely to account for these beneficial effects of allopurinol. However, as there are only moderate levels of xanthine oxidase activity in dog hearts (Muxfeldt &

Schaper, 1987), this may account for the incomplete protection by allopurinol against endothelium dysfunction, and implies that other mechanisms also contribute to ischaemic damage of dog coronary arteries.

The accumulation of activated leukocytes in ischaemic and reperfused coronary arteries (Mullane *et al.*, 1984; Sobey *et al.*, 1990a) and myocardium (Romson *et al.*, 1983; Engler *et al.*, 1983; Mullane *et al.*, 1984) is associated with tissue damage that is thought to be mediated by superoxide anions. Nifedipine (Jouvin-Marche *et al.*, 1983) and another dihydropyridine, nisoldipine, have been reported to inhibit neutrophil functions, including superoxide anion production (Irita *et al.*, 1986). Hence, an additional mechanism for the protection of endothelial function by amlodipine in this study might have occurred through the reduction of superoxide-mediated damage to the coronary arterial endothelium by neutrophils. Furthermore, leukocytes could increase the likelihood of local vasospasm occurring through the release of vasoconstrictor substances (Gillespie *et al.*, 1986; Lucchesi & Mullane, 1986; Ohlstein & Nicholls, 1989; Sobey *et al.*, 1990b; Nishida *et al.*, 1990; Sessa & Mullane, 1990). Therefore by reducing local vasoconstrictor release from leukocytes, administration of calcium antagonists might be valuable in preventing the reocclusion of a reperfused coronary artery.

It is well established that propranolol reduces cardiac work via β-adrenoceptor blockade and hence reduces myocardial oxygen demand, and that this drug is effective in the clinical treatment of ischaemic heart disease. The protective actions of propranolol against the effects of ischaemia have therefore been considered to be due to the improved balance between oxygen demand and delivery in the ischaemic myocardium (Mueller *et al.*, 1974). It is not clear whether the reduction of ischaemia-induced endothelial dysfunction by propranolol in this study was related to its reduction of heart rate, and hence of cardiac work. In addition to their respective established pharmacological actions, it is now known that both propranolol and the dihydropyridine, nifedipine, exert significant membrane antiperoxidative activity against superoxide-induced damage to canine isolated sarcolemmal membranes (Mak & Weglicki, 1988; 1990). It was proposed that this effect was related to the lipophilic properties of these agents. This mechanism could account for the known membrane stabilizing properties of propranolol, and also its reported protective effects on cardiodynamic and mitochondrial function in ischaemic and reperfused hearts (Nayler *et al.*, 1980; Ichihara & Abiko, 1983; Lu *et al.*, 1990) that are not related to β-adrenoceptor blockade (Lu *et al.*, 1990). Thus, the preservation of endothelial cell function by both propranolol and amlodipine in the present study may have been due, at least in part, to their reduction of endothelial membrane damage by oxygen-derived free radicals.

In conclusion, these experiments have demonstrated that the impairment of endothelium-dependent coronary artery relaxation that occurs following acute ischaemia and reperfusion can be prevented by administration of amlodipine, propranolol or allopurinol. These results suggest that (1) calcium entry and (2) superoxide anion-mediated damage to vascular endothelial cells may contribute to the endothelial dysfunction caused by ischaemia and reperfusion. The consequent improvement to myocardial perfusion by these drugs might be a significant factor in their ability to limit myocardial damage following ischaemia.

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Effect of sulphur containing amino acids on [^{3}H]-acetylcholine release from amacrine cells of the rabbit retina

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1 The effects of the sulphur containing amino acids, homocysteic acid, homocysteine sulphinic acid, cysteic acid and cysteine sulphinic acid on the release of [^{3}H]-acetylcholine ([^{3}H]-ACh) from the cholinergic amacrine cells of the rabbit retina were examined.

2 All the compounds stimulated the spontaneous resting release and abolished the light-evoked release of [^{3}H]-ACh. Except for homocysteine sulphinic acid these actions occurred at concentrations that did not affect the erg b-wave amplitude, indicating a site of action at the inner retina.

3 N-methyl-D-aspartate (in Mg^{2+}-containing medium) clearly blocked the effects of homocysteic acid and homocysteine sulphinic acid on the resting release of [^{3}H]-ACh but had no effect on the actions of cysteic acid and cysteine sulphinic acid.

4 Since N-methyl-D-aspartate is an antagonist of the light-evoked endogenous bipolar cell transmitter released onto cholinergic cells, these results are consistent with the suggestion that homocysteic acid or homocysteine sulphinic acid may be a transmitter released from this subpopulation of bipolar cells.

5 The present experiments indicate the existence of excitatory amino acids that have closer pharmacological properties to a bipolar cell transmitter than glutamate but it remains to be seen whether homocysteic acid or homocysteine sulphinic acid occur in these particular bipolar cells.

Keywords: Bipolar cell; transmitter; sulphur containing amino acids; retina; acetylcholine release

Introduction

It was discovered many years ago that the sulphur containing analogues of glutamate (homocysteic acid and homocysteine sulphinic acid) and aspartate (cysteic acid and cysteine sulphinic acid) have powerful excitatory actions in spinal neurones (Curtis & Watkins, 1960; Watkins & Evans, 1981). Subsequent studies revealed uptake processes for these compounds in the central nervous system (Recasens *et al.*, 1982; Wilson & Pastuszko, 1986) and their ability to release other transmitters from brain slices (Lehmann *et al.*, 1988), cultured neurones (Dunlop *et al.*, 1989a) and synaptosomes (Dunlop *et al.*, 1989b). The possibility that one or more of these sulphur containing amino acids might be a central excitatory transmitter was apparently strengthened by reports that potassium depolarization caused their release from brain slices in a calcium-dependent manner (Do *et al.*, 1986a,b). The evidence that a sulphur containing amino acid is a transmitter in the central nervous system is most complete for cysteine sulphinic acid (Griffiths, 1990) but in general the role of sulphur containing excitatory amino acids in the brain remains obscure and a possible transmitter role is doubted by some (Kilpatrick *et al.*, 1991). The excitatory sulphur containing amino acids have been little studied in the retina, but cysteine sulphinic acid has been shown to have excitatory effects on carp horizontal cells (Wu & Dowling, 1978), and homocysteic acid excites many neurones in the inner retina (Miller, personal communication).

In the retina, as in other areas of the central nervous system, glutamate is believed to be the major excitatory transmitter (see Massey, 1990 for review). In the dark, it is released continuously from photoreceptors at synapses with bipolar cells in the outer plexiform layer. One class of bipolar cell is hyperpolarized by glutamate acting on AP_4-type receptors (Slaughter & Miller, 1981), whilst another class is depolarized by an action on non-NMDA type receptors. When the retina is exposed to light, the release of glutamate from photoreceptors stops, so that the first class of bipolar cells is depolarized, whilst the second class is hyperpolarized (referred to as depolarizing bipolar cells and hyperpolarizing bipolar cells respectively). The bipolar cells synapse in the inner plexiform layer with ganglion cells and amacrine cells, where again the transmitter is generally believed to be glutamate (Massey, 1990). This suggestion is supported by recent immunocytochemical studies, which have revealed the presence of glutamate in the bipolar cells of the turtle (Ehinger *et al.*, 1988) and goldfish (Marc *et al.*, 1990). However, in the particular case of bipolar cells that synapse onto cholinergic amacrine cells in the rabbit retina, the pharmacology does not entirely fit with glutamate being the bipolar cell transmitter.

In the retina, acetylcholine (ACh) is a transmitter only in a small subpopulation of (intrinsic) amacrine cells (Neal, 1983, for review). Using a rabbit eye-cup preparation, we have shown that flickering light releases both [^{3}H]-ACh and endogenous ACh (Neal *et al.*, 1991) by a pathway involving mainly the depolarizing bipolar cells (Neal *et al.*, 1981). We have also shown that excitatory amino acids (glutamate and aspartate) and analogues (quisqualate and kainate) depolarize cholinergic amacrine cells and stimulate the release of ACh (Cunningham & Neal, 1985a). NMDA has a similar action in Mg-free medium, but in normal Krebs-bicarbonate medium, it blocks the light-evoked release of ACh at the bipolar cell/amacrine cell level by acting as an antagonist of the bipolar cell transmitter (Cunningham & Neal, 1985a; Neal & Cunningham, 1989). When NMDA was tested as an antagonist of possible bipolar cell transmitters and glutamate analogues, we found that it did not antagonize the actions of glutamate, aspartate or quisqualate, but did block the actions of kainate (Cunningham & Neal, 1985a). Thus, we argued that the bipolar cell transmitter released onto cholinergic amacrine cells was not glutamate or aspartate but was a different excitatory amino acid that acted on kainate receptors. Subsequently we found that homocysteic acid increased amacrine cell ACh release, and because the actions of this sulphur containing glutamate analogue were antagonized by NMDA in the same way as the light-evoked release of ACh, we suggested that homocysteic acid or a related compound might be the bipolar cell transmitter released onto cholinergic amacrine cells (Neal & Cunningham, 1989). In the present experiments, we have examined three other sulphur containing amino acids

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(homocysteine sulphinic acid (HCSA), cysteic acid (CA) and cysteine sulphinic acid (CSA)) on [^{3}H]-ACh release from the retina in order to see whether they fit the 'pharmacological profile' of the bipolar cell transmitter in a similar manner to HCA.

Methods

Rabbit eye-cup preparation

The methods have been described in detail previously (Massey & Neal, 1979; Neal & Cunningham, 1982). Briefly, adult albino rabbits were anaesthetized with urethane (1.5 g kg^{-1}, i.p.) and a stainless-steel ring was sutured to the episclera to prevent collapse of the eye-cup. The cornea and iris were removed and this was followed by an intracapsular lens extraction and vitrectomy. A syringe mounted on a micromanipulator was used to place in the eye-cup 0.5 ml of Krebs bicarbonate medium containing physostigmine sulphate (30 μM) and [^{3}H]-choline (0.5 μM). After 30 min this solution was removed and the eye-cup was continuously irrigated for 60 min with fresh medium. The solution in the eye-cup was replaced at 5 min intervals and the total radioactivity in the resulting samples was measured by liquid scintillation counting. In previous experiments, [^{3}H]-ACh and [^{3}H]-Ch were separated by high-voltage electrophoresis, before counting. The spontaneously released radioactivity was mainly [^{3}H]-Ch ([^{3}H]-ACh, 20%; [^{3}H]-Ch, 80%). However, it was found that [^{3}H]-ACh represented 95–100% of the increase in total radioactivity evoked by flickering light or the local application of excitatory amino acids (Neal & Cunningham, 1982). Therefore, the increase in total radioactivity produced by these stimuli was assumed to be due to an increase in the release of [^{3}H]-ACh.

The light source was a quartz halide bulb (100 W) and the initially dark-adapted retina was stimulated for 5 min periods with flickering light (25% duty cycle; retinal illuminance, 250 lux).

Electroretinography

The viability of the preparation was continuously assessed by recording the electroretinogram (erg) as described previously (Neal & Cunningham, 1982). The effect of each drug on the erg evoked by single flashes was recorded, usually during the peak effect of the drug. The b-wave amplitude was measured from the peak of the a-wave to the peak of the b-wave. Since the b-wave is believed to reflect activity of the depolarizing bipolar cells (Miller & Dowling, 1970; Newman & Odette, 1984), drug-induced depression of the b-wave amplitude was tentatively taken to indicate a site of drug action distal to the inner plexiform layer (i.e. photoreceptors, outer plexiform layer, bipolar cells).

Expression of results

The effects of light stimulation on the release of total radioactivity from the retina are expressed as the ratio of the peak-evoked release of radioactivity to the average release of radioactivity in the two samples immediately preceding stimulation. The resulting ratio is hereafter referred to as the 'light-evoked release of [^{3}H]-ACh'.

In a previous study (Cunningham & Neal, 1983) we established that when the retina was subjected to six periods of stimulation (3 Hz, 25% duty cycle, 650 lx), the light-evoked releases of [^{3}H]-ACh (as defined above) did not differ significantly in experiments lasting 2.5 h. These control studies also revealed that during the course of a 2.5 h release experiment, only 28% of the radioactivity initially present in the retina was lost, and that at the end of these experiments, 42% of the radioactivity remaining in the tissue was present as [^{3}H]-ACh (Cunningham & Neal, 1983).

The effects of sulphur containing excitatory amino acids on the spontaneous resting release of [^{3}H]-ACh are expressed as follows: percentage increase = peak release of radioactivity in presence of drug/average resting release of radioactivity in two samples immediately preceding drug $\times$ 100.

Analysis of results

In each rabbit experiment, the effect of a drug was examined at least twice and the mean values of the light-evoked release of [^{3}H]-ACh in the presence and absence of the drug (controls) were calculated. Each drug was tested in three to five separate rabbit experiments and the light-evoked release of [^{3}H]-ACh in the presence of the drug was expressed as a percentage of that obtained in the absence of drug (control light-evoked releases). The results are expressed as the mean $\pm$ s.e. of mean and the effects of the drugs were tested for significance by the use of Student's t test.

Medium and chemicals

Krebs bicarbonate Ringer solution of the following composition was used (mM): NaCl 118, KCl 4.74, $CaCl_2$ 2.52, $MgSO_4$ 1.18, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 9.5. The medium was continuously gassed with a mixture of 5% CO_2 in O_2.

[Methyl-^{3}H]-choline chloride (specific activity 78 Ci mmol^{-1}) was obtained from Amersham, U.K. NMDA was obtained from Cambridge Research Biochemicals. HCA, CA and CSA were obtained from Sigma. HCSA was obtained from Tocris Neuramin. Other chemicals (Analar grade) were obtained from BDH.

Results

L-Homocysteic acid (HCA) and homocysteine sulphinic acid (HCSA)

The effects of HCA and HCSA on [^{3}H]-ACh release in typical experiments are illustrated in Figure 1a and b respectively.

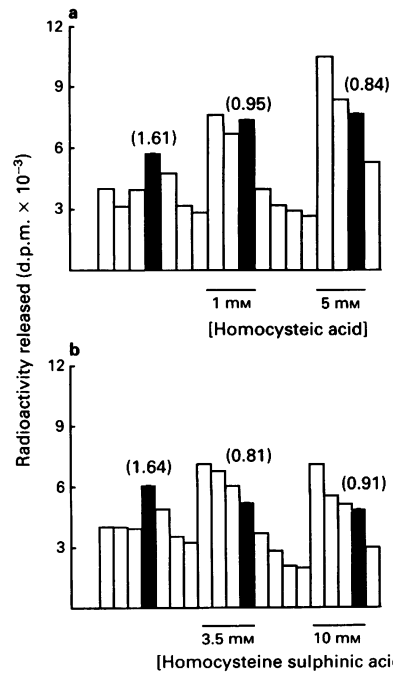

Figure 1 Effects of (a) homocysteic acid (HCA) and (b) homocysteine sulphinic acid (HCSA) on the release of radioactivity from the rabbit retina in a typical experiment. Each column is a 5 min collection period and solid columns indicate stimulation with flickering light (3 Hz). The numbers in parentheses indicate the ratio of light-evoked release/preceding resting release. Note that exposure of the retina to either HCA or HCSA increased the resting release of radioactivity, and at the concentrations used in this experiment, abolished the light-evoked release.

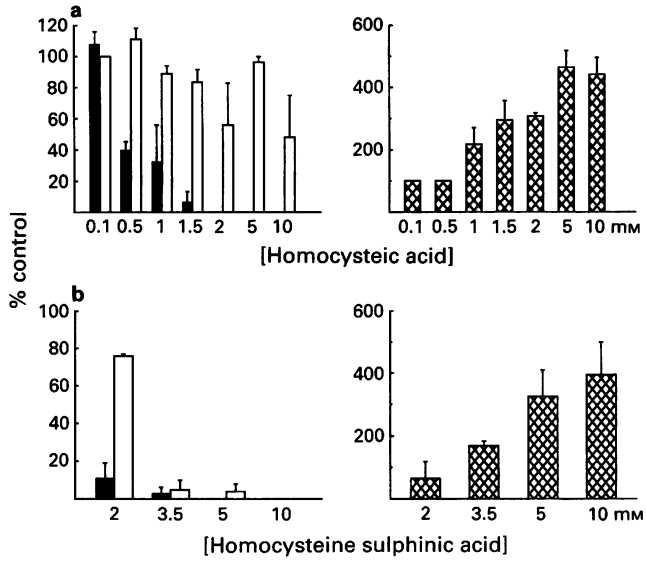

Figure 2 Summary of the effects of different concentrations of (a) homocysteic acid (HCA) and (b) homocysteine sulphinic acid (HCSA) on the light-evoked release of [^{3}H]-acetylcholine ([^{3}H]-ACh) (solid columns) and the erg b-wave amplitude (open columns, left panels) and on the resting release of [^{3}H]-ACh (cross-hatched columns, right panels). The results are expressed as percentages of control values obtained in the absence of drug. Each result is the mean of 3–5 experiments. The vertical bars indicate the s.e.means. The control values for the light-evoked release of [^{3}H]-ACh, erg b-wave amplitude, and resting release respectively were (a) 1.71 ± 0.06, $80 \pm 10\,\mu V$, $4.1 \pm 0.35 \times 10^3$ d.p.m. ($n = 5$); (b) 1.66 ± 0.13, $74 \pm 7\,\mu V$, $2.9 \pm 0.26 \times 10^3$ d.p.m. ($n = 5$). Note that HCSA but not HCA significantly depressed the erg b-wave amplitude at 3.5 mM ($P < 0.01$, $n = 3$).

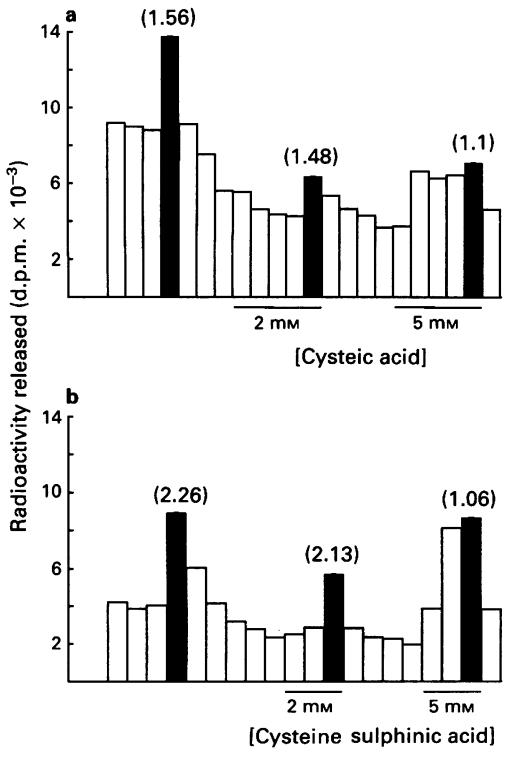

Figure 3 Effects of (a) cysteic acid (CA) and (b) cysteine sulphinic acid (CSA) on the release of radioactivity from the rabbit retina in a typical experiment. Each column is a 5 min collection period and solid columns indicate stimulation with flickering light (3 Hz). In these two experiments CA and CSA at a concentration of 2 mM had no significant effect, but at 5 mM, both compounds increased the resting release of [^{3}H]-acetylcholine ([^{3}H]-ACh) and virtually abolished the light-evoked release of [^{3}H]-ACh.

Exposure of the retina to HCA at concentrations of 1 mM and 5 mM increased the resting release of ACh and abolished the light-evoked release of ACh. The results of all the experiments performed with different concentrations of HCA (0.1–10 mM) are summarised in Figure 2a, and show that the light-evoked release of ACh was progressively reduced as the HCA concentration was increased from 0.5 mM to 2.0 mM. The resting release of ACh was progressively increased by HCA over approximately the same concentration range. The amplitude of the erg b-wave was not significantly depressed by HCA (0.1–5 mM) indicating that its main site of action was located in the inner retina.

HCSA (Figure 1b) at concentrations of 3.5 mM and 10 mM also increased the resting release of ACh and abolished the light-evoked release of ACh. The results of all the experiments with different concentrations of HCSA (2–10 mM) are summarized in Figure 2b. The main difference from the results obtained with HCA was that the concentration of HCSA (5 mM) that just produced the maximum increase in the resting release of ACh depressed the amplitude of the erg b-wave by over 90% (Figure 2b).

L-Cysteic acid (CA) and cysteine sulphinic acid (CSA)

The effects of the aspartate analogues, CA and CSA on [^{3}H]-ACh release in typical experiments are illustrated in Figure 3a and b respectively. Exposure of the retina to CA at a concentration of 5 mM increased the resting release of ACh and abolished the light-evoked release of ACh. The results of all the experiments performed with different concentrations of CA (2–10 mM) are summarised in Figure 4a and show that the light-evoked release of ACh was greatly reduced at concentrations of 3.5–10 mM CA. The resting release of ACh was increased by CA over the same concentration-range. At the highest concentration of CA examined (10 mM), the erg b-wave amplitude was severely depressed.

CSA (Figure 3b) at a concentration of 5 mM also increased the resting release of ACh and abolished the light-evoked release of ACh. The results of all the experiments with different concentrations of CSA (2–10 mM) are summarized in Figure 4b and show that the effects of CSA on ACh release

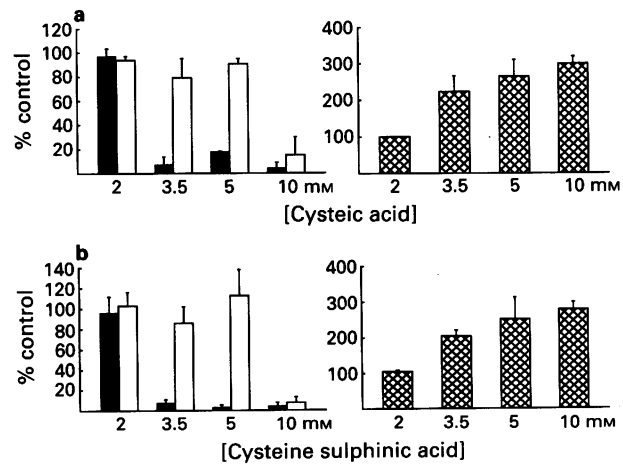

Figure 4 Summary of the effects of different concentrations of (a) cysteic acid (CA) and (b) cysteine sulphinic acid (CSA) on the light-evoked release of [^{3}H]-acetylcholine ([^{3}H]-ACh) (solid columns) and the erg b-wave amplitude (open columns, left panels) and on the resting release of [^{3}H]-ACh (cross-hatched columns, right panels). Each result is the mean of 3–5 experiments. The vertical bars indicate s.e.means. The control values for the light-evoked release of [^{3}H]-ACh, erg b-wave amplitude, and resting release were respectively (a) 1.62 ± 0.05, $72 \pm 10\,\mu V$, $3.7 \pm 0.31 \times 10^3$ d.p.m. ($n = 5$); (b) 1.85 ± 0.15, $83 \pm 9\,\mu V$, $4.1 \pm 0.27 \times 10^3$ d.p.m. ($n = 5$). Note that both CA and CSA almost abolished the light-evoked release of ACh and maximally increased the resting release of ACh at concentrations that did not significantly affect the erg b-wave amplitude.

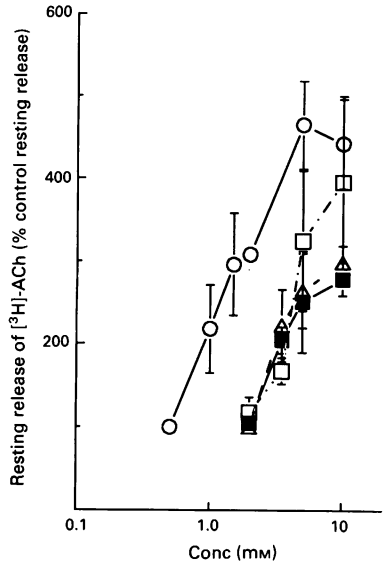

Figure 5 Concentration-response curves of the effect of sulphur-containing amino acids on the resting release of [^{3}H]-acetylcholine ([^{3}H]-ACh). Each point is the mean of 3–5 experiments. The vertical bars indicate the s.e.means. (○) Homocysteic acid; (■) cysteine sulphinic acid; (△) cysteic acid; (□) homocysteine sulphinic acid.

and on the erg b-wave amplitude were very similar to those obtained with CA.

Comparison of drug effects on resting release of acetylcholine

Concentration-response curves for the compounds tested are illustrated in Figure 5. HCA and HCSA produced the same maximum release of ACh (400–450% of control values) the EC_{50} values being 1.5 mM and 4 mM respectively. The concentration-response curves for CA and CSA were almost identical both producing maximum increases in ACh of approximately 300%. The EC_{50} values for CA and CSA were almost identical being 3.4 and 3.6 mM respectively.

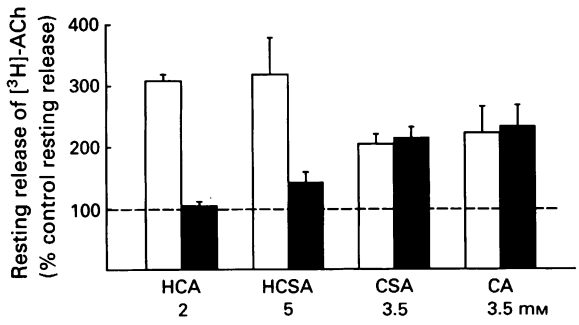

Figure 6 Summary of the effect of N-methyl-D-aspartate (NMDA, 5 mM) on the action of sulphur-containing amino acids on the resting release of [^{3}H]-acetylcholine ([^{3}H]-ACh). Open columns indicate effect of agonists on [^{3}H]-ACh in the absence of NMDA. Solid columns indicate effect of agonists on [^{3}H]-ACh in presence of NMDA. The concentration of NMDA chosen was that which just blocked the light-evoked release of [^{3}H]-ACh. The concentrations of agonists were those that produced approximately 50% of the maximum increase in [^{3}H]-ACh release. Each result is the mean of 3–5 experiments. The vertical bars indicate the s.e.means. Dashed line indicates 100% resting release. NMDA significantly inhibited the agonist-induced release of [^{3}H]-ACh only in the case of homocysteic acid (HCA) and homocysteine sulphinic acid (HCSA) ($P < 0.001$ and 0.02 respectively).

Effect of N-methyl-D-aspartate (NMDA) on the actions of agonists on resting acetylcholine release

NMDA in normal Mg-containing medium does not depolarize cholinergic amacrine cells and does not increase the release of ACh (Cunningham & Neal, 1985a; Neal & Cunningham, 1989; Linn & Massey, 1991). However, NMDA blocks the light-evoked release of ACh without depressing the erg b-wave (Cunningham & Neal, 1985a) an effect consistent with a blockade of the depolarizing bipolar cell transmitter. Since the depolarizing bipolar cell transmitter is blocked by NMDA, we tested this compound for antagonist activity against the sulphur containing amino acids. The concentration of each agonist was that which produced 50–60% of the maximum effect on ACh release. The concentration of NMDA (5 mM) was chosen because it just blocked the increase in ACh release caused by flickering light (3 Hz). The results are illustrated in Figure 6 and clearly show that NMDA antagonized the effects of HCA and HCSA on ACh release, whilst the actions of CA and CSA were completely unaffected.

Discussion

The present results indicate that the sulphur containing excitatory amino acids have actions on the retina that closely resemble those described previously for glutamate and the analogues, kainate and quisqualate (Cunningham & Neal, 1985a). All these compounds when applied locally to the retina at progressively increasing concentrations, increase the resting release of ACh, and reduce the light-evoked release of ACh. At high concentrations some of these agents (notably CA, CSA, HCSA and quisqualate) decrease the amplitude of the erg b-wave, suggesting that they may be activating the AP_4 sub-type of glutamate receptors on the depolarizing bipolar cells (activation of these receptors would hyperpolarize these cells and so prevent the light-evoked release of ACh). Previous studies with L-APB (2-amino-4-phosphonobutyric acid) have shown a close correlation between the amplitude of the erg b-wave and the light-evoked release of ACh (Cunningham & Neal, 1985a).

HCA, glutamate, and aspartate at concentrations up to 10 mM did not significantly affect the erg b-wave, strongly suggesting that their site of action is more likely to be localised in the inner retina. This is consistent with the fact that ACh recovered in the eye-cup preparation originates mainly from the displaced amacrine cells in the innermost neuronal (ganglion cell) layer of the retina (Neal *et al.*, 1981). Thus, it seems likely that the excitatory amino acids, sulphur containing excitatory amino acids, and analogues all increase the resting release of ACh by directly depolarizing the cholinergic amacrine cells. The light-evoked release of ACh is abolished because the amounts of bipolar cell transmitter released by flickering light cannot produce further depolarization of the amacrine cells. (As mentioned previously, at the highest concentrations used, some agents may have additional sites of action in the distal retina). The reduction in the light-evoked release of ACh caused by excitatory amino acids is not merely due to a ceiling effect on ACh release because bicuculline which causes a very large increase in the resting release of ACh does not prevent flickering light from evoking a further increase in ACh release (Cunningham & Neal, 1983).

The present results confirm our previous report (Neal & Cunningham, 1989) that HCA increases the resting release of ACh and is antagonized by NMDA. The sulphur containing aspartate analogues, CA and CSA, also increased the release of ACh but had a somewhat lower efficacy and were less potent. More importantly, the actions of CA and CSA at submaximal concentrations were not antagonized by NMDA and are therefore less likely to be transmitters released from bipolar cells onto cholinergic amacrine cells. HCSA had similar actions to HCA on ACh release, and although it was somewhat less effective than HCA, its actions were largely

antagonized by NMDA. Thus, HCSA is perhaps less likely than HCA to be a retinal transmitter, but such an action cannot be excluded from the present results. One problem with the eye-cup preparation used in the present study is that it is difficult to exclude indirect effects. Thus, it might be argued that the effects of the sulphur containing excitatory amino acids on ACh release result from indirect actions, for example, by causing glutamate release via homoexchange. This is probably more likely in the case of CA and CSA, which have a high affinity ($K_m \simeq 5$–$50\,\mu M$) for the glutamate transporter, rather than HCA and HCSA which have a low affinity for the glutamate transporter ($K_m \simeq 500$–$1500\,\mu M$). If CA and CSA did produce their effects on ACh release indirectly by releasing glutamate, then this would explain why their actions are not antagonized by NMDA. Whilst we cannot exclude this possibility, it seems unlikely because both CA and CSA almost abolish the erg at high concentrations, whilst similar concentrations of glutamate itself have no significant effect on the erg b-wave amplitude.

An important criterion for a substance to be a transmitter is that it should be present. The concentration of HCA in the rabbit retina is low, being about 1 nmol g^{-1} wet wt when measured by high performance liquid chromatography (h.p.l.c.) (Neal & Cunningham, 1989). This is approximately an order of magnitude less than the levels reported to exist in other areas of the central nervous system by Kilpatrick & Mozley (1986). In fact, the retinal levels of HCA may be less than the amounts we found if the recent controversial claim by Waller *et al.* (1991) that HCA measured in brain is a methodological artifact proves to be correct. In any event, it is clear that the concentration of HCA in the retina is much less than that of glutamate or aspartate (concentration around 2–10 μmol g^{-1} wet wt). Furthermore, neither Ehinger *et al.* (1988) nor Marc *et al.* (1990) found any evidence for glutamate-immunonegative bipolar cells. However, glutamate is an important intermediary metabolite and its presence does not prove that glutamate is always acting as a transmitter in these cells. Also in a tissue notorious for species variation, results obtained in the turtle and goldfish retina, cannot simply be extrapolated to the rabbit. A more serious question than the actual concentration of HCA in the retina, is its cellular localization. Immunohistochemical studies of HCA in the rat cerebellum, hippocampus and cerebral cortex have revealed a predominantly glial localization (Cuenod *et al.*, 1990). In the rat retina, HCA-like immunoreactivity has been observed by Ortega *et al.* (1991) but unfortunately, the immunoreactive cells, whether glial, neuronal or both could not be defined. The other possible bipolar cell transmitter suggested by the present pharmacological experiments is HCSA but we do not know whether this substance is present in the retina.

Our suggestion that the two sulphur containing glutamate analogues HCA and/or HCSA may be transmitter(s) in a small subpopulation of bipolar cells in the rabbit retina depends entirely on pharmacological evidence obtained by the use of NMDA as an antagonist. NMDA is normally considered to be an agonist, and indeed, in Mg-free medium, NMDA was found to have actions on retinal ACh release which were similar to those produced by other excitatory amino acids (Cunningham & Neal, 1985a). However, in normal Mg-containing medium, NMDA clearly blocked the effects of HCA (and HCSA) on retinal ACh release. In addition, NMDA has been shown to be a kainate antagonist in the outer retinas of fish (Ariel *et al.*, 1986), frog (Cunningham & Neal, 1985b) and in rat isolated retina ganglion cells (Karschin *et al.*, 1988).

In our hands, the use of NMDA as an antagonist in the rabbit eye-cup preparation has enabled us unequivocally to divide agonists into those whose actions are blocked (HCA, HCSA, kainate) and those whose actions are not (CA, CSA, glutamate, quisqualate, aspartate). Since NMDA blocks the light-evoked release of ACh (in a manner identical to that of PDA) we would argue that kainate and quisqualate aside, agents in the former group are more likely to be the bipolar cell transmitter released onto cholinergic amacrine cells than agents in the latter. However, in view of the recent controversy on the use of h.p.l.c. to measure sulphur-containing amino acids (Waller *et al.*, 1991), it remains to be seen whether the retina possesses any HCA or HCSA. The importance of the present results is perhaps not whether HCA or HCSA is an actual bipolar cell transmitter, but the fact that these agonists demonstrate the existence of compounds that agree with the pharmacology of the bipolar cell transmitter in a way that glutamate does not.

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Different *in vivo* properties of three new inhibitors of catechol *O*-methyltransferase in the rat

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1 We compared three new catechol *O*-methyltransferase (COMT) inhibitors (OR-611, Ro 40-7592 and CGP 28014; 10 and 30 mg kg^{-1}, i.p.) in male rats given levodopa (L-DOPA, 50 mg kg^{-1}, i.p.) and carbidopa ((−)-L-α-methyl dopa, 50 mg kg^{-1}, i.p.). In some studies pretreatment with pargyline (80 mg kg^{-1}, i.p.) was used to block the function of monoamine oxidase (MAO).

2 Decreases of hypothalamic and striatal 3-*O*-methyl-dopa (3-OMD) levels were used as measures of the inhibition of peripheral COMT. The inhibition of brain COMT activity was estimated by decreases of hypothalamic and striatal homovanillic acid (HVA) and 3-methoxytyramine (3-MT; after pargyline) levels.

3 The three COMT inhibitors studied had different individual characteristics. OR-611 was primarily a peripherally acting COMT inhibitor, decreasing 3-OMD levels in the striatum (to 31–52%) and in the hypothalamus (to 16–27%) both in the control and pargyline-treated animals at 1 and 3 h. It did not have any effect on brain HVA and 3-MT.

3 Ro 40-7592 was a broad spectrum COMT inhibitor decreasing striatal and hypothalamic 3-OMD (always to <30%), HVA (to <50%) and 3-MT levels (to <23%) significantly both at 1 and 3 h. It was more potent than OR-611.

4 CGP 28014 functioned as a weak COMT inhibitor in the periphery inhibiting 3-OMD formation only at 3 h. In contrast, it was fairly potent in decreasing the brain HVA and 3-MT levels at 1 h (to 37–22% and 42–35% in the striatum, and to 57–33% and 64–35% in the hypothalamus, respectively) but not at 3 h. Since CGP 28014, unlike OR-611 and Ro 40-7592, did not generally increase the brain DOPA, dopamine or DOPAC levels, it was not a typical COMT inhibitor.

Keywords: Catechol *O*-methyltransferase (COMT) inhibitors; brain catecholamines; DOPA metabolism; Parkinson's disease

Introduction

Catechol *O*-methyltransferase (COMT; EC 2.1.1.6) is a ubiquitous enzyme that has both soluble (S-COMT) and membrane-bound (MB-COMT) forms. COMT is one of the key enzymes in the metabolism of L-3,4-dihydroxyphenylalanine (DOPA) and catecholamines. DOPA is converted in the peripheral tissues and brain by dopa decarboxylase (DDC) to dopamine and by COMT to 3-*O*-methyldopa (3-OMD). If DDC is blocked, huge amounts of 3-OMD are formed (Cedarbaum, 1987). Dopamine in the rat (and human) brain is deaminated by monoamine oxidase (MAO) to 3,4-dihydroxy-phenylacetic acid (DOPAC). DOPAC is then *O*-methylated by COMT to the major final metabolite, homovanillic acid (HVA). If MAO is blocked, much 3-MT is formed by COMT from dopamine. Some conjugation reactions of dopamine, DOPAC and HVA also occur in the brain (Kopin, 1985).

The discovery of new COMT inhibitors (Männistö *et al.*, 1988; Borgulya *et al.*, 1989; Waldmeier *et al.*, 1990a) has greatly revitalized COMT research. Nitrocatechol is the key structure in the majority of these compounds. Several of them are very active COMT inhibitors *in vitro* with K_i values in the low nanomolar range. The compounds are highly selective without any action on other methyltransferases, or other catecholamine synthesizing or metabolizing enzymes (Männistö *et al.*, 1988; Borgulya *et al.*, 1989).

CGP 28014 is not a nitrocatechol but a nitropyridine derivative. It has no COMT inhibiting activity *in vitro*, while *in vivo* its action on the metabolism of DOPA and dopamine mimics that of the COMT inhibitors (Waldmeier *et al.*, 1990a,b). The mechanism of action of CGP 28014 is unknown.

We have compared the behaviour of the COMT inhibitors of different origins. There is some knowledge about the pharmacokinetic differences between some of these compounds. Some of them (Ro 41-0960; Ro 40-7592) penetrate the brain (Borgulya *et al.*, 1989), while others (e.g., OR-462; OR-611) enter the brain in very small amounts (Nissinen *et al.*, 1988; Männistö & Kaakkola, 1989; 1990). No similar data are available for CGP 28014. However, it seems to have central activity since it decreased HVA and 3-MT levels in the brain (Waldmeier *et al.*, 1990a,b).

Methods

Animals

Male Wistar rats (Wist/Kuo, 210–235 g; Department of Pharmacology and Toxicology, University of Helsinki) were housed 3–5 per cage at 21 ± 1°C in 12 h light and dark cycles (light on 07 h 00 min). Water and food were available *ad libitum*.

Protocols

A triple treatment of levodopa (50 mg kg^{-1}), carbidopa (50 mg kg^{-1}) and one of the COMT inhibitors (OR-611, Ro 40-7592 or CGP 28014; 0, 10 or 30 mg kg^{-1}) was given 60 or 180 min before the rats were killed. In some experiments, 80 mg kg^{-1} of pargyline hydrochloride was given 60 min before the triple treatment to block MAO and to redirect the dopamine metabolism in the brain. The drugs were injected intraperitoneally (i.p.) in a volume of 0.5 ml 100 g^{-1} animal weight. There were 5–8 animals per treatment group.

After decapitation by guillotine, the brains were removed within 30 s, cooled in liquid nitrogen, and the hypothalamus and striatum were dissected according to Glowinski & Iversen (1966). The dissected tissues were frozen in liquid nitrogen and stored at −80°C until analysed.

Analytical

DOPA, dopamine, noradrenaline (NA), 5-hydroxytryptamine (5-HT) and their metabolites in the hypothalamus and striatum were analysed by high performance liquid chromatography (h.p.l.c.) using electrochemical detection according to

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the method of Wagner et al. (1982) as modified by Männistö et al. (1990). Detection limits were 20 pg per injection for NA, normetanephrine (NMN), dopamine, DOPAC, 5-HT, 5-hydroxyindoleacetic acid (5-HIAA) and 3-MT and 100 pg per injection for 3-OMD and HVA.

Drugs

Carbidopa ((−)-L-α-methyldopa, Orion Pharmaceutica, Espoo, Finland) was suspended in a few drops of Tween 80 and then diluted with saline. Levodopa methylester hydrochloride (L-DOPA) and pargyline hydrochloride were from Sigma Chemical Company, MO, U.S.A. OR-611 (entacapone INN; N,N-diethyl-2-cyano-3-(3,4-dihydroxy-5-nitrophenyl) acrylamide), Ro 40-7592 (3,4-dihydroxy-4'-methyl-5-nitrobenzophenone) and CGP 28014 (N-(2-pyridone-6-yl)-N',N'-di-n-propylformamidine) were synthesized by Ms Aino Pippuri in Orion Pharmaceutica, Espoo, Finland. The purity of the compounds was checked with thin layer chromatography (t.l.c.) and nuclear magnetic resonance (n.m.r.) and was always better than 99%. These drugs were suspended in a few drops of Tween 80 and then diluted with saline. All doses refer to acids or bases.

The following chemicals for h.p.l.c. were purchased from Sigma (St. Louis, MO, U.S.A.): dopamine hydrochloride; DOPAC; HVA; 5-HT creatinine sulphate; NA hydrochloride; DOPA; 3-OMD; 3-MT; NMN; 3,4-dihydroxybenzylamine (internal standard); and 1-octane-sulphonic acid. Other chemicals were of analytical grade and obtained from Merck (Darmstadt, BRD).

Statistics

Arithmetic mean, s.e.mean and s.d. of each group were calculated. Results were analysed usually with one-way (treatment) analysis of variance, followed by a Tukey-Kramer post-hoc test. In case of deviation from the normal variation, a logarithmic transformation ($\log_{10}$) was made before statistics. In rare occasions when the variances between the treatment groups were far from equal, we used Kruskal-Wallis one-way analysis of variance instead. Systat statistical software was used in calculations (Wilkinson, 1990).

Results

Effect of COMT inhibitors on striatal and hypothalamic 3-OMD and HVA levels after levodopa and carbidopa treatment

At 1 h after levodopa (50 mg kg^{-1}) and carbidopa (50 mg kg^{-1}) and each of the three COMT inhibitors, brain 3-OMD levels were dose-dependently decreased by OR-611 and Ro 40-7592 but not by CGP 28014. OR-611 (10 and 30 mg kg^{-1}, respectively) decreased the 3-OMD levels to 52% and 39% in the striatum and to 27% and 10% in the hypothalamus ($P < 0.001$), and Ro 40-7592 (10 and 30 mg kg^{-1}) to 32% and 16% in the striatum and to 21% and 5% in the hypothalamus ($P < 0.001$) (Figure 1). These effects of OR-611 and Ro 40-7592 were sustained for at least 3 h, and even CGP 28014 decreased 3-OMD levels at 3 h (to 60–66%; Tables 1 and 2). Ro 40-7592 was the most effective of the three compounds at both time points.

Striatal and hypothalamic HVA levels, on the other hand, were not affected by OR-611 while Ro 40-7592 (10 and 30 mg kg^{-1}, respectively) decreased them to 44% and 24% in the striatum and to 49% in the hypothalamus. Even CGP 28014 (10 and 30 mg kg^{-1}) decreased HVA to 37% and 22% in the striatum and to 57% and 33% in the hypothalamus at 1 h ($P < 0.001$–0.01) (Figure 1). Ro 40-7592 was still active at 3 h, but CGP 28014 was not (Tables 1 and 2).

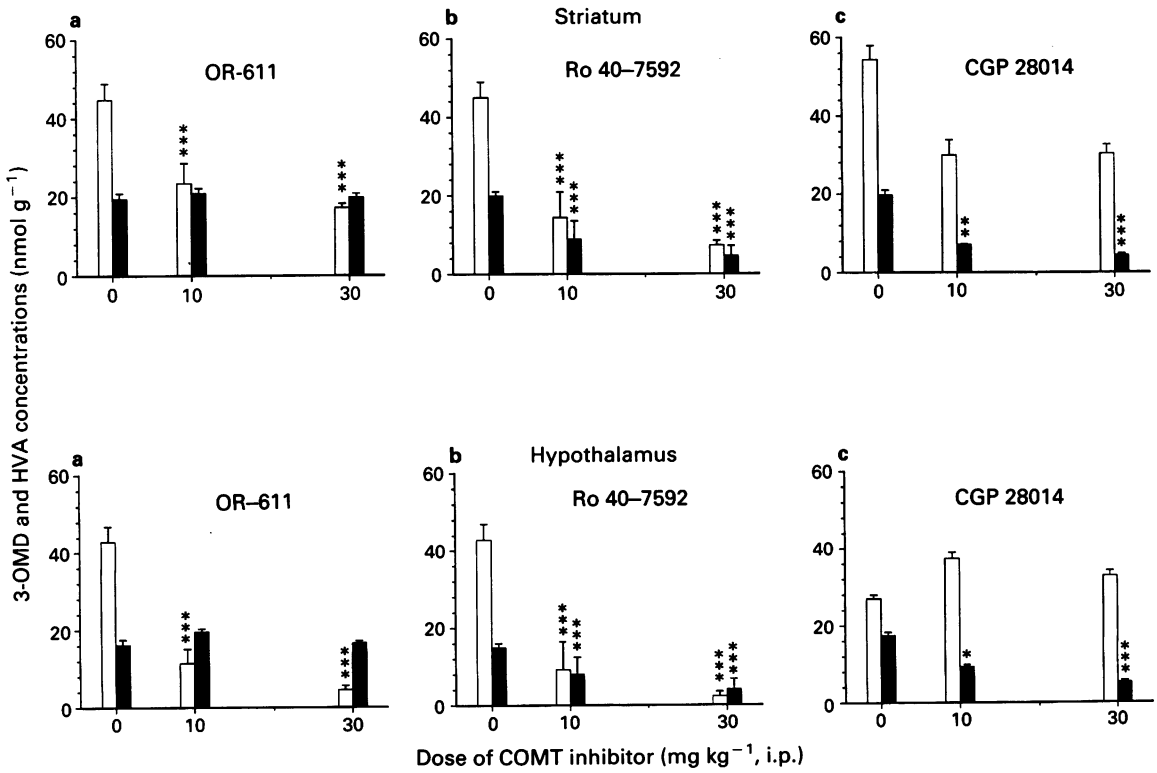

Figure 1 Striatal (upper part) and hypothalamic (lower part) 3-*O*-methyldopa (3-OMD, open columns) and homovanillic acid (HVA, solid columns) concentrations at 1 h after levodopa plus carbidopa (50 mg kg^{-1}, i.p.) and three catechol-*O*-methyltransferase (COMT) inhibitors ((a) OR-611; (b) Ro 40-7592 and (c) CGP 28014; all either 10 or 30 mg kg^{-1}, i.p.). Mean with s.e.mean shown by vertical bars. $n = 19$ in levodopa plus carbidopa group, otherwise $n = 5$–8. Statistics: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ versus levodopa and carbidopa treatment (effect of COMT inhibitors).

Table 1 Striatal DOPA, 3-O-methyldopa (3-OMD), dopamine and its metabolite concentrations at 3 h after levodopa (50 mg kg^{-1}, i.p.) plus carbidopa (50 mg kg^{-1}, i.p.) and three catechol-*O*-methyltransferase (COMT) inhibitors (10 or 30 mg kg^{-1}, i.p.)

	Striatal concentrations (nmol g^{-1})				
Treatment	*DOPA*	*3-OMD*	*Dopamine*	*DOPAC*	*HVA*
Levodopa (50 mg kg^{-1}) + carbidopa (50 mg kg^{-1})	3.5 ± 0.5	121.7 ± 5.7	104.5 ± 3.3	69.0 ± 4.6	48.4 ± 2.2
Levodopa + carbidopa + OR-611 (10 mg kg^{-1})	7.6 ± 1.5	32.7 ± 5.2***	166.7 ± 9.8***	168.9 ± 4.8***	62.6 ± 4.4**
Levodopa + carbidopa + OR-611 (30 mg kg^{-1})	13.2 ± 3.5	24.1 ± 8.0***	169.1 ± 13.1***	171.9 ± 19.0***	58.7 ± 1.6*
Levodopa + carbidopa + Ro 40-7592 (10 mg kg^{-1})	30.4 ± 5.6***	9.9 ± 0.9***	175.0 ± 13.7***	267.7 ± 7.7***	17.0 ± 1.6***
Levodopa + carbidopa + Ro 40-7592 (30 mg kg^{-1})	22.8 ± 2.0***	7.7 ± 0.5***	176.3 ± 13.1***	276.0 ± 7.7***	11.5 ± 0.5***
Levodopa + carbidopa + CGP 28014 (10 mg kg^{-1})	9.1 ± 2.5	72.9 ± 14.2**	109.0 ± 9.1	104.7 ± 22.0	39.0 ± 6.6
Levodopa + carbidopa + CGP 28014 (30 mg kg^{-1})	10.1 ± 4.1	80.5 ± 4.7***	109.7 ± 12.4	144.6 ± 15.5***	31.3 ± 2.7***

Mean ± s.e.mean. $n = 19$ in levodopa plus carbidopa group, otherwise $n = 5$–8. DOPAC: 3,4-dihydroxy-phenylacetic acid; HVA: homovanillic acid. Statistics: $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ versus levodopa plus carbidopa treatment.

Other amines, DOPA and amine metabolites after levodopa and carbidopa and each of the COMT inhibitors

At 1 h, DOPA and dopamine levels in the striatum and hypothalamus were generally increased (maximally about 2 fold) by OR-611 and Ro 40-7592 (except by 10 mg kg^{-1} in the striatum) but not by CGP 28014 (Tables 3 and 4). All COMT inhibitors elevated generally DOPAC levels (maximally 2 fold). However, the effect of CGP 28014 on striatal DOPAC was not significant at 1 h (Tables 3 and 4).

At 3 h, DOPA levels were significantly elevated only by Ro 40-7592 both in the striatum (7–9 fold) and hypothalamus (3.5–4.5 fold), and by 30 mg kg^{-1} of OR-611 in the latter (3 fold; Tables 1 and 2). Ro 40-7592 and OR-611 also increased dopamine (less than 2 fold) and DOPAC levels (2.5–4 fold) in both brain regions. Even CGP 28014, at 30 mg kg^{-1}, enhanced DOPAC levels (about 2 fold) both in the striatum and hypothalamus (Tables 1 and 2).

NA and NMN as well as 5-HT and 5-HIAA levels were not significantly affected by any treatment in either brain area (not shown).

Table 2 Hypothalamic DOPA, 3-O-methyldopa (3-OMD), dopamine and its metabolite concentrations at 3 h after levodopa plus carbidopa (50 mg kg^{-1}, i.p.) and three different catechol-*O*-methyltransferase (COMT) inhibitors (10 or 30 mg kg^{-1}, i.p.)

	Hypothalamic concentrations (nmol g^{-1})				
Treatment	*DOPA*	*3-OMD*	*Dopamine*	*DOPAC*	*HVA*
Levodopa (50 mg kg^{-1}) + carbidopa (50 mg kg^{-1})	9.6 ± 1.0	127.4 ± 3.8	15.0 ± 0.3	38.1 ± 2.4	25.8 ± 1.1
Levodopa + carbidopa + OR-611 (10 mg kg^{-})	20.8 ± 5.1	30.3 ± 4.3***	25.5 ± 2.6***	89.8 ± 17.8**	29.1 ± 3.8
Levodopa + carbidopa + OR-611 (30 mg kg^{-1})	29.4 ± 4.6***	29.3 ± 10.9***	28.1 ± 2.6***	111.2 ± 13.7***	41.7 ± 2.2***
Levodopa + carbidopa + Ro 40-7592 (10 mg kg^{-1})	43.6 ± 4.1***	8.0 ± 1.4***	30.0 ± 1.3***	163.6 ± 8.3***	12.6 ± 1.1***
Levodopa + carbidopa + Ro 40-7592 (30 mg kg^{-1})	34.0 ± 6.1***	2.4 ± 0.5***	28.3 ± 2.1***	162.4 ± 8.3***	7.1 ± 0.5***
Levodopa + carbidopa + CGP 28014 (10 mg kg^{-1})	13.7 ± 4.1	81.0 ± 14.7***	15.7 ± 3.3	54.1 ± 13.7	21.4 ± 4.9
Levodopa + carbidopa + CGP 28014 (30 mg kg^{-1})	18.3 ± 2.0	81.9 ± 4.3***	18.9 ± 1.3	80.3 ± 7.1	19.2 ± 1.6

Mean ± s.e.mean. $n = 19$ in levodopa plus carbidopa group, otherwise $n = 5$–8. DOPAC: 3,4-dihydroxy-phenylacetic acid; HVA: homovanillic acid. Statistics: $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ versus levodopa plus carbidopa treatment.

Table 3 Striatal DOPA, dopamine and 3,4-dihydroxy-phenylacetic acid (DOPAC) concentrations at 1 h after levodopa (50 mg kg^{-1}, i.p.) and carbidopa (50 mg kg^{-1}, i.p.) and three different catechol-*O*-methyltransferase (COMT) inhibitors (10 or 30 mg kg^{-1}, i.p.)

	Striatal concentrations (nmol g^{-1})		
Treatment	*DOPA*	*Dopamine*	*DOPAC*
Levodopa (50 mg kg^{-1}) + carbidopa (50 mg kg^{-1})	19.8 ± 2.5	109.7 ± 4.6	49.4 ± 3.6
Levodopa + carbidopa + OR-611 (10 mg kg^{-1})	35.5 ± 1.5*	163.3 ± 5.9***	74.4 ± 3.0*
Levodopa + carbidopa + OR-611 (30 mg kg^{-1})	36.5 ± 4.1**	176.3 ± 8.5***	95.2 ± 4.7**
Levodopa + carbidopa + Ro 40-7592 (10 mg kg^{-1})	37.5 ± 5.1	152.1 ± 17.0	82.1 ± 9.5*
Levodopa + carbidopa + Ro 40-7592 (30 mg kg^{-1})	40.6 ± 5.6**	153.4 ± 14.4*	95.2 ± 10.7
Levodopa + carbidopa + CGP 28014 (10 mg kg^{-1})	23.1 ± 4.3	114.9 ± 8.5	61.0 ± 6.2
Levodopa + carbidopa + CGP 28014 (30 mg kg^{-1})	27.5 ± 2.5	128.9 ± 7.2	66.0 ± 4.7

Mean ± s.e.mean. $n = 19$ in levodopa plus carbidopa group, otherwise $n = 5$–8. Statistics: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ versus levodopa plus carbidopa treatment.

Table 4 Hypothalamic DOPA, dopamine and 3,4-dihydroxy-phenylacetic acid (DOPAC) concentrations at 1 h after levodopa (50 mg kg^{-1}, i.p.) plus carbidopa (50 mg kg^{-1}, i.p.) and three different catechol-*O*-methyltransferase (COMT) inhibitors (10 or 30 mg kg^{-1}, i.p.)

Treatment	*Hypothalamic concentrations* (nmol g^{-1}) *DOPA*	*Dopamine*	*DOPAC*
Levodopa (50 mg kg^{-1}) + carbidopa (50 mg kg^{-1})	23.8 ± 2.5	14.4 ± 0.2	42.5 ± 3.9
Levodopa + carbidopa + OR-611 (10 mg kg^{-1})	42.6 ± 1.5**	26.8 ± 0.7***	81.1 ± 1.5**
Levodopa + carbidopa + OR-611 (30 mg kg^{-1})	50.2 ± 4.6***	27.4 ± 1.3***	99.9 ± 5.2**
Levodopa + carbidopa + Ro 40-7592 (10 mg kg^{-1})	45.3 ± 6.4**	22.2 ± 1.3***	91.0 ± 10.3
Levodopa + carbidopa + Ro 40-7592 (30 mg kg^{-1})	49.1 ± 5.0***	26.3 ± 1.8***	97.0 ± 10.9
Levodopa + carbidopa + CGP 28014 (10 mg kg^{-1})	33.8 ± 1.9	18.9 ± 8.5	61.3 ± 5.9
Levodopa + carbidopa + CGP 28014 (30 mg kg^{-1})	35.0 ± 2.0*	18.3 ± 1.3	75.0 ± 4.8**

Mean ± s.e.mean. $n = 19$ in levodopa plus carbidopa group, otherwise $n = 5$–8. Statistics: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ versus levodopa plus carbidopa treatment.

Effect of each of the COMT inhibitors on striatal and hypothalamic 3-OMD and 3-MT levels after levodopa and carbidopa in the pargyline pretreated rats

In pargyline pretreated rats, OR-611 (10 and 30 mg kg^{-1}, respectively) suppressed brain 3-OMD levels at 1 h after levodopa and carbidopa treatment to 41% and 31% in the striatum and to 16% and 18% in the hypothalamus ($P < 0.001$). Ro 40-7592 (10 and 30 mg kg^{-1}) was even more effective, decreasing the 3-OMD levels to 14% and 11% in the striatum and to 11% and 8% in the hypothalamus. Again the effect of CGP 28014 was not significant (Figure 2).

CGP 28014 ($P < 0.01$–0.001) and Ro 40-7592 ($P < 0.001$) decreased both striatal (CGP 28014 to 42% and 35% and Ro 40-7592 to 20% and 14%) and hypothalamic (CGP 28014 to 64% and 45% and Ro 40-7592 to 23% and 12%) 3-MT levels at 10 and 30 mg kg^{-1}, respectively. OR-611 had no effect on the brain 3-MT levels (Figure 2).

Few of the rats given pargyline with levodopa and carbidopa survived for 3 h in the pilot studies. Therefore, we did not perform 3 h experiments in the pargyline pretreated rats.

Other amines, DOPA and amine metabolites after levodopa and carbidopa and each of the COMT inhibitors in the pargyline pretreated rats

Even after the pargyline pretreatment, DOPA and dopamine levels in the striatum and hypothalamus were generally further increased by OR-611 (always less than 2 fold) and Ro 40-7592 (usually less than 3 fold) but not by CGP 28014. Both DOPAC and HVA levels were near the detection limit and no effect of any COMT inhibitor was noted (not shown).

NA and 5-HT levels in the hypothalamus were greatly increased by pargyline (2 fold and 5 fold, respectively) and NA levels further significantly elevated by OR-611 (1.2 fold) and Ro 40-7592 (1.5 fold), but never significantly elevated by CGP

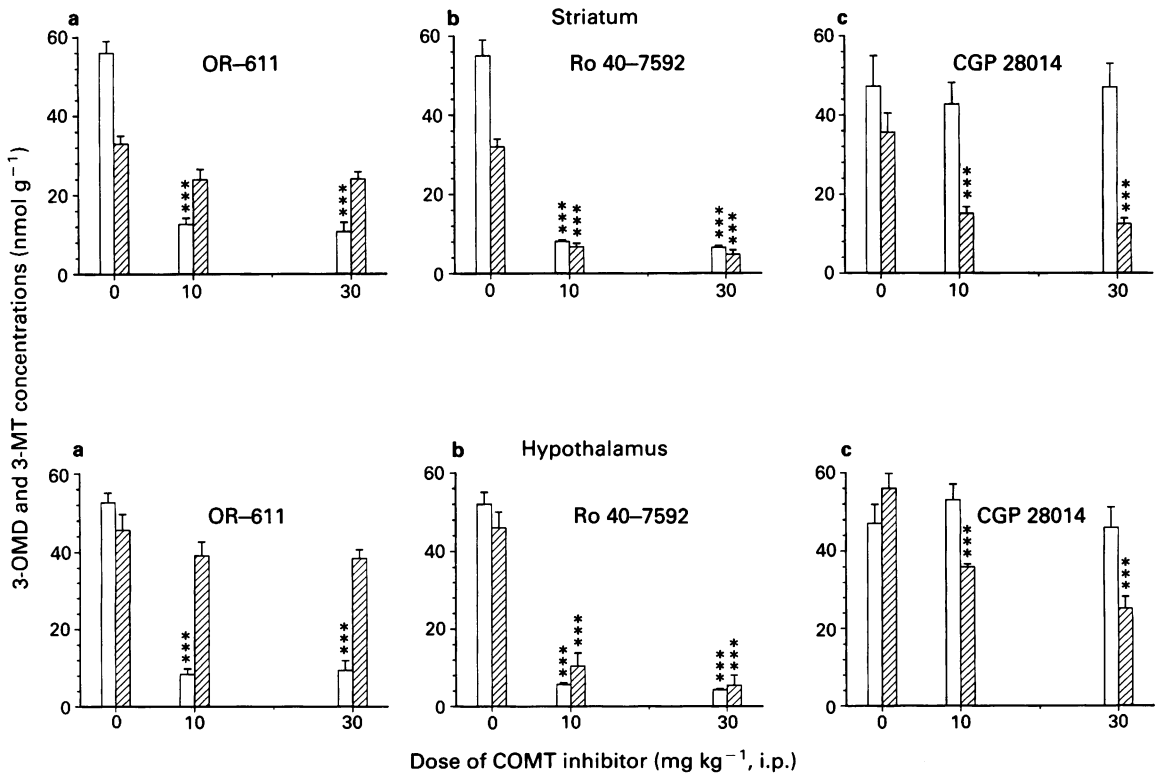

Figure 2 Striatal (upper part) and hypothalamic (lower part) 3-O-methyldopa (3-OMD, open columns) and 3-methoxytyramine (3-MT, hatched columns) concentrations at 1 h after levodopa plus carbidopa (50 mg kg^{-1}, i.p.) in the pargyline-treated (80 mg kg^{-1}, 1 h before levodopa and carbidopa) and three catechol-*O*-methyltransferase (COMT) inhibitors ((a) OR-611; (b) Ro 40-7592 and (c) CGP 28014; all either 10 or 30 mg kg^{-1}, i.p.). Mean with s.e.mean shown by vertical bars. $n = 19$ in levodopa plus carbidopa group, otherwise $n = 5$–8. Statistics: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ versus levodopa and carbidopa treatment (effect of COMT inhibitors).

28014. Corresponding NMN and 5-HIAA levels were significantly suppressed (not shown).

Pargyline elevated 5-HT levels and decreased 5-HIAA also in the striatum, while changes in NA levels were not significant. Only Ro 40-7592 further enhanced striatal noradrenaline levels (1.7 fold) over those caused by pargyline. Both OR-611 and Ro 40-7592 decreased significantly striatal NMN levels in the pargyline-treated rats (not shown).

Discussion

The major indicators of COMT function in the present study are the concentrations of 3-OMD, HVA and 3-MT in the striatal and hypothalamic tissues. Most of the peripherally administered levodopa does not enter the brain. Therefore most of the 3-OMD is also formed outside the brain. Since 3-OMD readily enters the brain (Kopin, 1985), its amount in the brain tissue is a measure of the peripheral action of COMT. HVA and 3-MT analysed from the brain samples can be considered purely as the COMT-produced brain metabolites of dopamine. They do not easily pass the blood-brain barrier, and therefore the peripherally formed HVA and 3-MT do not significantly affect levels in the brain. Normally, the brain levels of 3-MT, even after levodopa loading, are near or below the electrochemical detection limit. If MAO pathways are blocked (for instance by using pargyline), 3-MT concentrations become easily analysable (Kopin, 1985). Changes in 3-MT can then be used to support the results obtained by measuring HVA levels.

Based on the above definitions, each of the three COMT inhibitors studied behaved differently. OR-611 was principally a peripherally acting COMT inhibitor. It did not decrease but rather increased brain HVA. Even so it increased significantly brain dopamine levels apparently through enhanced DOPA supply. A moderate lowering effect on 3-OMD lasted at least 3 h. Although it produced little, if any, inhibition of brain COMT, it was able to potentiate the action of levodopa in behavioural studies (Etemadzadeh *et al.*, 1989).

Ro 40-7592 was a broad-spectrum COMT inhibitor acting effectively both in the periphery and brain, as described by Zürcher *et al.* (1990). It was also long-acting, still having nearly maximum efficacy at 3 h. Ro 40-7592 closely resembled one of its chemical relatives, Ro 41-0960, which has been thoroughly studied (Borgulya *et al.*, 1989).

CGP 28014 was a poor COMT inhibitor in the periphery but quite an efficient COMT inhibitor in the brain, preventing both HVA and 3-MT formation. It was, however, less effective than Ro 40-7592. This kind of brain selectivity of CGP 28014 has not been emphasized before. In the studies of Waldmeier and coworkers (1990a,b), CGP 28014 inhibited 3-OMD and HVA formation to a similar extent both after oral and intraperitoneal administration. Therefore the route of intake does not explain the difference between our and their results.

At the present time, we cannot resolve the disparity in our results and those of Waldmeier and coworkers (1991a,b). However, the weak and delayed effect of CGP 28014 on peripheral COMT was a constant finding. Most of the original experiments on CGP 28014 were done without levodopa treatment. Even in rare cases where levodopa was given, it was administered without the inhibitor of peripheral dopa decarboxylase (DDC) (Waldmeier *et al.*, 1990a,b). Therefore, most of the DOPA was metabolized to dopamine in the peripheral tissues. Only minor amounts of DOPA were metabolized to 3-OMD or transported to the brain. In the present study, levodopa and carbidopa were always given together. Levodopa coadministered with carbidopa increased DOPA and dopamine levels in the striatal microdialysis fluid 7.8 and 3.3 times, respectively, over those after levodopa alone (Nakashima *et al.*, 1991). Accordingly, in our studies the levels of 3-OMD in the brain were already high at 1 h and were still increasing at 3 h. Even so, the inhibitory effect of CGP 28014 on 3-OMD formation became significant only when at least 3 h had elapsed from the administration of carbidopa. At this time, DOPA levels in the brain were already decreased to one-half or less than those at 1 h.

It is hard to explain how the presence of carbidopa or DOPA would abolish the effect of CGP 28014 on peripheral COMT. The weak COMT inhibiting activity of carbidopa and/or DOPA in the peripheral tissues (Baldessarini, 1972; Kaakkola *et al.*, 1991) would rather have the opposite effect (LeWitt, 1989).

The mechanism of action of CGP 28014 is unknown. It inhibits COMT *in vitro* only in millimolar concentrations. The major metabolite of CGP 28014, 2-amino-6-hydroxypyridine, is not a COMT inhibitor (Waldmeier *et al.*, 1990a,b). In the present study, DOPA, dopamine and DOPAC levels were increased very little by CGP 28014 in the striatum or hypothalamus. In this respect CGP 28014 differed from the real COMT inhibitors, the nitrocatechol derivatives. CGP 28014 may inhibit the transfer of the COMT substrates to the enzyme, i.e., it may inhibit uptake$_2$, as already discussed by Waldmeier *et al.* (1991a). Since peripheral uptake$_2$ is not identical to the brain uptake$_2$ (Trendelenburg, 1989), we suggest that CGP 28014 preferentially inhibits the central type uptake$_2$.

The majority of dopamine in the rat brain is metabolized by neuronal monoamine oxidase-A (MAO-A, Oreland *et al.*, 1983; Kato *et al.*, 1986). Hence, if uptake$_2$ is inhibited, the subsequent shortage of dopamine in the glial cells, where MAO-B prevails (Kopin, 1985), would normally not decrease DOPAC formation. However, when animals are loaded with levodopa, as in the present study, the capacity of MAO-A can be surpassed, and MAO-B becomes important (Männistö & Tuomainen, 1991). Therefore, uptake$_2$ inhibition would decrease the entry of dopamine to glial MAO-B. Indeed, in the present study, less DOPAC was seen after CGP 28014 than after OR-611 or Ro 40-7592.

In conclusion, the three compounds studied behave differently: OR-611 is an effective, but mainly peripheral COMT inhibitor. Ro 40-7592 is even more potent and long acting, and has an additional inhibitory effect on COMT in the brain. CGP 28014 is probably not a COMT inhibitor at all. However, it preferentially inhibits HVA and 3-MT formation in the brain but inhibits only weakly 3-OMD formation in the periphery.

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Induction and potential biological relevance of a Ca^{2+}-independent nitric oxide synthase in the myocardium

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1 We have investigated whether the myocardium and isolated cardiac myocytes can express a Ca^{2+}-independent NO synthase after treatment with endotoxin or cytokines. Nitric oxide synthesis was measured in cytosols from the left ventricular wall from rats treated with endotoxin, or from freshly isolated myocytes from adult rats treated *in vitro* with cytokines.

2 Cytosols from the ventricle of saline-treated control animals showed only Ca^{2+}-dependent NO synthesis. After treatment with endotoxin, the expression of an inducible, Ca^{2+}-independent NO synthase was observed. The activity of this enzyme was maximal at 6 h and returned towards control levels by 18 h; no alterations occurred in the Ca^{2+}-dependent NO synthase activity. Parallel to this enzyme induction there was an increase in myocardial guanosine 3':5'-cyclic monophosphate (cyclic GMP) and plasma nitrite and nitrate (NO_x^-). All these changes were prevented by pretreatment of the rats with dexamethasone.

3 Myocytes possessed Ca^{2+}-dependent NO synthase activity and expressed, after treatment with tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), a Ca^{2+}-independent NO synthase, the induction of which was prevented by dexamethasone and cycloheximide.

4 Since increases in cyclic GMP levels in the heart are associated with reduced myocardial contractility, it is possible that the enhanced production of NO by a Ca^{2+}-independent enzyme accounts, at least in part, for the depression of myocardial contractility seen in septic shock, cardiomyopathies, allograft rejection, burn trauma, as well as during anti-tumour therapy with cytokines.

Keywords: Endotoxin; tumour necrosis factor; interleukin-1β; myocytes; heart; nitric oxide synthase; cyclic GMP; nitrites; nitrates; dexamethasone

Introduction

In the vasculature, the basal release of nitric oxide (NO) by a constitutive, Ca^{2+}-dependent NO synthase plays a role in the control of blood pressure and regional blood flow by elevating the levels of guanosine 3':5'-cyclic monophosphate (cyclic GMP) (see Moncada *et al.*, 1991a for review). Treatment with endotoxin or cytokines induces a Ca^{2+}-independent NO synthase in the endothelium (Radomski *et al.*, 1990) and vascular smooth muscle (Knowles *et al.*, 1990a; Rees *et al.*, 1990a; Busse & Mülsch, 1990). The expression of this enzyme leads to vasodilatation and hyporesponsiveness to vasoconstrictors (Rees *et al.*, 1990a; Smith *et al.*, 1991a) which explain the haemodynamic changes in endotoxin shock (Moncada *et al.*, 1991a). These changes, as well as the accompanying progressive increase in the synthesis of NO and cyclic GMP, are inhibited by N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of both Ca^{2+}-dependent and Ca^{2+}-independent NO synthases and by dexamethasone, which prevents the induction of the latter enzyme (see Moncada *et al.*, 1991a, for review).

Although delayed hypotension is a hallmark of septic shock, many studies have revealed that depression in myocardial contractility is a determinant pathophysiological event in the outcome of this condition (Solis & Downing, 1966; Kadowitz & Yard, 1970; Gunteroth *et al.*, 1982; Parker & Adams, 1985; Shepherd *et al.*, 1986; Suffredini *et al.*, 1989). Endotoxin does not have a direct effect on cardiac muscle but acts through the release of products (Abel, 1990), the nature of which and whether they are locally or systemically active have not been established. A myocardial depressant factor (Lefer & Martin, 1970), probably a protein, has been partially isolated (Parrillo *et al.*, 1985; Hallström *et al.*, 1991). However, more recently, cytokines which are released in response to endotoxin challenge (Beutler *et al.*, 1985) have been implicated in myocardial depression (Hollenberg *et al.*, 1989; Hosenpud *et al.*, 1989; Sobotka *et al.*, 1990; Wiechmann *et al.*, 1991).

Increases in cyclic GMP or treatment with its membrane-permeable analogue, 8-bromo-cyclic GMP, have been shown to decrease myocardial contractility (Smith *et al.*, 1991b; Shah *et al.*, 1991; Fort & Lewis, 1991). Furthermore, we have demonstrated in cultured endocardial cells the release of NO by a Ca^{2+}-dependent enzyme (Schulz *et al.*, 1991) which plays a role in the regulation of myocardial contractility (Smith *et al.*, 1991b).

In view of these findings and the possibility that the pathological release of NO might be involved in myocardial dysfunction, we have now investigated whether myocardial tissue *in vivo* and freshly isolated myocytes express the Ca^{2+}-independent, inducible NO synthase when treated with endotoxin or cytokines.

Some of these results were presented at the joint meeting of the British and Nordic Pharmacological Societies, Southampton, 18–20th September, 1991.

Methods

Treatment of rats and preparation of soluble tissue extracts

Male Wistar rats (250–300 g, Charles River) were injected intraperitoneally with: (a) endotoxin (4 mg kg^{-1}), (b) dexamethasone (1 mg kg^{-1}), (c) dexamethasone (1 mg kg^{-1}) followed 30 min later by endotoxin (4 mg kg^{-1}) or (d) with an equivalent volume of pyrogen-free saline. Animals were killed by cervical dislocation after 6 h or at the times indicated. The thoracic cavity was opened and a blood sample was taken immediately by cardiac puncture with a heparinized syringe. The blood sample was centrifuged (10,000 *g*, 1 min) and stored at −20°C for later determination of plasma NO_x^-. The heart was removed, rinsed and placed into cold Krebs buffer gassed with 5% CO_2 in O_2. A 3–4 mm size segment of myocardium

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from the left ventricular wall was excized, avoiding any major blood vessels and the endocardial surface. This was cut into segments, washed thoroughly with three exchanges of Krebs buffer and blotted. One piece (ca. 1–1.5 mm) was frozen separately in liquid nitrogen for determination of cyclic GMP content and the rest was freeze-clamped for determination of ventricular NO synthase activity. The freeze-clamped segments were homogenized in 0.5 ml of ice-cold buffer containing 320 mM sucrose, 10 mM HEPES, 0.1 mM EDTA, 1 mM DL-dithiothreitol, 100 $\mu g\,ml^{-1}$ phenylmethylsulphonyl fluoride, 10 $\mu g\,ml^{-1}$ leupeptin, 10 $\mu g\,ml^{-1}$ soybean trypsin inhibitor, and 2 $\mu g\,ml^{-1}$ aprotinin (adjusted to pH 7.2 at 20°C with HCl) in a Ystral homogenizer. The homogenate was centrifuged (100,000 g, 30 min) at 4°C and the cytosolic (supernatant) fraction was kept on ice for immediate assay of NO synthase activity.

Induction of NO synthase in cardiac myocytes

Cardiac myocytes from adult rats (250–350 g) were isolated by perfusion of the isolated heart with collagenase as described (Powell *et al.*, 1980; Stone *et al.*, 1989). The final cell preparation containing approximately 55% viable (i.e. trypan-blue-excluding, rod-shaped) cells was resuspended (approx. 30,000 rods ml^{-1}) in Dulbecco's MEM supplemented with 10% foetal calf serum, 2 mM glutamine, penicillin (100 u ml^{-1}) and streptomycin (100 $\mu g\,ml^{-1}$). Polymyxin B (10 $\mu g\,ml^{-1}$) was added to bind any endotoxin which may have contaminated the preparation during the myocyte isolation procedure. Cells were incubated for 24 h at 37°C in an atmosphere containing 5% CO_2 in the presence of (a) culture medium alone or (b) TNF-α (20 ng ml^{-1}) and IL-1β (5 ng ml^{-1}), plus (c) cycloheximide (10 μM) or (d) dexamethasone (3 μM) which was added 30 min before the cytokines. Cells were harvested by centrifugation (40 g, 2 min) and the cell pellet washed 3 times with phosphate-buffered saline (PBS). Cell viability was reduced to approx. 38% after 24 h incubations. A final spin (10,000 g, 15 s) was performed to compact the pellet which was resuspended in ice-cold homogenization buffer and sonicated twice for 3 s at 100 W. The cytosolic fraction was prepared as indicated above.

Assay of Ca^{2+}-dependent and Ca^{2+}-independent NO synthase

Nitric oxide formation in the cytosolic fractions of rat ventricular wall and isolated myocytes was measured by the formation of radiolabelled [^{14}C]-citrulline from [^{14}C]-L-arginine, essentially as described by Knowles *et al.* (1990b). Duplicate incubations for 10 min at 37°C were performed for each sample in the presence or absence of either EGTA (1 mM) or EGTA plus L-NMMA (1 mM each) to determine the level of the Ca^{2+}-dependent and Ca^{2+}-independent NO synthase activities, respectively, before terminating the reaction by addition of 0.1 vol of 20% (vol:vol) aqueous $HClO_4$. Samples were neutralized by addition of 0·23 vol of 1.9 M aqueous $KHCO_3$, cooled on ice for 5 min and centrifuged (10,000 g, 2 min). [^{14}C]-citrulline in the supernatant was separated from [^{14}C]-arginine by cation-exchange chromatography using AG 50W-X8 resin and quantified by liquid-scintillation counting.

Determination of cyclic GMP in left ventricular wall

Frozen sements of left ventricular wall were pulverized in a stainless steel pestle and mortar cooled in dry ice. Ice-cold 0.2 M perchloric acid in 80 mM HEPES (0.5 ml) was added. After mixing, homogenates were kept on ice for 10 min and centrifuged (10,000 g, 2 min). The supernatant was removed and neutralized with 1.1 M K_3PO_4 and centrifuged as described above; cyclic GMP levels were determined in duplicate, by specific radioimmunoassay (Moncada *et al.*, 1991b). The protein pellet was solubilized by boiling for 10 min in 2 M NaOH for protein determination.

Determination of plasma NO_x^-

Plasma samples were deproteinized by ultrafiltration (Centrifree micropartition system, Amicon). The nitrate content of the samples was reduced to nitrite with a nitrate reductase (Schmidt *et al.*, 1990). Nitrate reductase (20 mu), phosphate buffer (1.2 M, pH 7.5), FAD (120 mM) and NADPH (14.4 mM) were added to 100 μl of deproteinized plasma or appropriate dilutions in distilled water to give a final volume of 120 μl and incubated for 1 h at 37°C. 5-10 μl of the reduced sample was injected into a reaction vessel containing refluxing 6% aqueous sodium iodide/glacial acetic acid (1:5, vol/vol). Under these conditions, nitrite is reduced to NO and is removed in the gas phase by a constant stream of nitrogen. This was mixed with ozone to form a chemiluminescent product which was then measured in a detector coupled to an electronic integrator (see Palmer *et al.*, 1987). The area under the peak of the chemiluminescence signal is proportional to the amount of nitrite in the sample, as calibrated using a standard solution of $NaNO_2$ and is linear in the range of 5–100 μM. A standard curve of reduction of nitrate to nitrite was performed and used in each experiment as a reference. As plasma contains both nitrite and nitrate the concentrations are reported as total NO_x^- content.

Determination of protein

The protein content of the solubilized protein pellet from ventricular wall extracts for cyclic GMP determination or from the cytosols prepared from homogenates of ventricular wall or myocytes was measured with BCA protein reagent; bovine serum albumin was used as a standard.

Materials

Phenol-extracted lipopolysaccharide (LPS, endotoxin) from *Salmonella typhosa 0901* (Difco), L-[U-^{14}C]-arginine monohydrochloride (305 mCi $mmol^{-1}$), [8-^{3}H]-guanosine 3′:5′-cyclic monophosphate (cyclic GMP, 18.2 Ci $mmol^{-1}$), human recombinant IL-1β and TNF-α (Amersham), dexamethasone sodium phosphate (Decadron, Merck Sharpe and Dohme), AG 50W-X8 cation exchange resin (200–400 mesh, Bio-Rad), bicinchoninic acid protein assay reagent (Pierce), NADPH tetrasodium salt (Boehringer Mannheim), rabbit antiserum to cyclic GMP (Dr J. Garthwaite, University of Liverpool), collagenase type I (Worthington), cell culture materials (Flow and Gibco) and L-NMMA acetate (Wellcome) were obtained as indicated. All other reagents were obtained from either Sigma or BDH.

Statistics

Results are expressed as the mean ± s.e.mean for n experiments. Student's unpaired t test or analysis of variance followed by Fisher's least significant difference test to compare individual means were used, as appropriate. $P < 0.05$ was considered statistically significant.

Results

Effects of endotoxin on NO synthase in the left ventricular wall and plasma NO_x^-

Cytosols obtained from the left ventricular wall from rats injected with pyrogen-free saline showed a small but significant level of Ca^{2+}-dependent NO synthase activity (1.30 ± 0.16 pmol NO mg^{-1} protein min^{-1}, $n = 6$) which was not changed in cytosols obtained from rats at different times after injection of endotoxin (Figure 1a).

There was no significant Ca^{2+}-independent NO synthase activity in cytosols of left ventricular wall from rats 6 h after injection of pyrogen-free saline (Figure 1a). Thirty minutes

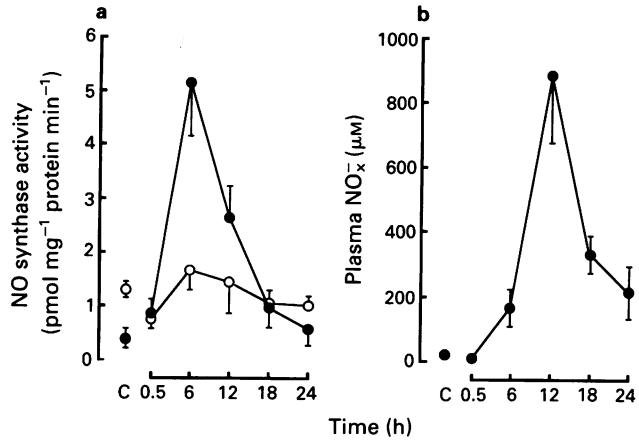

Figure 1 Time course of activation of left ventricular NO synthase and changes in plasma NO_x^- concentration after i.p. injection of endotoxin (LPS) or pyrogen-free saline in rats. (a) Ca^{2+}-dependent (○) and Ca^{2+}-independent (●) NO synthase activity in the left ventricular wall after treatment with LPS. C represents control values 6 h after injection of pyrogen-free saline. (b) Level of NO_x^- in plasma at time rats were killed after treatment with LPS. C represents control values 6 h after injection of pyrogen-free saline. Each point is the mean of heart or plasma samples from 4–6 animals; s.e.mean shown by vertical bars. If no error bars are shown the error lies within the height of the symbol.

after treatment with endotoxin, however, the level of Ca^{2+}-independent activity started to increase, reaching a maximum of 5.15 ± 1.00 pmol NO mg^{-1} protein min^{-1} ($n = 5$) at 6 h and declined thereafter towards the control level by 18 h (Figure 1a).

Rats injected with pyrogen-free saline had a plasma NO_x^- concentration of 22.6 ± 4.3 μM ($n = 7$) at the time when they were killed 6 h later (Figure 1b). Injection of endotoxin (4 mg kg^{-1}) caused a time-dependent increase in the concentration of NO_x^- in plasma which was significantly higher than the control value by 6 h, reached a peak of about 40 times the control value at 12 h and thereafter slowly declined towards control levels by 24 h (Figure 1b). The rise and fall in plasma NO_x^- coincided with the onset and disappearance of endotoxaemia-like symptoms in the rats, namely a huddled appearance, ruffled fur and lethargy, all of which were resolved by 24 h.

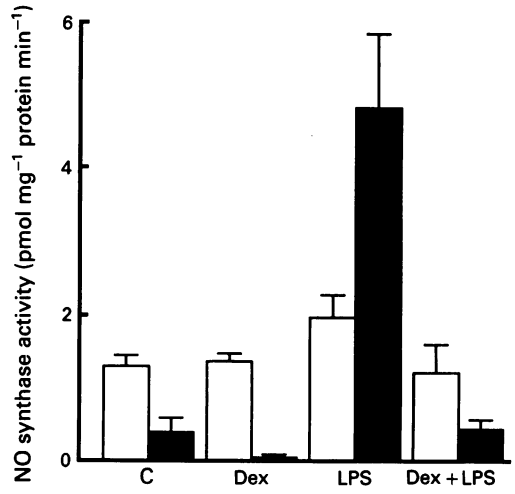

Figure 3 Effect of dexamethasone on the level of the Ca^{2+}-independent inducible (closed columns) and the Ca^{2+}-dependent constitutive (open columns) NO synthases in the left ventricular wall. The level of activity of the NO synthases 6 h after injection of pyrogen-free saline vehicle (C), dexamethasone (Dex), endotoxin (LPS), or LPS preceded 30 min by dexamethasone (Dex + LPS) is shown. The Ca^{2+}-dependent constitutive NO synthase activity was unaffected by the various treatments. LPS caused the expression of the Ca^{2+}-independent NO synthase activity which was abolished by dexamethasone. The results are the mean of values obtained from six animals per group; s.e.mean shown by vertical bars.

Six hours after treatment with endotoxin there was a 50% increase in the levels of cyclic GMP in the left ventricular wall compared with animals injected with saline (Figure 2).

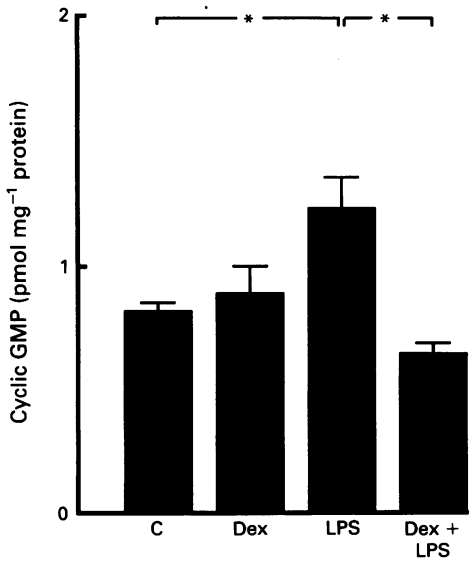

Figure 2 Effect of endotoxin (LPS) and dexamethasone on the level of cyclic GMP in the left ventricular wall 6 h after i.p. administration. Treatment with dexamethasone (Dex) had no effect on the basal level of cyclic GMP, whereas LPS raised the level of cyclic GMP significantly. Dexamethasone, given 30 min before LPS, abolished the increase in cyclic GMP by LPS (Dex + LPS). The columns represent the mean of samples taken from 4–6 animals; s.e.mean shown by vertical bars. * $P < 0.01$.

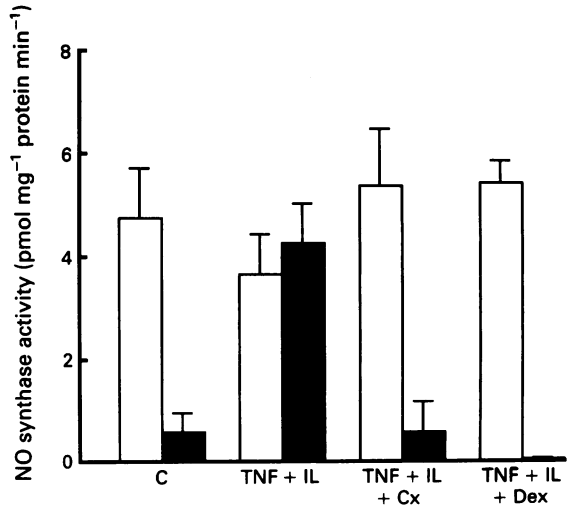

Figure 4 Constitutive and inducible NO synthase activity in isolated cardiac myocytes. Myocytes were kept for 24 h *in vitro* in control medium (C) alone or after various additions. Ca^{2+}-dependent constitutive NO synthase activity (open columns) seen in control myocytes is unaffected by treatment with tumour necrosis factor-α (TNF) and interleukin-1β (IL), alone or in combination with cycloheximide (Cx) or dexamethasone (Dex). In contrast, treatment with TNF and IL causes the expression of the Ca^{2+}-independent NO synthase (closed columns), which is abolished by Cx or Dex. The results are the mean of 3–5 experiments; vertical bars show s.e.mean.

Effect of dexamethasone

Treatment with dexamethasone alone did not significantly alter the plasma NO_x^- from the control level ($14.4 \pm 4.5\,\mu M$, $n = 6$, versus $22.6 \pm 4.3\,\mu M$, $n = 7$, respectively). Dexamethasone did, however, inhibit the increase in plasma concentration of NO_x^- induced by endotoxin from $361.6 \pm 39.1\,\mu M$ ($n = 6$) to $72.1 \pm 21.0\,\mu M$ ($n = 4$) at 6 h. Furthermore, dexamethasone prevented the endotoxin-stimulated expression of the Ca^{2+}-independent NO synthase (Figure 3) and the increase in cyclic GMP in the myocardium (Figure 2) without affecting the activity of the Ca^{2+}-dependent enzyme (Figure 3).

NO synthase in freshly isolated cardiac myocytes

Cardiac myocytes incubated in control medium for 24 h showed Ca^{2+}-dependent NO synthase activity of 4.73 ± 0.99 pmol mg^{-1} protein min^{-1} ($n = 4$) but no significant Ca^{2+}-independent NO synthase activity (Figure 4). Incubation with TNF-α and IL-1β induced the expression of Ca^{2+}-independent NO synthase activity in the myocytes which was prevented by cycloheximide ($10\,\mu M$), an inhibitor of protein synthesis, or by dexamethasone ($3\,\mu M$). The Ca^{2+}-dependent NO synthase activity was unaffected by treatment with the cytokines alone or in combination with cycloheximide or dexamethasone.

Discussion

Treatment of rats with endotoxin results, after a lag time, in the induction of a Ca^{2+}-independent NO synthase in the left ventricular myocardium. This enzyme, which is also expressed in isolated cardiac myocytes after treatment with TNF-α and IL-1β, is similar in properties and time course to that induced in many other cells and tissues in response to the same type of stimulus (Radomski *et al.*, 1990; Knowles *et al.*, 1990a; Rees *et al.*, 1990a; Busse & Mülsch, 1990; McCall *et al.*, 1991).

Depressed myocardial contractility in animals and man is known to occur in conditions associated with the release of cytokines, including endotoxin shock (Solis & Downing, 1966; Kadowitz & Yard, 1970; Gunteroth *et al.*, 1982; Velkov *et al.*, 1989; Suffredini *et al.*, 1989) or treatment with endotoxin *in vitro* (Macnicol *et al.*, 1973). Similarly, during antitumour therapy with cytokines (Deyton *et al.*, 1989; Nora *et al.*, 1989) or after exposure to cytokines *in vitro* (Hosenpud *et al.*, 1989; Hollenberg *et al.*, 1989; Sobotka *et al.*, 1990; Wiechmann *et al.*, 1991), a profound depression in cardiac function has been observed. Moreover, IL-1 and TNF have been shown to diminish the β-adrenoceptor-mediated increase in contractility in cultured cardiac myocytes (Gulick *et al.*, 1989). All these changes occur after a lag time of several hours suggesting that they involve *de novo* synthesis of protein.

Nitric oxide released from endocardial cells by the Ca^{2+}-dependent NO synthase (Schulz *et al.*, 1991) plays a role in the physiological modulation of myocardial contractility by increasing the level of cyclic GMP in cardiac muscle and thus exerting a negative inotropic effect (Smith *et al.*, 1991b; Shah *et al.*, 1991; Fort & Lewis, 1991). The mechanism for this is not clear; however, it may be due to the cyclic GMP-mediated inhibition of the entry of Ca^{2+} into the cell (Nawrath, 1977; Bkaily & Sperelakis, 1985; Hartzell & Fischmeister, 1986; Méry *et al.*, 1991) by activation of a cyclic GMP-dependent protein kinase (Méry *et al.*, 1991).

Induction by endotoxin or cytokines of a Ca^{2+}-independent enzyme in the myocardium leads to an excessive and prolonged release of NO which will result in diminished myocardial contractility. The negative inotropism in the heart during septic shock is accompanied by an increase in end-diastolic volume indicative of ventricular dilatation (see Parillo *et al.*, 1990), reflecting the change from a physiological (Ca^{2+}-dependent) release of NO by the endocardium to a pathological (Ca^{2+}-independent) NO release by the myocardium. This is comparable to the situation in the vascular wall, where the induction of a Ca^{2+}-independent release of NO in the endothelium and vascular smooth muscle leads to pathological vasodilatation (Rees *et al.*, 1990a; Smith *et al.*, 1991a; see Moncada *et al.*, 1991a for review). In this respect it is worth noting that we have recently observed the expression of a Ca^{2+}-independent enzyme in porcine endocardial cells *in vitro* (Schulz, Smith, Lewis and Moncada, unpublished observations).

Whether decreased contractility and ventricular dilatation are due to the stimulation of the soluble guanylate cyclase by NO (Moncada *et al.*, 1989) or to the direct cytotoxic actions of this molecule on iron-containing enzymes of the respiratory chain (Drapier & Hibbs, 1988), or both, is unknown. Our results show that the increased synthesis of NO in the myocardium is accompanied by an increase of 50% in the level of cyclic GMP. This moderate increase is comparable with a 90% increase in cyclic GMP levels observed in the rat isolated perfused heart 1 min after treatment with acetylcholine at a dose which produced a significant reduction in contractile response (George *et al.*, 1973). Further studies are required to determine whether, as in the vascular endothelium (Palmer *et al.*, 1992) and in EMT-6 adenocarcinoma cells (O'Connor & Moncada, 1991), prolonged induction of NO synthase in the myocardium leads to cytotoxicity.

Interestingly, we have observed a Ca^{2+}-dependent NO synthase activity in the myocardium and freshly isolated cardiac myocytes. Indeed, a continuous basal release of NO by this enzyme in the myocyte, acting on the guanylate cyclase, may regulate cardiac contractility in an autocrine fashion. This is different from vascular smooth muscle, which does not contain the constitutive NO synthase (Palmer *et al.*, 1987; Knowles *et al.*, 1990b; Rees *et al.*, 1990a; Busse & Mülsch, 1990) and in which the basal level of cyclic GMP is regulated by NO released from the endothelium (Rapoport & Murad, 1983; Martin *et al.*, 1986; Moncada *et al.*, 1991b). The physiological significance of this difference between cardiac and vascular muscle remains to be investigated.

Little is known, at present, about the consequences of inhibition of NO synthase on cardiac function. In the rat, administration of L-NMMA or other inhibitors of NO synthase leads to bradycardia, which is probably reflex in origin (Rees *et al.*, 1990b; Gardiner *et al.*, 1990). A negative inotropic effect has been observed following administration of L-nitroarginine methyl ester to the conscious rat; this may have been due to a direct action of this compound on the myocardium or to a reduction in coronary blood flow resulting from its potent vasoconstrictor actions (Gardiner *et al.*, 1990). More studies on the direct cardiac actions of inhibitors of NO synthase are required.

Induction of the myocardial Ca^{2+}-independent NO synthase by endotoxin caused a rise in plasma NO_x^- concentration, which followed the appearance of the enzyme in the ventricular wall. Inflammatory reactions *in vivo* are known to cause a marked increase in the excretion of nitrate (Wagner *et al.*, 1983; Stuehr & Marletta, 1985). Nitric oxide is rapidly oxidized in aqueous solution to nitrite (Palmer *et al.*, 1987), which is itself oxidized *in vivo* to nitrate (Witter & Balish, 1979). This is likely to occur by the stoichiometric reaction of nitrite with oxyhaemoglobin to form nitrate (Kosaka *et al.*, 1979). Urinary nitrate excretion was recently shown to be a sensitive indicator of the activity of NO synthase *in vivo* (Granger *et al.*, 1991). After endotoxin treatment, the NO synthase is induced in tissues other than the myocardium. Thus plasma NO_x^- levels reflect the production of NO in different organs as well as a balance between production and excretion of NO_x^-. The relative contribution of various tissues to the plasma level of NO_x^- and the fate of these metabolites remain to be studied.

Dexamethasone inhibited the induction of the Ca^{2+}-independent NO synthase in the myocardium and isolated

cardiac myocytes and the associated rise in myocardial cyclic GMP and plasma NO_x^- levels. The treatment of septic shock with glucocorticoids is controversial (Haynes & Murad, 1985). The benefits of these drugs in preventing the myocardial and haemodynamic consequences of septic shock are seen only if they are administered before endotoxin (Kadowitz & Yard, 1970). Interestingly, isolated papillary muscles taken from adrenalectomized rats are known to show a more rapid decline in contractile tension *ex vivo* than those from control animals and this can be prevented by treatment with dexamethasone *in vivo* or *in vitro* (Lefer, 1968). Whether the protection afforded by dexamethasone is due to its prevention of NO-stimulated increases in cyclic GMP levels or to inhibition of NO-mediated cytotoxicity (O'Connor & Moncada, 1991) is not yet known.

Our finding that endotoxin induces the myocardium to synthesize NO by a Ca^{2+}-independent enzyme is likely to have major consequences in our understanding of the cardiomyopathies associated with septic shock, inflammatory diseases of the heart such as endocarditis and myocarditis, idiopathic dilated cardiomyopathy, allograft rejection, burn trauma and anti-tumour therapy with cytokines (Deyton *et al.*, 1989). As endotoxin, exotoxin and infection with parasites have cardiac depressant effects similar to those seen in sepsis (Cunnion & Parillo, 1989) it may be that NO represents the final common pathway of disparate immunological challenges resulting in the pathophysiological alterations seen in these disorders. A better understanding of the two enzymes responsible for the synthesis of NO could lead to a selective inhibitor of the Ca^{2+}-independent NO synthase, which may prove useful in the treatment of these disorders.

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Bovine polymorphonuclear leukocytes increase sensitivity to noradrenaline in isolated mesenteric arteries

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1 The effects of polymorphonuclear leukocytes (PMN) on vascular function to (−)-noradrenaline were examined *in vitro*. Purified bovine PMN were incubated in siliconized organ baths containing rings of bovine mesenteric arteries, after which a concentration-effect curve in response to (−)-noradrenaline was obtained.

2 PMN-derived products induced a long lasting concentration-dependent contraction of the blood vessels generating 24.4 ± 6.8% of the maximal tension to (−)-noradrenaline at a cell concentration of 2.5×10^6 ml^{-1}. The contractile response was also found in endothelium-denuded vascular rings.

3 PMN present in the organ bath caused an increase in the sensitivity of vascular rings to (−)-noradrenaline. At a cell number of 2.5×10^6 PMN ml^{-1} the pD_2-value for (−)-noradrenaline was augmented 0.40 ± 0.05 ($P < 0.001$), while total contraction at the highest concentration (−)-noradrenaline was not affected. This increase in sensitivity was dependent on an intact endothelium.

4 The increase in sensitivity to (−)-noradrenaline by PMN was inhibited by superoxide dismutase, but not by catalase, dimethylthiourea, indomethacin or nordihydroguaiaretic acid. The non-stimulated bovine PMN produced oxygen radicals as measured by chemiluminescence.

5 Simultaneous incubation of PMN and (−)-noradrenaline with arterial rings induced an increase in the release of prostacyclin, measured by an elevated concentration of 6-keto-prostaglandin $F_{1\alpha}$ in the supernatant.

6 It is concluded that PMN can increase vascular tone directly or indirectly probably via the interaction of PMN-derived superoxide anions with endothelium-derived relaxing factor.

Keywords: Polymorphonuclear leukocytes; oxygen-derived radicals; mesenteric arteries; noradrenaline; endothelium

Introduction

Polymorphonuclear leukocytes (PMN) adhere to the vascular endothelium and accumulate at discrete sites within the circulation in many pathological states including acute inflammatory disorders, myocardial infarction, hypertension and haemorrhagic or endotoxin shock (Brigham & Meyrick, 1984; Chobanian *et al.*, 1984; Schmid-Schönbein & Engler, 1986; Carden *et al.*, 1990). The interaction of PMN and blood vessels may be important in the regulation of vascular function since activated granulocytes release a variety of factors that are capable of influencing vascular tone.

PMN-derived vasoactive compounds include reactive oxygen species, lipid metabolites, proteolytic enzymes and nitric oxide (Wright *et al.*, 1989). It has been suggested that superoxide anion may be a contracting factor (Katusic & Vanhoutte, 1989) and may also indirectly affect vascular tone by inactivating endothelium-derived relaxing factor (EDRF; Gryglewski *et al.*, 1986). Hydrogen peroxide induces vascular relaxation due to stimulation of guanylate cyclase (Burke & Wolin, 1987) and in addition, hydrogen peroxide enhances synthesis of prostacyclin (Harlan & Callahan, 1984). Lipid mediators, such as platelet-activating factor cause vascular relaxation (Siren & Feuerstein, 1989), while others, like leukotriene, constrict arteries (Feigen, 1983). Furthermore proteolytic enzymes are able to cleave vasoactive peptides (Wintroub *et al.*, 1984).

Endothelial cells play a critical role in maintaining vascular tone via generation of potent vasoconstrictor and dilator substances (Vanhoutte, 1989). The vasodilators prostacyclin and EDRF are released by a variety of neurohumoral substances. Endothelin is a potent vasoconstrictor.

Preliminary studies described in the literature show that neutrophils may either cause vasodilatation (Rimele *et al.*, 1988; Mehta *et al.*, 1989) or vasoconstriction *in vitro* (Gillespie *et al.*, 1986; Nishida *et al.*, 1990; Sessa & Mullane, 1990) and *in vivo* (Carden *et al.*, 1990). Besides direct responses on arteries, neutrophils have also been reported to augment platelet-induced contractions in rabbit aorta (Sessa & Mullane, 1990) and histamine-induced pig coronary vasoconstriction (Van Heuven-Nolsen *et al.*, 1990). The interaction of neutrophils with intact endothelium was necessary for some of these effects, but in most cases adhesion of neutrophils was not required.

In the present study we investigated the influence of bovine PMN *in vitro* on the response of bovine mesenteric arteries to (−)-noradrenaline. Adrenoceptors play a major role in the regulation of peripheral vascular resistance. In hypertension both sympathetic activity and contractile responsiveness of arteries to (−)-noradrenaline are enhanced (Michel *et al.*, 1990). We show that besides the ability of PMN to contract isolated arterial rings, PMN were also capable of increasing the sensitivity to (−)-noradrenaline.

Methods

Bovine PMN isolation (Henricks *et al.*, 1987)

Whole blood was obtained in heparinized tubes from cows. Plasma was withdrawn after centrifugation at 400 *g* for 10 min. Erythrocytes were lysed by hypotonic treatment with a phosphate buffer. The white blood cells were suspended in Krebs buffer. Krebs buffer consisted of (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.1, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 7.6 and was carbogenated with 95% O_2 and 5% CO_2. The cell suspension was layered onto Percoll (1.077 g ml^{-1}) and centrifuged for 20 min at 400 *g* to remove mononuclear cells. The neutrophil-rich pellet was suspended in Krebs buffer and was washed three times with Krebs buffer, and centrifuged once at 400 *g* and twice at 100 *g* to reduce platelet contamination. The PMN suspension was kept on ice at a

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concentration of 1×10^7 to 2×10^7 cells ml^{-1} and was washed once more just before use in the organ bath experiments. The PMN suspension contained 74 ± 10% neutrophils, 18 ± 11% eosinophils and 7.8 ± 0.9% mononuclear cells (mean ± s.e.). Viability was always greater than 95% as assessed by trypan blue exclusion. Platelet contamination of the PMN suspension was less than one platelet per PMN.

Bovine mesenteric arteries

Bovine mesenteric arteries were obtained from a local slaughterhouse and transported to the laboratory in ice-cold Krebs solution. Adherent tissue was removed and in some experiments the arteries were kept overnight at 4°C under a continuous overflow of carbogenated Krebs buffer. Preliminary experiments revealed that this storage procedure did not affect the contractility of the preparation to (−)-noradrenaline. Arteries from the third or fourth branch of the mesenteric artery with a diameter of 2 to 2.5 mm were cut into rings of 2 mm length. In some experiments the endothelium was removed by careful rubbing of the intima with cotton wool. Destruction of endothelial cells was confirmed by histological study.

Organ bath experiments

The isolated rings were suspended between two stainless steel wire hooks in 12 ml siliconized water-jacketed organ baths which were filled with Krebs buffer which was gassed with 95% O_2 and 5% CO_2. The temperature was maintained at 37°C. Tension was measured with a Harvard isometric transducer and registered by a computer. Preload of the mesenteric rings was adjusted during 30 min to 50 mN, which was optimal to generate a maximal contraction as determined with 50 mM KCl and (−)-noradrenaline (10^{-8}–10^{-5} M). The isolated mesenteric arteries were allowed to stabilize for 50 min during which the tissue was washed several times. The rings were exposed initially to a single concentration of 10^{-5} M (−)-noradrenaline. Krebs buffer was refreshed several times after which (−)-noradrenaline (10^{-9}–10^{-5} M) was added cumulatively to the organ bath. Again the arterial rings were washed several times. After a resting period the PMN suspension was added at several concentrations to the organ bath and incubated for 30 min. Thereafter a second cumulative concentration-effect curve was obtained with (−)-noradrenaline.

Some experiments were carried out in the presence of inhibitors of the arachidonic acid metabolism or oxygen-radical scavengers. The cyclo-oxygenase inhibitor indomethacin (1 μM) and the lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA, 10 μM) were used for inhibition of the arachidonic acid metabolism. Superoxide dismutase (SOD, 100 u ml^{-1}), catalase (1000 u ml^{-1}) or dimethylthiourea (10 mM) were used to scavenge respectively the oxygen-derived radicals superoxide anion, hydrogen peroxide or hydroxyl radical. These compounds were added to the organ bath 15 min before the incubation with PMN.

In one experiment, inhibition of EDRF synthesis was studied by adding N-monomethyl-L-arginine (L-NMMA; 100 μM) to the organ bath 15 min before the second cumulative concentration-effect curve with (−)-noradrenaline was obtained. In this experiment PMN were not added to the organ bath.

Radioimmunoassay experiments

Mesenteric arterial rings were prepared as described above. Arterial segments and PMN suspension (2.5×10^6 cells ml^{-1} Krebs buffer) were preincubated for 20 min at 37°C. Then (−)-noradrenaline (3×10^{-7} M) was added and the tissues were incubated for 10 min after which the rings were removed. Reaction tubes were stirred throughout the experiment. Incubation fluid was collected and centrifuged at 400 *g* for 5 min at 4°C to remove the PMN. The supernatant was stored at −80°C until assayed. The experiment was also performed in the presence of both the oxygen-radical scavengers SOD (100 u ml^{-1}) and catalase (1000 u ml^{-1}). 6-keto-prostaglandin $F_{1\alpha}$ and thromboxane B_2, stable metabolites of prostacyclin and thromboxane A_2 respectively, were measured in the supernatant by means of a radioimmunoassay (Dupont de Nemour, NEN research products, Boston, U.S.A. and Amersham, Buckinghamshire, U.K. respectively).

Chemiluminescence

Superoxide anion production by PMN was measured by lucigenin enhanced chemiluminescence, with a LKB-Wallac 1251 luminometer (Finland). PMN suspension (final cell number 2.5×10^6 ml^{-1} Krebs buffer) and lucigenin (end concentration 0.5 mM) were added to Krebs buffer in a cuvette at a total volume of 0.5 ml. The cuvettes were placed in the luminometer and chemiluminescence was measured every minute during 1 h. The temperature was maintained at 37°C. To investigate the effect of arterial segments on the superoxide anion production by PMN, chemiluminescence was also determined in the presence of arterial rings with and without endothelium. The generation of superoxide radicals by PMN stimulated with phorbol myristate acetate (PMA; 100 ng ml^{-1}) was also determined according to the above described method.

Drugs

(−)-Noradrenaline (OPG, Utrecht, the Netherlands), superoxide dismutase (SOD; Sigma, St. Louis, U.S.A.), N-methyl-L-arginine (L-NMMA; Sigma, St. Louis, U.S.A.), catalase (Boehringer Mannheim, Germany), and dimethylthiourea (Merck, Suchardt, Germany) were dissolved in saline. Nordihydroguaiaretic acid (NDGA; 1 mM; Sigma, St. Louis, U.S.A.) was dissolved in 0.01 M NaOH. Indomethacin (10 mM; Merck Sharp & Dome, Haarlem, the Netherlands) was dissolved in 1 M Tris (2-amino-2-hydroxymethyl-propane-1,3-diol) buffer (pH = 7.8). All further dilutions were in saline. Phorbol myristate aceate (PMA) and lucigenin (both Sigma, St. Louis, U.S.A.) were dissolved and diluted in Krebs buffer.

Data analysis

All data are presented as mean ± s.e.mean. pD_2-values (−log EC_{50}) are used to indicate sensitivity to (−)-noradrenaline-induced vasoconstriction (Ariëns & Van Rossum, 1957). Peak chemiluminescence and total chemiluminescence integrated over a period of 0 to 60 min were determined and presented as the percentage of the response of the PMN after PMA stimulation. Statistical analysis was performed using the two-tailed Student's *t* test for paired and unpaired observations, as appropriate.

Results

Effect of PMN incubation

The addition of PMN (2.5×10^5–5×10^6 ml^{-1}) to the organ bath elicited a sustained contraction of bovine mesenteric arteries at the highest cell concentrations (Figure 1). The vasoconstriction was endothelium-independent at the concentrations tested (2.5×10^5–2.5×10^6 PMN ml^{-1}). The contraction of intact bovine mesenteric arteries due to incubation with 2.5×10^6 PMN ml^{-1} was 24.4 ± 6.8% of the maximal (−)-noradrenaline contraction. This contractile effect was not significantly influenced by incubation with the oxygen-radical scavengers SOD (100 u ml^{-1}) and catalase (1000 u ml^{-1}) together (12.5 ± 7.8%), SOD alone (32.2 ± 13.5%), catalase

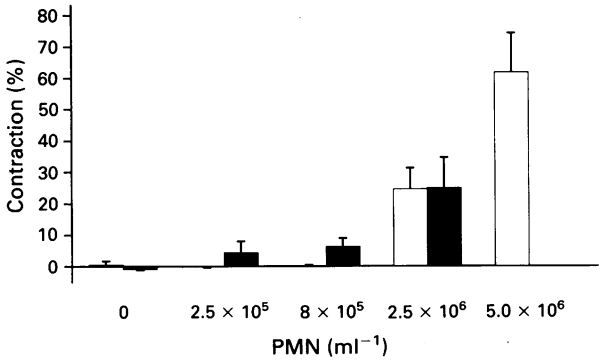

Figure 1 Contractions of bovine mesenteric arterial rings after 30 min incubation with different numbers of polymorphonuclear leukocytes (PMN). The open columns represent the response in intact arteries and the closed columns in endothelium-denuded rings. Data are expressed as the percentage of the maximal response to (−)-noradrenaline. Mean are given ($n \geqslant 7$) with s.e.mean shown by vertical bars.

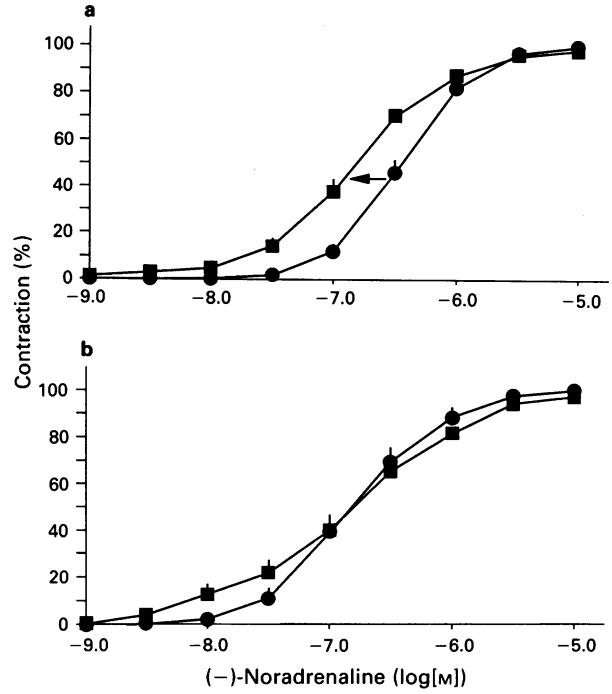

Figure 2 Concentration-effect curves to (−)-noradrenaline in bovine mesenteric arteries in the absence (●) and presence of 2.5×10^6 polymorphonuclear leukocytes (PNM) ml^{-1} (■) are shown. The experiment was performed in intact arterial rings (a, $n = 18$) and in arteries denuded of their endothelium (b, $n = 8$). Data are expressed relative to the maximal increase in force by (−)-noradrenaline. Means are given with s.e.mean shown by vertical lines.

alone (43.7 ± 8.8%) or dimethylthiourea (10 mM; 44.1 ± 10.5%), or with the lipoxygenase inhibitor NDGA (10 μM; 38.2 ± 12.5%). By contrast, in the presence of the cyclo-oxygenase inhibitor indomethacin (1 μM), PMN caused a contraction at a lower cell concentration (8×10^5 PMN ml^{-1}, 19.6 ± 5.5%) and the contraction observed on incubation with 2.5×10^6 PMN ml^{-1} was significantly augmented (55.0 ± 10.3%, $P < 0.01$, $n = 8$).

Effect of PMN on (−)-noradrenaline-induced contraction

Different numbers of PMN in the organ bath increased the sensitivity of bovine mesenteric arteries to (−)-noradrenaline, as indicated by an enhanced pD_2-value (Table 1). The pD_2 for (−)-noradrenaline was increased maximally (ΔpD_2 (PMN − control): 0.40 ± 0.05, $P < 0.001$), when the concentration-effect curve was obtained in the presence of 2.5×10^6 PMN ml^{-1} (Figure 2). The maximal contraction to (−)-noradrenaline in control arteries was 252 ± 18 mN. The maximal response was not altered after incubation with 2.5×10^6 PMN ml^{-1} (257 ± 18 mN). Removal of the endothelium also increased the sensitivity to (−)-noradrenaline (pD_2; 6.77 ± 0.07, $n = 32$ without endothelium vs 6.43 ± 0.03, $n = 60$ with endothelium, $P < 0.001$). However, addition of PMN to endothelum-denuded vascular rings in the organ bath caused no further enhancement of the pD_2 value (Figure 2). Only at a cell concentration of 2.5×10^5 PMN ml^{-1} was a small but significant enhancement observed (Table 1).

Effect of N-monomethyl-L-arginine on (−)-noradrenaline contraction

For comparison, the modulatory role of EDRF in the vasoconstriction induced by (−)-noradrenaline was investigated. Inhibition of EDRF synthesis by L-NMMA (100 μM) resulted in an increase in the pD_2-value for (−)-noradrenaline (ΔpD_2 (L-NMMA − control): 0.30 ± 0.08, $P < 0.01$, $n = 12$), but had no effect on the maximal contraction. No differences in the contractile response to (−)-noradrenaline were observed after L-NMMA in endothelium-denuded preparations (ΔpD_2 (L-NMMA − control): 0.02 ± 0.18, $n = 4$).

Table 1 Effect of polymorphonuclear leukocytes (PMN) on sensitivity to (−)-noradrenaline in bovine isolated mesenteric arteries

PMN (ml^{-1})	n	pD_2 (*control*)	pD_2 (*PMN*)	ΔpD_2	
With endothelium					
0	18	6.45 ± 0.06	6.50 ± 0.07	0.05 ± 0.03	
2.5×10^5	7	6.51 ± 0.12	6.65 ± 0.09	0.15 ± 0.05§	$P < 0.05$
8×10^5	11	6.35 ± 0.07	6.52 ± 0.07	0.18 ± 0.06§	$P < 0.01$
2.5×10^6	17	6.46 ± 0.06	6.86 ± 0.07	0.40 ± 0.05§	$P < 0.001$
5×10^6	7	6.37 ± 0.07	6.60 ± 0.07	0.23 ± 0.09§	$P < 0.01$
Without endothelium					
0	7	6.67 ± 0.14	6.58 ± 0.14	−0.08 ± 0.07	
2.5×10^5	7	6.72 ± 0.16	6.88 ± 0.18	0.15 ± 0.06§	$P < 0.05$
8×10^5	10	6.83 ± 0.13	6.95 ± 0.15	0.12 ± 0.06	
2.5×10^6	8	6.83 ± 0.11	6.86 ± 0.12	0.03 ± 0.10	

Mean values ± s.e.mean are given. Two (−)-noradrenaline concentration-effect curves were obtained in bovine mesenteric arterial rings with and without endothelium. The control pD_2-values were obtained from the first curve. The second concentration-effect curve was obtained after 30 min incubation of the tissues with different numbers of PMN (pD_2(PMN)). The difference between the pD_2-values of PMN-incubation and control curves is depicted as ΔpD_2.

§ Significance was calculated by a Student's *t* test for paired results between PMN-incubation and control.

Table 2 Influence of inhibitors of the arachidonic acid pathway and oxygen radical scavengers on polymorphonuclear leukocyte (PMN)-induced increase in sensitivity to (−)-noradrenaline

	$\Delta pD_2 \pm$ s.e.mean[a]		
Treatment[b]	*Without PMN*	*PMN* 8×10^5 ml^{-1}	*PMN* 2.5×10^6 ml^{-1}
None	0.05 ± 0.03	0.18 ± 0.06§	0.40 ± 0.05§
Inhibitors of arachidonic acid pathway			
NDGA	−0.04 ± 0.04	0.38 ± 0.08§	0.35 ± 0.15§
Indomethacin	0.28 ± 0.07†	0.64 ± 0.09§†	0.35 ± 0.11
Oxygen-radical scavengers			
SOD and catalase	−0.01 ± 0.05		0.04 ± 0.05†
SOD	−0.09 ± 0.07		−0.03 ± 0.13†
Catalase	−0.03 ± 0.07		0.25 ± 0.09§
Dimethylthiourea	−0.01 ± 0.08		0.31 ± 0.15

[a] ΔpD_2: difference between pD_2-values of (−)-noradrenaline concentration-effect curves after and before incubation. For explanation see also Table 1.
[b] $n \geqslant 6$. The concentrations used were 10 μM nordihydroguaiaretic acid (NDGA), 1 μM indomethacin, 100 u ml^{-1} superoxide dismutase (SOD), 1000 u ml^{-1} catalase and 10 mM dimethylthiourea.
§ $P < 0.05$ in unpaired t test vs incubation with no PMN.
† $P < 0.01$ in unpaired t test vs PMN-incubation without treatment.

Influence of inhibitors of arachidonic acid metabolism and oxygen radical scavengers

To examine the mechanism of action by PMN on the vasoconstriction to (−)-noradrenaline, the influence of inhibitors of the arachidonic acid pathway were investigated. The data are summarized in Table 2. The lipoxygenase inhibitor NDGA (10 μM) did not diminish the increase in pD_2 by PMN. In the absence of PMN the cyclo-oxygenase inhibitor indomethacin (1 μM) itself exerted an increase in pD_2 for (−)-noradrenaline. The pD_2-value was further enhanced by incubation of the arterial rings with indomethacin and 8×10^5 PMN ml^{-1}, but not with 2.5×10^6 PMN ml^{-1}.

In order to examine a possible role of oxygen-derived radicals, free radical scavengers were added to the organ bath (Table 2). The increase in pD_2 for (−)-noradrenaline in bovine mesenteric arteries was entirely abolished by SOD (100 u ml^{-1}) and catalase (1000 u ml^{-1}) or SOD (100 u ml^{-1}) alone. However, the increase in the pD_2-value for (−)-noradrenaline was not inhibited by catalase (1000 u ml^{-1}) alone and only partially inhibited by dimethylthiourea (10 mM). The used inhibitors and scavengers had no marked effect on the basal tone of the arterial rings or on the maximal contractions elicted by (−)-noradrenaline (data not shown).

Effect of PMN on 6-keto-prostaglandin $F_{1\alpha}$ and thromboxane B_2 production

The ratio of prostacyclin and thromboxane A_2 (TxA_2) formation, two products of the cyclo-oxygenase pathway, is important for the regulation of vascular tone *in vivo*. Prostacyclin produced by vascular cells causes relaxation, while TxA_2 causes vasoconstriction. TxB_2, a stable metabolite of TxA_2, was not detected in the supernatants of any studied group (detection limit: 50 pg ml^{-1}). In contrast 6-keto-$PGF_{1\alpha}$ was already produced by non-treated arterial rings (Table 3). This basal level was not increased significantly by incubations with 2.5×10^6 PMN ml^{-1} or (−)-noradrenaline (3×10^{-7} M). However, simultaneous incubation of PMN and (−)-noradrenaline with arterial rings resulted in a twofold increase of the level of 6-keto-$PGF_{1\alpha}$ in the supernatant. This elevated production was inhibited by SOD (100 u ml^{-1}) and catalase (1000 u ml^{-1}). SOD and catalase had no effect on the 6-keto-$PGF_{1\alpha}$ levels produced by (−)-noradrenaline-treated vascular rings.

Table 3 6-keto-Prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) (pg mg^{-1} tissue wet weight)

	No cells	*PMN*
Control	85 ± 15	101 ± 16
(−)-Noradrenaline (3×10^{-7} M)	111 ± 16	244 ± 57§
with SOD and catalase (100 u ml^{-1} and 1000 u ml^{-1})	111 ± 27	102 ± 23

Data represent mean ± s.e.mean ($n = 7$). Arterial rings were incubated with 2.5×10^6 polymorphonuclear leukocytes (PMN) ml^{-1} and (−)-noradrenaline. 6-keto-$PGF_{1\alpha}$ levels were determined in the supernatant by means of radioimmunoassay.
§ $P < 0.05$ vs control, no cells or superoxide dismutase (SOD) and catalase treatment (unpaired t test).

Chemiluminescence

Superoxide anions were produced by stimulated and non-stimulated PMN, as determined by lucigenin enhanced chemiluminescence which is selective for superoxide radicals. Peak time, maximal level and total chemiluminescence are listed in Table 4. Stimulation of 2.5×10^6 PMN ml^{-1} with PMA (100 ng ml^{-1}) resulted in a sharp rise of the chemiluminescence to a maximum of 565 ± 71 mV after 4.2 ± 0.2 min. Maximal chemiluminescence by non-stimulated PMN was reached at 15.6 ± 1.7 min after which it gradually declined. Peak chemiluminescence was 9.6 ± 3.1% of the maximum by PMA-stimulated PMN. The chemiluminescence pattern, after addition of intact or endothelium-denuded arterial rings to the cuvette with PMN before measurement was started, showed no differences from that of non-stimulated PMN. No chemiluminescence could be detected in arterial rings in the absence of PMN.

Table 4 Oxygen-radical production by polymorphonuclear leukocytes (PMN): chemiluminescence measurements

	Peak (%)	*Total* (%)	t_{max} (min)
PMA-stimulated	100	100	4.2 ± 0.2
Non-stimulated	9.2 ± 3.1	15.0 ± 3.9	13.3 ± 1.1
With arterial rings			
intact	10.7 ± 3.1	18.3 ± 4.7	15.4 ± 1.7
denuded	8.2 ± 2.3	13.5 ± 2.9	13.6 ± 1.2

The cell number used was 2.5×10^6 PMN ml^{-1}. Lucigenin-enhanced chemiluminescence was measured every min during 1 h. The response of PMN stimulated with phorbol myristate acetate (100 ng ml^{-1}) was used as reference (100%). Peak chemiluminescence, time to reach maximal chemiluminescence and total chemiluminescence integrated from 0 to 60 min are given as mean ± s.e.mean ($n = 7$).

Discussion

The present study demonstrates that addition of bovine PMN to organ baths containing segments of bovine mesenteric arteries provoked a concentration-dependent contraction and an increase in the sensitivity to (−)-noradrenaline induced contractions. The PMN-dependent increase in sensitivity to (−)-noradrenaline was apparently not mediated by eicosanoids or leukotrienes derived from PMN and vascular cells, because neither inhibitors of cyclo-oxygenase nor lipoxygenase attenuated the effect of PMN. On the other hand, oxygen radicals may play a role since the effect on (−)-noradrenaline responsiveness was inhibited by SOD.

The isolation of PMN by percoll procedure resulted in platelet contamination. It is well recognized that platelets can induce contractions by the release of vasoconstrictive mediators like TxA_2 and 5-hydroxytryptamine (Houston *et al.*, 1986). Consequently, the direct contracting effect of PMN might be due to platelet contamination. However, the number of platelets required to elicit contractions of a similar magnitude were reported to be 10 to 20 fold higher than those found in the PMN cell suspension (Sessa & Mullane, 1990). Moreover, removal of the endothelium did not modify the direct contractile effect which is in contrast to the platelet-mediated response (Houston *et al.*, 1989). The contractile effect can also not be attributed to the release of TxA_2 from platelets since the effect was not attenuated by inhibitors of cyclo-oxygenase and TxB_2 was not detected after incubation of PMN with arterial rings. Taken together, these observations do not support a role for platelets as a source of mediators for contractions of bovine mesenteric arteries.

A short review of the literature reveals several reports suggesting that PMN may influence vascular function. In isolated hearts of the rabbit perfused with artificial buffer containing neutrophils, activation of the cells by PMA produced an increase in coronary perfusion pressure, indicative of a vasoconstriction (Gillespie *et al.*, 1986). Sessa & Mullane (1990) found that supernatant from rabbit neutrophils stimulated with calcium ionophore release a factor which directly augments vascular tone. Rimele *et al.* (1988) showed that rat PMN can release a relaxing factor similar to that reported for the endothelium. Also in isolated lungs, neutrophils increased pulmonary arterial pressure and vascular permeability (Carpenter *et al.*, 1987). Our present finding that bovine neutrophils can directly contract isolated bovine mesenteric arteries again establishes the direct effects of neutrophils in influencing vascular smooth muscle tone.

In addition, we found that PMN increased the potency of (−)-noradrenaline contractions. It is important to define the mechanisms by which neutrophil suspensions can induce this effect. The effect of neutrophils on the sensitivity to (−)-noradrenaline is probably not due to an effect of neutrophils directly on smooth muscle. Addition of PMN to endothelium-denuded vascular rings did not influence the (−)-noradrenaline-induced contractions.

There is the possibility that PMN produce potent cytotoxic substances, such as active oxygen species and proteolytic enzymes, which may injure endothelial cells (Ward & Varani, 1990). Because endothelial cells release vasodilator substances, endothelial damage by PMN may result in an increase in sensitivity to (−)-noradrenaline. The contribution of the endothelium to the response to (−)-noradrenaline is demonstrated by the fact that endothelium removal increased the sensitivity to (−)-noradrenaline. This indicates that (−)-noradrenaline can induce the release of a vasodilator factor from the endothelium.

The chemiluminescence studies of the present investigation show that superoxide anion radicals were produced by non-stimulated PMN. Since the chemiluminescence pattern was not altered in the presence of arterial rings, stimulation of PMN by allogenic tissue in the organ bath is not likely to occur. From the literature it is known that oxygen radicals may influence vasodilator substances from the endothelium, such as prostacyclin and EDRF (Harlan & Callahan, 1984; Rubanyi & Vanhoutte, 1986).

Measurement of 6-keto-$PGF_{1\alpha}$, the stable metabolite of prostacyclin, revealed that even under basal conditions arterial rings released prostacyclin. However, the production of prostacyclin was not influenced by (−)-noradrenaline at a concentration which causes half-maximal contraction in the organ bath experiments. The addition of PMN did not influence the basal level of 6-keto-$PGF_{1\alpha}$ in the supernatants. In contrast the release of prostacyclin was found to be increased by simultaneous incubation with PMN and (−)-noradrenaline. Therefore, the increase in (−)-noradrenaline sensitivity after addition of PMN is probably not due to an inhibition of prostacyclin production.

Endothelial cells can release a labile, local factor, EDRF that has chemical properties identical to those of nitric oxide (Palmer *et al.*, 1987). Cocks & Angus (1983) have shown that noradrenaline can induce endothelium-dependent relaxations. We confirmed in our experiments that inhibition of EDRF synthesis by L-NMMA resulted in a potentiation of the sensitivity to (−)-noradrenaline. EDRF has a short half-life and its activity is inhibited by agents which generate superoxide anion. Conversely, superoxide dismutase scavenges superoxide anion and increases the half-life of EDRF (Gryglewski *et al.*, 1986; Rubanyi & Vanhoutte, 1986). In the present study we found that the effect of PMN could be inhibited by superoxide dismutase, which suggests that oxygen radicals are involved in increasing the potency of (−)-noradrenaline. Since (−)-noradrenaline can release EDRF from the endothelium, it is suggested that oxygen radicals generated by the PMN inactivate EDRF, thereby increasing the sensitivity of (−)-noradrenaline contractions. Neither catalase nor dimethylthiourea diminished the augmented response after addition of the PMN, indicating that neither hydrogen peroxide nor hydroxyl radicals contribute to EDRF inactivation. Since it has been reported that hydrogen peroxide and hydroxyl radicals can induce or facilitate relaxations of vascular smooth muscle, it can be predicted that such an effect would have decreased the sensitivity of vascular rings to (−)-noradrenaline-induced contractions (Rubanyi & Vanhoutte, 1986). The fact that non-stimulated PMN were able to influence the contractions to (−)-noradrenaline supports the notion that a small amount of oxygen radicals is sufficient to inactivate EDRF.

In summary, the present study indicates that PMN can modulate vascular smooth muscle tone directly and indirectly, probably via EDRF inactivation.

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Mechanism of action of MY-1250, an active metabolite of Repirinast, in inhibiting histamine release from rat mast cells

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1 When MY-1250 (3.6×10^{-5} M) was added to mast cells, it caused a rapid increase in adenosine 3':5'-cyclic monophosphate (cyclic AMP) and decrease in adenosine 5'-triphosphate (ATP), both of which recovered to their original levels within 2 min. The accumulation of cyclic AMP was maximal at 20 s after challenge with MY-1250. The minimum level of ATP was observed at 20 s after addition of MY-1250.

2 The initial rise in $[Ca^{2+}]_i$ and the histamine release induced by DNP-AS antigen ($40\,\mu g\,ml^{-1}$) was most strongly inhibited at 20 s after incubation of the mast cells with MY-1250.

3 MY-1250 strongly and dose-dependently inhibited the histamine release from rat mast cells induced by antigen. Moreover, MY-1250 strongly inhibited calcium ion mobilization from the intracellular Ca^{2+}-store.

4 These results suggested that the inhibitory mechanism of MY-1250 on the initial rise in $[Ca^{2+}]_i$ and histamine release induced by antigen was due to the inhibition of ATP-dependent Ca^{2+}-release from the intracellular Ca^{2+}-stores.

Keywords: MY-1250; histamine release; intracellular Ca^{2+}-mobilization; cyclic AMP; ATP; mast cells

Introduction

MY-5116 (Repirinast), isoamyl 5,6-dihydro-7,8-dimethyl 4,5-dioxo-4H-pyrano [3,2-C] quinoline-2-carboxylate, is a new orally active drug for the treatment of allergic disease (Takahashi, 1986). The mechanism of this effect is based on the inhibition of the release of chemical mediators from mast cells by its main active metabolite MY-1250 (5,6-dihydro-7,8-dimethyl-4,5-dioxo-4H-pyrano [3,2-C] quinoline-2-carboxylic acid) (Takahashi, 1986). Kono & Ohata (1986) also reported that MY-1250 dose-dependently inhibited the release of both histamine and slow-reacting substance of anaphylaxis from passively sensitized human lung fragments. Moreover, we reported previously that MY-1250 dose-dependently inhibits Ca^{2+}-mobilization from intracellular Ca^{2+}-stores as well as histamine release from rat mast cells activated by antigen, the effects on both of these phenomena being closely correlated (Takei *et al.*, 1990). However, the inhibitory effect of MY-1250 on histamine release was only observed when the mast cells were incubated with the drug for a short period of time. The drug lost its inhibitory effect with a prolonged period of incubation (Yamada *et al.*, 1986). The mechanism of this effect is still unknown.

It is known that the addition of agents such as theophylline or dibutyryl cyclic AMP, which elevate intracellular cyclic AMP concentrations, prior to receptor bridging suppresses Ca^{2+}-influx and histamine release (Sullivan *et al.*, 1975). Recently, Lo *et al.* (1987) reported in experiments with rat basophilic cells (2H3 cells) that hydrolysis of inositol phospholipids, elevation of the Ca^{2+} signal and secretion of histamine stimulated by antigen and A23187 depended on the intracellular ATP concentration, and that depletion of ATP suppressed these events. This study was undertaken to evaluate the mechanism of action of MY-1250 on histamine release from rat isolated peritoneal mast cells and, in particular, to examine the role of cyclic AMP, intracellular ATP and Ca^{2+}-mobilization in the secretory process.

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Methods

Preparation of mast cells

Mast cells were obtained from peritoneal washings of male Wistar rats (200–300 g) (Saeki, 1964) and were purified by the method of Németh & Röhlich (1980). The purity of the mast cell preparation was about 90%. The viability of the cells was >97% as assessed by trypan blue exclusion.

Preparation of antigen and anti-dinitrophenylated Ascaris rat antiserum

Dinitrophenylated Ascaris antigen (DNP-AS) was prepared according to the method of Eisen *et al.* (1953). *Ascaris* protein was coupled with dinitrophenyl groups and used as antigen; 1.0 mg of DNP-AS mixed with 10^{10} killed *B. pertussis* organisms in 1 ml of physiological saline was injected into the four footpads of male Wistar rats. Five days later the rats were boosted with 1 ml of DNP-AS ($0.5\,mg\,ml^{-1}$) in physiological saline in the back muscle. Three days after the second injection, blood specimens were collected from the abdominal vein and anti-DNP-AS rat antiserum containing IgE antibody was prepared according to the method of Toda & Okumura (1971). The antisera were stored at −80°C. The passive cutaneous anaphylaxis (PCA) titer for the obtained antisera was 1:256.

Assay of histamine

Purified mast cells from normal rats were incubated for passive sensitization with anti-DNP-AS rat antisera for 1 h at 37°C (Yamada *et al.*, 1986). The mast cell suspensions in Tyrode-HEPES solution (pH 7.4) were incubated in duplicate at 37°C with or without MY-1250 for various times before the addition of antigen. Tyrode-HEPES solution contained (mM): NaCl 124, KCl 4.0, $CaCl_2$ 1, $NaHCO_3$ 10, glucose 5.6, NaH_2PO_4 0.64, $MgSO_4$ 0.5, 4-(2-hydroxyethyl)-1-piperazine

ethane-sulphonic acid (HEPES) 5, phosphatidylserine 50 mg ml^{-1} and 50 mg bovine serum albumin (BSA) liter^{-1}. BSA was omitted from the solution in experiments on histamine release. At intervals, the reaction was stopped by the addition of 1 ml of Tris-EDTA buffer. Tris-EDTA buffer contained (mM): Tris 25, NaCl 120, KCl 5, EDTA 1 and 0.2 g of human serum albumin per liter. Then, the cells were separated from the released histamine in the supernatant by centrifugation at 1300 *g* for 10 min at 4°C. Residual histamine remaining in the cells was released by disrupting the cells with 100% TCA (final concentration 10% TCA), and centrifugation at 1500 *g* for 15 min at 4°C. Histamine was determined fluorometrically (Shore *et al.*, 1959). The amount of histamine released was calculated as a percentage of the total histamine present in a control mast cell suspension.

Assay of cyclic AMP

Purified mast cells (2×10^6 cells/ml) were suspended in 55 μl of Tyrode-HEPES solution. They were then incubated at 37°C with MY-1250. The reaction was stopped by the addition of 1 ml of acid ethanol (1 ml of 1 M HCl/100 ml of ethanol) and frozen in dry ice/acetone. The reaction mixture was evaporated to dryness under nitrogen. Cyclic AMP was determined by the double antibody radioimmunoassay using the [^{125}I]-cyclic AMP assay kit (Amersham).

Determination of ATP

Thirty μl of purified mast cells suspension (2×10^5 cells ml^{-1}) in Tyrode-HEPES solution was incubated at 37°C with 20 μl of MY-1250. The reaction was stopped by addition of 1 ml of water and boiled for 5 min. ATP content was determined by the luciferin/luciferase reaction as described by Stanley & Williams (1969).

Measurement of intracellular calcium concentration

Quin-2 fluorescence was recorded by use of a model 650-40 Fluorescence 100 (Hitachi, Japan) with a temperature-controlled cuvette and a magnetically driven stirrer. Purified mast cells (1×10^6 cells ml^{-1}) were incubated at 37°C for 10 min with 100 μM Quin-2/AM (2-[(2-amino-5-methylphenoxy)methyl]-6-methoxy-8-aminoquinoline-N,N,N′,N′-tetraacetic acid, tetraacetoxymethyl ester) in Tyrode-HEPES solution. The cell suspension was diluted 1:10 with Tyrode-HEPES solution, kept for 60 min, and then washed twice with the complete solution. A cell suspension (1×10^5 cells ml^{-1}) was placed in the cuvette described above, and agents were added by a microsyringe directly into the cuvette, without interrupting the recording. The cell suspension was incubated at 37°C for various times with MY-1250, and challenged with an appropriate amount of antigen. Fluorescence excitation and emission wavelengths were 339 nm and 492 nm, respectively. The concentration of intracellular Ca^{2+} was calculated by the method described by Tsien *et al.* (1982).

MY-1250 was synthesized by Mitsubishi Kasei Corporation, Japan. L-α-Phosphatidylserine was purchased from Sigma Chemical Company, St. Louis, MO., U.S.A. and Quin-2/AM was obtained from Wako Pure Chemical Industries, Osaka, Japan. *Bordetella pertussis* vaccine was purchased from the Chiba Serum Institute, Chiba, Japan.

Results

MY-1250 (3.6×10^{-5} M) was added to passively sensitized mast cells, and the fluctuations of cyclic AMP and ATP were determined at intervals. As shown in Figure 1, the addition of MY-1250 caused a rapid increase in cyclic AMP levels in mast cells; the maximal value was 8.1 ± 0.12 pmol/10^6 cells after 20 s, followed by a rapid fall within 1 min.

On the other hand, the intracellular level of ATP rapidly decreased. The minimum level of ATP was observed at 20 s after addition of MY-1250 (Figure 1). The minimum value was 1.6 mM at 20 s after challenge by MY-1250. Both cyclic AMP and ATP recovered to their original levels after 2 min.

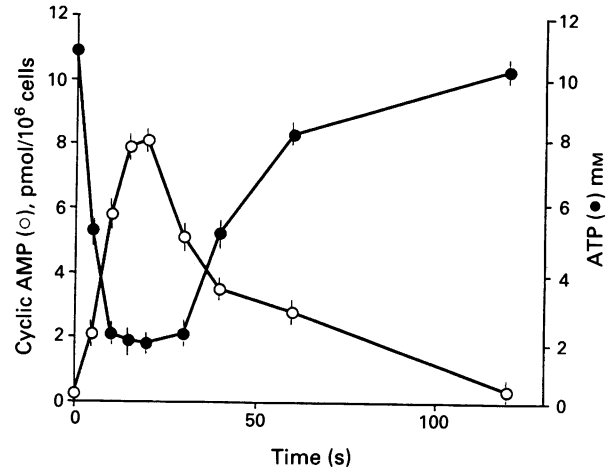

Figure 1 Effect of MY-1250 (3.6×10^{-5} M) on ATP (●) and cyclic AMP (○) levels in mast cells. All determinations are described in the text. The level of cyclic AMP in untreated mast cells was 1.3 ± 0.2 pmol/10^6 cells. These values were subtracted from experimental values. Each point represents the mean of 5 experiments and vertical bars indicate s.e.mean.

The DNP-AS antigen, at its optimal concentration of 40 μg ml^{-1}, induced a time-dependent release of histamine from rat peritoneal mast cells. The histamine release was essentially complete within 1–2 min, and 68 ± 3% of the total histamine was released after 10 min. MY-1250 (3.6×10^{-5} M) was incubated with the cells for various times before the addition of antigen. When MY-1250 was preincubated with the mast cells for 15 s and 60 s, the degree of inhibition of histamine release at 10 min after challenge with antigen was 73 ± 3% and 24 ± 2%, respectively (Figure 2). The histamine release induced by antigen was most strongly inhibited at 20 s after incubation of the mast cells with MY-1250, and 87 ± 3% inhibition was achieved.

MY-1250 strongly and dose-dependently inhibited histamine release from rat mast cells induced by antigen (Figure 3). When MY-1250 at concentrations of 3.6×10^{-9}, 3.6×10^{-8} and 3.6×10^{-7} M was preincubated with the mast cells for 20 s before the addition of antigen, the degree of inhibition was 15 ± 2, 23 ± 2 and 52 ± 2%, respectively. At 3.6×10^{-4} M, 85 ± 2% of inhibition was achieved. The IC_{50} value of MY-1250 on histamine release induced by antigen was 0.3 μM.

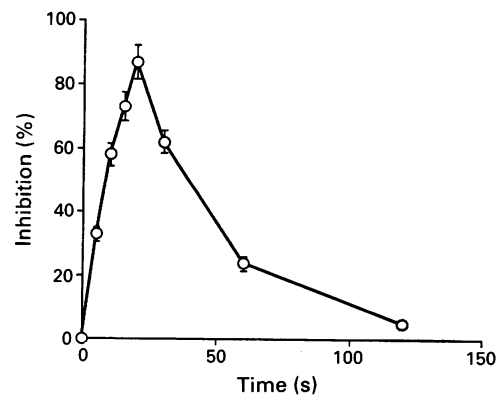

Figure 2 Effect of MY-1250 (3.6×10^{-5} M) on histamine release from mast cells induced by antigen (40 μg ml^{-1}). Passively sensitized mast cells were incubated with MY-1250 for various times before the addition of antigen. Each point represents the mean of 5 experiments and vertical bars indicate s.e.mean. The abscissa scale represents the time of incubation with MY-1250 before the addition of antigen.

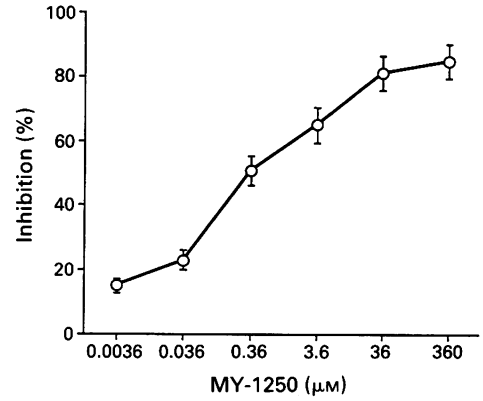

Figure 3 Inhibitory effect of MY-1250 on histamine release from a passively sensitized rat mast cells induced by antigen ($40\,\mu g\,ml^{-1}$). Spontaneous histamine release from mast cells was $5.3 \pm 0.2\%$ and this value was subtracted from experimental values. After preincubation with MY-1250 for 20 s, the mast cell suspension was incubated with antigen for 10 min at 37°C. Each point represents the mean of 5 experiments and vertical bars indicate s.e.mean.

Purified passively sensitized rat mast cells were challenged with $40\,\mu g\,ml^{-1}$ of antigen. This treatment induced a rapid initial increase in $[Ca^{2+}]_i$ within several seconds which was followed by a further slower increase of $[Ca^{2+}]_i$ (second rise)

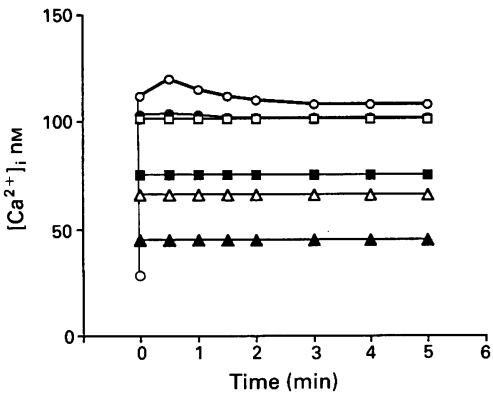

Figure 4 Effect of MY-1250 (3.6×10^{-5} M) on the initial rise in $[Ca^{2+}]_i$ induced by antigen ($40\,\mu g\,ml^{-1}$). Passively sensitized mast cells were incubated with MY-1250 (3.6×10^{-5} M) for various times before the addition of antigen. Each point represents the mean of 5 experiments: (○) antigen ($40\,\mu g\,ml^{-1}$); (●) incubated with MY-1250 for 5 s; (□) incubated with MY-1250 for 60 s; (■) incubated with MY-1250 for 30 s; (△) incubated with MY-1250 for 15 s; (▲) incubated with MY-1250 for 20 s.

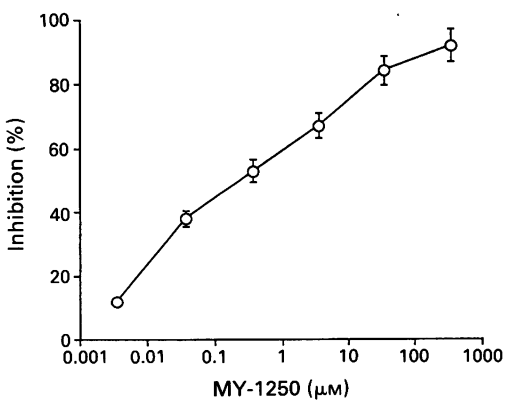

Figure 5 Inhibitory effect of MY-1250 on the increase in $[Ca^{2+}]_i$ induced by antigen. After preincubation with MY-1250 for 20 s, mast cells (1×10^5 cells ml^{-1}) containing various concentrations of MY-1250 were challenged with $40\,\mu g\,ml^{-1}$ of antigen and incubated at 37°C. Each point represents the mean of 5 experiments and vertical bars indicate s.e.mean.

reaching a maximum at 1 min after challenge with antigen (Figure 4). Afterwards $[Ca^{2+}]_i$ began gradually to decrease. Histamine release followed the initial rise in $[Ca^{2+}]_i$ and, in parallel, reached a maximum with 1 min. The initial and maximal increase were 85 and 93 nM of $[Ca^{2+}]_i$, respectively.

The effects of the various incubation times of mast cells with MY-1250 (3.6×10^{-5} M) on the initial rise in $[Ca^{2+}]_i$ in antigen-activated mast cells were examined (Figure 4). When MY-1250 was preincubated with the mast cells for 15 s and 60 s, the initial rise in $[Ca^{2+}]_i$ reached a maximum of only 39 ± 3 and 74 ± 2 nM, respectively (Figure 4); the inhibitory effect was 55 ± 3 and $13 \pm 2\%$ for the initial rise, respectively. The initial rise in $[Ca^{2+}]_i$ was strongly inhibited at 20 s after incubation of the mast cells with MY-1250, and the initial rise in $[Ca^{2+}]_i$ reached a maximum of only 18 ± 1 nM. The inhibitory effect was $79 \pm 3\%$ for the initial rise.

The inhibition by MY-1250 of both antigen-induced histamine release and the initial rise in $[Ca^{2+}]_i$ was most marked when the drug was preincubated with the cells for 20 s. This coincided with the peak time for cyclic AMP production induced by MY-1250.

MY-1250 dose-dependently inhibited the initial rise in $[Ca^{2+}]_i$. In the presence of 3.6×10^{-8}, 3.6×10^{-7} and 3.6×10^{-6} M MY-1250, the initial rise in $[Ca^{2+}]_i$ reached a maximum of only 52 ± 2, 37 ± 2 and 25 ± 1 nM, respectively (Figure 5). The inhibitory effect was 39 ± 2, 57 ± 3 and $65 \pm 4\%$ for the initial rise, respectively. At 3.6×10^{-4} M, the initial rise in $[Ca^{2+}]_i$ was inhibited by $92 \pm 4\%$ and reached a maximum of only 7 ± 0.5 nM. The IC_{50} value for this effect was $0.25\,\mu M$.

Discussion

These experiments were undertaken to clarify the effect of MY-1250 on antigen-induced histamine release from rat peritoneal mast cells. MY-1250 strongly and dose-dependently inhibited this release. The effect was maximal after preincubation with the mast cells for 20 s but disappeared at 60 s after the addition of MY-1250.

We previously reported that MY-1250 inhibits Ca^{2+}-mobilization from the intracellular Ca^{2+}-store as well as histamine release from mast cells activated by antigen, the effects on both of these phenomena being closely correlated (Takei *et al.*, 1989). Moreover, Ca^{2+}-trapping experiments with EGTA showed that the intracellular Ca^{2+} mobilization induced by anti-IgE and Con A is closely correlated with histamine release, whereas the influx of extracellular Ca^{2+} is not. From these results, we suggested that the initial rise in $[Ca^{2+}]_i$, due to Ca^{2+}-mobilization, correlates with the histamine release promoted by the various secretagogues (Takei *et al.*, 1989).

In this study, MY-1250 strongly inhibited the initial rise in $[Ca^{2+}]_i$ which may be explained by inhibition of Ca^{2+}-mobilization from the intracellular Ca^{2+}-store, after incubation with the mast cells for 20 s. After preincubation with MY-1250 for 60 s the inhibitory effects disappeared.

Similarly, the accumulation of cyclic AMP induced by MY-1250 was maximal at 20 s, followed by a rapid fall within 1 min. The elevation of cyclic AMP levels induced by MY-1250 correlated with the inhibitory effect of MY-1250 on the intracellular Ca^{2+}-mobilization and the histamine release induced by antigen.

A dual role has been assigned to cyclic AMP in IgE-dependent histamine release from mast cells. One is the implication of the initial and indomethacin-resistant increase in cyclic AMP in IgE-Fc receptor-dependent induced secretion (Ishizaka, 1982), and the other is the inhibitory effect of cyclic AMP on the secretion reaction (Sullivan *et al.*, 1975; Ishizaka *et al.*, 1981). It is widely accepted that cyclic AMP serves as second messenger that mediates the inhibition of activated cells. Theophylline, dibutyryl cyclic AMP and isoprenaline inhibit histamine release from mast cells (Hayashi *et al.*, 1976).

These facts suggest that cyclic AMP acts as a regulator of histamine release.

It has been reported that Amlexanox, an anti-allergic agent, inhibits histamine release by augmenting the cyclic AMP content in mast cells both by enhancing cyclic AMP generation and by inhibiting cyclic AMP phosphodiesterase (Makino *et al.*, 1987). In this study, MY-1250 induced a tentative and rapid increase in cyclic AMP and a concomitant decrease in ATP in mast cells, and strongly inhibited histamine release induced by antigen.

MY-1250 also inhibited phosphodiesterase, but the IC_{50} value was 2 mM (data not shown). The IC_{50} value of MY-1250 for histamine release was in the order of 0.3 μM. The inhibitory effects of MY-1250 on histamine release from mast cells activated by antigen and on phosphodiesterase are thus not correlated. The accumulation of cyclic AMP induced by MY-1250 may hence be caused by stimulation of adenylate cyclase.

Recently, Lo *et al.* reported that the increases in $[Ca^{2+}]_i$ in response to A23187 as well as antigen are dependent on cellular ATP in 2H3 cells (Lo *et al.*, 1987). They found that secretion from mast cells and 2H3 cells utilized intracellular ATP and that high intracellular levels of ATP are required for the generation of the calcium response in 2H3 cells, irrespective of the need for ATP in the formation of polyphosphoinositides.

From these results, we suggest that events in mast cells stimulated by antigen, such as Ca^{2+}-mobilization from intracellular Ca^{2+}-stores and histamine release, are modulated by ATP. In particular, the regulation of Ca^{2+}-release from the intracellular Ca^{2+}-store by ATP seems to be most important for histamine release from mast cells induced by antigen. Some workers have suggested that the inhibitory effect of cyclic AMP on secretagogue-dependent histamine release was due to the chelation of intracellular calcium by increased cyclic AMP (Austen & Humphrey, 1963). Alternatively, the cyclic nucleotide might induce protein phosphorylation and thereby block Ca^{2+} influx or promote the intracellular sequestration or compartmentalization of the cation (Theocharides *et al.*, 1980).

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CI988, a selective antagonist of cholecystokinin$_B$ receptors, prevents morphine tolerance in the rat

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1 The effect of chronic treatment with CI988, a recently developed selective antagonist of cholecystokinin type-B receptors (CCK_B receptors) on the tolerance to morphine analgesia was studied in rats with the hot plate test.

2 Morphine tolerance was induced with the use of two paradigms. Morphine was injected i.p. either in a schedule of increasing doses (1–32 mg kg^{-1}) twice daily for 6 days or at a fixed dose (3 mg kg^{-1}) daily for 29 days.

3 In both series of experiments, tolerance to the analgesic effect of morphine was prevented by simultaneous treatment with i.p. CI988. Chronic treatment with only CI988 daily for up to 29 days did not reduce the analgesic effect of a weekly injection of morphine.

4 CI988 did not diminish the physical dependence to morphine, as examined with naloxone precipitated withdrawal.

5 The present results provide evidence that chronic treatment with a selective CCK_B receptor antagonist could prevent tolerance to the analgesic effect of morphine without affecting morphine-induced physical dependence. Application of CCK antagonists may be clinically important in treating chronic pain patients by preventing morphine tolerance and by eliminating the need to increase morphine doses to unacceptable levels.

Keywords: Morphine; tolerance; dependence; cholecystokinin$_B$ receptor; hot plate test

Introduction

The peptide cholecystokinin (CCK) occurs in many areas of the CNS, primarily as the sulphated octapeptide CCK-8 (Vanderhaeghen *et al.*, 1975; Dockray, 1976; Rehfeld, 1978; Williams *et al.*, 1987). It fulfils many of the criteria for a neurotransmitter and may have a role in various CNS functions, including pain modulation (see Vanderhaeghen & Crawley, 1985; Baber *et al.*, 1989 for review). It has been suggested that CCK is an endogenous opioid antagonist. Thus, exogenously applied CCK antagonizes the analgesic effect of morphine and endogenous opioids (Itoh *et al.*, 1982; Faris *et al.*, 1983; Han *et al.*, 1985; Watkins *et al.*, 1985; Wiesenfeld-Hallin & Duranti, 1987), CCK antagonists potentiate morphine analgesia (Watkins *et al.*, 1984; 1985; Katsuura & Itoh, 1985; Dourish *et al.*, 1988; 1990) and reverse or prevent morphine tolerance (Watkins *et al.*, 1984; Tang *et al.*, 1984; Panerai *et al.*, 1987; Dourish *et al.*, 1988; 1990).

Two subtypes of CCK receptors exist, which have been denoted as CCK_A and CCK_B (Innis & Snyder, 1980; Saito *et al.*, 1980; Moran *et al.*, 1986). CCK_A receptors are located principally in the peripheral nervous system of rodents, whereas the majority of CCK receptors in brain and spinal cord of rodents belong to the B type (Moran *et al.*, 1986; Hill & Woodruff, 1990). Recently, selective antagonists for CCK_A (MK-329, L-365,031) and CCK_B (L-365,260, CI988) receptors have been developed (Evans *et al.*, 1986; Hughes *et al.*, 1990), making it possible to examine the subtypes of CCK receptor involved in CCK-opioid interaction. Thus, Dourish *et al.* (1988, 1990) have shown that MK-329, L-365,031 and L-365, 260 potentiate morphine analgesia and prevent morphine tolerance in rats with the rank order of potency L-365, 260 > MK-329 > L-365,031, which corresponds to their potency in blocking the central CCK_B receptors, indicating a critical role for these receptors in modulation of the opioid system. We have also recently demonstrated that CI988 (formerly PD 134308), another highly selective and potent CCK_B receptor antagonist (Hughes *et al.*, 1990), strongly enhances the analgesic effect of morphine, as examined in both electrophysiological and behavioural studies (Wiesenfeld-Hallin *et al.*, 1990).

The object of the present study was to examine the effect of chronic treatment with CI988 on the development of tolerance to the analgesic effect of morphine. We used two experimental paradigms to induce morphine tolerance, during 6 and 29 days respectively, in order to evaluate the short and long term effect of CCK_B receptor blockade on morphine analgesia. The latter is particularly interesting since Kellstein & Mayer (1990) have recently reported that chronic administration of the weak and non-selective CCK receptor antagonists, proglumide and lorglumide, reverses the enhancement of morphine analgesia induced by acute pretreatment with the antagonists. Furthermore, the possible effect of chronic treatment with CI988 on the physical dependence induced by chronic morphine was also examined by means of naloxone-precipitated withdrawal. Since it has been shown previously that repeated nociceptive testing could induce behavioural tolerance to morphine analgesia (Advokat, 1981; Sherman *et al.*, 1982; Advokat & Isaac, 1983; Milne *et al.*, 1989), we also tested whether behavioural tolerance developed in our experimental paradigm over long term, repeated nociceptive testing.

Methods

Animals

Eighty male Sprague-Dawley rats weighing 200 g at the beginning of the experiments were used (Alab AB, Stockholm, Sweden) and housed and maintained in groups of five in standard translucent plastic laboratory cages under a 12 h light/dark cycle (on at 06 h 00 min). Water and laboratory chow were available *ad libitum*.

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Analgesia testing

The analgesic effect of morphine was tested with a hot plate (IITC, Woodland Hills, CA, U.S.A.) at 54.0 ± 0.2°C. The latency to licking a hindpaw was determined to an accuracy of 0.1 s. Before any drugs were administered, the rats were habituated by being tested on the hot plate for 5 days to obtain a stable control response value (Figure 1). Our previous experience indicated this procedure is very important to obtain a stable baseline response since the lick latency of rats decreased as a factor of repeated testing in the hot plate test. On the day of testing for analgesia, the rats were pre-tested three times on the hot plate. The values from the last two pre-tests were averaged and taken as baseline latency (BL). The animals were tested on single trials at 10 min intervals for 60 min after 1 mg kg^{-1} morphine i.p., or at 20 or 30 min intervals for 150 min after 3 mg kg^{-1} morphine i.p., and the postdrug latency (DL) was recorded. The drug effect was calculated as the percentage change in reaction time from baseline latency values by use of the formula [(DL-BL)/BL] × 100%.

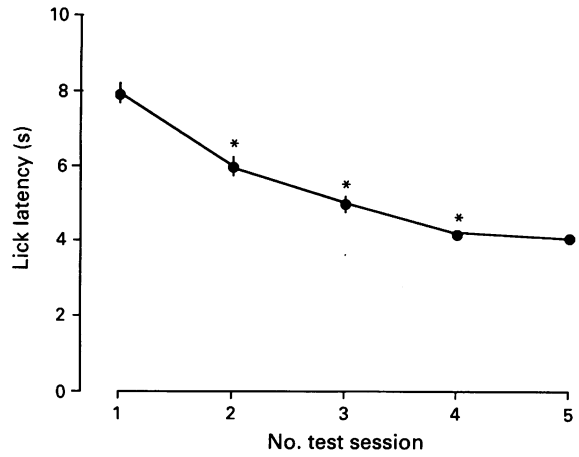

Figure 1 The effect of habituation on the lick latency of rats in the hot plate test in 5 test sessions performed daily at the same time each day. Each test session consists of two or three trials and the lick latencies in each session were averaged. ANOVA indicated a significant difference in the response latency across test sessions ($F_{4,395}$ = 134.437, $P < 0.0001$). The Newman-Keuls test indicated that up to the 4th test session, the lick latency is significantly shorter than in the previous session. * $P < 0.01$ compared to latency on the previous day.

Experimental design

Two series of experiments were conducted. In series I, thirty rats were randomly divided into 4 treatment groups as indicated in Table 1. Morphine tolerance was induced by a 6 day schedule of twice daily injection of incremental doses of morphine, beginning at 1 mg kg^{-1} and culminating with 32 mg kg^{-1} on the morning prior to the analgesia test (see Table 1 for details). Animals were injected daily with i.p. saline, CI988 (0.5 mg kg^{-1}), saline plus morphine or CI988 plus morphine. On the afternoon of day 6 the rats were challenged with 1 mg kg^{-1} morphine and the analgesic effect was evaluated.

In series II, opioid tolerance was induced with a daily i.p. injection of 3 mg kg^{-1} morphine. Fifty rats were divided into five groups, as indicated in Table 2. The analgesic effect of 3 mg kg^{-1} i.p. morphine was assessed on day 1 in four groups. The rats were then injected daily with saline plus saline, CI988 (1 mg kg^{-1}) plus saline, CI988 plus morphine, or saline plus morphine (see Table 2 for details). The analgesic effect of morphine was tested in these four groups of rats on days 8, 15, 22 and 29 to follow the development of tolerance. In group 5, ten rats received daily i.p. injection of saline and weekly analgesic testing without morphine. On day 29, these rats were injected i.p. with 3 mg kg^{-1} morphine and its analgesic effect was examined.

All testing was carried out in a 'blind' fashion.

Naloxone-precipitated withdrawal

The method of naloxone-precipitated withdrawal was used to assess the effect of CI988 on opioid dependence. On day 7 of series I and day 30 of series II, all rats were injected with 1 mg kg^{-1} i.p. naloxone and the appearance of various characteristic withdrawal symptoms were noted and recorded during the following 20 min.

Drugs

CI988 (Parke-Davis, Cambridge, U.K., see Hughes *et al.*, 1990 for structure), morphine hydrochloride (Kabi, Stockholm, Sweden) and naloxone (Dupont, Wilmington, Del., U.S.A.)

Table 1 Chronic treatment regime used in series I to study the effects of CI988 on morphine tolerance and dependence

	Treatment (twice daily)			
Day	*Group 1*	*Group 2*	*Group 3*	*Group 4*
1	Morphine 1 mg kg^{-1} + saline	Saline + saline	CI988 + morphine	CI988 + saline
2	Morphine 2 mg kg^{-1} + saline	Saline + saline	CI988 + morphine	CI988 + saline
3	Morphine 4 mg kg^{-1} + saline	Saline + saline	CI988 + morphine	CI988 + saline
4	Morphine 8 mg kg^{-1} + saline	Saline + saline	CI988 + morphine	CI988 + saline
5	Morphine 16 mg kg^{-1} + saline	Saline + saline	CI988 + morphine	CI988 + saline
6 (08 h 00 min)	Morphine 32 mg kg^{-1} + saline	Saline + saline	CI988 + morphine	CI988 + saline
6 (14 h 00 min)	All groups challenged with 1 mg kg^{-1} morphine i.p.			
7	All groups challenged with 1 mg kg^{-1} naloxone i.p.			

n = 5–10 animals in each group. The dose of CI988 used was 0.5 mg kg^{-1}.

Table 2 Chronic treatment regime used in series II

	Treatment (daily)				
Day	*Group 1*	*Group 2*	*Group 3*	*Group 4*	*Group 5*
1	Morphine + test + saline	Morphine + test + CI988	Morphine + test + CI988	Morphine + test + saline	Saline + test
2–7	Saline + morphine	CI988 + morphine	CI988 + saline	Saline + saline	Saline
8	Morphine + test + saline	Morphine + test + CI988	Morphine + test + CI988	Morphine + test + saline	Saline + test
9–14	Saline + morphine	CI988 + morphine	CI988 + saline	Saline + saline	Saline
15	Morphine + test + saline	Morphine + test + CI988	Morphine + test + CI988	Morphine + test + saline	Saline + test
16–21	Saline + morphine	CI988 + morphine	CI988 + saline	Saline + saline	Saline
22	Morphine + test + saline	Morphine + test + CI988	Morphine + test + CI988	Morphine + test + saline	Saline + test
23–28	Saline + morphine	CI988 + morphine	CI988 + saline	Saline + saline	Saline
29	Morphine + test + saline	Morphine + test + CI988	Morphine + test + CI988	Morphine + test + saline	Morphine + test
30	All groups of rats were challenged with 1 mg kg^{-1} naloxone				

$n = 10$ animals in each group. The dose of morphine was 3 mg kg^{-1} and CI988 was 1 mg kg^{-1}. The temporal order of treatments is shown in the table.

were dissolved in 0.9% saline and injected i.p. in a volume of 1 ml kg^{-1}.

Statistics

All data are expressed as mean ± s.e.mean. Statistics were calculated with analysis of variance (ANOVA) followed by Dunnett's test or Newman-Keuls test.

Results

Effect of CI988 on morphine tolerance in series I

Morphine 1 mg kg^{-1}, i.p., elicited moderate analgesia for 50 min in group 2, which received twice daily injections of saline (Figure 2). The effect was similar to that seen in normal rats (Wiesenfeld-Hallin *et al.*, 1990). In contrast, in group 1, which received incremental doses of morphine for 6 days, the challenge dose of 1 mg kg^{-1} morphine did not evoke an analgesic effect (Figure 2). Morphine caused analgesia in rats in group 3, which received chronic treatment with CI988 prior to injection with morphine (Figure 2). Chronic administration of CI988 by itself (group 4) did not reduce morphine-induced analgesia (Figure 2). ANOVA with repeated measures indicated that the analgesic effect of morphine was significantly different when all 4 groups were compared ($F_{3,130} = 9.669$, $P < 0.0001$), but there was no difference among groups 1, 3 and 4 ($F_{2,110} = 2.856$, $P > 0.05$), indicating a lack of morphine tolerance in rats of group 3, which received CI988 along with morphine.

Effect of CI988 on morphine tolerance in series II

Morphine at a dose of 3 mg kg^{-1}, i.p., had a strong analgesic effect for about 2 h on the first testing occasion. Daily injection of i.p. saline and weekly analgesic testing for 4 weeks (group 5) did not reduce the analgesic effect of morphine on day 29. After daily i.p. injection of 3 mg kg^{-1} morphine (group 1), the analgesic effect of morphine was significantly diminished on day 8 and totally disappeared by day 15 and thereafter (Figure 3). In the other three groups of rats in which morphine plus CI988, CI988 or saline were injected daily (groups 2, 3 and 4 respectively), the analgesic effect of morphine was unchanged on day 8. A significant reduction of morphine's analgesic effect was observed in all three groups of rats on day 15 (Figure 3). The effect of morphine in rats in these three groups were still, however, significantly stronger than that in group 1, which received daily injections of morphine, and no difference between these three groups of rats was found ($F_{2,27} = 0.399$, $P > 0.05$). There was no further reduction of the analgesic effect of morphine in these three groups of rats up to 29 days, when the experiments were terminated. Two factor ANOVA (treatment groups vs time) indicated that the difference in the analgesic effect of morphine

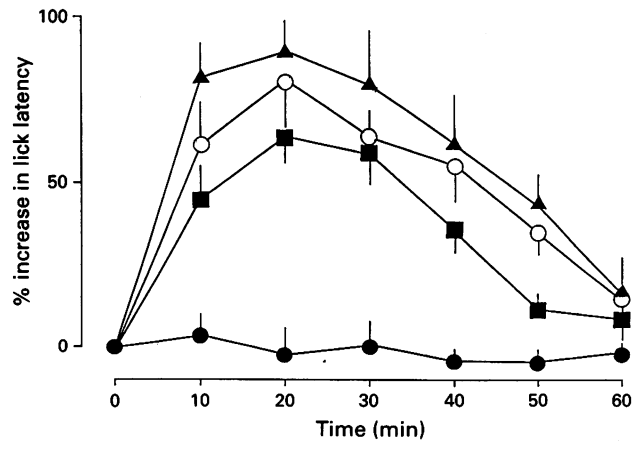

Figure 2 Effect of i.p. morphine (1 mg kg^{-1}) on the lick latency of rats on the hot plate on day 6 of series I in groups of rats which received twice daily i.p. injection of increasing doses of morphine (●), saline (○), morphine plus CI988 (0.5 mg kg^{-1}) (■) or CI988 (▲). The challenge dose of morphine was injected at time 0 and vertical bars represent s.e.mean $n = 5$–10 in each group. ANOVA with repeated measures indicated that the analgesic effect of morphine was significantly different when all 4 groups were compared ($F_{3,130} = 9.669$, $P < 0.0001$), but there was no difference in groups which received chronic saline, CI988 plus morphine or CI988 ($F_{2,110} = 2.865$, $P > 0.05$).

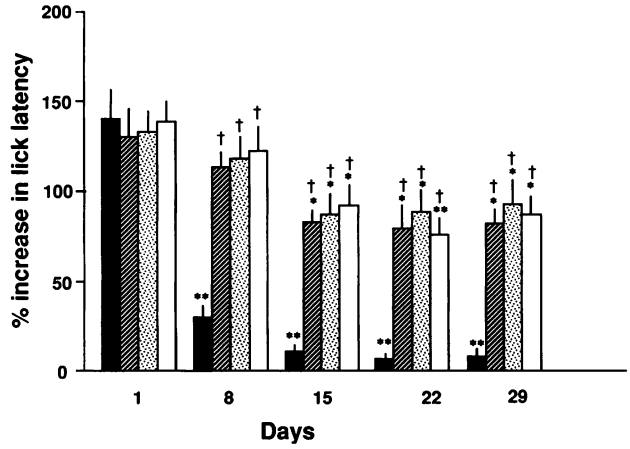

Figure 3 Effect of i.p. morphine (3 mg kg^{-1}) on the lick latency on the hot plate in groups of rats in series II which received daily injection of morphine (solid columns), morphine plus CI988 (1 mg kg^{-1}) (hatched columns), CI988 (stippled columns) or saline (open columns). The time elapsed from the beginning of the chronic treatment is indicated under the columns. The maximal increase of lick latency for each rat, which is usually achieved 40 or 60 min after injection of morphine, is presented. The vertical lines indicate s.e.mean. $n = 10$ in each group. † $P < 0.005$ compared to the group which received daily injection of morphine; * $P < 0.05$ and ** $P < 0.01$ compared to the respective group on day 1.

among the four groups over the entire experimental period was significant ($F_{3,180} = 38.176$, $P < 0.001$). The reduction of the analgesic effect of morphine over time was also significant ($F_{4,180} = 30.688$, $P < 0.001$). ANOVA applied to data from rats in groups 2, 3 and 4 revealed that there was no significant difference in the analgesic effect of morphine ($F_{2,135} = 0.645$, $P > 0.5$), but the decrease of the analgesic effect of morphine over time was significant ($F_{4,135} = 10.731$, $P < 0.001$), among these three groups (Figure 3).

There was no significant change in baseline reaction latency in all groups of rats over the entire period of observation (not shown).

Effect of chronic treatment with CI988 on morphine dependence

Severe characteristic withdrawal symptoms were induced in group 1 of series I after i.p. injection of 1 mg kg^{-1} naloxone (Table 3). Chronic treatment with CI988 had no effect on the appearance or intensity of these symptoms (Table 3). Very slight withdrawal symptoms were observed in some animals that only received daily injection of CI988, which on average were not significantly different from saline controls (Table 3).

The expression of withdrawal symptoms was much weaker in group 1 of series II, which may be related to the administration of a lower dose of morphine (3 mg kg^{-1}) during chronic treatment (Table 4). CI988 again failed to prevent the occurrence of these symptoms and in rats which received a daily injection of CI988 and weekly injection of morphine, slight withdrawal symptoms were also present, which again were not significantly different from the saline group (Table 4). There were no detectable withdrawal symptoms in rats which received a daily injection of saline and a weekly injection of morphine.

Discussion

The present study presents clear evidence which indicates that chronic treatment with CI988, a highly selective antagonist of

Table 3 Effects of CI988 on naloxone-precipitated morphine withdrawal in rats in series I

	Groups			
Withdrawal symptoms	*Morphine + saline*	*Saline + saline*	*Morphine + CI988*	*Saline + CI988*
Ptosis	100	0	90	20
Irritability	100	0	100	20
Diarrhoea	80	0	60	10
Teeth chattering	100	0	100	20
Writhing	60	0	40	0
Wet dog shakes	8.0 ± 1.4*	0	6.7 ± 1.1*	2.4 ± 0.6

Rats were injected twice daily with increasing doses of morphine (group 1), saline (group 2), morphine plus CI988 (group 3) and CI988 (group 4) for 6 days (see Table 1 for details). Naloxone-precipitated withdrawal was assessed on day 7. All rats were injected i.p. with naloxone 1 mg kg^{-1} prior to a 20 min observation period. The frequency of wet dog shakes (mean ± s.e.mean) during 20 min is indicated, other values are the percentages of animals in each group showing the symptom during the test ($n = 5$–10 in each group). ANOVA indicated that the frequency of wet dog shakes is significantly different among 4 groups ($F_{3,26} = 12.785$, $P < 0.001$). * $P < 0.01$ compared to group 2 and group 4 with Newman-Keuls test.

Table 4 Effects of CI988 on naloxone-precipitated morphine withdrawal in series II

	Groups			
Withdrawal symptoms	*Morphine + saline*	*Morphine + CI988*	*Saline + CI988*	*Saline + saline*
Ptosis	70	70	20	0
Irritability	60	60	20	0
Diarrhoea	0	0	0	0
Teeth chattering	80	80	0	0
Writhing	10	0	0	0
Wet dog shakes	3.9 ± 1.4*	3.3 ± 0.9*	1.7 ± 0.6	0.2 ± 0.2

Rats were injected daily with morphine (group 1), CI988 plus morphine (group 2), CI988 (group 3) and saline (group 4) for 29 days (see Table 2 for details). Naloxone precipitated withdrawal was assessed on day 30. All animals were injected i.p. 1 mg kg^{-1} naloxone prior to a 20 min observation. Wet dog shakes are frequency (mean ± s.e.mean) during the test, other values are the percentages of animals in each group showing the symptom during the test ($n = 10$ in each group). ANOVA indicated that the frequency of wet dog shakes is significantly different among the 4 groups ($F_{3,36} = 3.457$, $P < 0.05$). * $P < 0.05$ compared with group 4.

the CCK_B receptor, prevents the development of tolerance to the analgesic effect of morphine in rats. These data support the results from previous studies in which a number of selective and non-selective CCK antagonists were shown to prevent or reverse tolerance to morphine (Tang *et al.*, 1984; Watkins *et al.*, 1984; Panerai *et al.*, 1987; Dourish *et al.*, 1988; 1990). Since CI988 is a highly selective and potent CCK_B antagonist, our results also support the notion that the CCK-opioid interaction occurs through CCK_B receptors in rodents (Dourish *et al.*, 1990).

In series II of the present study, we observed that the analgesic effect of morphine is reduced over time in rats that received daily injection of CI988 plus morphine. Since similar phenomena also occur in rats which receive daily injection of saline, it is clear that this effect does not reflect the inefficacy of CI988 in preventing morphine tolerance over a long period of time, but some other factors. A number of previous studies have shown that repeated nociceptive testing could induce behavioural tolerance to morphine analgesia (Advokat, 1981; Sherman *et al.*, 1982; Advokat & Isaac, 1983; Milne *et al.*, 1989). We tested for the occurrence of behavioural tolerance by including another group of rats which received daily injection of saline and a weekly nociceptive test and were challenged with morphine only once, after 4 weeks. The observation that the analgesic effect of morphine is similar in this group to that in rats examined at the beginning of the experiments fails to support the hypothesis of behavioural tolerance to nociceptive testing in rats which were injected with morphine weekly, but not daily. Thus, it is possible that a weekly injection of morphine causes the development of some tolerance, albeit significantly less than that caused by daily injection. Tolerance development was not prevented by CI988, as on the day of nociceptive testing, CI988 was injected 2–3 h later than morphine to avoid the acute potentiation of morphine's analgesic effect by the CCK_B antagonist (Wiesenfeld-Hallin *et al.*, 1990). However, we cannot exclude the possibility that the single morphine injection (group 5) had a discriminative stimulus property, which led to the full analgesic effect of the opioid. Nonetheless, the analgesic effect of morphine was not totally diminished in rats which received a weekly injection of morphine and was significantly stronger than in rats which received a daily injection during the entire 29 day experimental period (Figure 3). It should be noted that our procedure of habituating the animals on the hot plate test for five days before data collection may have contributed to the increased sensitivity of this assay of morphine's analgesic effect, since unhabituated animals would respond with a longer latency at the first testing session than in later sessions, which may be incorrectly interpreted as tolerance.

The observation that there is no difference between groups of rats which receive daily CI988, saline and CI988 plus morphine therefore indicates that (1) tolerance induced by daily morphine injection is prevented by CI988 and (2) chronic treatment with CI988 *per se* does not reduce morphine analgesia. The latter conclusion is at variance with the recent report by Kellstein & Mayer (1990), who showed that chronic treatment with the CCK antagonists proglumide and lorglumide reduces the analgesic effect of morphine. Although difference in route of drug administration (intrathecal vs systemic) or in nociceptive tests used (tail flick vs hot plate) may contribute to this difference, it should be noted that the use of proglumide and its analogues as tools to study the function of CCK has been questioned because of the poor potency and low selectivity of such drugs for the CCK_B receptor (Makovec *et al.*, 1986; Rezvani *et al.*, 1987; Gaudreau *et al.*, 1990).

The mechanism for the prevention of morphine tolerance by CCK antagonists has not been studied in detail. Since exogenously applied CCK antagonizes morphine-induced analgesia and injection of morphine increases the release of CCK in the spinal cord *in vivo* (Tang *et al.*, 1984), it is possible that chronic treatment with morphine may induce up-regulation of the endogenous CCK system, which antagonizes the action of morphine, and tolerance occurs. Chronic treatment with CCK antagonists may suppress such an up-regulation, thus preventing the development of morphine tolerance. Neurochemical studies examining changes in CCK levels in CNS after injection of morphine have given inconsistent results. While Faris *et al.* (1986) reported that acute or chronic morphine increases the level of CCK in the hypothalamus in rats, Rosén & Brodin (1989) failed to observe significant changes in CCK content in brain and spinal cord after acute morphine injection. However, it should be pointed out that examination of changes in CCK synthesis after acute and chronic morphine treatment may be more relevant in this context, since changes in tissue CCK content may have been masked by increased release of this neuropeptide.

There are, however, other possibilities by which a CCK_B antagonist may prevent morphine tolerance. For example, it has been suggested that tolerance to the analgesic effects of morphine may be related to classical conditioning (Siegel, 1976; Siegel & MacRae, 1984; Dafters & Odber, 1989). According to this theory, the administration of morphine can be viewed as a conditioning trial, with environmental cues present at the time of drug administration constituting the conditioned stimulus, and the acute pharmacological stimulation constituting the unconditioned stimulus. Tolerance is interpreted in this theory as a manifestation of the acquisition of a compensatory conditioned response between the pharmacological effects of the drug and those environmental cues which always precede these pharmacological effects. Since CI988 is a very strong anxiolytic agent (Hughes *et al.*, 1990), it is therefore possible that it may depress the reaction and memory of animals to negative environmental cues, resulting in the prevention of tolerance.

Chronic treatment with the CCK_B antagonist did not diminish naloxone-precipitated withdrawal symptoms induced by chronic morphine, indicating that CCK receptor blockade is ineffective in preventing morphine dependence. A similar conclusion has been reached by Panerai *et al.* (1987) and Dourish *et al.* (1988, 1990). It seems likely that the development of morphine dependence is different from that of tolerance and does not involve changes in the endogenous CCK system (see Ling *et al.*, 1984). The weak withdrawal symptoms observed in rats that were daily injected only with CI988 supports our previous results that CI988 causes a weak depression of the spinal flexor reflex in a naloxone-reversible manner. Since CI988 has negligible affinity for all three subtypes of opioid receptors (Hughes *et al.*, 1990), it is unlikely that this effect of CI988 results from a direct action on opioid receptors, but suggests a tonic CCK-ergic inhibition of the endogenous opioid system by the CCK_B antagonist. Nonetheless, the withdrawal symptoms seen in CI988-treated rats were weak, not significantly different from animals which received chronic treatment with saline and were even less apparent after long term than after short term treatment (Tables 3 and 4).

In conclusion, the present results demonstrate that CI988 effectively prevents morphine tolerance in rats over an extended testing period. Provided that similar mechanisms operate in man, this and other CCK receptor antagonists may be clinically useful in managing chronic pain by preventing morphine tolerance. It should be noted, however, that although the dominant receptor subtype for CCK is the B-type in rat CNS, recent studies have reported the existence of a substantial level of CCK_A binding sites in primate brain and spinal cord (Hill *et al.*, 1990; Hill & Woodruff, 1990). Therefore, one cannot rule out the possibility that a CCK_A antagonist may be more relevant in man.

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Comparisons of the effects of ryanodine on catecholamine secretion evoked by caffeine and acetylcholine in perfused adrenal glands of the guinea-pig

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1 The effect of ryanodine on catecholamine secretion induced by caffeine and muscarinic receptor activation was investigated in perfused adrenal glands of the guinea-pig.

2 Caffeine (40 mM) caused only a small increase in catecholamine secretion during perfusion with standard Locke solution. Caffeine-induced catecholamine secretion was markedly enhanced after removal of $CaCl_2$ together with replacement of NaCl with sucrose.

3 In the absence of $CaCl_2$ and NaCl, 50 μM ryanodine had no effect on the resting catecholamine secretion. Caffeine (40 mM) administered 15 min after treatment with ryanodine caused an increase in catecholamine secretion similar to that prior to application of ryanodine, but failed to have any effect thereafter. Combined application of ryanodine and caffeine also prevented catecholamine secretion induced by caffeine applied subsequently.

4 Catecholamine secretion induced by 100 μM acetylcholine (ACh) was only partially inhibited after treatment with ryanodine plus caffeine under Ca^{2+}-free, Na^+-deficient conditions.

5 Preferential influence of ryanodine on the response to caffeine was also confirmed in catecholamine secretion evoked by paired stimuli with caffeine and ACh alternately, during perfusion with either Ca^{2+}-free Locke or sucrose-substituted solutions.

6 These results indicate that caffeine increases catecholamine secretion by mobilizing Ca^{2+} from intracellular Ca^{2+} stores through ryanodine-sensitive mechanisms in guinea-pig adrenal chromaffin cells. Ca^{2+} stores sensitive to caffeine and muscarinic receptor activation may not overlap entirely.

Keywords: Adrenal chromaffin cells; caffeine; calcium store; catecholamine secretion; muscarinic receptor; ryanodine

Introduction

Caffeine is known to be a potentiator of Ca^{2+}-induced Ca^{2+} release (CICR) from the sarcoplasmic reticulum of muscle cells (Endo, 1977). On the other hand, ryanodine was considered to lock Ca^{2+} channels of sarcoplasmic reticulum in an open state in skeletal and cardiac muscle (Fleischer *et al.*, 1985; Rousseau *et al.*, 1987). It was also demonstrated that ryanodine blocks the Ca^{2+} channels responsible for CICR, but not those responsible for the release induced by inositol 1,4,5-trisphosphate (IP_3) in the sarcoplasmic reticulum of smooth muscle cells (Iino *et al.*, 1988; Iino, 1990).

We have argued that muscarinic, but not nicotinic, receptor-mediated catecholamine secretion from adrenal chromaffin cells may result from mobilization of Ca^{2+} from intracellular storage sites (Nakazato *et al.*, 1984; 1988; Yamada *et al.*, 1988; 1989a). Also, caffeine was much more effective in increasing catecholamine secretion in the absence of extracellular Ca^{2+} than in its presence in perfused adrenal glands of the cat (Yamada *et al.*, 1988; 1989b). Recently, it was found that the combined application of ryanodine and caffeine causes inhibition of subsequent caffeine-induced increases in both the cytosolic free Ca^{2+} level ($[Ca^{2+}]_i$) and catecholamine secretion in bovine cultured adrenal chromaffin cells (Teraoka *et al.*, 1991). In addition, Ca^{2+} stores responsible for caffeine- and muscarinic receptor-mediated catecholamine secretion were assumed to be distinct, because muscarinic stimuli were still effective even after caffeine caused a substantial secretory response in the absence of extracellular Ca^{2+} (Yamada *et al.*, 1989b). However, it remains to be determined whether ryanodine-sensitive Ca^{2+} stores are involved in the initiation of these evoked catecholamine secretions.

In the present experiments, the effect of ryanodine on catecholamine secretion induced by caffeine and acetylcholine (ACh) was investigated in perfused adrenal glands of the guinea-pig. The results show that ryanodine inhibits the secretory response to caffeine much more potently than that to ACh in the absence of extracellular Ca^{2+}. The results of this study have been presented in part at the XIth International Congress of Pharmacology, 1990, Amsterdam (Nakazato *et al.*, 1990).

Methods

Guinea-pigs of either sex, weighing 400–700 g, were anaesthetized intraperitoneally with sodium pentobarbitone (40 mg kg^{-1}). Both adrenal glands were perfused and isolated following the general procedure described previously (Ito *et al.*, 1979). The glands were perfused at a flow rate of 0.5 to 0.6 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). For Na^+-deficient solution, NaCl was replaced by an osmotically equivalent concentration of sucrose. In the sucrose-substituted solution containing $CaCl_2$, phosphate buffer was replaced by 2.5 mM Tris aminomethane buffer to avoid precipitation. For Ca^{2+}-free solutions, both $CaCl_2$ and $MgCl_2$ were omitted. When ACh was used as a secretagogue, perfusion solutions contained 0.2 μM physostigmine and 500 μM hexamethonium. All solutions used were bubbled with pure O_2 throughout the experiments.

Experiments were started about 40 min after isolation of adrenal glands. Secretagogues were administered for 1 min beginning 1 min before the 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.

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Adrenaline and noradrenaline were separated by high performance liquid chromatography (h.p.l.c., Jasco) and detected by an electrochemical detector (840-EC, Jasco). The detection limit was 10 nM (0.01 nmol ml^{-1}) for both adrenaline and noradrenaline. The treatment of samples for h.p.l.c. was carried out according to the method described by Salzman & Sellers (1982).

The following materials were used: acetylcholine chloride (Ovisort, Daiichi, Tokyo), caffeine monohydrate (Wako Pure Chem, Tokyo), hexamethonium chloride dihydrate (Wako Pure Chem.), physostigmine sulphate (Wako Pure Chem.) and ryanodine (Research Biochemicals).

The data are presented as arithmetic mean ± s.e.mean. Significance was assessed with Student's t test.

Results

Effects of removal of Mg^{2+}, Ca^{2+} and/or Na^+ on caffeine-induced catecholamine secretion

The effectiveness of caffeine in increasing catecholamine secretion was investigated in the presence or absence of Mg^{2+}, Ca^{2+} and/or Na^+ (Table 1). In the presence of all these cations, 40 mM caffeine caused only a small increase in catecholamine secretion. When Mg^{2+} was removed, no apparent changes occurred in the size of the caffeine-induced secretory response, which was slightly enhanced after subsequent removal of Ca^{2+}. When NaCl was replaced by sucrose, there appeared to be a significant increase in catecholamine secretion as observed previously (Teraoka *et al.*, 1990) (data not shown). Therefore, caffeine was applied after the termination of this Na^+-deficient secretory response. Under these conditions, caffeine caused only a small increase in catecholamine secretion, as long as Ca^{2+} and/or Mg^{2+} were contained in sucrose-substituted solution. However, when both Ca^{2+} and Mg^{2+} were removed subsequently, the response to caffeine was greatly enhanced (Table 1).

Effects of ryanodine on caffeine-induced catecholamine secretion

The effect of ryanodine on caffeine-induced catecholamine secretion was investigated under Ca^{2+}- and Mg^{2+}-free (referred to simply as Ca^{2+}-free in the following), and Na^+-deficient conditions. After the termination of Na^+-deficient catecholamine secretion, 40 mM caffeine was applied sequentially for 1 min usually at 15 min intervals. This caused repeated increases in catecholamine secretion which declined gradually on repetition, but which were restored after readmission of Ca^{2+} for 25 min (Figure 1a). When 50 μM ryanodine was applied simultaneously with 40 mM caffeine, catecholamine secretion was still increased, though the amounts of catecholamine released were slightly lower than those obtained when caffeine was administered alone. The combined application of ryanodine and caffeine, however, prevented caffeine applied subsequently from evoking the secretory response (Figure 1b).

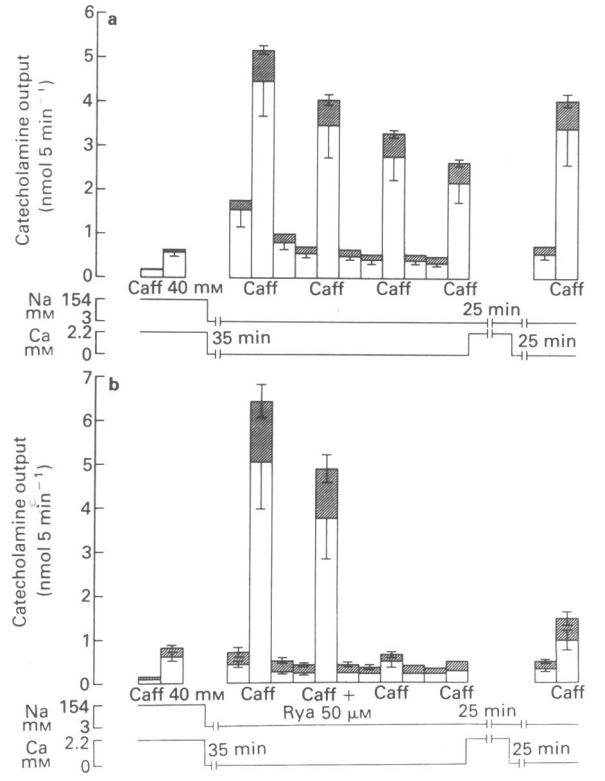

Figure 1 Effect of combined application of ryanodine and caffeine on caffeine-induced catecholamine secretion. Caffeine (Caff, 40 mM) was sequentially applied for 1 min before and after changing the perfusion solution from standard Locke solution to Ca^{2+}-free, Na^+-deficient solution (a). In (b), caffeine was replaced with 50 μM ryanodine plus 40 mM caffeine (Caff + Rya) at the second stimulus with caffeine during perfusion with the Na^+-deficient solution. At the end of the experiments, 40 mM caffeine was also applied after readmission of 2.2 mM Ca^{2+} for 25 min in (a) and (b). Columns show the mean secretion (and, if vertical lines are present, s.e.mean) of adrenaline (open) and noradrenaline (hatched) obtained from 6(a) and 5(b) experiments.

When 50 μM ryanodine was applied alone, there was no change in either the resting or caffeine-evoked catecholamine secretion which was obtained 15 min after administration of ryanodine. In the subsequent period, however, caffeine was prevented from evoking a secretory response, though the effectiveness of caffeine was partially restored after readmission of Ca^{2+} for 25 min (Figure 2).

Effects of ryanodine on acetylcholine-induced catecholamine secretion

The effect of ryanodine on catecholamine secretion induced by muscarinic receptor activation was also observed under Na^+-deficient conditions. First, the effectiveness of ACh was deter-

Table 1 The effectiveness of caffeine in releasing catecholamine in the presence and absence of Ca^{2+} and/or Mg^{2+} in Na^+-rich (Locke) and Na^+-deficient solutions

Ionic conditions		*Na^+-rich*		*Na^+-deficient*	
Ca^{2+}	*Mg^{2+}*	*NA*	*Ad*	*NA*	*Ad*
+	+	0.05 ± 0.01	0.43 ± 0.08 (11)	0.17 ± 0.09	0.57 ± 0.14 (4)
+	−	0.08 ± 0.04	0.36 ± 0.09 (9)	0.18 ± 0.03	0.45 ± 0.10 (5)
−	−	0.08 ± 0.02	0.73 ± 0.11* (6)	0.58 ± 0.11*	3.13 ± 0.65** (8)

Values indicate the mean (±s.e. mean) of adrenaline (Ad) and noradrenaline (NA) released by 40 mM caffeine in nmol 5 min^{-1} under the various ionic conditions indicated. The values of caffeine-induced catecholamine secretion were obtained by subtracting the values of resting release from respective evoked release of adrenaline and noradrenaline. In Na^+-deficient solution, NaCl was replaced by an osmotically equivalent concentration of sucrose. Numbers in parentheses indicate the numbers of experiments. *$P < 0.05$, **$P < 0.01$, when compared with respective values obtained in the presence of both Ca^{2+} and Mg^{2+}.

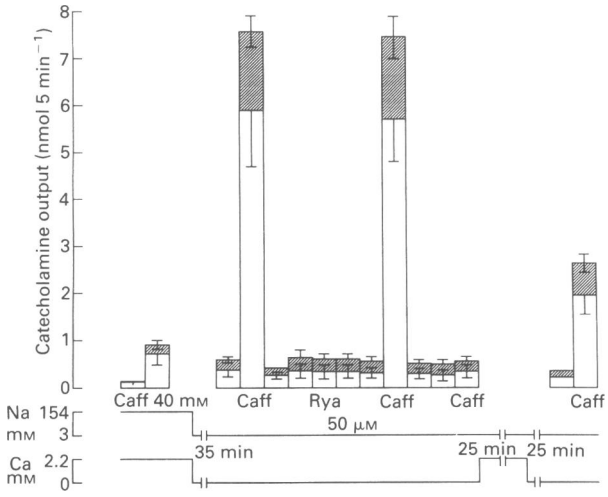

Figure 2 Effect of application of ryanodine alone on resting and caffeine-induced catecholamine secretion. Caffeine (Caff, 40 mM) was sequentially applied for 1 min before and after changing the perfusion solution from standard Locke solution to Ca^{2+}-free, Na^+-deficient solution. Ryanodine (50 μM, Rya) was applied instead of caffeine at the second stimulus during exposure to the Na^+-deficient solution. At the end of the experiments, 40 mM caffeine was also applied after readmission of 2.2 mM Ca^{2+} for 25 min. Columns show the mean secretion (and, if vertical lines are present, s.e.mean) of adrenaline (open) and noradrenaline (hatched) obtained from 6 experiments.

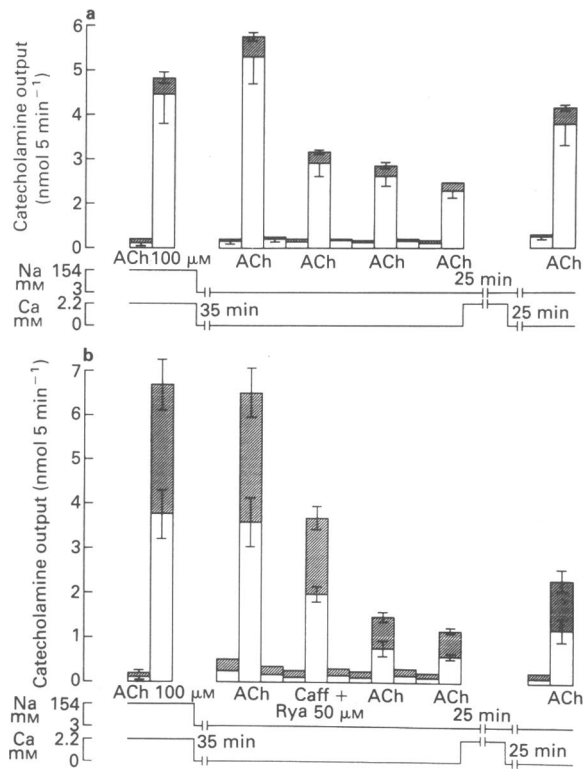

Figure 3 Effect of combined application of ryanodine and caffeine on acetylcholine-induced catecholamine secretion. Acetylcholine (ACh, 100 μM) was sequentially applied for 1 min before and after change of the perfusion solution from standard Locke solution to Ca^{2+}-free, Na^+-deficient solution (a). In (b) ACh was replaced with 40 mM caffeine plus 50 μM ryanodine (Caff + Rya) at the second stimulus with ACh during perfusion with the Na^+-deficient solution. At the end of the experiments, 100 μM ACh was also applied after readmission of 2.2 mM Ca^{2+} for 25 min in (a) and (b). Physostigmine (0.2 μM) and hexamethonium (500 μM) were present throughout the experiments. Columns show the mean secretion (and, if vertical lines are present, s.e.mean) of adrenaline (open) and noradrenaline (hatched) obtained from 3(a) and 4(b) experiments.

mined by applying it before and after the change of perfusion medium from standard Locke solution to Ca^{2+}-free, Na^+-deficient solution in the presence of 0.2 μM physostigmine plus 500 μM hexamethonium. After exposure to Na^+-deficient solution, the first application of 100 μM ACh caused an increase in catecholamine secretion of similar magnitude to that obtained during perfusion with standard Locke solution. However, the response to the second dose of ACh was decreased to about 50% of the first one and was followed thereafter by gradually declining responses (Figure 3a). Readmission of Ca^{2+} for 25 min caused a partial restoration of the response to ACh.

Caffeine (40 mM) and 50 μM ryanodine were simultaneously applied 15 min after the first application of ACh under Na^+-deficient conditions. As mentioned above, the combined application of caffeine and ryanodine caused a substantial increase in catecholamine secretion. Unlike the response to caffeine, however, ACh-induced catecholamine secretion was not abolished subsequently (Figure 3b).

To compare the effectiveness of ryanodine on caffeine- and ACh-induced catecholamine secretions, the amounts of total catecholamine released by both drugs illustrated in Figures 1 and 3 were expressed as a percentage of the first response under Na^+-deficient conditions. Figure 4 shows that ryanodine inhibited caffeine-induced catecholamine secretion to a much greater extent than ACh-induced secretion.

Effect of ryanodine on catecholamine secretion evoked by paired stimuli with caffeine and acetylcholine

Caffeine (40 mM) and ACh (100 μM) applied at 15 min intervals caused increases in catecholamine secretion repeatedly during perfusion with Ca^{2+}-free, Na^+-deficient solution, as long as the adrenal glands were loaded with 2.2 mM Ca^{2+} for 15 min between paired stimuli (Figure 5a). When 50 μM ryanodine was administered with the second dose of caffeine, the secretory response to the third dose of caffeine was significantly decreased (Figure 5b). There was practically no inhibition of ACh-induced secretory responses.

Similarly, the effect of ryanodine on catecholamine secretions induced by paired stimuli with caffeine and ACh was observed during perfusion with Ca^{2+}-free Locke solution (Na^+-rich). Under these conditions, both caffeine and ACh were also repeatedly effective in evoking catecholamine secretion (Figure 6a). In addition, combined application of ryanodine and caffeine again inhibited preferentially the caffeine-induced response in the subsequent time periods (Figure 6b). The normalized plotting of the data illustrated in Figures 5 and 6 shows clearly the preferential inhibition of the caffeine-induced response after treatment with caffeine plus ryanodine (Figure 7).

Discussion

The present experiments confirmed that caffeine was much more effective in repeatedly increasing catecholamine secretion in the absence of extracellular Ca^{2+} and Na^+ than in their presence in perfused adrenal glands of the guinea-pig (Teraoka *et al.*, 1990). In addition, ACh was also found to cause repetitive increases in catecholamine secretion under Ca^{2+}-free, Na^+-deficient conditions, although the response declined to a greater degree than that to caffeine on repetition. In any case, the repetitiveness of the secretory response was reliable enough to investigate the effect of ryanodine. Thus, ryanodine was found to inhibit the secretory response to caffeine much more potently than that to ACh under Ca^{2+}-free, Na^+-deficient conditions. Although the secretory response in the presence of a standard concentration of Na^+ was small, ryanodine also preferentially inhibited the caffeine-induced response indicating that the effect of ryanodine was not critically influenced by Na^+. Similarly, we already found in bovine cultured adrenal chromaffin cells that caffeine stimulated catecholamine secretion in the absence of extracellular Ca^{2+} and

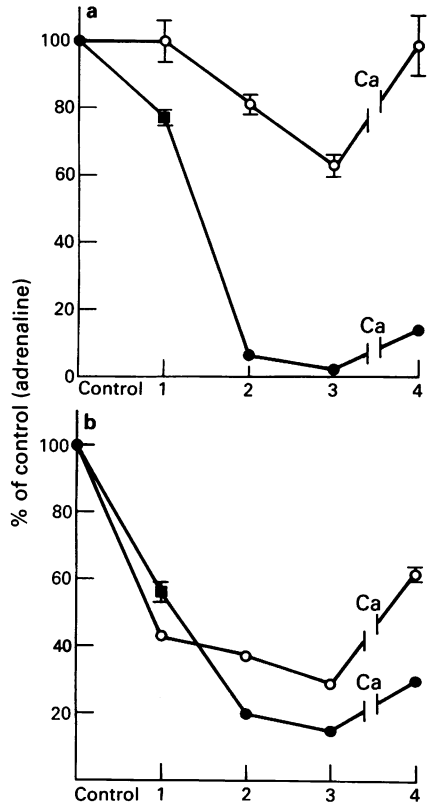

Figure 4 Comparison of the effect of ryanodine on caffeine- (a) and acetylcholine (b) -induced catecholamine secretions. The results for the effect of combined application of caffeine and ryanodine on total catecholamine (adrenaline + noradrenaline) secretion sequentially (1, 2, 3 and 4) evoked by caffeine (a) and ACh (b) in Figures 1 and 3 were expressed as percentages of the first secretory responses (Control) during perfusion with Ca^{2+}-free, Na^+-deficient solution. The symbols indicate the mean percentage values (and, if vertical lines are present, s.e.mean) in control (○) and ryanodine-treated (●) experiments; (■) values obtained by combined application of caffeine and ryanodine. Ca indicates the readmission of Ca^{2+} for 25 min.

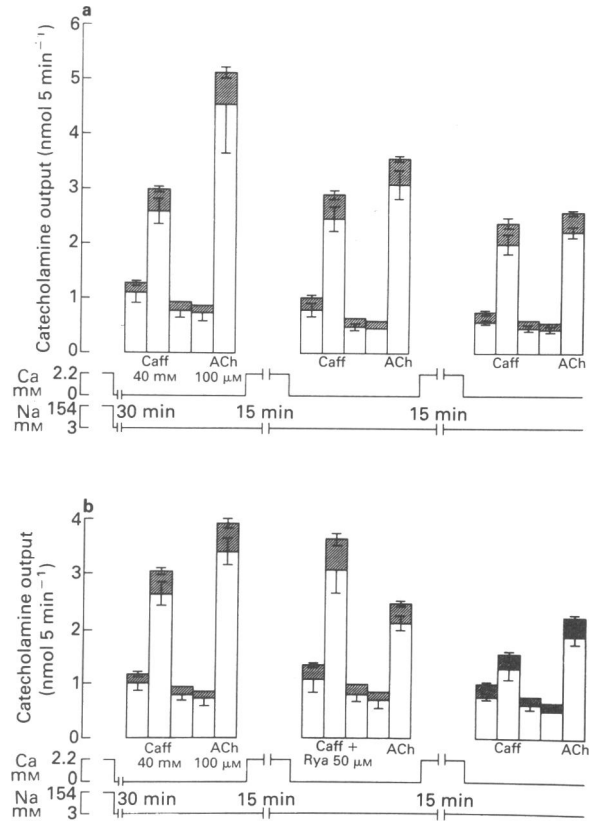

Figure 5 Effect of ryanodine on catecholamine secretion evoked by paired stimuli with caffeine and acetylcholine under Na^+-deficient conditions. Caffeine (40 mM) and ACh (100 μM) were applied for 1 min each at 15 min intervals during perfusion with Ca^{2+}-free, Na^+-deficient solution. Between paired stimuli with caffeine and ACh, 2.2 mM Ca^{2+} was reintroduced for 15 min (a). In (b), caffeine was replaced with 50 μM ryanodine plus 40 mM caffeine (Caf + Rya) for the second stimulus with caffeine. Columns show the means (and, if vertical lines are present, s.e.mean) of adrenaline (open) and noradrenaline (hatched) obtained from 4 experiments.

Na^+ more effectively than in their presence, and that the caffeine-induced increase in both catecholamine secretion and $[Ca^{2+}]_i$ was equally inhibited after combined application of caffeine and ryanodine (Teraoka *et al.*, 1991).

It has been established that caffeine (Endo, 1977) and ryanodine (Rousseau *et al.*, 1987; Iino *et al.*, 1988) are powerful modulators of the CICR mechanism of sacroplasmic reticulum in muscle cells. Recently, Malgaroli *et al.* (1990) demonstrated that caffeine generated spontaneous fluctuations of $[Ca^{2+}]_i$ in rat adrenal chromaffin cells, which was prevented after treatment with ryanodine. They proposed that the caffeine-induced fluctuation arose from CICR from intracellular stores. In agreement with the results of the present experiment, caffeine-evoked catecholamine secretion from adrenal glands of the dog (Sumikawa *et al.*, 1987) and cat (Yamada, Teraoka & Nakazato, unpublished observation) was also inhibited by procaine or dantrolene, a drug used clinically for the treatment of malignant hyperthermia (Kolb *et al.*, 1982) and was reported to inhibit selectively CICR from sarcoplasmic reticulum in skeletal muscle (Ohta *et al.*, 1990). In the present experiments, the effect of ryanodine on $[Ca^{2+}]_i$ was not investigated because of limitations in experimental conditions. However, as far as caffeine-induced catecholamine secretion is concerned, the effect of ryanodine in perfused adrenal glands of guinea-pig agrees with that obtained from bovine cultured adrenal chromaffin cells (Teraoka *et al.*, 1991). Therefore, if caffeine-induced catecholamine secretion strictly reflects the change in $[Ca^{2+}]_i$, then the results of the present experiments may indicate that guinea-pig adrenal chromaffin cells possess intracellular Ca^{2+} stores sensitive to both caffeine and ryanodine. In addition, it appears that caffeine-evoked catecholamine secretion may result from mobilization of Ca^{2+} from intracellular Ca^{2+} stores, probably through the CICR mechanism.

Caffeine and IP_3 release Ca^{2+} from the same types of intracellular Ca^{2+} stores in skeletal and cardiac muscles, but from different stores in smooth muscle of the gastrointestinal tract (Iino *et al.*, 1988; Iino, 1989; 1990). It was also reported that the caffeine-sensitive Ca^{2+} store is distinct from those susceptible to agonists, such as histamine, noradrenaline or bradykinin in vascular smooth muscle (Kanaide *et al.*, 1987; Matsumoto *et al.*, 1990) and sensory neurones (Thayer *et al.*, 1988). The present experiments show that combined application of caffeine and ryanodine inhibited caffeine-induced catecholamine secretion to a much greater extent than ACh-induced catecholamine secretion in the subsequent time periods. Furthermore, ACh still caused a substantial increase in catecholamine secretion even when applied immediately after the caffeine-induced response. It therefore seems likely that Ca^{2+} stores responsible for caffeine- and muscarinic receptor-mediated catecholamine secretion may not overlap entirely in the adrenal chromaffin cells of the guinea-pig.

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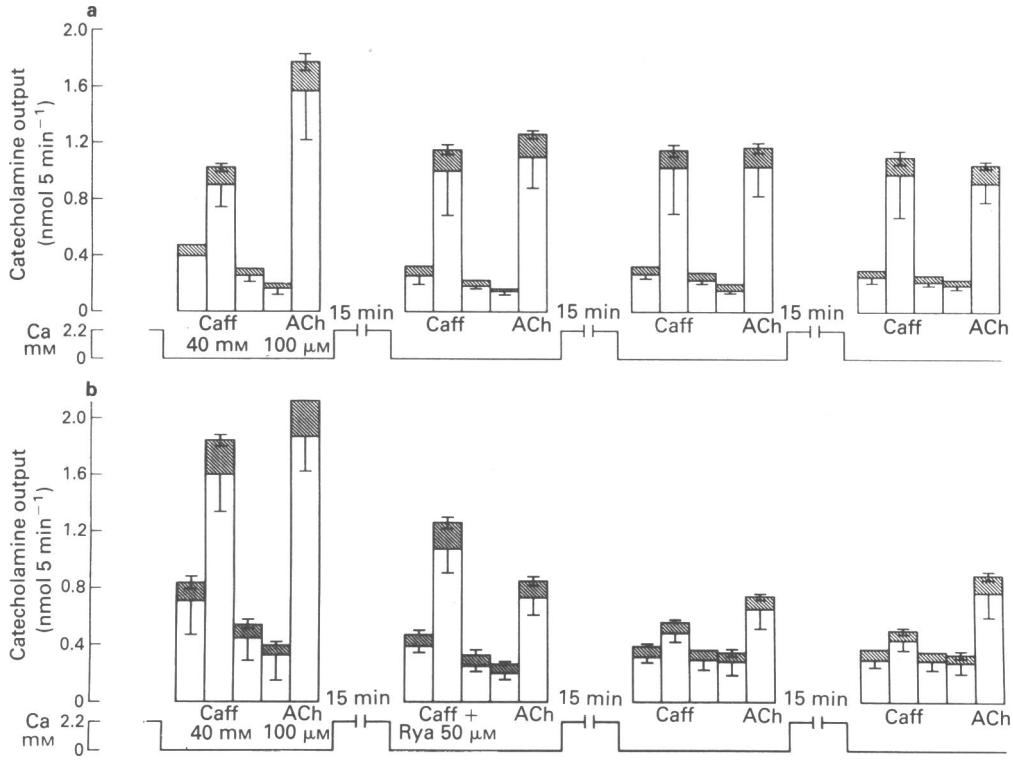

Figure 6 Effect of ryanodine on catecholamine secretion evoked by paired stimuli with caffeine and acetylcholine (ACh) under Na^+-rich conditions. Caffeine (40 mM) and ACh (100 μM) were applied for 1 min each at 15 min intervals during perfusion with Ca^{2+}-free Locke solution (Na^+-rich). Between paired stimuli with caffeine and ACh, 2.2 mM Ca^{2+} was reintroduced for 15 min (a). In (b) caffeine was replaced with 50 μM ryanodine plus 40 mM caffeine (Caff + Rya) at the second stimulus with caffeine. Columns show the mean secretion (and, if vertical lines are present, s.e.mean) of adrenaline (open) and noradrenaline (hatched) obtained from 3(a) and 4(b) experiments.

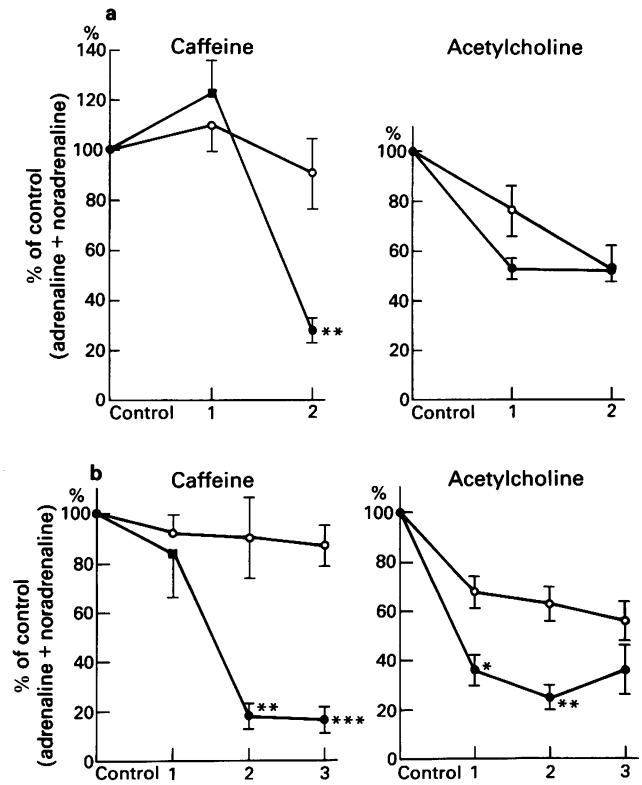

Figure 7 Comparison of the effect of ryanodine on catecholamine secretions evoked by paired stimuli with caffeine and acetylcholine (ACh). The results for the effect of combined application of caffeine and ryanodine on total catecholamine (adrenaline + noradrenaline) secretion sequentially (1, 2 and/or 3) evoked by paired stimuli with caffeine (left) and ACh (right) in Figures 5 and 6 were expressed as percentages of the first secretory responses (Control) under Na^+-deficient (a) and Na^+-rich (b) conditions. The symbols indicate the mean percentage values (and, if vertical lines are present, s.e.mean) in control (○) and ryanodine-treated (●) experiments; (■) shows the values obtained by combined application of caffeine and ryanodine.

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Studies on the mechanism of 5-HT_1 receptor-induced smooth muscle contraction in dog saphenous vein

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1 We have investigated the mechanism of smooth muscle contraction evoked by activation of 5-HT_1-like receptors in dog isolated saphenous vein.

2 In the presence of the 5-HT_2 receptor antagonist, ritanserin (0.1 μM), concentration-effect curves (10 nM–300 μM) for 5-hydroxytryptamine (5-HT)-induced smooth muscle contraction were biphasic. This could be attributed to a direct action on 5-HT_1-like receptors at low concentrations of 5-HT (10 nM–10 μM) and an indirect (through the release of noradrenaline from sympathetic neurones) activation of postjunctional α-adrenoceptors at higher 5-HT concentrations. In contrast, concentration-effect curves (100 nM–100 μM) for sumatriptan-induced contractions were not biphasic, and were due solely to activation of 5-HT_1-like receptors.

3 Smooth muscle contractions evoked either by low concentrations of 5-HT or by sumatriptan were abolished by removal of extracellular calcium and were markedly inhibited, but not abolished, by the calcium channel blocker, verapamil (1–30 μM). In contrast, contractions evoked by high concentrations of 5-HT were markedly less sensitive to removal of extracellular calcium or to verapamil.

4 5-HT and sumatriptan also inhibited (to a maximum of about 50%) prostaglandin E_2 (PGE_2, 5 μM)-stimulated adenosine 3′:5′-cyclic monophosphate (cyclic AMP) formation. This effect was mimicked by the α_2-adrenoceptor agonist, azepexole (B-HT933) but not by the α_1-adrenoceptor agonist, methoxamine.

5 In contrast to mediation of smooth muscle contraction, the 5-HT_1-like receptor-mediated inhibition of PGE_2-stimulated cyclic AMP formation evoked by 5-HT or sumatriptan was not attenuated by removal of extracellular calcium or by verapamil (1 μM).

6 A directly-acting inhibitor of adenylyl cyclase, 2′,3′-dideoxyadenosine (1 mM) inhibited PGE_2-stimulated cyclic AMP formation but did not produce smooth muscle contraction.

7 These results suggest that contractile responses of dog isolated saphenous vein arising through activation of 5-HT_1-like receptors are associated with both an influx of extracellular calcium ions (to a large extent via voltage-dependent channels) and an inhibition of adenylyl cyclase. However, although these two responses are coupled to the same receptor, they appear to be independent.

Keywords: Sumatriptan; 5-HT_1-like receptor; dog saphenous vein; adenylyl cyclase; calcium; cyclic AMP

Introduction

Sumatriptan is a novel and selective 5-HT_1-like receptor agonist (Humphrey *et al.*, 1988) which is clinically effective in the treatment of migraine (Patten, 1991). Sumatriptan produces both smooth muscle contraction (Humphrey *et al.*, 1988) and inhibition of prostaglandin E_2 (PGE_2)-stimulated adenosine 3′:5′-cyclic monophosphate (cyclic AMP) formation (Sumner & Humphrey, 1990) in dog isolated saphenous vein.

We have attempted to explore further the mechanism whereby sumatriptan evokes smooth muscle contraction in the dog saphenous vein preparation. In this regard, the roles of intracellular and extracellular calcium in the contractile responses elicited by α_1- and α_2-adrenoceptor agonists have been investigated previously (Mathews *et al.*, 1984; reviewed in Bülbring & Tomita, 1987 and Docherty, 1989). These studies have demonstrated that smooth muscle contraction evoked by α_2-adrenoceptor activation is more sensitive to calcium channel bockers, both *in vitro* (Mathews *et al.*, 1984) and *in vivo* (Van Meel *et al.*, 1983), than is that evoked through activation of α_1-adrenoceptors. Although the receptor-coupling mechanisms underlying these responses in dog saphenous vein are not known, in other tissues, α_1- and α_2-adrenoceptors appear to be coupled to activation of phospholipase C or to inhibition of adenylyl cyclase respectively (Fain & Garcia-Sainz, 1980). In keeping with this, α_2-adrenoceptor-mediated pressor responses *in vivo* can be attenuated by pretreatment with pertussis toxin (Boyer *et al.*, 1983). These observations would suggest that α_2-adrenoceptor-mediated smooth muscle contraction may result from the influx of extracellular calcium (largely through voltage-dependent channels) and the inhibition of adenylyl cyclase. We therefore sought to explore whether the same may be true for 5-HT_1-like receptor-mediated smooth muscle contraction in dog isolated saphenous vein.

In addition, sumatriptan has also been shown to act as an agonist at brain 5-HT_{1D} receptors (Schoeffter & Hoyer, 1989). These receptors are negatively coupled to adenylyl cyclase (Waeber *et al.*, 1988; Schoeffter & Hoyer, 1989) and as such are somewhat similar to dog saphenous vein 5-HT_1-like receptors (Summer & Humphrey, 1990). Accordingly, we have attempted to characterize in more detail the receptor mediating inhibition of adenylyl cyclase in dog saphenous vein with a view to comparing it with brain 5-HT_{1D} receptors.

Methods

Lengths (3–5 cm) of lateral saphenous vein were removed from Beagle dogs of either sex (7–10 kg body weight) which had been anaesthetized with barbitone (300 mg kg^{-1}, i.p.) following induction with thiopentone (25 mg kg^{-1}, i.v.) and pentobarbitone (60 mg, i.v.). These blood vessels were then used for measurements of smooth muscle contraction or cyclic AMP formation.

Measurement of smooth muscle contraction

This was performed essentially as described by Apperley *et al.* (1980). Briefly, four spirally-cut strips (2 mm wide, 2–3 cm long) were mounted individually in 20 ml organ baths for isometric recording of tension changes and were maintained at

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37°C in a gassed (95% O_2, 5% CO_2), modified Krebs solution (composition, mM: NaCl 118, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 0.6, D-glucose 11.0, containing $CaCl_2$ 1.3 mM and ritanserin 0.1 μM (5-HT_2 receptor antagonist)). Tissues were subjected to an initial resting tension of 0.5 g and were allowed to equilibrate for 60 min with changes of Krebs solution every 15 min. During this time, preparations were contracted once with a single concentration (30 mM) of KCl. Subsequently, a cumulative concentration-effect curve to either 5-hydroxytryptamine (5-HT) or sumatriptan was established for all preparations. In all cases, the preparations developed a sustained contraction and responses were measured at the plateau tension.

In experiments exploring the calcium sensitivity of contractile responses to 5-HT or sumatriptan, the Krebs solution was then replaced with a similar solution containing either 2.6 mM, 1.3 mM, 0.65 mM, 0.33 mM or nominally 0 mM $CaCl_2$ (not containing EGTA), and a cumulative concentration-effect curve to 5-HT or sumatriptan was repeated 30 min later. In this way, cumulative concentration-effect curves for each agonist were generated in the presence of varying concentrations of calcium. Responses were then calculated as a percentage of the maximal contraction evoked during the determination of the original concentration-effect curve to 5-HT or sumatriptan (which was obtained in Krebs solution containing 1.3 mM $CaCl_2$, a concentration which reflects plasma free calcium concentrations).

To examine the effects of verapamil, following an initial concentration-effect curve to 5-HT or sumatriptan, tissues were exposed for 30 min to vehicle (control) or verapamil (1, 3, 10 or 30 μM) before an agonist concentration-effect curve was repeated.

Measurement of cyclic AMP formation

Rings of dog isolated saphenous vein (2–3 mm in length) were incubated for 30 min at 37°C in gassed (95% O_2, 5% CO_2), Krebs solution (1.3 mM $CaCl_2$) containing the cyclic nucleotide phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 100 μM) and antagonists where appropriate.

Rings were then co-incubated for 2 min at 37°C with 5 μM prostaglandin E_2 (PGE_2) and either vehicle (control) or test agonist, before being rapidly frozen in liquid nitrogen to terminate cyclic AMP formation. Rings were subsequently extracted for the measurement of protein content and cyclic AMP as previously described (Sumner *et al.*, 1989). The cyclic AMP assay employed a commercially available kit (Amersham International, England) and was based upon the competition between [^{3}H]-cyclic AMP and unlabelled cyclic AMP for a finite number of sites on a binding protein. Separation of bound and unbound cyclic AMP was achieved by charcoal sedimentation. The sensitivity of this assay (non-acetylated protocol) was 1–2 pmol per tube.

Statistics

Results are presented as arithmetic means with standard error of the mean from n observations, which is also the number of veins used. Student's unpaired t test was used to assess the significance of differences between mean values, significance being defined by a P value less than 0.05.

Drugs

Drugs were obtained from the following sources: verapamil hydrochloride (Sigma), 2',3'-dideoxyadenosine (Sigma), 3-isobutyl-1-methylxanthine (Sigma), forskolin (Sigma), spiperone ketanserin and ritanserin (Janssen), methiothepin maleate (Hoffman La-Roche), metergoline (Farmitalia), methysergide hydrogen maleate and mesulergine (Sandoz), azepexole (B-HT933; Boehringer-Ingelheim) and prostaglandin E_2 (Upjohn).

The following compounds were synthesized by Glaxo Group Research: sumatriptan, 5-carboxamidotryptamine, cyanopindolol and ondansetron.

Drug stocks were prepared and diluted in distilled water or Krebs buffer, with the exception of cyanopindolol, ketanserin, spiperone and ondansetron which were dissolved in 1 M acetic acid and diluted in Krebs buffer.

Results

Smooth muscle contraction

Concentration-effect curves for 5-HT (10 nM–300 μM)-induced contraction of strips of dog isolated saphenous vein were biphasic, the second phase being evident at 5-HT concentration greater than 10 μM (Figure 1). Decreasing the extracellular calcium concentration in the Krebs solution from 2.6 to 0 mM reduced the maximal contractile response to low concentrations of 5-HT in a concentration-dependent manner (Figure 1), with little or no response being evident in the absence of extracellular calcium. In contrast, contractions evoked by high concentrations of 5-HT were markedly less sensitive to decreasing the extracellular calcium concentration, with contractile responses still being evident even in the absence of calcium (Figure 1).

In contrast to those obtained with 5-HT, concentration-effect curves for sumatriptan (100 nM–100 μM)-induced smooth muscle contraction were not biphasic (Figure 2). The magnitude of the contraction induced by sumatriptan, like that evoked by low concentrations of 5-HT, was markedly reduced by decreasing the extracellular calcium concentration and was abolished in the absence of calcium (Figure 2).

In the presence of 1.3 mM calcium, verapamil (1–30 μM) inhibited smooth muscle contraction evoked either by low concentrations of 5-HT (Figure 3) or by sumatriptan (Figure 4). However, this inhibition was not complete, with a substantial proportion of the response to 5-HT (low concentrations) or to sumatriptan being resistant to verapamil. Contractions evoked by high concentrations of 5-HT were less susceptible to blockade by verapamil, although some inhibition was apparent in the presence of 30 μM verapamil (Figure 3).

Inhibition of prostaglandin E_2-stimulated cyclic AMP formation

Sumatriptan, 5-HT and 5-carboxamidotryptamine (5-CT) all inhibited (at 10 μM) PGE_2-stimulated cyclic AMP formation

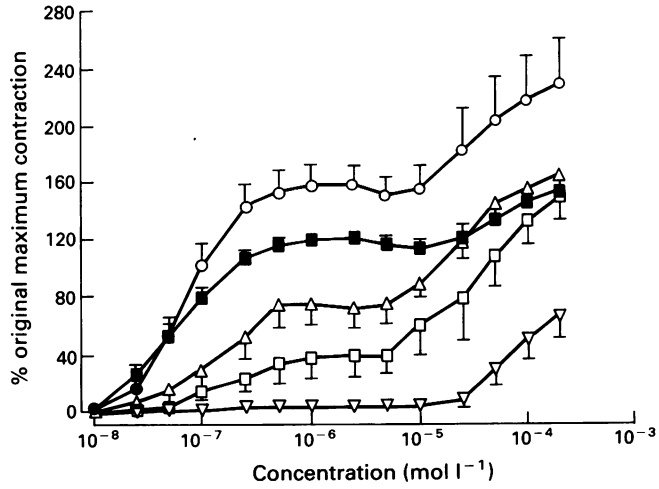

Figure 1 Effect of altering the extracellular calcium concentration on the contraction of dog isolated saphenous vein evoked by 5-hydroxytryptamine (5-HT). An initial cumulative concentration-effect curve to 5-HT was established in the presence of 1.3 mM $CaCl_2$. The Krebs solution was then replaced with one containing either 2.6 mM (○), 1.3 mM (■), 0.65 mM (△), 0.33 mM (□) or 0 mM (▽) calcium and, 30 min later, a cumulative concentration-effect curve to 5-HT was repeated. Results are mean of 4 determinations, and are expressed relative to the initial maximal response to 5-HT obtained in Krebs containing 1.3 mM $CaCl_2$; vertical bars show s.e.mean. The 5-HT_2 receptor antagonist, ritanserin (0.1 μM) was present throughout.

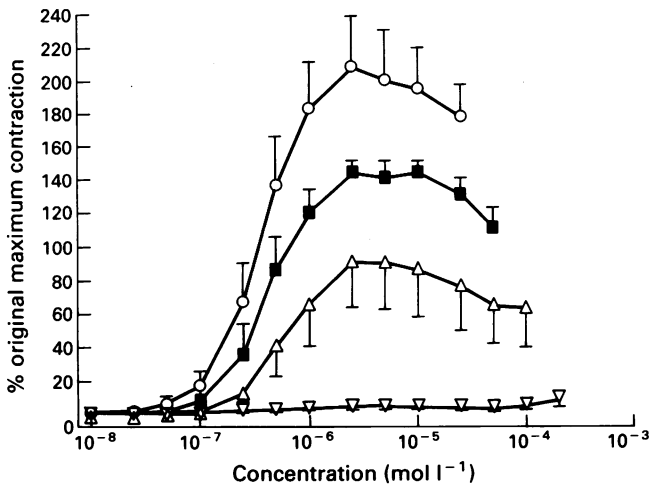

Figure 2 Effects of altering extracellular calcium concentrations on the contraction of dog isolated saphenous vein by sumatriptan. An initial cumulative concentration-effect curve to sumatriptan was established on all preparations in the presence of 1.3 mM $CaCl_2$. The Krebs solution was then replaced with one containing 2.6 mM (○), 1.3 mM (■), 0.65 mM (△) or 0 mM (▽) calcium and, 30 min later, a cumulative concentration-effect curve to sumatriptan was repeated. Results are means of 4 determinations, and are expressed relative to the original maximum contraction to sumatriptan obtained in Krebs containing 1.3 mM $CaCl_2$; vertical bars show s.e.mean. The 5-HT_2 receptor antagonist, ritanserin (0.1 μM) was present throughout.

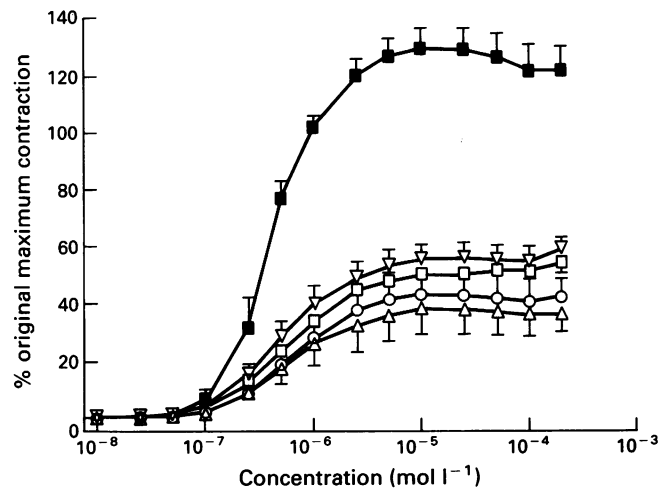

Figure 4 Contraction of dog isolated saphenous vein by sumatriptan in the presence of verapamil. Initial cumulative concentration-effect curves to sumatriptan were established on all preparations. Each preparation was then exposed to vehicle (■) or to verapamil at 1 μM (□), 3 μM (▽), 10 μM (○) or 30 μM (△) for 30 min before repeating the curves to sumatriptan. Results are means of 4 determinations, and are expressed relative to the initial maximum response to sumatriptan; vertical bars show s.e.mean. The calcium concentration was 1.3 mM and ritanserin (0.1 μM) was continuously present throughout.

(Table 1). This response was mimicked by the α_2-adrenoceptor agonist, azepexole (10 μM) but not by the α_1-adrenoceptor agonist, methoxamine (10 μM) (Table 1). In addition to its effects on PGE_2-stimulated cyclic AMP formation, sumatriptan (10 μM) also reduced both basal (51% reduction) and forskolin (5 μM)-stimulated (65% reduction) cyclic AMP formation (Table 2).

Sumatriptan (10 μM)-induced inhibition of PGE_2-stimulated cyclic AMP formation was antagonized by methiothepin (1 μM), but not by spiperone, ondansetron, cyanopindolol, or mesulergine (all at 1 μM) or by metergoline at 0.1 μM (Table 3). At these concentrations, none of the antagonists tested had any effect on PGE_2-stimulated cyclic AMP formation in their

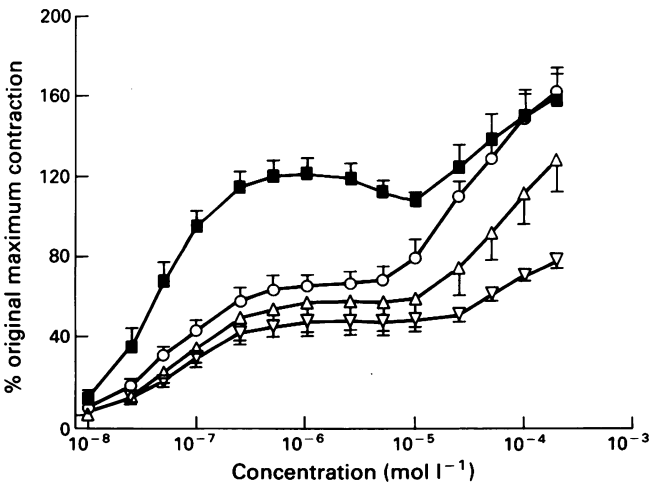

Figure 3 Contraction of dog isolated saphenous vein by 5-hydroxytryptamine (5-HT) in the presence of verapamil. An initial cumulative concentration-effect curve to 5-HT was established in all preparations. Each preparation was then exposed to vehicle (■) or verapamil at 3 μM (○), 10 μM (△) or 30 μM (▽) for 30 min before repeating the concentration-effect curve to 5-HT. Results are means of 4 determinations and are expressed relative to the initial maximum response to 5-HT; vertical bars show s.e.mean. The Krebs solution contained 1.3 mM $CaCl_2$ and 0.1 μM ritanserin throughout.

Table 1 Inhibition of prostaglandin E_2 (PGE_2)-stimulated adenosine 3′:5′-cyclic monophosphate (cyclic AMP) formation in rings of dog isolated saphenous vein

Treatment	*Cyclic AMP* (pmol mg^{-1} protein min^{-1})
None	3.18 (±0.62)
PGE_2 (5 μM) controls	6.87 (±1.00)
PGE_2 + sumatriptan (10 μM)	4.07 (±0.84)*
PGE_2 + 5-HT (10 μM)	4.16 (±0.70)*
PGE_2 + 5-carboxamidotryptamine (10 μM)	4.40 (±0.81)*
PGE_2 + azepexole (10 μM)	3.92 (±0.26)*
PGE_2 + methoxamine (10 μM)	5.94 (±0.77)
PGE_2 + dideoxyadenosine (1 mM)	5.03 (±0.45)*

Results are mean (±s.e.mean) of 3–6 determinations. Note that all of the agonists tested inhibited PGE_2-stimulated cyclic AMP formation with the single exception of methoxamine.
* Significantly different from PGE_2 control ($P < 0.05$).

Table 2 Effect of sumatriptan on basal, prostaglandin E_2 (PGE_2)-stimulated and forskolin-stimulated adenosine 3′:5′-cyclic monophosphate (cyclic AMP) formation in rings of dog isolated saphenous vein

Treatment	*Cyclic AMP* (pmol mg^{-1} protein min^{-1})
Basal control	3.18 (±0.62)
Basal + sumatriptan (10 μM)	1.56 (±0.47)*
PGE_2 (5 μM) control	6.70 (±0.47)
PGE_2 (5 μM) + sumatriptan (10 μM)	3.98 (±0.44)*
Forskolin (5 μM) control	14.58 (±1.01)
Forskolin (5 μM) + sumatriptan (10 μM)	5.15 (±0.96)*

Results are mean (±s.e. mean) of 4–6 determinations.
* Significantly different from the corresponding control ($P < 0.05$).

Table 3 Effect of sumatriptan on prostaglandin E_2 (PGE_2)-stimulated adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation in rings of dog isolated saphenous vein in the absence and presence of various 5-hydroxytryptamine (5-HT) receptor antagonists

Treatment	*Cyclic AMP* (pmol mg^{-1} protein min^{-1})
Basal control	3.18 (±0.62)
Basal + sumatriptan (10 μM)	1.56 (±0.30)
PGE_2 control	6.70 (±0.47)
PGE_2 + sumatriptan (10 μM)	3.98 (±0.44)*
PGE_2 + sumatriptan + spiperone (1 μM)	4.10 (±0.26)*
PGE_2 + sumatriptan + ondansetron (1 μM)	3.60 (±0.42)*
PGE_2 + sumatriptan + cyanopindolol (1 μM)	3.08 (±0.34)*
PGE_2 + sumatriptan + mesulergine (1 μM)	3.11 (±0.39)*
PGE_2 + sumatriptan + metergoline (0.1 μM)	3.65 (±0.44)*
PGE_2 + sumatriptan + methiothepin (1 μM)	6.42 (±0.98)

Results are mean (±s.e.mean) of 6 determinations. All experiments were performed in the presence of PGE_2 (5 μM), with the exception of the basal determinations in the absence or presence of sumatriptan.
* Significantly different from PGE_2 control, $P < 0.05$.
† At concentrations above 0.1 μM, metergoline displayed agonist activity (see results).

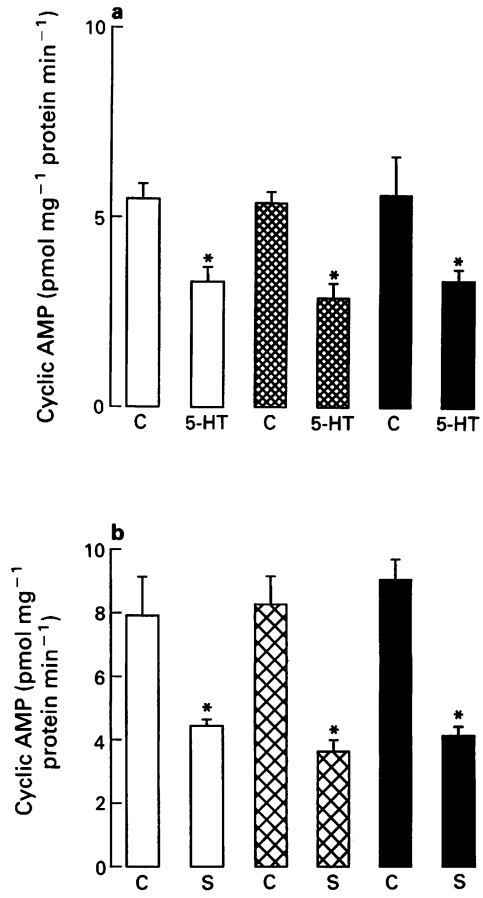

Figure 5 Effect of 5-hydroxytryptamine (5-HT) (a) and sumatriptan (b) on prostaglandin E_2 (PGE_2)-stimulated adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation in dog isolated saphenous vein in the absence of calcium or in the presence of verapamil. Rings of dog isolated saphenous vein were incubated for 30 min in Krebs solution containing 100 μM 3-isobutyl-1-methylxanthine (IBMX) and 1.3 mM $CaCl_2$, in the absence (open columns) or presence of 1 μM verapamil (cross hatched columns), or in calcium-free Krebs containing 100 μM IBMX and 1 mM EGTA (solid columns). Rings were then exposed for 2 min to PGE_2 (5 μM) and either vehicle (C) or 10 μM 5-HT (a) or 10 μM sumatriptan (denoted S in b) before extracting and assaying the protein and cyclic AMP content of each ring. Results are means of 4 (5-HT) or 5 (sumatriptan) determinations; vertical bars show s.e. mean.
* $P < 0.05$ relative to the appropriate controls.

own right. However, at higher concentrations, metergoline (1 μM) and methysergide (10 μM) also inhibited PGE_2-stimulated cyclic AMP formation, although only the effect of metergoline proved to be significant (mean inhibition of PGE_2-stimulated cyclic AMP formation was 23 ± 5% for metergoline (1 μM) and 23 ± 15% for methysergide (10 μM) compared to 33 ± 3% for sumatriptan (10 μM), $n = 4$). The effects of these antagonists on the inhibition of basal cyclic AMP formation produced by sumatriptan were not examined.

Incubation of rings of dog saphenous vein in Ca^{2+}-free Krebs solution containing 1 mM EGTA or in Krebs solution containing 1.3 mM Ca^{2+} and 1 μM verapamil did not affect the inhibition of PGE_2-stimulated cyclic AMP formation produced either by 5-HT (Figure 5a) or by sumatriptan (Figure 5b).

A directly-acting inhibitor of adenylyl cyclase, 2',3'-dideoxyadenosine (Haslam *et al.*, 1978) also inhibited (only significantly at 1 mM) PGE_2-stimulated cyclic AMP formation (Table 1), but unlike sumatriptan, did not evoke contraction of strips of dog isolated saphenous vein (results not shown).

Discussion

The intracellular mechanism of 5-HT_1-like receptor-mediated smooth muscle contraction in the dog isolated saphenous vein is not known (Apperley *et al.*, 1980; Humphrey *et al.*, 1988). In this study we have demonstrated that sustained smooth muscle contraction arising through activation of 5-HT_1-like receptors by either 5-HT or sumatriptan is dependent upon extracellular calcium, and is largely attenuated, but not abolished, by verapamil, a blocker of voltage-dependent calcium channels (Fleckenstein 1977). Interestingly, the contractile effects of high concentrations of 5-HT, which arise indirectly through noradrenaline release and activation of post-junctional α-adrenoceptors (Humphrey, 1978), probably of the α_1-subtype (Mathews *et al.*, 1984), were far less sensitive to either removal of extracellular calcium or to verapamil.

These results would argue against a non-specific effect of verapamil, and are in keeping with those obtained with selective adrenoceptor agonists in this tissue (Mathews *et al.*, 1984; Bülbring & Tomita, 1987; Docherty, 1989) and are compatible with a major role for intracellular calcium mobilization in α_1-adrenoceptor-mediated responses (Fain & García-Sainz, 1980). Furthermore, these observations would also suggest that 5-HT_1-like and α_1-adrenoceptor-mediated smooth muscle contraction arise largely through different mechanisms, involving the influx of extracellular calcium (largely through voltage-dependent channels) or the mobilization of intracellular calcium respectively. It is recognised, however, that the initial contractile response to 5-HT_1-like receptor activation may be more dependent upon mobilisation of intracellular calcium stores, with the subsequent sustained phase of contraction being supported by an influx of extracellular calcium. Although this possibility was not specifically addressed in this study, there was no evidence for any contraction to 5-HT or sumatriptan in nominally calcium-free Krebs solution, irrespective of whether cumulative concentration-effect curves (see Results) or single concentrations (results not shown) were examined.

As well as causing smooth muscle contraction, 5-HT and sumatriptan also reduced PGE_2-stimulated cyclic AMP formation, possibly due to an inhibition of adenylyl cyclase. Since agents which increase cyclic AMP concentrations in this

tissue also cause smooth muscle relaxation (Kikkawa *et al.*, 1986), it is conceivable that reducing cyclic AMP levels could lead, at least in part, to smooth muscle contraction.

In keeping with this, the α_2-adrenoceptor agonist, azepexole (B-HT933), also inhibited cyclic AMP formation (this study) and caused smooth contraction (Ruffolo & Zeid, 1985; Rhodes & Waterfall, 1987) in this tissue. A number of adenosine analogues have been shown to inhibit adenylyl cyclase through a direct action at the so-called 'P-site' on the enzyme (Haslam *et al.*, 1978). One such compound, 2',3'-dideoxyadenosine, although significantly reducing cyclic AMP levels (Table 1), failed to elicit smooth muscle contraction. It is possible that cyclic AMP concentrations need to be reduced beyond a critical level in order to evoke contraction. Indeed, the 5-HT$_1$-like receptor agonists, which both caused contraction of dog saphenous vein, also elicited a greater reduction in cyclic AMP levels than did dideoxyadenosine. Nevertheless, this result would suggest that a reduction in cyclic AMP levels *per se* is not likely to be directly responsible for smooth muscle contraction.

An alternative mechanism might be one in which a rise in intracellular calcium levels brings about both smooth muscle contraction and a fall in cyclic AMP levels. In this regard, calcium can clearly modulate cyclic AMP formation and breakdown (Rasmussen & Barrett, 1984; Resink *et al.*, 1986). However, such a mechanism appears unlikely in view of the lack of effect of either reducing the extracellular calcium concentration, or of verapamil, on 5-HT$_1$-like receptor-mediated reductions in cyclic AMP concentrations, compared with the marked effect that was observed on 5-HT$_1$-like receptor-mediated smooth muscle contraction.

Any mechanism which attempts to account for 5-HT$_1$-like receptor-mediated smooth muscle contraction and inhibition of cyclic AMP formation must accommodate the experimental observations that contraction: (1) is totally dependent upon extracellular calcium, a large component of which appears to enter the cell via voltage-dependent calcium channels, (2) is clearly different from α-adrenoceptor-mediated contraction (which probably involves α_1-receptor-mediated mobilization of intracellular calcium stores) and (3) is accompanied by a concomitant inhibition of adenylyl cyclase which appears to be an independent event, despite being evoked via the same receptor. A mechanism which could account for all these observations is one in which 5-HT$_1$-like receptor-mediated inhibition of adenylyl cyclase and activation of a cationic channel occur independently. The ensuing cellular depolarisation would open voltage-dependent calcium channels thereby propagating the calcium influx, leading to smooth muscle contractions. The latter response may be facilitated by the accompanying fall in cyclic AMP levels (Rasmussen & Barrett, 1984).

Sumatriptan has been reported to act as an agonist at brain 5-HT$_{1D}$ receptors (Schoeffter & Hoyer, 1989). These brain receptors are similar to dog saphenous vein 5-HT$_1$-like receptors, in that both appear to be negatively coupled to adenylyl cyclase and both are sensitive to 5-HT, 5-CT and sumatriptan but not to spiperone (Waeber *et al.*, 1988; Schoeffter & Hoyer, 1989; Sumner & Humphrey, 1990; this study).

However, metergoline appears to differentiate between brain 5-HT$_{1D}$ receptors, for which it shows a high affinity (Waeber *et al.*, 1988), and the dog saphenous vein 5-HT$_1$-like receptor, at which it appears to act as a weak partial agonist (Perren *et al.*, 1991). The results of this study confirm the weak partial agonist activity of metergoline at the dog saphenous vein 5-HT$_1$-like receptor. It is of interest to note, however, that studies using a different central 5-HT$_{1D}$ receptor model (Middlemiss *et al.*, 1988) found methysergide and metergoline to be weak antagonists or partial agonists. These data are in keeping with those obtained in this study, and support the notion that brain 5-HT$_{1D}$ receptors and the dog saphenous vein 5-HT$_1$-like receptor may be similar. Nevertheless, the definitive characterisation of these two important 5-HT$_1$ receptor sites, both of which are negatively coupled to adenylyl cyclase, must await the identification of specific and selective receptor antagonists.

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The role of cyclic AMP in non-adrenergic non-cholinergic contraction in guinea-pig bronchi

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1 We investigated the role of adenosine 3':5'-cyclic monophosphate (cyclic AMP) in non-adrenergic non-cholinergic (NANC) contraction in guinea-pig bronchial strips.

2 Forskolin (3 nM to 1 μM) reduced NANC contraction induced by electrical field stimulation (EFS) in a concentration-dependent fashion ($-\log EC_{50}$ was 7.22 ± 0.12 M and maximum inhibition was $100 \pm 0.01\%$). However, forskolin (< 1 μM) did not alter the contraction induced by substance P (SP, 1 μM).

3 Dibutyryl cyclic AMP (1 mM) also reduced NANC contractions induced by EFS ($100 \pm 0.01\%$) without significant effect on SP (1 μM)-induced contractions. In contrast, dibutyryl cyclic GMP (1 mM) was without effect against either NANC or SP-induced contractions.

4 Both the β_2-adrenoceptor agonist, procaterol (0.1 nM to 3 nM) and theophylline (100 nM to 1 mM) concentration-dependently reduced EFS-induced NANC contractions without significant effect on SP (1 μM)-induced contractions.

5 In contrast to forskolin, procaterol and theophylline, both sodium nitroprusside and cromakalim inhibited the EFS-induced contractions only at those concentrations that similarly reduced the contractions induced by SP (1 μM).

6 These results suggest that cyclic AMP may mediate pre-junctional inhibition of NANC contractions in guinea-pig bronchi.

Keywords: Airway smooth muscle; cyclic AMP; forskolin; β_2-adrenoceptor agonist; theophylline

Introduction

There is considerable evidence that adenosine 3':5'-cyclic monophosphate (cyclic AMP), acting as a second messenger of activated β-adrenoceptors, causes relaxation of airway smooth muscle (Lefkowitz *et al.*, 1984). Cyclic AMP is also thought to inhibit neurotransmitter release in both adrenergic nerve (Langer, 1977) and motor nerve endings (Hirsh *et al.*, 1990). Because prostaglandin E_1 (PGE_1) stimulates adenylate cyclase, thereby producing a rise in cyclic AMP levels (Colucci, 1986), the prejunctional inhibitory action of PGE_1 on non-adrenergic non-cholinergic (NANC) contraction (Aikawa *et al.*, 1990) may be mediated by elevation of intracellular cyclic AMP in sensory nerve endings.

To test this hypothesis, we investigated the effects of forskolin a potent adenylate cyclase stimulator (Seamon *et al.*, 1981), and those of dibutyryl cyclic AMP (db cyclic AMP) on NANC contractions evoked by electrical field stimulation (EFS) to determine whether cyclic AMP has an inhibitory action on sensory neurones. We have also investigated the effects of the β_2-adrenoceptor stimulant, procaterol, (Himori & Taira, 1977) and of theophylline (both of which are known to increase intracellular cyclic AMP in various tissues) on EFS-induced NANC contraction.

Methods

Male Hartley guinea-pigs (250–350 g) were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.). Lungs were removed rapidly and were immersed in Krebs-Henseleit solution equilibrated with 95% O_2 and 5% CO_2. The two main bronchi were dissected free of loose connective tissue, opened longitudinally along the anterior side, and cut into strips. These strips were mounted in organ baths containing 5 ml Krebs-Henseleit solution maintained at 37°C. Isometric tension was continuously measured with a force transducer (TB612-T, Nihon Kohden, Japan) connected to a pen recorder (HORIZ-8K, San-ei Co., Japan). Bronchial strips were placed between two rectangular platinum electrodes (6 × 40 mm) for EFS. The tissues were stretched initially to a tension of 1 g for 30 s and thereafter maintained for 60 min under a resting tension of 0.25 g, which was found to be optimal for measuring changes in tension. EFS (supramaximal voltage of 50 V, a frequency of 10 Hz and 1 ms duration) was applied for 20 s. All experiments were carried out in the presence of atropine (1 μM), indomethacin (1 μM), phentolamine (10 μM) and propranolol (1 μM) except that propranolol was not used in the studies on procaterol. The contractile responses induced under these conditions were completely abolished by tetrodotoxin (1 μM), confirming the neural nature of the response.

Experimental protocol

After the baseline response to EFS was obtained, we added increasing concentrations of forskolin (from 3 nM to 1 μM) and repeated the stimulation 5 min after the administration of each concentration of forskolin, when the effect of forskolin on the response to EFS reached its maximum. Forskolin was administered after the preceding contraction had returned to the baseline. Likewise, we tested the effects of procaterol (from 0.1 nM to 3 nM), theophylline (from 100 nM to 1 mM) and db cyclic AMP (1 mM) on EFS-induced contractions. We also tested the effects of forskolin (0.3 μM) on contractions induced by capsaicin (0.1 μM). After the baseline response to capsaicin (0.1 μM) was obtained, the bath was rinsed for 15 min. Five minutes after the administration of either forskolin (0.3 μM) or the vehicle for forskolin, capsaicin (0.1 μM) was administered again. We compared the second response to the first one (control).

The effects of forskolin (from 30 nM to 1 μM), procaterol (from 0.1 nM to 3 nM), theophylline (from 100 nM to 1 mM) and db cyclic AMP (1 mM) on contractile responses to exogenous substance P (SP) (1 μM, which produced a contractile response equivalent to that produced by EFS) were also evaluated. To construct concentration-response curves to SP, cumulative

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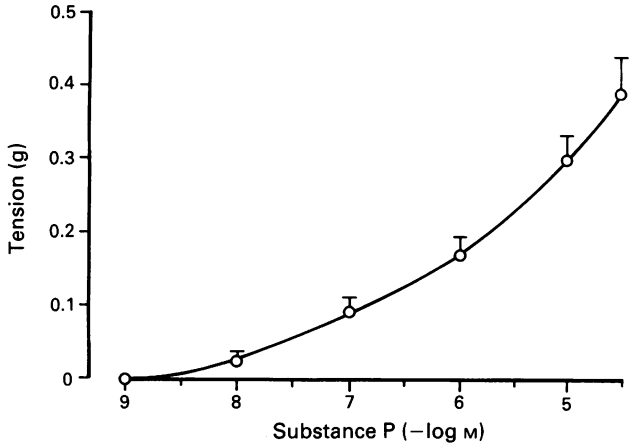

Figure 1 Concentration-response curves of guinea-pig bronchi to substance P. Data are shown as mean of values from 6 guinea-pigs; s.e.mean indicated by vertical bars.

concentration-response curves to SP were obtained with concentrations ranging from 10^{-9} M to 3×10^{-5} M. Each succeeding concentration of SP was added after the previous contraction had reached a plateau.

To test compounds acting via systems other than cyclic AMP, we studied the effects of sodium nitroprusside (1 nM to 1 μM) and cromakalim (0.1 nM to 100 nM) on EFS- and SP (1 μM)-induced contraction. To evaluate further whether ATP-sensitive potassium channels are coupled with forskolin-induced effects or dibutyryl guanosine 3':5'-cyclic monophosphate (db cyclic GMP) modified EFS-induced contractions, we tested either the effects of glibenclamide (10 μM) on forskolin (0.3 μM)-induced inhibitory actions or those of db cyclic GMP (10 μM to 1 mM) on contractions induced by EFS.

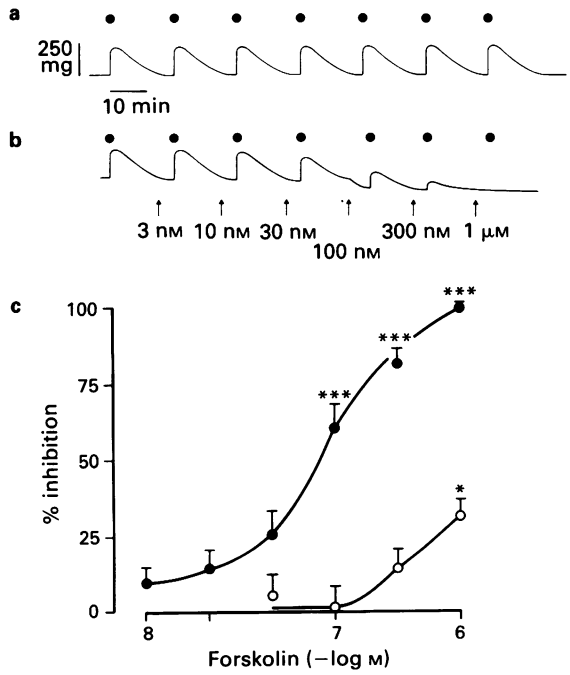

Figure 2 Typical tracing showing the contraction induced by electrical field stimulation (●) (EFS) in the presence of atropine (1 μM), propranolol (1 μM), phentolamine (10 μM) and indomethacin (1 μM) in guinea-pig bronchi *in vitro*. (a) Long-lasting contraction caused by EFS was reproducible several times and (b) inhibited by forskolin. (c) Forskolin inhibited EFS- (●) and substance P (1 μM)- (○) induced contractions concentration-dependently. Data are shown as mean of values from 6 guinea-pigs; s.e.mean indicated by vertical bars. Significant differences from control values are indicated by $*P < 0.05$ and $***P < 0.001$.

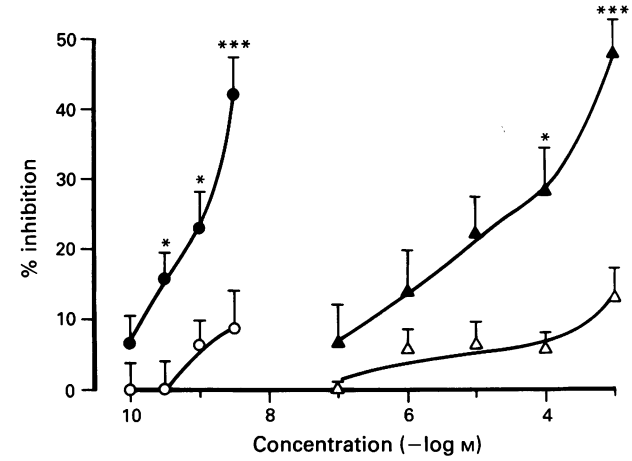

Figure 3 Concentration-response curves to procaterol (circles) and theophylline (triangles) on electrical field stimulation (EFS)-induced (closed symbols) and substance P-induced (open symbols) contractions. Data are shown as mean of values from 7 guinea-pigs; s.e.mean indicated by vertical bars. Significant differences from control values are indicated by $*P < 0.05$ and $***P < 0.001$.

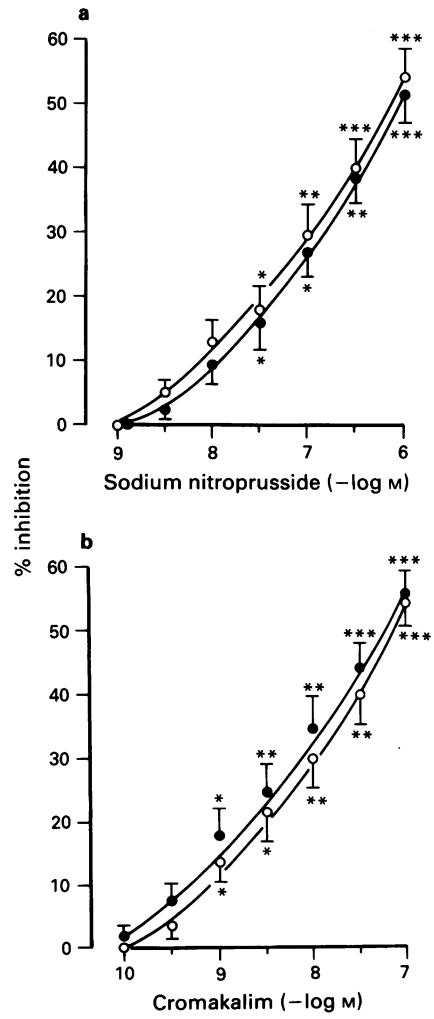

Figure 4 Concentration-response curves to sodium nitroprusside (a) and cromakalim (b) on electrical field stimulation (EFS)-induced (●) and substance P-induced (○) contractions. Data are shown as mean of values from 7 guinea-pigs; s.e.mean indictated by vertical bars. Significant differences from control values are indicated by $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

The following drugs were used: tetrodotoxin, forskolin, N^6-2'-0-dibutyryl adenosine 3':5'-cyclic monophosphate, N^2,2'-0-dibutyryl guanosine 3':5'-cyclic monophosphate, theophylline, atropine sulphate, capsaicin, glibenclamide (Sigma), and indomethacin, (±)-propranolol hydrochloride, sodium nitroprusside dehydrate (Wako Pure Chemicals), procaterol (a gift from Otsuka Pharmac. Co.), phentolamine (Ciba Geigy) and substance P (Peptide Institute. Inc.), Cromakalim was kindly given by the Beecham Pharmaceutical Co. Ltd.

Values are expressed as mean ± s.e.mean. Statistical analysis was performed by one way analysis of variance. Significance was accepted at $P < 0.05$.

Results

SP (0.01–30 μM) caused concentration-dependent contractions of guinea-pig bronchial strips. A concentration of 1 μM was used in all subsequent experiments. This concentration was submaximally effective (Figure 1).

EFS produced a long lasting NANC contraction that was reproducible on repeated stimulation within any given tissue (Figure 2a). The contractions produced by SP (1 μM) were also reproducible, and were not significantly different in amplitude from those elicited by EFS (165 ± 20 mg vs. 200 ± 28 mg, $n = 6$ in forskolin; 192 ± 24 mg vs. 180 ± 25 mg, $n = 6$ in db cyclic AMP; 188 ± 29 mg vs. 172 ± 26 mg, $n = 7$ in procaterol; 190 ± 24 mg vs. 198 ± 30 mg, $n = 7$ in theophylline; 175 ± 23 mg vs. 168 ± 20 mg, $n = 7$ in sodium nitroprusside; 174 ± 23 mg vs. 182 ± 27 mg, $n = 7$ in cromakalim) ($P > 0.20$). Forskolin decreased the amplitude of both EFS-induced (Figure 2b) and SP-induced contractions. The inhibition of each was concentration-dependent but only that of EFS achieved 50% inhibition ($-\log EC_{50} = 7.22 \pm 0.12$ M for EFS) (Figure 2c). Forskolin at 0.3 μM inhibited EFS-induced contractions (by 77.6 ± 5.3%; $P < 0.001$) but did not significantly alter the contractile response to exogenously administered SP (by 14.9 ± 5.0%; $P > 0.20$). At 1 μM, forskolin inhibited EFS-induced NANC contractions (by 100 ± 0.01%; $P < 0.001$) and SP-induced contraction (by 31.9 ± 5.2%; $P < 0.05$). Likewise, capsaicin (0.1 μM)-induced contractions (an average contraction = 186 ± 21 mg, $n = 5$) were significantly inhibited by forskolin (0.3 μM) (by 78.9 ± 10.9% with forskolin and 23.0 ± 7.4% in the presence of the vehicle for forskolin; $P < 0.01$, $n = 5$). Capsaicin (0.1 μM)-induced contractile responses were not significantly different from those by EFS ($P > 0.30$).

With the concentrations used in the present study, procaterol and theophylline did not alter the baseline tension but they reduced EFS-induced NANC contractions in a dose-dependent fashion (Figure 3). Procaterol at 3 nM and theophylline at 1 mM inhibited EFS-induced NANC contractions (by 41.9 ± 5.7% and 47.9 ± 4.8%, respectively; $P < 0.001$) but they had no significant effect on the contractile response to exogenously administered SP (by 8.3 ± 2.4% and 13.0 ± 4.9%, respectively; $P > 0.30$).

Db cyclic AMP (1 mM) completely inhibited EFS-induced contractions (by 100 ± 0.01%; $P < 0.01$, $n = 6$) without affecting SP-induced contractions (by 3.3 ± 5.5%; $P > 0.50$, $n = 6$).

Both sodium nitroprusside and cromakalim inhibited the EFS- and SP-induced contraction in a concentration-dependent fashion. However, the inhibitory effects of sodium nitroprusside and cromakalim on contractile responses did not differ between EFS and SP at any concentration ($P > 0.20$) (Figure 4). Likewise, glibenclamide (10 μM) failed to alter the forskolin (0.3 μM)-induced inhibitory effect on contractions induced by EFS (by 81.7 ± 2.3% and 78.1 ± 3.3% in the presence and absence of glibenclamide, respectively; $P > 0.30$, $n = 5$). Thus, the inhibitory action of forskolin on EFS-induced contractions was not affected by glibenclamide.

In contrast to db cyclic AMP, db cyclic GMP (from 10 μM to 1 mM) had no effect on EFS-induced contractions ($P > 0.30$, $n = 5$).

Discussion

Our study shows that forskolin, procaterol, theophylline and db cyclic AMP reduce the neurally-mediated constrictor response evoked by EFS in guinea-pig bronchi. This excitatory response resulting from nerve stimulation in the presence of atropine is NANC in nature, and probably due to the release of neuropeptides such as SP and neurokinin A from sensory nerve endings (Lundberg *et al.*, 1983).

In order to establish whether forskolin elicits its inhibitory effects prejunctionally on neuronal terminals or postjunctionally on the tachykinin receptors of the airway smooth muscle, we compared the effect that forskolin had on the contractile response to EFS and capsaicin with the effect that it had on the contractile response to SP. Forskolin, in concentrations up to 0.3 μM, inhibited NANC contractions without having an inhibitory effect on the contractile response to SP. Likewise, db cyclic AMP, procaterol and theophylline reduced NANC contractions without a significant effect on similar contractions induced by SP.

The facilitation of neurotransmitter release triggered by the activation of presynaptic β-adrenoceptors is mediated through an increase in the level of cyclic AMP in adrenergic nerves (Langer, 1977). Likewise, forskolin activates adenylate cyclase in the presynaptic adrenergic nerve endings, resulting in the facilitation of neurotransmitter release without changing the vascular smooth muscle cell function (Ishikawa & Sperelakis, 1989). In contrast, in the airways, β-adrenoceptor stimulants have been reported to reduce cholinergic neurotransmission (Danser *et al.*, 1987; Ito, 1988) as well as NANC contractions (Kamikawa & Shimo, 1990). β-Adrenoceptors are linked to the adenylate cyclase system (Lefkowitz *et al.*, 1984), and xanthines such as theophylline have an inhibitory effect on cyclic nucleotide phosphodiesterase (Katsuki & Murad, 1977), resulting in increases in cyclic AMP in various tissues. Therefore, forskolin, procaterol and theophylline may increase cyclic AMP levels in sensory nerve endings, resulting in the inhibition of NANC contractions via pre-junctional mechanisms, an effect mimicked by the stable cyclic AMP analogue, db cyclic AMP.

Sodium nitroprusside and cromakalim relax smooth muscles by activating guanylate cyclase and elevating cyclic GMP levels (Ignarro & Kadowitz, 1985) and by enhancing potassium efflux from the cell and thereby hyperpolarizing the plasma membrane (Allen *et al.*, 1986; Hamilton *et al.*, 1986), respectively. However, while sodium nitroprusside and cromakalim both inhibited NANC contractions, they were equally potent in inhibiting SP-induced contractions, suggesting that prejunctional inhibition of NANC contractions is not mediated via the cyclic GMP system or cromakalim-sensitive potassium channels. This idea is also supported by the findings that db cyclic GMP had no effect on NANC contractions and glibenclamide, an inhibitor of ATP-sensitive potassium channels (Cook & Hales, 1984) failed to inhibit forskolin-induced inhibition of NANC contractions.

Our study suggests that the elevation of cyclic AMP in nerve endings inhibits the release of sensory neuropeptides from nerves. Therefore, it may be possible that cyclic AMP is a second messenger in the inhibitory action of drugs on sensory nerves.

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The effect of lipopolysaccharide, interleukin-1 and tumour necrosis factor on the hepatic accumulation of 5-hydroxytryptamine and platelets in the mouse

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1 Injection of lipopolysaccharide (LPS; 0.5–500 $\mu g\,kg^{-1}$) into mice induced a dose-dependent, slowly developing increase in hepatic content of 5-hydroxytryptamine (5-HT). This sustained increase could not be attributed to an LPS-induced alteration of the pharmacokinetic handling of 5-HT by stimulation of its uptake or inhibition of its degradation.

2 Regional differences were apparent in the tissue content of histamine and 5-HT between mast cell-deficient (W/W^v) and normal (+/+) mice. LPS administration (0.5 $mg\,kg^{-1}$) gave comparable increases in the hepatic level of 5-HT in mast cell-deficient and normal mice.

3 Reserpine pretreatment (1 $mg\,kg^{-1}$) selectively reduced 5-HT levels in the blood, spleen, liver, brain and lung of normal mice. Prior treatment with this agent also abolished the LPS (0.5 $mg\,kg^{-1}$)-induced hepatic accumulation of 5-HT.

4 Accumulation of 5-HT in the liver by LPS (0.1 $mg\,kg^{-1}$) was temporally associated with both a fall in the levels of circulating platelets, and a reduction in the concentration of 5-HT in the blood. The LPS dose-dependent (0.5–500 $\mu g\,kg^{-1}$) increase in hepatic 5-HT content was associated with a similar dose-dependent reduction in the circulating levels of 5-HT.

5 Interleukin-1, α and β (10 $\mu g\,kg^{-1}$) and tumour necrosis factor α (TNFα) (1 $mg\,kg^{-1}$) significantly enhanced the accumulation of 5-HT within the liver. Administration of TNFα (10 $\mu g\,kg^{-1}$) potentiated the increase in hepatic 5-HT content seen with IL-1β (10 $\mu g\,kg^{-1}$).

6 Electron microscopy revealed numerous platelets in the sinusoidal and perisinusoidal Disse spaces within the liver, in animals pretreated with LPS (0.1 $mg\,kg^{-1}$). The platelets retained their intact structure and showed no evidence of degranulation.

7 These data suggest that the LPS and cytokine-induced mobilization of 5-HT in the liver is associated with the hepatic translocation of platelets. This migration appears to be independent of platelet aggregation.

Keywords: Lipopolysaccharide; platelet; interleukin-1; tumour necrosis factor; 5-hydroxytryptamine; liver; endotoxin

Introduction

In the pathogenesis of septic shock, vasoactive amines such as histamine and 5-hydroxytryptamine (5-HT) have been implicated as endogenous mediators contributing to liver dysfunction (Halpern *et al.*, 1963; Iff & Vas, 1966). Previous data have demonstrated that injection of lipopolysaccharides (LPS) into mice induces a rise in both histamine and 5-HT levels within the liver (Endo, 1982, 1983a). The LPS-induced increase in hepatic histamine levels has been attributed to an induction of histidine decarboxylase, an enzyme involved in its synthesis. This induction has also been reported in other tissues such as the lung, spleen and bone marrow (Endo, 1982; 1983b; 1989; Endo *et al.*, 1986). However, the mechanisms responsible for the accumulation of 5-HT within the liver remain to be established. It is known that the rise in 5-HT observed after LPS injection occurs after a time lag of 1 h, reaches a maximum at 4.5–6 h and returns to baseline within 2 days (Endo 1983a). This rise is extremely sensitive to LPS, significant accumulation occurring at concentrations as low as 0.1 $\mu g\,kg^{-1}$ (Endo, 1984). Unlike histamine, the increase in 5-HT appears to be relatively specific for the liver and does not occur in other tissues such as the lung, spleen, intestine or kidney (Endo, 1983a). In addition, the accumulation of 5-HT is not attributable to an enhancement of its synthesis (Endo, 1987), which suggests this amine is transported into the liver from the surrounding tissues.

The aim of the present study was to investigate the mechanisms and identify the possible sources responsible for the hepatic accumulation of 5-HT. Mast cells in rodents contain and release 5-HT in addition to histamine (Moran *et al.*, 1962; Prouvost-Danon *et al.*, 1966). Thus, we used mice known to be deficient in mast cells (Kitamura *et al.*, 1978; Suda *et al.*, 1985) to ascertain the role played by these cells in the mobilization of 5-HT into the liver. Recently, it has been suggested that cytokines released by macrophages may play a role in the LPS-induced rise in hepatic 5-HT (Endo *et al.*, 1985; Endo, 1991) and we have also therefore investigated the effects of recombinant interleukin-1 (IL-1, α and β) and tumour necrosis factor α (TNFα).

Methods

Animals

Male, 6 week old WBB6F1 (B-W/+ xC57BL/6-W^v/+)-W/W^v (mast cell-deficient), WBB6F1-+/+ (normal litter mates of W/W^v) and ddY mice were obtained from Shizuoka Agricultural Association, Japan.

Determination of 5 hydroxytryptamine and histamine

To avoid the rapid degradation of 5-HT in the blood, preweighed tubes containing 3 ml of 0.4 M $HClO_4$, 2 mM EDTA and 0.1% cysteine-HCl were used to collect 5 drops of blood, which was then rapidly mixed and weighed. The tubes were then quickly transferred into a dry ice/ethanol bath for freezing, where they were kept for up to 2 days. For the estimation of tissue content of 5-HT and histamine, the respective organs

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were rapidly removed, weighed and frozen in dry ice and ethanol. 5-HT was measured according to a previously described method (Tadano *et al.*, 1980) modified to allow the simultaneous measurement of many samples. Briefly, 5-HT within the tissues and blood was extracted in 0.4 M HClO4 containing 2 mM EDTA and 0.1% cysteine-HCl in an Ultra Turrax homogenizer. The extract was neutralized to approximately pH 4.5 with 2 M KOH before 1 ml was applied to a phosphylated-cellulose packed column and equilibrated with 0.01 M phosphate buffer (pH 6.2). The column was washed with 4 ml of the phosphate buffer and 0.8 ml of 0.1 M HCl containing 0.1% cysteine-HCl, before the 5-HT was eluted by another 1.5 ml of 0.1 M HCl containing 0.1% cysteine-HCl. The 5-HT content of the elutate was determined fluorometrically.

Histamine content was determined as described previously (Endo, 1983c).

Estimation of platelet count

Approximately 5 drops of blood from stunned, decapitated mice were collected into a preweighed test tube containing 0.5 ml of 4 mM EDTA in 0.01 M phosphate buffered saline. The tube was weighed and the number of platelets was automatically counted in a Celltac MEK-4150 cell counter.

Effect of lipopolysaccharide on 5-hydroxytryptamine synthesis and degradation

To examine whether LPS administration alters the pharmacokinetic handling of 5-HT, normal mice were injected with either saline or LPS ($0.5\,mg\,kg^{-1}$) i.v. and 4 h later various doses of 5-HT ($5–20\,mg\,kg^{-1}$) were given i.p. After 2 h, the mice were killed and their livers analysed for differences in 5-HT content. The effect of pargyline, an irreversible inhibitor of monoamine oxidase, was examined on the LPS-induced rise in hepatic content of 5-HT. Saline or pargyline ($100\,mg\,kg^{-1}$) were given by intraperitoneal injection, followed 1 h later by various doses of LPS ($0.1–10\,\mu g\,kg^{-1}$) i.p. The mice were killed 4 h later and their livers analysed for 5-HT content.

Examination of possible sources of 5-hydroxytryptamine

The 5-HT content of a variety of tissues was examined in mast cell-deficient (W/W^v) and normal (+/+) mice to assess the role of the mast cell as a possible source of 5-HT by examining its tissue distribution. To ascertain whether the LPS-induced rise in 5-HT was attributable to an influx of mast cells, LPS was administered ($0.5\,mg\,kg^{-1}$, i.v.) to mast cell-deficient and normal litter mate controls. The mice were killed 4.5 h later and their livers analysed for 5-HT content.

Effect of reserpine

To assess the relative levels of 5-HT in organs which could be mobilized by LPS, a variety of tissues were examined in ddY mice, 24 h after saline or reserpine ($1\,mg\,kg^{-1}$) pretreatment. To examine whether tissues depleted of their 5-HT content contributed to the LPS-induced liver accumulation, mice pretreated with either saline or reserpine ($1\,mg\,kg^{-1}$) were injected 24 h later with either saline or LPS ($0.5\,mg\,kg^{-1}$, i.v.). After 4.5 h the mice were killed and their livers analysed for 5-HT content.

Relationship between hepatic and circulating levels of 5 hydroxytryptamine

Various doses of LPS ($0.5–500\,\mu g\,kg^{-1}$) were injected i.v. into normal mice, to examine the dose-dependent relationship between circulating and hepatic levels of 5-HT. The mice were killed 4.5 h later and the respective tissues analysed for 5-HT content. Assessment of the temporal relationship between hepatic and circulating levels of 5-HT was performed as follows. At various time intervals after the injection of $0.1\,mg\,kg^{-1}$ LPS, ddY mice were killed and their blood and livers analysed for 5-HT content. To calculate the total amount of 5-HT in the blood, its volume was calculated according to the method of Wish *et al.* (1950).

Lipopolysaccharide-induced thrombocytopenia

Since platelets are recognized as blood elements containing significant amounts of 5-HT, the temporal relationship between the levels of circulating platelets and hepatic 5-HT content was examined in normal mice. The mice were killed at various times after LPS administration ($0.1\,mg\,kg^{-1}$) when platelet counts and hepatic 5-HT content were measured.

Effect of cytokine administration

IL-1α, IL-1β and TNFα at doses of $10\,\mu g\,kg^{-1}$ or $1\,mg\,kg^{-1}$, alone and in combination, were injected intraperitoneally into ddY mice. After 4 h their liver content of 5-HT was measured and cellular structure was examined by electron microscopy.

Electron microscopy

For visualization of the hepatic cellular events, 4.5 h after saline or LPS ($0.1\,mg\,kg^{-1}$) injection i.v., the livers from stunned, decapitated mice were rapidly removed and chopped into small pieces. The specimens were fixed with a mixture of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cocodylate buffer, pH 7.4, post-fixed with 1% aqueous OsO_4, and stained *en bloc* with 1% aqueous uranyl acetate for 1 h at 4°C. The samples were dehydrated with a graded series of ethanols, passed through propylene oxide and embedded in EPON 812. Ultra-thin sections mounted on copper grids were stained with uranyl acetate and lead citrate and examined with a Hitachi H-700 transmission electron microscope. For the visualization of possible fibrin disposition, the staining procedure according to Carstairs (1965) was followed.

Materials

Lipopolysaccharide (*E. coli*; 055B5) was purchased from Difco Lab., Detroit, U.S.A.; reserpine (Apoplon) from Daiichi Seiyaku Co. Ltd., Tokyo, Japan and pargyline hydrochloride from Sigma Chemical Co., St. Louis, U.S.A. Recombinant human interleukin-1α (IL-1α) and recombinant human tumour necrosis factor α (TNFα) were provided by Dainippon Pharmaceutical Co., Osaka, Japan, prepared according to Furutani *et al.*, (1985) and Yamada *et al.* (1985). Recombinant human IL-1β was donated by the Ohtsuka Pharmaceutical Co., Tokushima, Japan and prepared according to Kikumoto *et al.* (1987). On a SDS-polyacrylamide gel, electrophoresis of each of the cytokines gave a single band. The contaminants of LPS in the preparations of IL-1α, IL-1β and TNFα were less than 0.02, 0.1 and 0.04 ng per mg protein respectively (*Limulus* test). The remainder of the laboratory reagents were obtained from Wako Pure Chemical Industries Ltd., Tokyo, Japan. The drugs and cytokines were dissolved in sterile saline and injected in a volume of 0.1 ml per 10 g body weight.

Statistical analysis

Experimental values shown in the text, figures and tables are given as mean ± standard deviation. The statistical significance of these differences were analysed by Student's unpaired t test and P values less than 0.05 were considered to be significant.

Results

Lipopolysaccharide-induced alteration of 5-hydroxytryptamine uptake/degradation

There was no significant difference between saline or LPS ($0.5\,mg\,kg^{-1}$) pretreated mice in the amount of 5-HT that was

increased in the liver by the prior administration of 5-HT (data not shown). In addition, there was no effect of pargyline (100 mg kg^{-1}) on the LPS-induced accumulation of 5-HT in the liver.

Examination of possible sources of 5-hydroxytryptamine

The histamine and 5-HT content of various tissues in mast cell-deficient mice and their normal litter mates is shown in Table 1. In mast cell-deficient mice, histamine levels were significantly lower in the spleen, lung, thymus and skin; in contrast, only the cutaneous 5-HT content was found to be significantly lower in these animals ($P < 0.01$).

Lipopolysaccharide-induced accumulation of 5-hydroxytryptamine in the liver of mast cell-deficient mice

Accumulation of hepatic 5-HT following injection of LPS into normal and mast cell-deficient mice is shown in Figure 1. LPS (0.5 mg kg^{-1}) induced a significant accumulation of 5-HT in the liver of normal and mast cell deficient mice ($P < 0.01$).

Effect of reserpine pretreatment

Reserpine pretreatment (1 mg kg^{-1}, i.p.) administered 24 h before tissue 5-HT analysis, revealed that various organs, noteably the brain, liver, spleen, lung, thymus and blood, but not intestine, had been significantly depleted of their 5-HT content (Table 2). Administration of LPS (0.5 mg kg^{-1}), 24 h after reserpine pretreatment failed to induce the rise in hepatic 5-HT seen in saline pretreated mice (Figure 2). Prior administration of reserpine depleted circulating levels of 5-HT, which were unaffected by the subsequent addition of LPS (Figure 2).

Table 1 Histamine and 5-hydroxytryptamine (5-HT) levels in normal (+/+) and mast cell deficient (W/W^v) mice

	Histamine (nmol g^{-1})		*5-HT* (nmol g^{-1})	
	+/+	W/W^v	+/+	W/W^v
Brain	0.62 ± 0.02	0.56 ± 0.02	3.7 ± 0.3	3.8 ± 0.3
Liver	0.79 ± 0.04	0.69 ± 0.03	2.3 ± 0.1	2.6 ± 0.2
Spleen	6.0 ± 0.6	0.9 ± 0.1*	88 ± 11	86 ± 20
Lung	2.3 ± 0.2	0.3 ± 0.1*	5.7 ± 1.8	5.5 ± 1.9
Thymus	10.0 ± 2.0	0.8 ± 0.3*	1.3 ± 0.1	1.4 ± 0.2
Skin	80.0 ± 7.0	2.0 ± 0.4*	0.9 ± 0.2	<0.3*
Intestine	5.0 ± 0.8	4.3 ± 0.5	46 ± 5	41 ± 4
Blood	0.29 ± 0.05	0.21 ± 0.04	19 ± 2	22 ± 2

* $P < 0.01$. Values represent the mean and standard deviation of results from 4 mice.

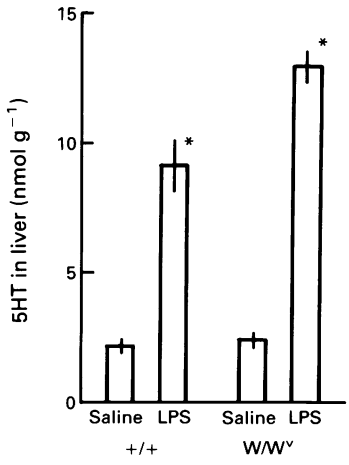

Figure 1 Lipopolysaccharide (LPS, 0.5 mg kg^{-1})-induced 5-hydroxytryptamine (5-HT) accumulation within the liver of normal (+/+) and mast cell deficient (W/W^v) mice. Each column represents the mean and vertical lines indicate the standard deviation of results from 4 animals. *$P < 0.01$.

Table 2 5-Hydroxytryptamine (5-HT) levels in saline and reserpine-pretreated ddY mice

	5-HT (nmol g^{-1})	
	Saline	*Reserpine-treated*
Brain	3.4 ± 0.1	2.0 ± 0.4*
Liver	1.9 ± 0.1	0.2 ± 0.1*
Spleen	50 ± 6	1.3 ± 0.2*
Lung	6.5 ± 0.8	0.3 ± 0.1*
Thymus	1.0 ± 0.1	0.3 ± 0.1*
Muscle	0.5 ± 0.1	0.4 ± 0.1
Skin	0.8 ± 0.2	0.5 ± 0.1
Ear	1.4 ± 0.3	1.0 ± 0.2
Intestine	50 ± 6	52 ± 16
Blood	14 ± 2	0.3 ± 0.1*

* $P < 0.01$. Values represent the mean and standard deviation of results from 4 mice.

Dose and time-dependent lipopolysaccharide-induced mobilization of 5-hydroxytryptamine

LPS (0.5–500 μg kg^{-1}) injection induced a dose-dependent rise in hepatic 5-HT content within the liver. This mobilization was maximal between 5–50 μg kg^{-1} and was reflected in a dose-dependent reduction in circulating levels of 5-HT (Figure 3). Measurement of the kinetics underlying the translocation of 5-HT revealed a significant rise in the hepatic content 2 h after the LPS (0.1 mg kg^{-1}) was administered (Figure 4). Examination of the kinetics of the circulating levels of 5-HT following LPS (0.1 mg kg^{-1}) injection showed a significant depletion within 1 h (Figure 4), circulating and hepatic levels

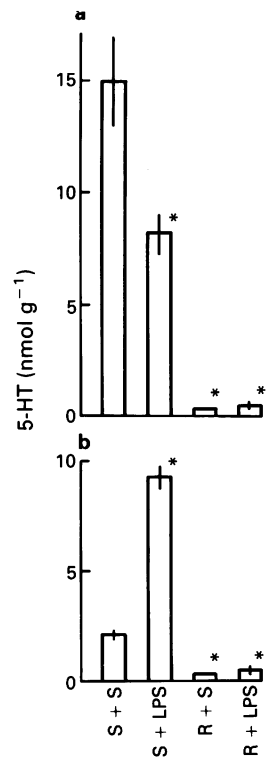

Figure 2 The effect of lipopolysaccharide (LPS, 0.5 mg kg^{-1}) on 5-hydroxytryptamine (5-HT) levels in the blood (a) and liver (b) of saline (S) or reserpine (R) pretreated ddY mice. Each column represents the mean and vertical lines indicate the standard deviation of results from 4 animals. *$P < 0.01$.

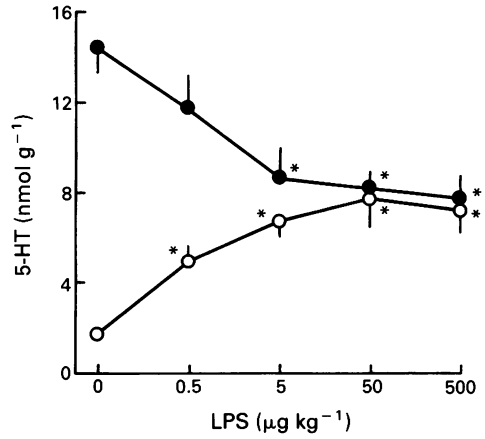

Figure 3 Dose dependent relationship of lipopolysaccharide (LPS, 0.5–500 $\mu g\,kg^{-1}$) administration on the level of 5-hydroxytryptamine (5-HT) in the blood (●) and liver (○) in ddY mice. Each point represents the mean and vertical lines indicate standard deviation of results from 4 animals. $^*P < 0.01$.

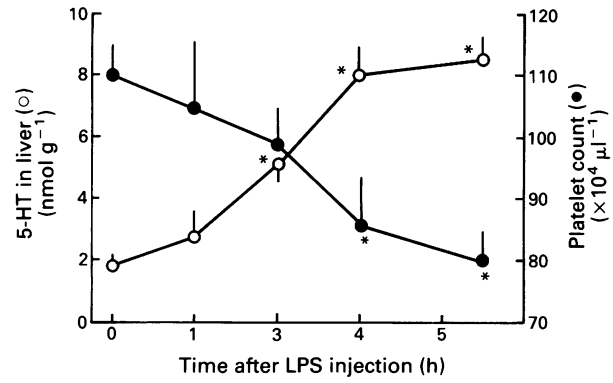

Figure 5 Temporal relationship between the increase in 5-hydroxytryptamine (5-HT) content in the liver (○) and the decrease in platelet count in the blood (●) following lipopolysaccharide (LPS, 0.1 $mg\,kg^{-1}$) administration. Each point represents the mean and vertical lines indicate the standard deviation of results from 4 normal mice. $^*P < 0.01$.

plateaued within 4.5 h. An analysis of the amount of 5-HT lost from the blood 4.5 h after LPS administration indicated that approximately 70% accumulated within the liver (amount lost from the blood, 13.1 nmol; amount accumulated within the liver, 9.4 nmol: $n = 8$).

Lipopolysaccharide-induced thrombocytopenia

The temporal relationship between circulating levels of platelets and hepatic accumulation of 5-HT stimulated by LPS (0.1 $mg\,kg^{-1}$) is shown in Figure 5. A close reciprocal relationship was evident, significant liver accumulation of 5-HT and platelet depletion occuring within 3 and 4 h of LPS injection, respectively. Maximal hepatic accumulation of 5-HT and thrombocytopenia were evident at 5.5 h.

Effects of interleukin-1α, -1β and tumour necrosis factor α

As demonstrated in Figure 6, IL-1α, IL-1β (10 $\mu g\,kg^{-1}$) and TNFα (1 $mg\,kg^{-1}$) all induced a rise in the hepatic accumulation of 5-HT. These effects showed a similar time course to that observed on LPS administration (data not shown). The concurrent administration of IL-1α and IL-1β produced an accumulation of 5-HT within the liver which was equal to the administration of either cytokine alone. However, TNFα potentiated the effects of both IL-1α and IL-β. The combination of TNFα (10 $\mu g\,kg^{-1}$) and IL-1 (α or β; 10 $\mu g\,kg^{-1}$) resulted in an accumulation of 5-HT that corresponded to the level induced by 1 $mg\,kg^{-1}$ TNFα, or to the maximal level obtained on LPS injection.

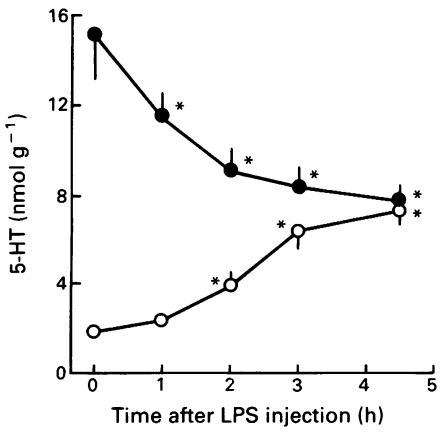

Figure 4 Temporal relationship between the 5-hydroxytryptamine (5-HT) content in the blood (●) and liver (○) after lipopolysaccharide (LPS, 0.1 $mg\,kg^{-1}$) administration in ddY mice. Each point represents the mean and vertical lines indicate the standard deviation of results from 4 animals. $^*P < 0.01$.

Electron microscopy

A notable feature of the livers taken from LPS-treated mice was the presence of numerous platelets, located in the sinusoidal and perisinusoidal spaces between hepatocytes and endothelial cells. An estimation of the platelet numbers in 5 objective fields gave values of 0.8 ± 0.7 for normal liver and 5.8 ± 2.1 for the livers of LPS-pretreated mice. The platelets seen within the liver still retained the characteristic dense granules and microtubules indicating that these cells had not undergone degranulation.

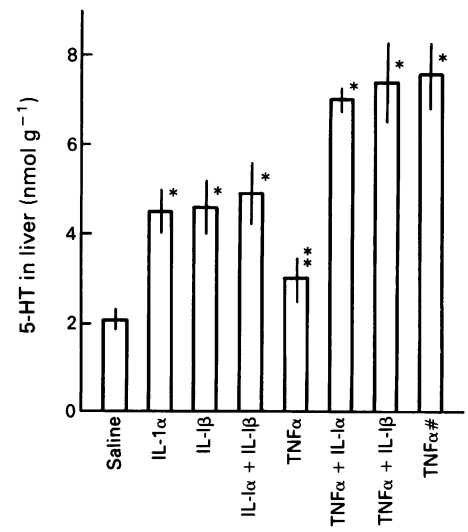

Figure 6 The effect of various cytokines, alone and in combination, on hepatic 5-hydroxytryptamine (5-HT) accumulation in ddY mice. Interleukin (IL) 1α and 1β were administered at 10 $\mu g\,kg^{-1}$, i.p., tumour necrosis factor α (TNFα) was administered at 10 $\mu g\,kg^{-1}$ and 1 $mg\,kg^{-1}$, i.p. (#). Each column represents the mean and vertical lines indicate the standard deviation of results form 4 animals. $^*P < 0.01$; $^{**}P < 0.1$.

Additionally, there was no apparent deposition of fibrin in the perisinusoidal spaces as detected by Carstairs' staining method. Figure 7a displays the typical lack of interaction between a platelet and a Kupffer's cell (KC) in a normal liver. In contrast, the majority of the platelets in the sinusoidal spaces of the liver in LPS-treated mice (Figure 7b–d) were surrounded by well developed cell processes from Kupffer's cells and there were electron dense sites between platelets and Kupffer's cells. All of the KC in the specimens from LPS-treated mice were Mac-1 positive, indicating that they had been previously activated.

In the liver of LPS-treated mice, many more polymorphonuclear neutrophils were observed in the sinusoidal rather than the perisinusoidal spaces. However, there was no obvious attachment of the platelets to the neutrophils. A similar profile of cell accumulation was observed with the combination of IL-1α and TNFα (both 10 $\mu g\,kg^{-1}$).

Discussion

These studies, whilst confirming previous results showing LPS-induced accumulation of 5-HT in the liver (Endo, 1982; 1983a), have attempted to investigate the underlying mechanisms. Previous data have demonstrated that the increase in 5-HT within the liver cannot be attributed to the stimulation

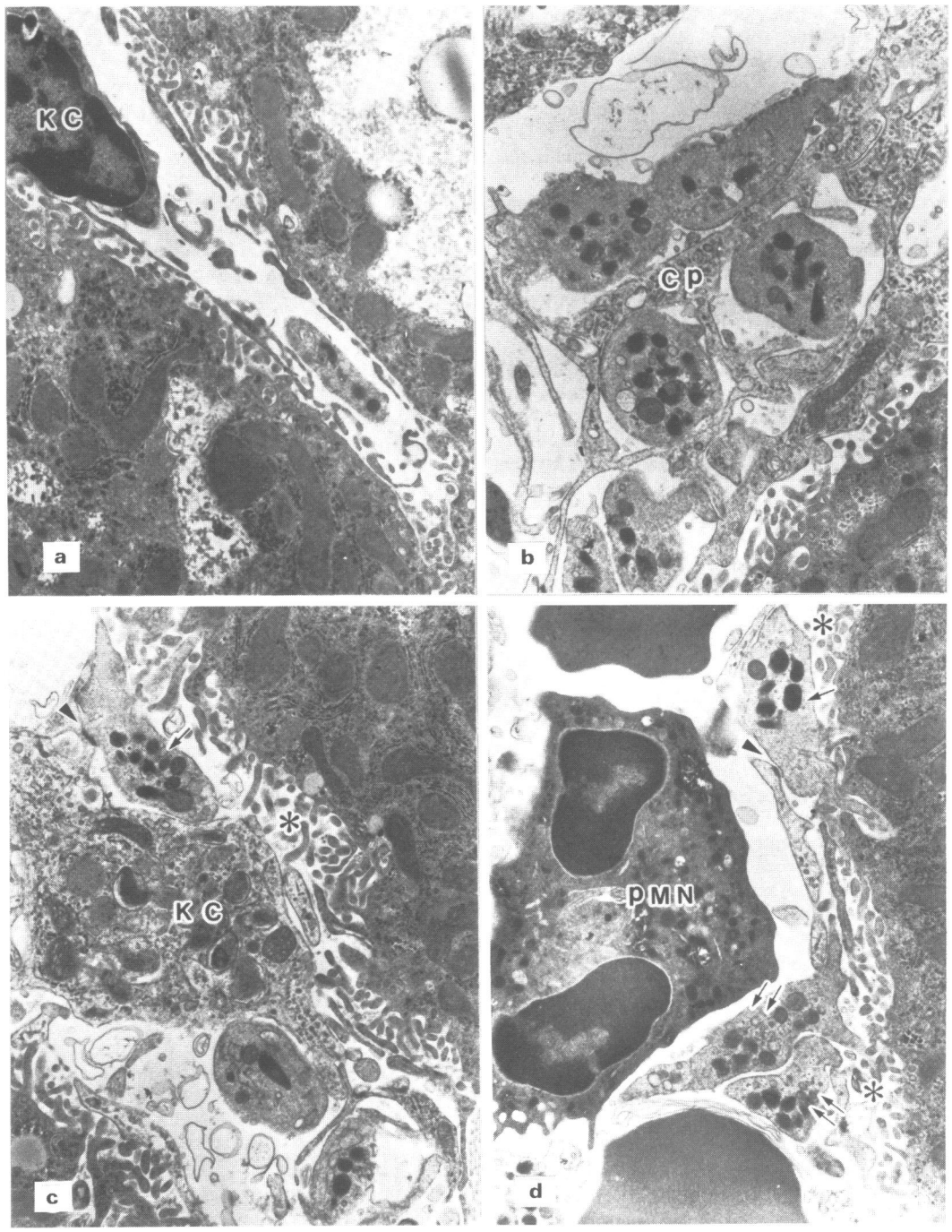

Figure 7 Electron micrographs of hepatic sinusoidal spaces in normal (a) and lipopolysaccharide (LPS)-treated (b–d) ddY mice. (a) A platelet and a Kupffer's cell (KC) are seen in the sinusoidal space. (b) Platelets in the sinusoidal space are surrounded by the cell processes (CP) of a Kupffer's cell. (c) A platelet (arrow) is localized in a space of Disse (*). There is an electron dense site (arrow head) suggesting intercellular contact. (d) Two platelets (arrows) are penetrating into a space of Disse. A platelet (an arrow) is seen in a space of Disse, and there is a presumed cell contact site (arrow head) between the Kupffer's cell and the platelet, and an extension of the platelet pseudopod into the hepatocyte. A polymorphonuclear neutrophil (PMN) is also present in the sinusoidal space.

of its synthesis (Endo, 1987). Furthermore, no evidence was obtained in this study to suggest that LPS enhanced stimulation of 5-HT uptake into the liver or inhibited its degradation. As such, the hepatic increase observed is postulated to be the result of mobilization from 5-HT containing tissues.

The histamine content in various tissues was shown to be lower in mast cell-deficient (W/W^v) than normal litter mate control (+/+) mice (Yamatodani *et al.*, 1982; present study). However, there is little difference in these two groups of mice with respect to tissue 5-HT content except for the skin. This may suggest that only cutaneous mast cells contain significant amounts of 5-HT. Since mast cell deficient and normal mice have comparable increases in liver 5-HT content following LPS administration, it is concluded that mast cells *per se* do not contribute to the 5-HT accumulation observed.

Despite containing the highest concentration of 5-HT per gram of tissue and its almost total depletion by reserpine-pretreatment, the spleen is not thought to be responsible for the mobilization of 5-HT into the liver, because in some experiments the 5-HT level in the spleen was enhanced by LPS (Endo, 1983a). The intestine is a rich source of 5-HT, however, it is not depleted by reserpine-pretreatment. If the intestine is the source of 5-HT for the liver, possibly via the connecting portal system, then reserpine pretreatment might be expected to have no effect on the LPS-induced stimulation of hepatic 5-HT accumulation. Thus, the intestine is probably not responsible for the increase in 5-HT observed.

From the inverse relationship between hepatic and circulating 5-HT levels, it is suggested that 5-HT is mobilized, following LPS administration, from the blood into the liver. Furthermore, reserpine pretreatment caused an almost total depletion of 5-HT from the blood and prevented the LPS-induced rise in 5-HT within the liver. From the data it can be calculated that 70% of the 5-HT lost from the blood accumulates within the liver. This could be explained simply by the engorgement or congestion of the liver with blood; however, no increase in total liver mass was observed on LPS administration (data not shown). From the time-dependent studies examining the levels of circulating platelets and 5-HT accumulation within the liver, the reciprocal relationship was highly suggestive of hepatic platelet accumulation. Furthermore, the total amount of 5-HT accumulated within the liver expressed as a percentage of the total blood content (32%) is almost identical to the amount of platelets lost from the circulation (30%). Whether these platelets represent a separate subpopulation of cells able to accumulate within the liver in response to LPS or cytokines, without degranulating, remains to be established. Although there is little information concerning the effect of LPS on platelets, it is known that 5-HT is released within 1 min of LPS injection from rabbit platelets (Davies *et al.*, 1963). This acute release of 5-HT may be responsible for the loss of circulating 5-HT from the blood within 1 h in the absence of significant hepatic platelet accumulation. This would also help explain why only 70% of the 5-HT lost from the circulation accumulates in the liver.

Examination of liver sections confirmed the presence of platelets within the sinusoidal and perisinusoidal spaces. Many platelets in sinusoidal spaces were surrounded by cell processes from Kupffer's cells, and there were electron dense sites between platelets and Kupffer's cells, which indicate that cellular interactions may play an important role in the migration of platelets from the sinusoidal into the Disse spaces.

In addition to LPS, various types of inflammatory agents also promote the accumulation of 5-HT within the liver of the mouse (Endo, 1983a; 1984). LPS is known to stimulate macrophages to produce cytokines such as IL-1α, IL-1β and TNFα (Oppenheim *et al.*, 1986; Old, 1987). In the present study, it was shown that each of these cytokines was able to induce accumulation of 5-HT within the liver. Endo (1991) showed that the effects of IL-1 (α and β) were significant at doses as little as $0.1\,\mu g\,kg^{-1}$ with maximum accumulation occurring at $10\,\mu g\,kg^{-1}$. Since no additional increase in 5-HT levels within the liver was observed with a combination of these two cytokines, it may be concluded that the respective pathways are closely interlinked if not identical. Moreover, there was an apparant synergism between IL-1 (α and β) and TNFα, corresponding to the maximum level induced by LPS. These results suggest that the increase in platelet and 5-HT content in the liver by LPS may be mediated via a combination of these cytokines.

Injection of LPS into experimental animals with immune adherence positive platelets has previously been shown to induce a biphasic response in the numbers of circulating platelets (Ulevitch *et al.*, 1975; Morrison & Ulevitch, 1978). The acute thrombocytopenia, occurring within several minutes is known to be accompanied by the release of 5-HT and histamine (Davies *et al.*, 1966) and platelet aggregation (Davies, 1966). However, since abrogation of this response does not prevent disseminated intravascular coagulation (DIC), it is the slowly developing phase which is believed to be imporant in the DIC induced by LPS or Gram-negative sepsis (Ulevitch *et al.*, 1975; 1978; Mathison & Ulevitch, 1981). This delayed thrombocytopenia is independent of complement (Ulevitch *et al.*, 1975; 1978; Morrison & Ulevitch, 1978) and occurs even in primates which lack immune adherence sites on their platelets (Ulevitch *et al.*, 1978). However, the role of platelets in this phenomenon has not been fully investigated. This study has proposed that the slowly developing thrombocytopenia following LPS administration in the mouse is associated with the accumulation of platelets into the sinusoidal and Disse spaces of the liver.

Due to its central role in thermal regulation, gluconeogenesis and in the production of clotting factors, the liver is intrinsically involved in the symptomology of Gram negative sepsis, i.e. fever, hypoglycaemia and stimulation of the coagulation system resulting in thrombocytopenia or DIC, hypotension or shock and death. Furthermore it has been previously shown that D-galactosamine, an agent which attacks the liver in a similar manner to human viral hepatitis, in combination with a small amount of LPS induces a lethal, fulminant hepatitis (Galanos *et al.*, 1979; Tiegs *et al.*, 1989). It has already been established that the toxic principle of Gram negative bacteria is LPS or endotoxin and that the actions of LPS may be mediated via IL-1 or TNFα (Oppenheim *et al.*, 1986; Old, 1987; Lehmann *et al.*, 1987; Wallach *et al.*, 1988, Mathison *et al.*, 1988; Endo, 1989; 1991). It is also known that platelets contain many factors such as hepatocyte growth factor, platelet-derived growth factor, and transforming growth factor β (Nakamura *et al.*, 1986) which may be important in the physiological defence system against bacterial invasion. Although the mechanisms for platelet accumulation in the liver remain to be clarified, the present findings suggest a new role for these cellular elements in the pathophysiological response to LPS.

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Pharmacological and histological examinations of regional differences of guinea-pig lung: a role of pleural surface smooth muscle in lung strip contraction

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1 Parenchymal lung strip preparations have been widely used as an *in vitro* model of peripheral airway smooth muscle. The present study examined functional responses of 4 consecutive guinea-pig lung parenchymal strips isolated from the central region (segment 1) to the distal edge (segment 4) of the lower lung lobe. The middle two segments were designated as segments 2 and 3.

2 Lung segments 1 and 4 exhibited significantly greater contraction than the other 2 segments to KCl when responses were expressed as mg force per mg tissue weight. Contractile responses to bronchospastic agents including histamine, carbachol, endothelin-1, leukotrienes (LT) B_4 and D_4, and the thromboxane A_2-mimetic U46619 demonstrated no significant difference in EC_{50} values among the 4 lung segments.

3 Contractile responses of segments 1 and 4 to antigen-challenge (ovalbumin), ionophore A23187 and substance P were significantly greater than the other 2 segments with respect to either sensitivity or maximum responsiveness.

4 U46619-induced contractions of the 4 lung segments were relaxed in similar manner by papaverine and theophylline up to 100%, salbutamol up to 80%, and sodium nitroprusside by only 20%. In contrast, sodium nitroprusside markedly reversed U46619-induced contraction of pulmonary arterial rings and bronchial rings.

5 Histological studies identified 2–4 layers of smooth muscle cells underlying the lung pleural surface. Mast cells were prominent in this area. Moreover, morphometric studies showed that segment 4 possessed the least amount of smooth muscle structures from bronchial/bronchiolar wall and vasculatures as compared to the other 3 segments, and a significant difference in this respect was evident between segment 1 and segment 4.

6 Since lung segments 1 and 4 are covered with larger surface area of lung pleura, the present results suggest that the significantly greater intrinsic contractile responses of segments 1 and 4 are associated with the presence of increased lung pleural surface possibly together with more mast cells. Thus, a primary contribution to the net contraction of the lung parenchymal strips may be smooth muscle from the lung pleura, alveolar ducts and interstitial contractile cells rather than from bronchi/bronchioles and microvasculatures.

Keywords: Peripheral airway smooth muscle; lung pleural surface; ovalbumin; A23187; eicosanoids; thromboxane A_2 mimetic; sodium nitroprusside

Introduction

Isolated lung parenchymal strip preparations were developed by Lulich *et al.* (1976), and Drazen & Schneider (1978) to study the function of peripheral airway smooth muscle. This *in vitro* model of the peripheral lung contracts to bronchospastic substances thought to be associated with airway hypersensitivity reactions including various eicosanoids, neurohormones, and peptides (Lulich *et al.*, 1976; Gerard, 1987; Burka, 1988). Antigen-induced contractions of immunologically sensitized parenchymal lung strips have provided further understanding of the pathogenic mechanisms of allergic asthma and have aided in development of potential antiasthmatic agents (Chand & Eyre, 1978; Fleisch *et al.*, 1982; 1985).

Controversies exist regarding the validity of the lung parenchymal strip as a true representation of peripheral airway smooth muscle. The lung parenchymal strip is a complex tissue consisting of many cell types including smooth muscle from bronchial and bronchiolar wall, pulmonary vasculature, alveolar ducts, and interstitial contractile cells (Kapanci *et al.*, 1974; Drazen & Schneider, 1978; Evans & Alder, 1981; Goldie *et al.*, 1984). Moreover, Halonen *et al.* (1990) have recently demonstrated the significance of smooth muscle layers underlying guinea-pig lung pleura as the primary site of action of platelet activating factor (PAF)-induced parenchymal lung strip contraction. All of these components are potential elements for the net contraction of lung strips. Systematic regional study of the peripheral lung has not been performed to determine which segment should be isolated to study contractile and relaxant responses to various pharmacological agonists, to ionophore, or to anaphylactic substances. The present investigation evaluated the regional differences in contractile and relaxant responses of 4 consecutive longitudinal parenchymal segments in order to establish the contribution of lung pleural smooth muscle to guinea-pig lung strip contractions.

Methods

Tissue preparation

Male Hartley guinea-pigs (Charles River, Portage, MI, U.S.A.) weighing 350–500 g were killed by decapitation under CO_2 narcosis. After thoracotomy, the heart and lungs were excised *en bloc* and perfused with 50 ml Krebs-bicarbonate solution via the pulmonary artery. Four longitudinal segments of similar size (3 mm × 20 mm) were cut from the right lower lobe of the lung and were designated from central to peripheral regions as segments 1, 2, 3 and 4, respectively (Figure 1).

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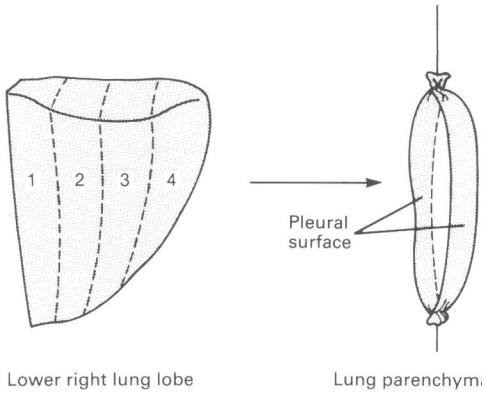

Figure 1 Diagram illustrating the isolation of 4 consecutive longitudinal parenchymal strips from the lower right lobe of guinea-pig lung. Lung strips possessed both the anterior and posterior pleural surfaces (shaded area).

The thickness of these lung strips varied from 2–3 mm for segments 1 and 4, and was 3–5 mm for the other two segments. All lung strips possessed both anterior and posterior pleural surfaces which accounted for approximately 75% and 46% of the total surface area of segments 1 and 4, and segments 2 and 3, respectively.

Lung strips were suspended isometrically under the previously determined optimum resting tension of 1 g (see below) in organ baths containing 10 ml Krebs-bicarbonate solution aerated with 95% O_2 and 5% CO_2 at 37°C. In some experiments, ring segments (~3 mm in length) from the main pulmonary artery and the right bronchi were isolated and mounted under the same conditions as the lung strips, except for the resting tension being 2 g. Composition of the Krebs-bicarbonate solution in mmol l^{-1} was: NaCl 118.2, KCl 4.6, $NaHCO_3$ 24.8, $CaCl_2 \cdot 2H_2O$ 2.5, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2 and dextrose 10.0. Contractile responses were monitored with a force displacement transducer (Grass FT-03) coupled to a Grass Model 7D polygraph recorder (Grass Instrument Co., Quincy, MA). A 60 min equilibration period was allowed before any experimentation was begun, during which time the bath fluid was changed every 10–15 min.

Sensitization procedures

In the antigen-induced contraction study (Schultz Dale reaction; Schultz, 1910; Dale, 1913; Chand & Eyre, 1978), parenchymal strips were isolated from guinea-pigs passively sensitized by a single intraperitoneal (i.p.) injection of 1 mg kg^{-1} rabbit IgG antibody against chicken egg albumin (Organon Teknicka Corp, West Chester, PA, U.S.A.). The animals were killed 2 days post injection (see tissue preparation).

Optimum resting tension determination

To determine the passive force required to produce maximum contractile responses of the 4 different lung segments, a series of length-tension studies was conducted. An initial resting force of 250 mg was applied to the parenchyma strips followed by a 60 min equilibration period before contractions were induced by 30 mM KCl. After repeated washings and returning to baseline tension, the resting force applied to the lung strips was increased stepwise by 250 mg after each KCl-induced contraction until contractile responses were weaker than preceding ones. At the end of the experiment, parenchymal strips were blotted and weighed on a Mettler H31 balance. Contractile responses were expressed as percentage of maximal contractions evoked by 30 mM KCl and some data were illustrated as mg force per mg weight of the tissue strips (mg F mg^{-1} wt).

Contractile studies

After the 60 min equilibration period, parenchymal strips were contracted by addition of single concentrations of 30 mM and 60 mM KCl. Since preliminary data indicated that contractile responses to a single dose of 60 mM KCl were consistently greater than those produced by a single dose of 160 mM KCl, the former was used as the 100% standard control response for all subsequent agonist-evoked contraction. Except for the concentration-response curves to histamine and carbachol, which were obtained on the same parenchymal strips, individual lung strips were used to produce a single concentration-response curve to a specific agonist. Whereas increasing concentrations of KCl (5–160 mM), histamine (0.01–100 μM), carbachol (0.01–100 μM), A23187 (0.1–100 μM), ovalbumin (OA, 0.1–3000 ng ml^{-1}), leukotriene D_4 (LTD_4, 0.1–100 nM), endothelin-1 (0.1–300 nM), or U46619 (a thromboxane A_2-mimetic, 0.1–3000 nM) were administered cumulatively into the organ baths, substance P (0.01–10 μM) and LTB_4 (2–3000 nM) were added sequentially to avoid potential development of tachyphylaxis. To prevent enzymatic degradation of the peptide agonists peptodes, endothelin-1- and substance P-induced contractions were elicited in the presence of 3×10^{-6} M thiorphan, an inhibitor of neutral endopeptidase (Gerard, 1987), which was added to the organ baths for 20 min before the administration of the peptides.

Relaxation studies

To determine if the 4 parenchymal segments exhibited a similar relaxation pattern to various smooth muscle relaxants, a series of spasmolytic experiments was performed. Histamine (1×10^{-4} M, a dose that produced maximum contractile response to this agonist) and carbachol (2×10^{-5} M, a dose that produced at least 80% of the maximum contractile response to this agonist) were first chosen to pre-contract parenchymal strips. However, contractions elicited by these agonists waned within 20 min. In contrast, 3×10^{-6} M U46619, a concentration that evoked maximal responses of the lung strips to this agonist, generated sustained contractions for at least 2 h. As a result, all parenchyma were pre-contracted with U46619 for 20 min (time required to reach plateau contraction) before one of the four relaxing agents including papaverine (0.001–300 μM), theophylline (0.01–100 μM), salbutamol (0.01–100 μM), or sodium nitroprusside (SNP, 0.01–100 μM) was added cumulatively to the organ baths. Concentration-relaxation responses to these agonists were obtained by injecting the next higher dose into the baths when the reduction of contractile force by the preceding dose was less than 25 mg in 5 min. Only one curve to a specific relaxant was generated from each lung strip. The relaxant effect was expressed as percentage reduction of the U46619-induced peak contraction of the parenchymal strips. In the salbutamol-mediated relaxation study, 1×10^{-6} M phentolamine was preincubated with the lung tissues for 20 min before the β-adrenoceptor agonist was added to prevent potential interaction of salbutamol (at high doses) with α-adrenoceptors in the lung strips. To differentiate between the contributory role of vascular and bronchial smooth muscle in lung parenchymal contraction, the relaxant effects of SNP (0.001–100 μM) on pulmonary arterial and bronchial rings pre-contracted with 3×10^{-6} M U46619 were studied and compared to that on the lung strips.

Histological studies

Lungs were removed and infused tracheally with either 10% neutral buffered formalin (3 animals) or Carnoy's fixative (2 animals), cut into strips, as described above, and prepared for routine paraffin embedding. A 6 μm tissue section was cut sagitally from the midpoint (~1.5 mm) of the thickness of each lung segment. Formalin fixed tissues were stained with haematoxylin and eosin or Masson's trichrome stain. Carnoy's fixed

tissue was stained with toluidine blue. Haematoxylin and eosin stained sections from each of the 4 lung segments of the left and right lower lobes of 3 animals were evaluated for number of bronchi (with cartilagenous rings), number of bronchioles (without cartilaginous rings) containing 3 or more layers of smooth muscle cells and number of blood vessels containing at least 3 layers of myocytes. Since smooth muscle structures in each lung segment from both left and right lung lobes were essentially the same, data were pooled together for statistical analysis. The total area for each section was determined by use of a Bioquant Image Analysis system and numbers of each structure per cm^2 of tissue determined. The mean values for each of the four strips were calculated by adding the numbers obtained from each of the six sections (right and left lung of 3 animals). Trichrome stained sections were evaluated for distribution and amount of smooth muscle. Toluidine blue stained sections were examined for distribution of mast cells.

Data analysis

All data are presented as mean ± s.e.mean. Sensitivities (EC_{50}) of the tissues were determined at 50% maximum response to a stimulant or a relaxant using a 4-parameter logistic model of nonlinear regression analysis. Comparisons of mean contractile or relaxant responses, mean EC_{50} values, and mean numbers of smooth muscle structures per cm^2 of tissue section were performed by one-way analysis of variance followed by either Tukey's honestly significant difference test (HSD) or Dunnett's two-tail t test. Significance level was set at $P < 0.05$.

Drugs and chemicals

Histamine dihydrochloride, carbamylcholine chloride (carbachol), substance P, ovalbumin, salbutamol hemisulphate, sodium nitroprusside and DL-thiorphan were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), endothelin-1 (human, porcine) from Peninsula Lab. Inc. (Belmont, CA, U.S.A.), IgG fraction rabbit anti-ovalbumin from Organon Teknicka Corp. (West Chester, PA, U.S.A.), U46619 (11α, 9α-epoxymethano-prostaglandin H_2) from Upjohn Co. (Kalamazoo, MI, U.S.A.) and phentolamine hydrochloride from Ciba Pharmaceutical Co. (Summit, N.J., U.S.A.). LTB_4, LTD_4, A23187, papaverine hydrochloride and theophylline were obtained from Eli Lilly and Co. (Indianapolis, IN, U.S.A. and Windlesham, U.K.). Leukotrienes B_4 and D_4 were stored in ethanol, U46619 in methyl acetate and IgG antibody in sterile saline at −20°C, and prepared freshly by serial dilution in distilled H_2O. A23187 and DL-thiorphan were dissolved in dimethyl sulphoxide, theophylline in hot (60–70°C) distilled H_2O, and all other drugs were prepared in distilled H_2O.

Results

Optimum resting tension determination

The 4 longitudinal lung segments rapidly contracted to 30 mM KCl, a dose previously shown to elicit near maximum contractile responses of guinea-pig lung parenchymal strips under a resting tension of 500 mg (Fleisch *et al.*, 1982). As the amount of resting force applied to the lung strips was increased from an initial tension of 250 mg, the magnitude of KCl-induced contractile responses increased correspondingly (Figure 2a). Maximum contractions for lung segments 3 and 4 were achieved with 1 g resting tension while those for segments 1 and 2 were attained with 1.25 g and 1.5 g resting force, respectively. To avoid excessive stretching of the parenchymal strips, an optimum resting force of 1 g was chosen for all lung segments to assure near maximum active contractile responses (~90%) in all subsequent experiments. When contractions of

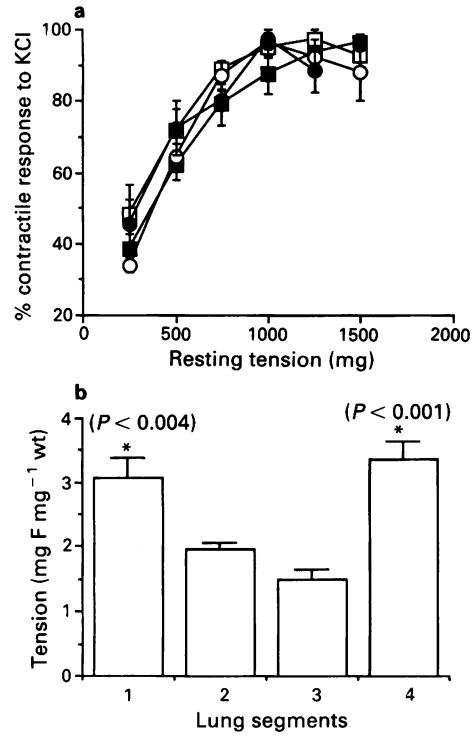

Figure 2 (a) Percent active contractile force developed in lung segments 1 (□), 2 (■), 3 (○) and 4 (●) in response to 30 mM KCl at a stepwise increase in resting tension applied to the tissues. Each point represents the mean (s.e.mean shown by vertical bars) of 4 experiments. (b) Contractile responses of the 4 lung segments to 30 mM KCl at 1 g resting tension were expressed as mg active force generated per mg weight of the tissues (mg F mg^{-1} wt). Each column represents mean (s.e.mean shown by vertical bars) of 4 experiments.

the parenchymal strips were expressed as mg force produced per mg weight of each lung strip at 1 g resting tension, values for segments 1 and 4 were 3.06 ± 0.33 and 3.35 ± 0.28 respectively, both of which were significantly greater ($P < 0.004$ and $P < 0.001$) than those for segments 2 and 3, being 1.96 ± 0.11 and 1.50 ± 0.16 (Figure 2b).

Contractile studies

Concentration-response relationships of the 4 parenchymal segments to KCl, histamine, carbachol and substance P are depicted in Figure 3. Although the lung strips exhibited similar sensitivity to increasing concentrations of KCl with −log molar EC_{50} values ranging from 1.71 ± 0.02 to 1.58 ± 0.03, and reached maximum contractile responses at 160 mM KCl, a significant leftward shift ($P < 0.05$, 1.35 fold) of the concentration-effect curve for segment 4 from that for segment 3 was noted (Figure 3a). However, the pharmacological and physiological significance of this small shift is questionable. The 4 parenchymal segments also demonstrated equivalent sensitivities to either histamine or carbachol with −log molar EC_{50} values ranging from 6.10 ± 0.12 to 5.73 ± 0.16 for the former and from 5.66 ± 0.08 to 5.43 ± 0.09 for the latter. Detectable contractions were observed at 1×10^{-8} M for histamine and at 1×10^{-7} M for carbachol, and peak contraction was achieved at 1×10^{-4} M for both agonists (Figure 3b, c). The maximum contractile response of the 4 lung segments produced by histamine, being 100% of the 60 mM KCl control response, was considerably higher than those induced by carbachol, the latter being only 70% of the KCl control. There was no significant difference in sensitivity among the 4 lung segments to substance P with −log molar EC_{50} values ranging from 6.91 ± 0.12 to 6.69 ± 0.16; however, the magnitude of contractions of segments 1 and 4 to this agonist was significantly ($P < 0.05$) higher than that

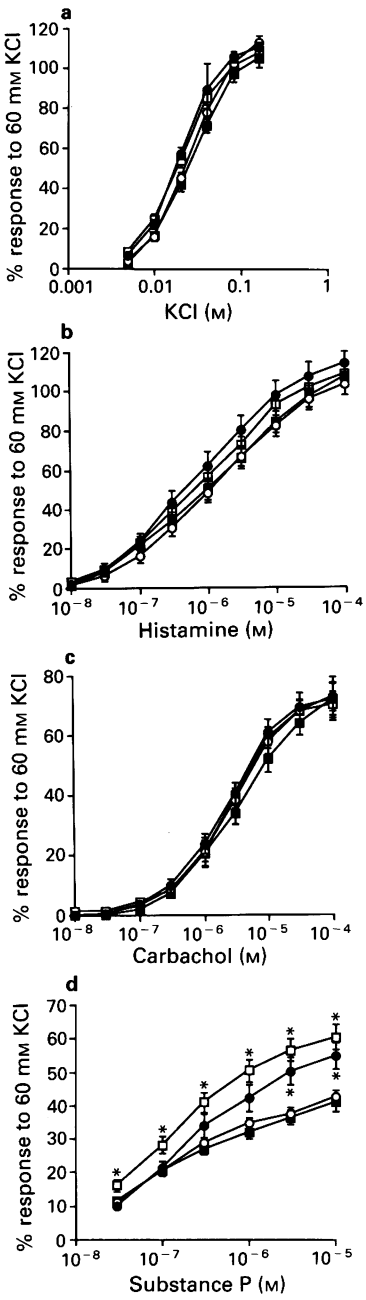

Figure 3 Log concentration-response curves of lung segments 1 (□), 2 (■), 3 (○), and 4 (●) to (a) KCl (5–160 mM), (b) histamine (0.01–100 μM), (c) carbachol (0.01–100 μM), and (d) substance P (0.01–10 μM). Results are expressed as percentage of contractile responses to 60 mM KCl. Each point represents the mean of 5–12 experiments; vertical bars show s.e.mean. * indicates $P < 0.05$.

produced by the other 2 strips throughout the entire range of concentrations (Figure 3d).

In tissue from sensitized animals, the ovalbumin concentration-response curve for lung segment 1 was significantly shifted ($P < 0.05$) to the left as compared to the other 3 segments which exhibited similar sensitivity to increasing concentrations of ovalbumin (Figure 4a). The $-\log g\,ml^{-1}$ EC_{50} value of ovalbumin for segment 1 was 8.67 ± 0.18 as compared to that for the other 3 segments ranging from 8.13 ± 0.05 to 7.80 ± 0.18, which represents a 4–7 fold increase in sensitivity. Moreover, the magnitude of contractile responses of segment 1 was significantly greater than that of the other 3 segments at concentrations of ovalbumin between 3×10^{-10} M and 1×10^{-6} M. The maximum contractile response produced by ovalbumin was about 120% of the KCl control response.

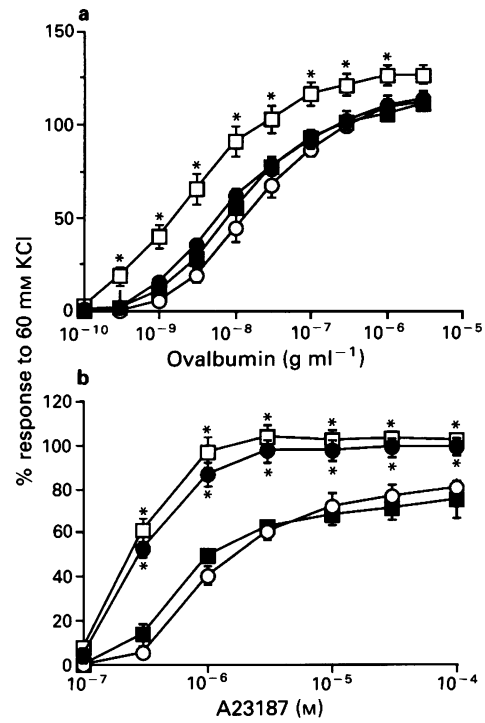

Figure 4 Log concentration-response curves of lung segments 1 (□), 2 (■), 3 (○), and 4 (●) to (a) ovalbumin ($10^{-10} - 3 \times 10^{-6}$ g ml^{-1}) and (b) A23187 (0.1–100 μM). Results are expressed as percentage of contractile responses to 60 mM KCl. Each point represents the mean of 5–6 experiments; vertical bars show s.e.mean. * indicates $P < 0.05$.

The concentration-response curves of segments 1 and 4 to A23187 were significantly shifted ($P < 0.05$) to the left with significantly higher maximum contractile responses ($P < 0.05$) as compared to the curves for the other two segments (Figure 4b). The $-\log$ molar EC_{50} values of A23187 for segments 1 and 4 were 6.72 ± 0.06 and 6.64 ± 0.07, whereas those for segments 2 and 3 were 6.34 ± 0.12 and 6.01 ± 0.12 respectively.

Table 1 summarizes the potencies ($-\log$ molar EC_{50}) and % maximal responses of LTB_4, LTD_4, U46619 and endothelin-1 on the 4 lung segments. There was no significant difference ($P > 0.05$) in $-\log$ molar EC_{50} to these agonists among the 4 lung parenchymal strips.

Relaxation studies

All lung segments contracted strongly to a single dose of 3×10^{-6} M U46619, and maximum contractions were achieved at 20 min and lasted for at least 2 h with negligible decreases in tension. Papaverine and theophylline produced similar concentration-dependent relaxation of all lung segments with very steep slopes within a narrow range of concentrations. Complete reversal (100% decrease) of U46619-induced contraction was obtained at 3×10^{-4} M for papaverine and at 3×10^{-3} M for theophylline. Theophylline exhibited equivalent potency ($P > 0.05$) in relaxing the 4 lung strips, whereas significant differences in sensitivities to papaverine ($P < 0.05$, 15–19 fold) were observed among lung segment 2 and segments 1 and 4 (Table 2). Salbutamol, 1×10^{-4} M, elicited approximately 65% maximum reduction of contraction for lung segments 1 and 2, and at 3×10^{-5} M, about 85% maximum reversal of contraction for lung segments 3 and 4 was observed. No further relaxation was observed in any lung segments beyond these concentrations. There was no significant difference ($P < 0.05$) in the potency of salbutamol among the 4 lung strips (Table 2).

In contrast to the above mentioned smooth muscle relaxants, sodium nitroprusside produced only a moderate relaxant effect on U46619-precontracted lung strips with a maximum

Table 1 Potencies and % maximal responses of various agonists on 4 lung segments

Agonists	Lung segments[a] 1	2	3	4
LTB_4	7.9 ± 0.1[b, c]	7.9 ± 0.1	7.8 ± 0.0	7.7 ± 0.0
	(68.4 ± 3.5)[d]	(59.0 ± 6.8)	(53.7 ± 5.7)	(69.4 ± 10.3)
LTD_4	8.0 ± 0.3	7.4 ± 0.2	7.3 ± 0.2	7.9 ± 0.2
	(111.0 ± 7.6)	(102.0 ± 4.3)	(91.7 ± 4.8)	(105.4 ± 4.3)
U46619	8.2 ± 0.8	7.7 ± 0.9	7.1 ± 0.8	7.9 ± 0.7
	(96.6 ± 7.2)	(92.3 ± 10.9)	(79.1 ± 8.4)	(95.2 ± 7.6)
Endothelin	7.3 ± 0.1	7.1 ± 0.1	7.1 ± 0.1	7.2 ± 0.1
	(81.5 ± 7.1)	(78.1 ± 3.1)	(80.5 ± 2.1)	(75.0 ± 3.2)

[a] Lung parenchymal strips were isolated longitudinally from the central to the peripheral region of the lower lung lobe and were designated as lung segments 1, 2, 3, and 4 respectively (see Methods).
[b] Each value represents the mean ± s.e.mean of 5–6 experiments.
[c] $-\log$ molar EC_{50}.
[d] % response to 60 mM KCl.
LTB_4, LTD_4: leukotrienes B_4 and D_4.

relaxation of 20–25% at 3×10^{-5} M (Figure 5a). In contrast, in pulmonary arterial and bronchial rings pre-contracted with 3×10^{-6} M U46619, sodium nitroprusside totally relaxed the pulmonary artery contraction at 3×10^{-6} M with $-\log$ molar EC_{50} value of 7.45 ± 0.05 and reversed the bronchial smooth muscle contraction by 90% with a $-\log$ molar EC_{50} value of 5.87 ± 0.08 (Figure 5b).

Histological studies

Morphometric analyses of the 4 lung segments are summarized in Table 3. Lung segment 2 contained significantly more bronchi ($P < 0.05$) than the other 3 segments, whereas lung segment 1 had more bronchioles ($P < 0.05$) than the other 3 segments. There were no significant differences in the distribution of pulmonary vasculature among the 4 segments. Despite the similarity in contractile responses observed between segments 1 and 4, the latter segment possessed significantly ($P < 0.05$) less total numbers of bronchi, bronchioles, and blood vessels than the former. On the other hand, 2–4 layers of smooth muscle cells with oblique orientation were identified to be associated with the visceral pleura surrounding the parenchyma (Figure 6). In toluidine blue-stained sections, mast cells were found not only in the connective tissue surrounding airways and vasculature, but also in that along the pleural surface.

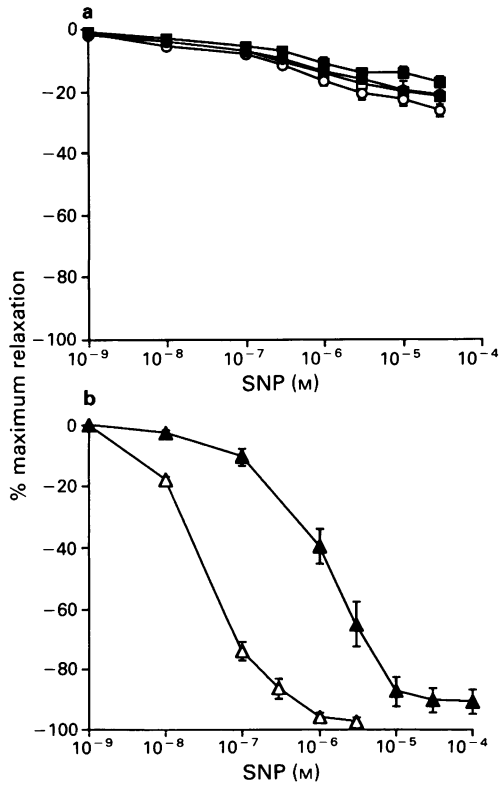

Figure 5 Log concentration-relaxation curves of (a) lung segments 1 (□), 2 (■), 3 (○), and 4 (●) and (b) pulmonary arterial ring segments (△) and right bronchial smooth muscle ring segments (▲) pre-contracted with 3×10^{-6} M U46619 to sodium nitroprusside (SNP) (0.01–100 μM). Results are expressed as percentage decreases in U46619-evoked maximum response of the respective tissues. Each point represents the mean of 5–6 experiments; vertical bars show s.e.mean.

Discussion

The present investigation demonstrated that parenchymal strip preparations isolated from different regions of guinea-pig lung responded in a qualitatively similar but quantitatively distinct manner to various pharmacological agents. When active tension was expressed as mg force produced per mg weight of tissue, lung segments 1 and 4 generated equivalent contractile responses to KCl which were significantly greater than those produced by the other two segments. The greater magnitude of contractile responses observed in segments 1 and 4 could not be attributed to additional smooth muscle structures including bronchi, bronchioles, or pulmonary vasculatures located in these lung strips. Morphometric analyses revealed that segment 4 consisted of significantly fewer ($P < 0.05$) contractile structures per cm^2 section area

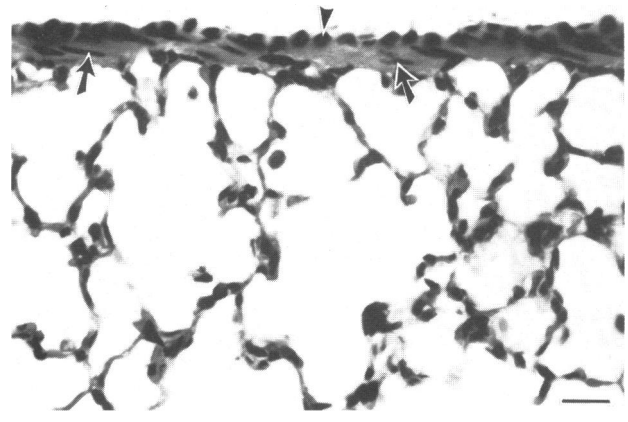

Figure 6 Photomicrograph of guinea-pig lung showing mesothelium (arrow head) with underlying smooth muscle cells (arrows). Haematoxylin and eosin, bar = 25 μm.

Table 2 Potencies and % maximal relaxation of various relaxants on 3×10^{-6} M U46619-precontracted lung segments

	Lung segments[a]			
Relaxants	*1*	*2*	*3*	*4*
Papaverine	4.6 ± 0.2[b,c,*]	3.4 ± 0.5	3.9 ± 0.2	4.7 ± 0.2*
	(100.0 ± 0.0)[d]	(100.0 ± 0.0)	(100.0 ± 0.0)	(100.0 ± 0.0)
Theophylline	3.0 ± 0.1	3.1 ± 0.2	3.1 ± 0.1	3.4 ± 0.2
	(100 ± 0.0)	(100.0 ± 0.0)	(100.0 ± 0.0)	(100.0 ± 0.0)
Salbutamol	6.5 ± 0.0	6.6 ± 0.1	6.7 ± 0.1	6.6 ± 0.1
	(62.8 ± 2.0)	(65.7 ± 6.2)	(87.9 ± 4.6)	(79.3 ± 5.4)

[a] Lung parenchymal strips were isolated longitudinally from the central to the peripheral region of the lower lung lobe and were designated as lung segments 1, 2, 3, and 4 respectively (see Methods).
[b] Each value represents the mean ± s.e.mean of 5–6 experiments.
[c] $-\log$ molar EC_{50}.
[d] % relaxation to 3×10^{-6} M U46619-induced precontraction of the lung segments.
* $P < 0.05$ indicates significant difference from lung segment 2.

than segment 1. The larger contractions produced by segments 1 and 4 appeared to be associated with pleural surface smooth muscle since both segments 1 and 4 had a considerably larger pleural surface area (75%) than segments 2 and 3 (46%), a finding that supports a recent study by Halonen *et al.* (1990). Contractile responses to various bronchospastic substances including histamine, carbachol, endothelin-1, LTB_4, LTD_4, or U46619 demonstrated no significant difference in sensitivity or maximum responsiveness among the 4 parenchymal lung strips. This homogeneity among the lung segments is probably due to the normalization procedure of transforming contractile response as percent maximum KCl control response.

The Schultz-Dale reaction describes an immunologically-mediated contraction of smooth muscle, in this case, parenchymal lung strips, due to the release of anaphylactic substances from sensitized effector cells such as the mast cell (Schultz, 1910; Dale, 1913; Chand & Eyre, 1978; Fleisch *et al.*, 1982). A23187, a divalent cationic ionophore, has been shown to release nonimmunologically anaphylactic mediators from lung with subsequent smooth muscle contraction (Piper & Seale, 1979; Fleisch *et al.*, 1982; Burka, 1983; Cheng *et al.*, 1987). In addition, A23187 can directly activate smooth muscle contraction by increasing cytoplasmic free Ca^{2+} concentration via enhancement of transmembraneous Ca^{2+} influx and intracellular Ca^{2+} release (Kondo, 1989). The concentration-response curve for IgG-sensitized lung segment 1 to ovalbumin was to the left of those for the other lung segments. Moreover, concentration-response relationships for segments 1 and 4 to A23187 were significantly ($P < 0.05$) more sensitive with maximum responsiveness substantially greater than segments 2 and 3. Thus, it appears that the increased sensitivity and maximum responsiveness of segment 1 to ovalbumin, and segments 1 and 4 to A23187 were probably associated with more mast cell degranulation upon challenge and, in addition, enhanced availability of Ca^{2+} to the smooth muscle in the pleural surface in response to A23187. Histological observations from the present study were reinforced by an additional report from our laboratory (Wong *et al.*, 1991) indicating that there is a widespread distribution of mast cells beneath the pleural surface. Similarly, concentration-response curves for segments 1 and 4 exhibited significantly higher maximum responses to substance P, which may be linked to the ability of this peptide to degranulate mast cells (Foreman, 1987).

All 4 parenchymal lung segments developed sustained tonic contractile responses to 3×10^{-6} M U46619, a thromboxane A_2-mimetic. This permitted evaluation of smooth muscle relaxants as a function of the various lung segments. The precontracted lung segments exhibited similar patterns of concentration-dependent relaxation to papaverine and theophylline with 100% reversal achieved at 3×10^{-4} M for the former and 3×10^{-3} M for the latter. Papaverine-induced relaxation of smooth muscle is mainly due to inhibition of both Ca^{2+} influx and of internal Ca^{2+} release (Fujioka, 1984; D'ocon, 1989); whereas theophylline-mediated relaxation is highly correlated with its inhibitory action against phosphodiesterase activity subsequent to an increase in adenosine 3′:5′-cyclic monophosphate (cyclic AMP) level (Obianime *et al.*, 1989; Brackett *et al.*, 1990). Salbutamol also caused a relatively uniform degree of relaxation of the 4 lung segments. However, this β-adrenoceptor agonist elicited only 60–80% maximum relaxation of the lung segments even at high concentrations between 1×10^{-4} M and 3×10^{-4} M.

Unexpectedly, sodium nitroprusside, which relaxes vascular smooth muscle by activating guanylate cyclase and subsequently increasing cyclic GMP levels (Murad *et al.*, 1985), barely reversed the U46619-induced contraction of the lung strips (by no more than 25%) at a concentration as high as 3×10^{-5} M. In contrast, near complete relaxation of pulmonary artery rings and bronchial rings contracted by U46619 was obtained by the same compound at 3×10^{-6} M for the artery and 1×10^{-4} M for the bronchi. These data indicate that sodium nitroprusside had the lowest efficacy in relaxing lung strips as compared to papaverine, theophylline, and salbutamol, and exhibited differential relaxing actions on lung parenchyma, pulmonary vascular smooth muscle and airway smooth muscle. Similarly, Dale & Obianime (1985) reported that sodium nitroprusside, at a concentration of 1×10^{-5} M, produced only minimal inhibitory effects on histamine-induced contraction of guinea-pig parenchymal lung strips and it actually increased the contraction of lung strips induced by phorbol ester. In contrast, sodium nitroprusside at the same concentration produced marked relax-

Table 3 Morphometric analysis of number of profiles of smooth muscle structures per cm^2 section area[a] of 4 lung segments

Segments[b]	*No. of bronchi*	*No. of bronchioles*	*No. of vessels*	*Total number*
1	2.4 ± 1.0**	35.9 ± 2.0	36.9 ± 2.3	75.1 ± 3.8
2	8.7 ± 1.2	23.9 ± 1.2***	33.4 ± 2.7	65.9 ± 3.4
3	3.6 ± 1.8*	26.7 ± 3.5*	28.9 ± 3.7	59.2 ± 4.9
4	0.4 ± 0.4***	25.4 ± 4.2*	29.8 ± 4.9	55.6 ± 7.6*

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$: indicate significant difference from the largest value in each column.
[a] Mean section areas (cm^2) of segments 1, 2, 3 and 4 were 0.56 ± 0.05, 0.56 ± 0.06, 0.56 ± 0.04 and 0.39 ± 0.02, respectively.
[b] 6 sections per segment group.

ation of bovine tracheal smooth muscle pre-contracted with carbachol (Katsuki & Murad, 1977). These results suggest that either the responsiveness of the pulmonary artery and bronchus to sodium nitroprusside is quite different from that of the pulmonary microvasculature and bronchioles in the parenchymal strips, or the pulmonary microvasculature and bronchioles play a minor role in lung parenchymal strip contraction. Szarek & Evans (1988) demonstrated differential responses between parenchymal strips and bronchioles in that LTC_4 contracted only the parenchymal strips but not the bronchioles. Taken together, these data suggest that U46619-mediated parenchymal lung strip contraction is primarily due to force development by smooth muscle from the alveolar ducts, interstitial contractile cells and pleural surface rather than from the bronchioles and microvasculature. Recent observations by Halonen *et al.* (1990) showed that smooth muscle underlying the visceral pleura is the primary contractile element for PAF-induced contraction of guinea-pig peripheral lung strips. In these experiments, removal of the pleural surface rendered the lung strips unresponsive to PAF. between pleural surface strips and parenchymal strips devoid Comparison of responses to various pharmacological agents between pleural surface strips and parenchymal strips devoid of pleural surface are underway and preliminary results have revealed that the smooth muscle of the pleural surface contracts to a variety of bronchoconstrictive mediators including

In summary, the present study demonstrated that parenchymal lung strips isolated from the most proximal region (segment 1) and the most distal region (segment 4) of guinea-pig lungs elicited intrinsically greater contractile capacity to a variety of agonists than the middle segments. We attribute the differences in contractile response to the larger surface area of smooth muscle layers beneath the lung pleura with additionally higher mast cells distribution. Taken together, histological and pharmacological studies suggest that pulmonary vasculature and bronchioles may not play a major role in parenchymal lung strip contraction, whereas smooth muscle from the pleural surface and alveolar ducts in addition to contractile interstitial cells may significantly contribute to the net contraction of the lung strips.

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Okadaic acid inhibits activation of phospholipase C in human platelets by mimicking the actions of protein kinases A and C

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1 The effect of okadaic acid, a potent inhibitor of protein phosphatases 1 and 2A (PP1 and PP2A), on human platelets has been investigated.

2 Okadaic acid exerts a general increase in phosphorylation of platelet proteins but did not induce aggregation or secretion of 5-hydroxytryptamine (5-HT). Okadaic acid, however, did inhibit thrombin-induced functional responses.

3 Maximally effective concentrations of prostacyclin, to elevate adenosine 3':5'-cyclic monophosphate (cyclic AMP), or phorbol dibutyrate, to activate protein kinase C, inhibited the formation of inositol phosphates by thrombin by approximately 60%. When used in combination, prostacyclin and phorbol dibutyrate reduced the levels of inositol phosphates induced by thrombin to 11%.

4 Okadaic acid (1 μM) decreased thrombin-induced formation of inositol phosphates by approximately 55% and increased the inhibitory action of prostacyclin or phorbol dibutyrate. Okadaic acid had no further effect when prostacyclin and phorbol dibutyrate were used in combination.

5 These results suggest that protein kinases A and C act to inhibit phospholipase C by distinct mechanisms and that their action is reversed by PP1 and/or PP2A.

Keywords: Human platelets; okadaic acid; protein phosphatases; adenylyl cyclase; protein kinase C

Introduction

In platelets, the major stimulatory second messengers are inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DG) produced by hydrolysis of phosphatidylinositol 4,5-bisphosphate (Seiss, 1989). IP3 releases Ca^{2+} from intracellular stores and DG activates protein kinase C. In addition to other actions, Ca^{2+} activates calmodulin-dependent kinases which phosphorylate several platelet proteins including myosin light chains (20 kDa). Protein kinase C also phosphorylates a number of substrates in platelets, the predominant of which has a molecular mass of 47 kDa but no known function (Tyers *et al.*, 1988). Ca^{2+} and protein kinase C interact synergistically to induce secretion; however, little is known about the mechanism of this process or proteins involved (Seiss & Lapetina, 1988).

In the present study, we have used the selective phosphatase inhibitor okadaic acid, a major toxic component in diarrhetic shellfish poisoning and a potent tumour promoter (Cohen, 1990), to probe the role of protein phosphatases 1 and 2A (PP1 and PP2A) in the activation of human platelets. Karaki *et al.* (1989) have reported that okadaic acid inhibits activation of rabbit platelets by thrombin, possibly by potentiation of adenosine 3':5'-cyclic monophosphate (cyclic AMP)-mediated events. In the present study, we show that okadaic acid inhibits activation of human platelets by thrombin through potentiation of protein kinase A- and protein kinase C-mediated inhibition of phosphoinositide hydrolysis.

Methods

Platelets were obtained from two sources. Blood was either drawn on the day of the experiment from aspirin-free volunteers with sterile 20 mM sodium citrate as anticoagulant and platelet-rich plasma obtained by centrifugation at 200 *g* for 20 min, or platelet concentrates were obtained from the Blood Transfusion Service, John Radcliffe Hospital, and used within 20 h of donation. Platelets were isolated from platelet-rich plasma or the platelet concentrate by centrifugation at 1000 *g* for 10 min in the presence of prostacyclin to prevent aggregation. Platelets were resuspended in 1 ml of modified Tyrodes-HEPES (composition in mM: NaCl 138, NaH_2PO_4 0.36, KCl 2.9, $NaHCO_3$ 12, HEPES 20, glucose 5, EGTA 1, $MgCl_2$ 1; pH 7.3) and labelled with [^{3}H]-inositol (50 μCi ml^{-1} for 3 h), [^{32}P]-orthophosphate (2 mCi ml^{-1} for 1 h) or [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT, 5 μCi ml^{-1} for 1 h). Platelets were then centrifuged in the presence of prostacyclin at 1000 *g* for 10 min and resuspended at a concentration of 2–8 $\times$ 10^8 ml^{-1} in the above buffer, containing indomethacin (10 μM) to inhibit cyclo-oxygenase. EGTA was omitted in studies of aggregation; LiCl (10 mM), which inhibits conversion of inositol monophosphate to free inositol, was added to [^{3}H]-inositol-labelled platelets. Platelets were left at least 30 min before experimentation.

Platelet suspensions (0.49 ml) were prewarmed at 37°C for 5 min before addition of compounds as appropriate. Experiments were stopped by transfer to 940 μl of chloroform/methanol/HCl (50:100:1) for analyses of inositol phosphates, to 0.5 ml of 6% (v/v) glutaraldehyde in phosphate buffer (pH 7.3) for analyses of 5-HT secretion, or to Laemmli buffer for analyses of protein phosphorylation. Inositol phosphates, protein phosphorylation and 5-HT secretion were measured as described previously (Nunn & Watson, 1987).

To prepare membrane and cytosol fractions, platelets were resuspended in 3 vol of lysis buffer (10 mM Tris, 1 mM EDTA, 0.1% (v/v) β-mercaptoethanol, 1 mM EGTA, pH 7.4) frozen rapidly in liquid nitrogen and thawed at 37°C; this process was repeated and the final suspension cooled to 4°C. The suspension was then sonicated on ice for 4 $\times$ 15 s before centrifugation at 1000 *g* for 10 min. The supernatant was removed and stored at 4°C; the pellet was resuspended in 3 vol of lysis buffer and the lysis procedure repeated. When lysis of all platelets had been achieved, after which no pellet formed when the suspension was centrifuged at 1000 *g* for 10 min, the pooled supernatants were centrifuged at 100,000 *g* at 4°C for 1 h. The supernatant represents the cytosol fraction while the pellet, resuspended in the original volume of lysis buffer, the membrane fraction. Protein phosphatase assays were performed on both membrane and cytosol fractions as described by Cohen *et al.* (1989) with ^{32}P-labelled phosphorylase a and casein as substrates.

Okadaic acid and labelled substrates for phosphatase assays were kindly given by Professor P. Cohen, Dundee. [^{3}H]-inositol (15.4 Ci mmol^{-1}), [^{32}P]-orthophosphate (8500–

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9120 Ci mmol^{-1}) and [^{3}H]-5-HT (21.0 Ci mmol^{-1}) were from N.E.N., Du Pont (U.K.) Ltd., Stevenage. Thrombin and phorbol dibutyrate were from Sigma Chemical Company Ltd., Poole. Prostacyclin was kindly donated by Wellcome Laboratories (Beckenham, Kent, U.K.) All other reagents were of Analytical grade.

Results

Protein phosphatase assays

The activities of PP1 and PP2A were assayed in both particulate and cytosolic fractions of human platelets by use of specific radiolabelled substrates. The use of a small heat-stable protein termed inhibitor-2 allowed selective inhibition of PP1 activity whilst okadaic acid (1 μM) inhibited both PP1 and PP2A activity; PP2C is unaffected by okadaic acid and has a requirement for Mg^{2+} (10 mM). The distribution of PP1 in platelets was similar in both particulate and cytosol fractions (Table 1). The specific activities of PP2A and PP2C were found to be higher in the cytosolic fraction than the particulate fraction (Table 1), which is in contrast to rabbit skeletal muscle where almost all PP2A and PP2C activity is found in the cytosolic fraction (Ingebritsen *et al.*, 1983).

Phosphorylation studies

Okadaic acid (1 μM) induced a general increase in phosphorylation of proteins over 60 min (Figures 1 and 2) similar to that observed in other cell types e.g. rat hepatocytes and adipocytes (Haystead *et al.*, 1989). The rate of phosphorylation varied greatly between proteins (Figure 2) while, in marked contrast, a protein of mol. wt. ~ 24 kDa was unaffected by okadaic acid (Figure 1). The threshold dose of okadaic acid was 0.3 μM (Figure 1a).

Action of okadaic acid on secretion and aggregation

Okadaic acid (1 μM) did not induce aggregation or secretion of 5-HT from platelets despite the marked changes in protein phosphorylation which occur over 60 min. Preincubation with okadaic acid (1 μM) for 10 min, however, inhibited thrombin-induced aggregation and secretion (Figures 3 and 4). This was particularly marked for low concentrations of thrombin, for example, okadaic acid reduced the secretion of 5-HT by 0.1 unit ml^{-1} thrombin from 38% to 4% (Figure 4).

Formation of inositol phosphates

Okadaic acid exerted a dose- and time-dependent inhibition of thrombin-induced formation of total [^{3}H]-inositol phosphates (Figure 5). Pre-incubation with 1 μM okadaic acid for 10 min inhibited thrombin-induced formation of inositol phosphates by approximately 56%. Moreover, significant inhibition was observed at the earliest time point monitored (5 s)

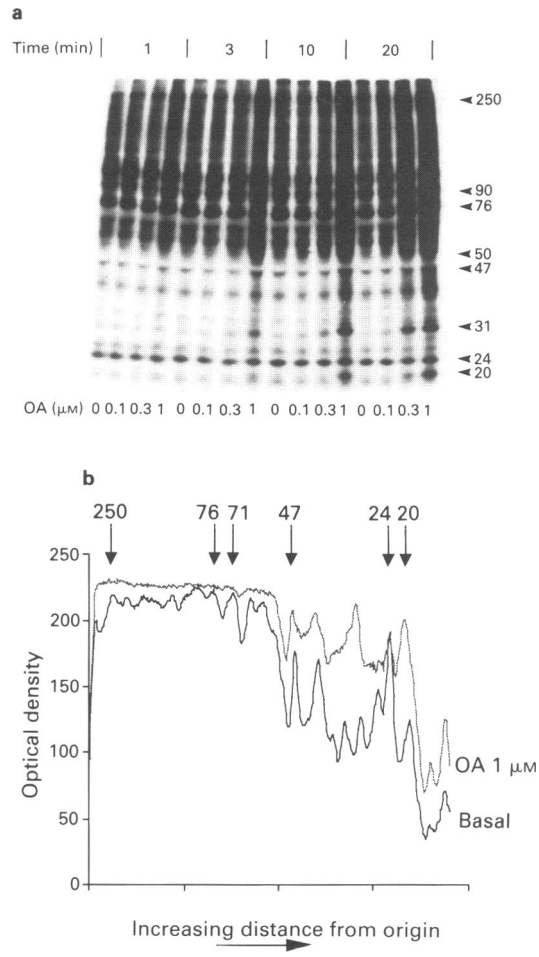

Figure 1 (a) Effect of okadaic acid on protein phosphorylation. Platelets were prelabelled with ^{32}P Pi and incubated with okadaic acid (1 μM) for varying times. Separation of proteins was achieved by gradient polyacrylamide gel electrophoresis (7.5%–15%), and autoradiography of the resulting gel indicates the extent of phosphorylation. Incubation time in the presence of okadaic acid (OA) is indicated at the top of the figure and the concentration indicated at the bottom. Relative molecular weights (in kDa) of distinct proteins are shown to the right of the figure. (b) Densitometric scan of basal protein phosphorylation (lower) and protein phosphorylation in the presence of okadaic acid (OA, 1 μM), pre-incubated for 10 min (upper). Increased phosphorylation is expressed as an increase in optical density. Molecular weights of distinct proteins are indicated in kDa at the top of the figure.

following exposure to thrombin (Figure 5) making it likely that this effect contributes to the inhibition of thrombin-induced aggregation and secretion by okadaic acid. Staurosporine, the non-selective kinase inhibitor (Tamaoki *et al.*,

Table 1 Protein phosphatase activity in human platelet cytosol and membrane fractions

	Particulate (mu mg^{-1} protein)	*Cytosol* (mu mg^{-1} protein)	*Rabbit skeletal muscle* (mu mg^{-1} protein)
PP1	0.27 ± 0.05	0.19 ± 0.005	1.13 ± 0.09
PP2A	0.22 ± 0.04	0.35 ± 0.01	0.35 ± 0.04
PP2C	0.03 ± 0.005	0.06 ± 0.01	0.022 ± 0.002

Activity is expressed as mean mu mg^{-1} protein ± s.e.mean from 6 experiments (one unit of activity is that amount of enzyme which catalyses the dephosphorylation of 1 μmol of substrate in 1 min). The activities of protein phosphatases 1 and 2A (PP1 and PP2A) were assayed with [^{32}P]-phosphorylase a, PP1 can be selectively blocked with inhibitor-1 whilst PP1 and PP2A can be blocked by okadaic acid (1 μM); assays were carried out as described in Methods. Casein is a substrate for both PP2A and PP2C therefore the assay for PP2C contained not only [^{32}P]-casein but also okadaic acid (5 μM) to block any PP2A activity and Mg^{2+} (10 mM) as PP2C has a requirement for this divalent cation. Specific activity of phosphatases in rabbit skeletal muscle was reported by Ingebritsen *et al.* (1983), in this case expressed as mu mg^{-1} protein.

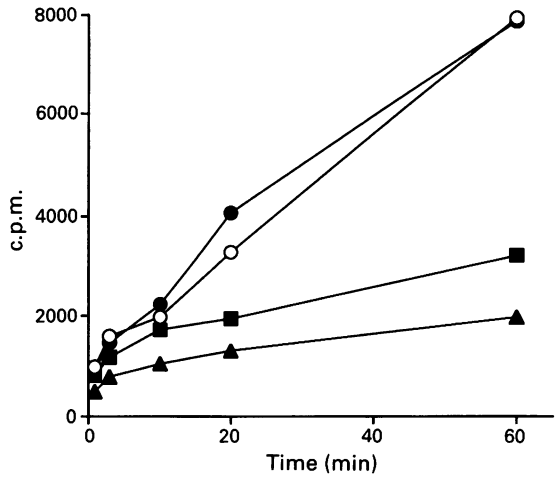

Figure 2 Effect of okadaic acid on the rate of protein phosphorylation. Platelets were prepared as described for Figure 1a, and incubated with okadaic acid (1 μM) for 10 min. Results are expressed as c.p.m. (■) 250 kDa; (▲) 50 kDa; (●) 76 kDa; (○) 90 kDa. These symbols correspond to proteins indicated in Figure 1a.

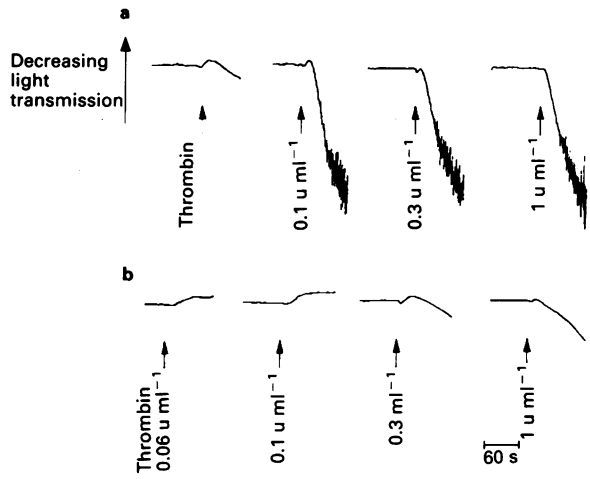

Figure 3 Effect of okadaic acid on thrombin-induced aggregation. A typical aggregation trace induced by a range of thrombin concentrations (0.06 units ml^{-1}–1 unit ml^{-1}) (a) was repeated in the presence of okadaic acid (1 μM, 10 min pre-incubation) (b). This is representative of three other similar experiments.

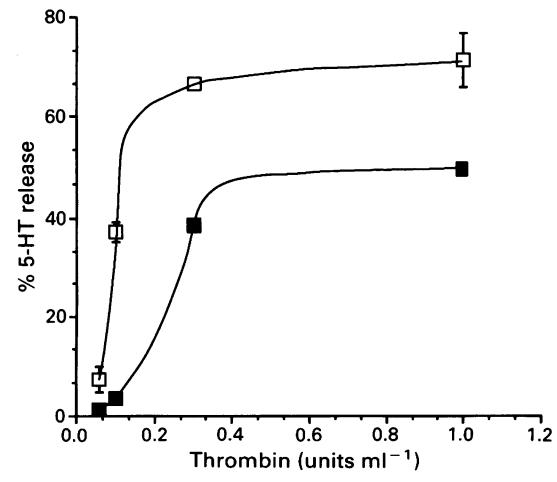

Figure 4 Effect of okadaic acid on thrombin-induced 5-hydroxytryptamine (5-HT) secretion. Platelets were prelabelled with [^{3}H]-5-HT and stimulated with thrombin concentrations for 1 min, in the presence (■) or absence (□) of okadaic acid (1 μM, 10 min pre-incubation). Results are from 3 experiments and are shown as mean % 5-HT release with s.e.mean shown by vertical bars.

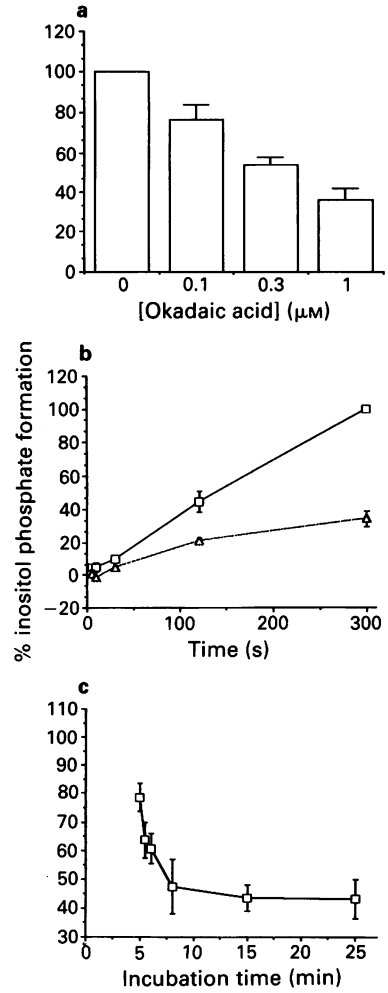

Figure 5 Effect of okadaic acid on thrombin-induced formation of inositol phosphates. (a) Dose-response curve for inhibition by okadaic acid. Platelets were prelabelled with [^{3}H]-inositol and pre-incubated with okadaic acid (0.1–1 μM, as indicated) for 10 min. Platelets were then stimulated with a maximal dose of thrombin (1 unit ml^{-1}) for 5 min. Experiments were performed in triplicate and results are from 3 experiments and expressed as % of control inositol phosphate formation with s.e.mean shown by vertical bars. (b) Platelets were prelabelled with [^{3}H]-inositol and pre-incubated with okadaic acid (1 μM) for 10 min as indicated: (△) presence of okadaic acid; (□) absence of okadaic acid. Platelets were then stimulated with a maximal dose of thrombin (1 unit ml^{-1}) for varying times. Experiments were performed in triplicate and results are expressed as % inositol phosphate formation with s.e.mean shown by vertical bars; this is a representative experiment with similar results to two others. (c) Time-dependent effect of okadaic acid on thrombin-induced inositol phosphate formation. Platelets were prelabelled with [^{3}H]-inositol and pre-incubated with okadaic acid (1 μM). Pre-incubation times with okadaic acid were as follows: 10 s, 30 s, 1 min, 3 min, 10 min and 20 min. Platelets were then stimulated with a maximal dose of thrombin (1 unit ml^{-1}) for 5 min. The time scale relates to the total incubation time in the presence of okadaic acid. Experiments were performed in triplicate and results are expressed as % inositol phosphate formation with s.e.mean shown by vertical bars; this is a representative experiment with similar results to two others.

1986), inhibited the action of okadaic acid on thrombin-induced formation of inositol phosphates (Table 2). This is consistent with the mechanism of action of okadaic acid being through potentiation of phosphorylation rather than acting as an antagonist at the thrombin receptor.

Pretreatment of platelets with maximally effective concentrations of prostacyclin or phorbol dibutyrate reduced thrombin-induced formation of inositol phosphates to approximately 40% of control values in each case. In the presence of 1 μM okadaic acid, these responses were reduced to

Table 2 Inhibition of thrombin-induced formation of total inositol phosphates

	d.p.m.	(%)
Thrombin	1067 ± 23	100
Thr + PGI_2	454 ± 62	42.5 ± 5.8
Thr + PDBu	434 ± 46	40.7 ± 4.3
Thr + OA	484 ± 49	45.4 ± 4.6
Thr + PGI_2 + OA	216 ± 36	20.2 ± 3.4
Thr + PDBu + OA	221 ± 36	22.6 ± 3.1
Thr + PGI_2 + PDBu	121 ± 19	11.3 ± 1.8
Thr + PGI_2 + PDBu + OA	94 ± 29	8.8 ± 2.7
Thr + staurosporine	1116 ± 48	104.6 ± 4.5
Thr + staurosporine + OA	1078 ± 96	101 ± 9.0

Results are expressed as d.p.m. or % ± s.e.mean of maximal formation of inositol phosphates, induced by thrombin during 5 min stimulation ($n \geq 4$). Compounds are as follows: thrombin (Thr, 1 unit ml^{-1}), prostacyclin (PGI_2) 2.5 $\mu g\,ml^{-1}$, phorbol dibutyrate (PDBu) 300 nM, okadaic acid (OA) 1 μM, staurosporine (3 μM). Platelets were pre-incubated in the presence of okadaic acid for 10 min prior to stimulation by thrombin.

approximately 20% of controls (Table 2). The inhibitory actions of prostacyclin and phorbol dibutyrate were partially additive, reducing the formation of inositol phosphates to 110% of control values (Table 2). This was not significantly altered in the presence of 1 μM okadaic acid ($P > 0.05$; Table 2). These results suggest that prostacyclin and phorbol dibutyrate inhibit the activation of phospholipase C (PLC) by thrombin through predominantly different mechanisms and that okadaic acid may be able to mimic both of these actions.

Discussion

Effect of okadaic acid on protein phosphorylation

Okadaic acid produces an increase in phosphorylation of a large number of platelet proteins which may reflect a net increase in activity of a single kinase or, more likely, a family of protein kinases. The net increase in kinase activity could be brought about in two ways. The kinases may be intrinsically active and their action terminated by PP1 and/or PP2A or they may be activated by increased phosphorylation which is under regulation from PP1 and/or PP2A. Recently eleven novel serine/threonine protein kinases were identified in platelets, ten of which show increased activity following challenge with thrombin, possibly as a result of autophosphorylation (Ferrell & Martin, 1989). The general increase in phosphorylation may also reflect an alteration in the balance between kinases and phosphatases leading to increased activity of several kinases, the cascade being regulated at one or more points by PP1 and/or 2A.

Action of okadaic acid on phospholipase C

It has been reported previously that okadaic acid mimics the inhibitory action of cyclic AMP-dependent protein kinase on vascular smooth muscle (Karaki *et al.*, 1989). However okadaic acid did not alter the levels of cyclic AMP suggesting that it does not activate adenylyl cyclase or inhibit cyclic AMP phosphodiesterase. Karaki *et al.* (1989) proposed that inhibition of protein phosphatases by okadaic acid resulted in an increase in the effects caused by cyclic AMP-dependent protein kinase (protein kinase A).

Prostacyclin mediates its inhibitory action on platelets through elevation of cyclic AMP and activation of protein kinase A (Seiss & Lapetina, 1989). One action of cyclic AMP is the inhibition of phosphoinositide hydrolysis following receptor activation (Watson *et al.*, 1984). Phorbol esters also inhibit formation of inositol phosphates by cell surface receptors and it has been postulated that this is a result of a negative feedback regulation by protein kinase C (Watson & Lapetina, 1985). The observation that the effect of phorbol dibutyrate is partly additive with that of prostacyclin suggests that they produce their effects by separate mechanisms.

A number of classes of phospholipase C (PLC) have been identified in a variety of tissues, α, β, γ and δ (Rhee *et al.*, 1989); cDNA sequencing and immunoreactivity analyses suggest that subtypes of these classes exist (Wahl & Carpenter, 1991). Activation of protein kinase C has been reported to phosphorylate selectively the β isoform of PLC in a number of cell lines as well as in bovine brain (Ryu *et al.*, 1990). In contrast, protein kinase A selectively phosphorylates the γ isoform of PLC in C_6Bu1 cells (Kim *et al.*, 1989). Phosphorylation of these isoforms of PLC has no apparent effect on their catalytic activity but may interfere with G protein interactions and therefore attenuate phosphoinositide hydrolysis in response to receptor activation (Kim *et al.*, 1989; Ryu *et al.*, 1990). Characterization of PLC enzymes in platelets has identified a number of subtypes, both cytosolic and membrane-bound (Baldassare *et al.*, 1989; Banno *et al.*, 1986; 1988; 1990). The relationship between platelet isozymes of PLC and those identified in other cell types has still to be established. Prostacyclin and phorbol ester may work at least, in part, by distinct mechanisms, possibly through selective phosphorylation of specific PLC isozymes. Alternatively cyclic AMP and protein kinase C may increase GTPase activity of the α subunit of the putative G protein Gp (Knight & Scrutton, 1987; Krishnamurthi *et al.*, 1989). It has also been demonstrated that agents which raise cyclic AMP levels decrease the ability of thrombin to bind to its receptor, this correlates closely with the inhibition of thrombin-induced functional responses (Lerea *et al.*, 1987). The observation that okadaic acid potentiates the effect of protein kinase A and protein kinase C but has no effect when both pathways are maximally activated suggests that the protein phosphatases PP1 and/or PP2A act to limit the actions of protein kinase A and protein kinase C on receptor-regulation of PLC.

Role of protein phosphatases in platelet activation

The role of kinases in stimulus-secretion coupling has been extensively investigated (Hanks *et al.*, 1988; Kikkawa *et al.*, 1989; Taylor *et al.*, 1990). More recently, attention has also focussed on the possible control of secretion by protein phosphatases. It has been reported that a platelet protein of 18 kDa molecular mass is dephosphorylated when platelets are stimulated with thrombin (Imaoka *et al.*, 1983). It is known that dephosphorylation in some cell types is a critical event for cellular responses. For example, in *Paramecium*, rapid and marked dephosphorylation of a 65 kDa protein correlates temporally with synchronous exocytosis: both events peak within 1–3 s and are complete by 5 s (Zeiseniss & Plattner, 1985). The possibility that activation of a phosphatase may be a requirement in the initial stages of stimulus-secretion coupling has been suggested (Zeiseniss & Plattner, 1985). In platelets, little is known of the importance of this effect. It has been proposed that platelet protein phosphatases may be involved in down-regulation of platelet function (Sakon *et al.*, 1990). However, we believe that the role of protein phosphatases is more complex, made evident by the ability of okadaic acid to attenuate functional responses in human platelets. Okadaic acid and other specific protein phosphatase inhibitors will allow investigation of the precise roles which PP1 and PP2A play in signal transduction.

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Increased secretion of glandular-kallikrein in the bronchial washings induced by intravenous injection of leukotriene C_4 in guinea-pigs

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1 Intravenous administration of leukotriene C_4 (LTC_4) and LTD_4 (1–10 nmol kg^{-1}) caused a dose-dependent increase in secretion of glandular-kallikrein in the bronchial washings of guinea-pigs, as measured by cleavage of a synthetic substrate and the formation of kinin. LTC_4 was more potent than LTD_4 and pilocarpine was much less potent than peptide leukotrienes on a molecular basis.

2 The increases in levels of glandular-kallikrein in the bronchial washings that were induced by LTC_4 (3 nmol kg^{-1}, i.v.) were almost completely inhibited by pretreatment with an antagonist of leukotrienes (ONO-1078), with an antagonist of thromboxane (S-1452), with an inhibitor of thromboxane synthetase (OKY-046), with indomethacin, with atropine or with scopolamine. These results indicate that the LTC_4-induced increase in levels of glandular-kallikrein may have been mediated by the formation of thromboxane and the release of acetylcholine.

3 The increases in levels of glandular-kallikrein in the bronchial washings induced by STA_2 (20 pmol kg^{-1}, i.v.), a stable analogue of thromboxane A_2, were completely blocked by pretreatment with atropine, whereas increases induced by pilocarpine (41 μmol kg^{-1}, i.v.) were not blocked by pretreatment with indomethacin, although such increases were inhibited by atropine. This result indicates that secretion of kallikrein stimulated by LTC_4 may have been mediated by the successive formation of thromboxane A_2 and release of acetylcholine.

4 Intravenous administration of bradykinin (3–30 nmol kg^{-1}) caused a dose-dependent increase in levels of glandular-kallikrein in the bronchial washings. This increase was completely inhibited by pretreatment with atropine, with indomethacin or with an antagonist of thromboxane.

5 The increases in levels of glandular-kallikrein in the bronchial washings induced by LTC_4 (3 nmol kg^{-1}, i.v.) and pilocarpine (41 μmol kg^{-1}, i.v.) were significantly inhibited by pretreatment with an antagonist of bradykinin. These results suggest that intravenous LTC_4 may increase secretion of glandular-kallikrein via formation of thromboxane A_2 and release of acetylcholine in that order, and kinin released by kallikrein may enhance the rate of secretion of glandular-kallikrein.

Keywords: Leukotriene C_4; glandular-kallikrein; bronchial washing; thromboxane A_2; acetylcholine; asthma

Introduction

Bronchial asthma has long been recognized as a disease of reversible airway obstruction, which is induced not only by bronchoconstriction but also by increases in the secretion of mucus. Leukotrienes, released from lungs of sensitized guinea-pig, were reported to be potent bronchoconstrictors (Kellaway & Trethewie, 1940). Bronchoconstriction produced by leukotrienes was almost completely inhibited by pretreatment with indomethacin (Piper & Samhoun, 1981) and an inhibitor of thromboxane synthetase (Piper & Samhoun, 1981; Ueno *et al.*, 1982; Austen *et al.*, 1983). In addition to bronchoconstriction, leukotrienes have been reported to increase bronchial secretion of mucus in guinea-pigs and man (Peatfield *et al.*, 1982; Marom et al., 1982; Johnson & McNee, 1983). As in the case of bronchoconstriction, the increase in bronchial secretion of mucus was blocked to a significant extent by pretreatment with indomethacin and with an inhibitor of thromboxane synthetase (Yanni & Smith, 1986).

Levels of glandular-kallikrein, a kinin-generating enzyme, increased in lavage fluid after provocation with allergen of the nasal (Proud *et al.*, 1983) and bronchial mucosa (Christiansen *et al.*, 1987) of atopic subjects. Here we show that intravenous injections of leukotrienes increase the levels of glandular-kallikrein in bronchial washings of guinea-pigs via the successive formation of thromboxane and release of acetylcholine.

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Methods

Collection of bronchial washings

Male Hartley guinea-pigs (specific pathogen-free, weighing 450–550 g, from Shizuoka Laboratory Animal Center, Hamamatsu, Japan) were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, s.c.) before the start of experimental procedures. Anaesthesia was maintained by occasional subcutaneous administration of sodium pentobarbitone (20 mg kg^{-1}). The right jugular vein was cannulated with a polyethylene cannula (PE-50, Clay Adams, Parsippany, NJ, U.S.A.). LTC_4 and other solutions of drugs were injected through this cannula. To avoid contamination by saliva of the bronchial washings during induction of bronchial secretion, the trachea was cannulated with a polyethylene cannula (size 6, Hibiki, Tokyo, Japan) through a short incision made just beneath the thyroidal cartilage bone, and a cotton ball containing silicon grease (High vacuum, Dow Corning, Midland, Mich., U.S.A.) was plugged into the oral side of the cut end of the trachea.

After completion of the experimental set-up, leukotriene C_4 (LTC_4, 3–30 nmol ml^{-1} saline kg^{-1}), LTD_4 (3–30 nmol ml^{-1} saline kg^{-1}), pilocarpine (12–120 μmol ml^{-1} saline kg^{-1}), STA_2 (9,11-epithio-11,12-methanothromboxane A_2, 1–10 nmol ml^{-1} saline kg^{-1}) or bradykinin (3–30 nmol ml^{-1} saline kg^{-1}) was injected as a single bolus through the jugular vein. Twenty minutes later, each animal was killed by exsanguination and the lung and trachea with cannula were isolated. After washing the outside of the lung with chilled physiological saline, half of the peripheral portion of the lung

was removed by cutting with small scissors. Physiological saline (0.4 ml), injected through the cannula inserted into the trachea, was able to flow out through the cut surface of the lung. This washing procedure was repeated 6 times at 1 min intervals and washings were collected in plastic tubes. The samples of bronchial washings were kept on ice until measurements of levels of glandular-kallikrein.

Pretreatment with drugs

Guinea-pigs were pretreated with ONO-1078 ([8-(p-phenylbutyloxy)benzoyl]amino-2-(tetrazol-5-yl)-4-oxo-4H-1-benzopyran hemihydrate, an antagonist of LTC_4, LTD_4 and LTE_4, 20–200 nmol ml^{-1} saline kg^{-1}, i.p.) (Obata *et al.*, 1987), indomethacin (an inhibitor of cyclo-oxygenase, 8.3–83 μmol ml^{-1} saline kg^{-1}, i.p.), OKY-046 ((E)-3-[4-(1-imidazolymethyl) phenyl]-2-propenoic acid hydrochloride monohydrate, an inhibitor of thromboxane synthetase, 10–100 μmol ml^{-1} saline kg^{-1}, i.v.) (Nato *et al.*, 1984), S-1452 (an antagonist of thromboxane A_2, 0.3–1.0 μmol ml^{-1} saline kg^{-1}, i.v.) (Hanasaki *et al.*, 1988), atropine (4.2–42 μmol ml^{-1} saline kg^{-1}, i.v.), scopolamine (10–100 μmol ml^{-1} saline kg^{-1}, i.v.) or an antagonist of bradykinin (Thi^{5,8}-D-Phe^{7}-bradykinin, 100–1000 nmol ml^{-1} saline kg^{-1}, i.v.). LTC_4, pilocarpine or STA_2 was administered intravenously 30 min after the intraperitoneal injection and 10 min after the intravenous injection of the above mentioned drugs.

Measurements of the activity of glandular-kallikrein

Immediately after the collection of bronchial washings, the activity of glandular-kallikrein was determined by use of a selective substrate for glandular-kallikrein. Fifty μl of bronchial washing was incubated with 950 μl of the solution of methylcoumaril amide (MCA) synthetic substrate (Pro-Phe-Arg-MCA, 2×10^{-4} M, in 50 mM Tris-HCl buffer that contained 0.1 M NaCl and 0.01 M $CaCl_2$, pH 8.0) at 37°C for 30 min. The reaction was terminated by addition of 2 ml of a 17% solution of acetic acid. The amount of amidomethylcoumarine (AMC) released was measured fluorometrically (excitation, 380 nm; emission, 460 nm) as described elsewhere (Morita *et al.*, 1977; Oh-ishi & Katori, 1979). A mean value from the six washings was calculated and taken as the activity of glandular-kallikrein in the bronchial washings.

Kinin-generating activity in bronchial washings

To assess the kinin-generating activity in the bronchial washings, purified low-molecular-weight (LMW) kininogen from bovine plasma, a natural substrate for glandular-kallikrein, was incubated with the bronchial washings. An aliquot of 300 μl of bronchial washings, 100 μl of 0.2 M Tris-HCl buffer (pH 8.0) and 100 μl of a solution of purified LMW kininogen (1 mg ml^{-1}, in physiological saline) were incubated together at 37°C for 60 min in the presence of an inhibitor of kininase I, DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid (10 μM) and an inhibitor of kininase II, captopril (30 μM) (Majima *et al.*, 1988). After addition of 100 μl of a 20% solution of trichloroacetic acid to the reaction mixture, the mixture was centrifuged at 1500 *g* for 15 min at 4°C. Four hundred μl of the supernatant were neutralized with the same volume of buffer B from an enzyme-immunoassay kit for bradykinin (Markit-A, Dainippon Pharmaceutical Co., Osaka, Japan). The amounts of bradykinin in the neutralized solution were determined with the enzyme-immunoassay kit, as previously described (Uchida *et al.*, 1986). Aprotinin (Vogel, 1979) was added to the incubation mixture (final concentration; 5000 Kiu ml^{-1}) in order to ensure that the kinin-generating activity was due to glandular-kallikrein.

Drugs

Drugs used were as follows: sodium pentobarbitone (Nembutal, Abbot Lab., North Chicago, IL, U.S.A.), peptidyl fluorogenic substrate, Pro-Phe-Arg-MCA (Peptide Institute Inc., Osaka, Japan), bradykinin (Peptide Institute Inc.), bradykinin B_2 receptor antagonist, Thi^{5,8}-D-Phe^{7}-bradykinin (Peptide Institute Inc.), DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid (Calbiochem., La Jolla, CA, U.S.A.), pilocarpine, atropine and scopolamine (Wako Pure Chemical Ind., Osaka, Japan). LTC_4, LTD_4, STA_2 and ONO-1078 were generous gifts from Ono Pharmaceutical Co. Ltd., Osaka, Japan. Captopril was a gift from Sankyo, Co., Tokyo, Japan. S-1452 was a gift from Shionogi Pharmaceutical Co., Osaka, Japan. OKY-046 was a gift from Kissei Pharmaceutical Co., Matsumoto, Japan. Indomethacin was a gift from Merck, Sharp and Dohme, Rahway, NJ, U.S.A. Aprotinin was a gift from Bayer A.G., Leverkusen, Germany. Purified LMW kininogen was a gift from Seikagaku Kogyo, Tokyo, Japan. Other reagents were all of analytical grade and were obtained from commercial sources.

Statistical analysis

When variances were not heterogeneous, Student's *t* test was used to evaluate the significance of differences. When variances were heterogeneous, statistical analysis was performed by the Aspine-Welch method or by the Wilcoxon's rank sum test. A value of *P* of less than 0.05 was regarded as significant.

Results

Increases in glandular-kallikrein activity in bronchial washings caused by pilocarpine

The activity of glandular-kallikrein activity increased in a dose-dependent manner with increasing doses of pilocarpine (12–120 μmol kg^{-1}) (Figure 1a). The increase in the level of glandular-kallikrein caused by pilocarpine (41 μmol kg^{-1}) was inhibited by pretreatment with atropine in a dose-dependent manner (Figure 1b).

Increases in glandular-kallikrein activity in bronchial washings caused by leukotriene

As shown in Figure 2, the activity of glandular-kallikrein in bronchial washing increased dose-dependently in response to 1, 3 and 10 nmol kg^{-1} of LTC_4. The magnitude of increase caused by the 3 doses was 132%, 193% and 271% of the activity of the saline control respectively, whereas those induced by LTD_4 (1, 3 and 10 nmol kg^{-1}) were 121%, 152% and 155% of the saline control. Thus, LTC_4 was more potent than LTD_4 in enhancing the secretion of glandular-kallikrein into the bronchial washings.

Kinin-generating activity in the bronchial washings induced by pilocarpine and leukotriene C_4

To test whether the amidase activity in bronchial washings was due to kinin-generating activity, bronchial washings were incubated with low-molecular-weight kininogen (Figure 3). The kinin-generating activity in bronchial washings after injection of saline was 22 ± 6 pmol min^{-1} ml^{-1} washing ($n = 6$), which was increased to 500% after injection of pilocarpine and to 840% after injection of LTC_4. The addition of aprotinin to reaction mixtures caused marked inhibition of the kinin-generating activity.

Inhibition of leukotriene C_4-induced increases in the activity of glandular-kallikrein in washings by pretreatment with inhibitors

Pretreatment of guinea-pigs with an antagonist of leukotriene, ONO-1078 (20–200 μmol kg^{-1}) caused a dose-dependent decrease in the activity of glandular-kallikrein that was

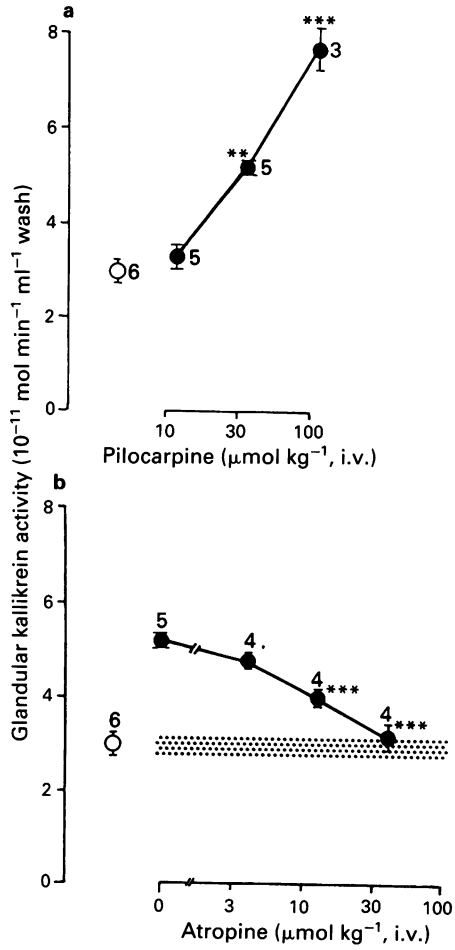

Figure 1 Dose-dependent increases in levels of glandular-kallikrein in bronchial washings in guinea-pigs caused by pilocarpine and the inhibitory effect of atropine. The ordinate scale shows the levels of activity of glandular-kallikrein in bronchial washings: (○) and the shaded areas indicate the control levels when only saline was injected. Dosage of pilocarpine used in (b) was 41 μmol kg^{-1} (i.v.). The numbers beside each point show the numbers of experiments. Each point represents the mean value (s.e.mean shown by vertical bar) and is compared with the value of the saline control (a) and the value without atropine treatment (b). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

induced by LTC_4 (3 nmol kg^{-1}). The antagonist at 200 μmol kg^{-1} completely blocked the increase in the glandular-kallikrein activity.

The pretreatment of animals with indomethacin (an inhibitor of cyclo-oxygenase) or OKY-046 (an inhibitor of thromboxane synthetase) inhibited the increase in the activity of glandular-kallikrein in bronchial washings and complete inhibition was observed with the higher doses of both inhibitors, indicating the involvement of the formation of thromboxane A_2 in the stimulation of secretion of kallikrein by LTC_4. The inhibition of the increase in the kallikrein activity caused by pretreatment with S-1452 (an antagonist of thromboxane) provided further evidence for the involvement of thromboxane A_2 in this process (Table 1).

When animals were pretreated with atropine or scoplamine, the increases in activity of glandular-kallikrein caused by LTC_4 were also inhibited by as much as 80% to 100%.

Inter-relationship between thromboxane A_2 and cholinergic nerves

In order to clarify the sequence of the actions of thromboxane A_2 and acetylcholine in the increased secretion of glandular-kallikrein into the bronchial washings caused by LTC_4, guinea-pigs were pretreated with indomethacin (83 μmol kg^{-1},

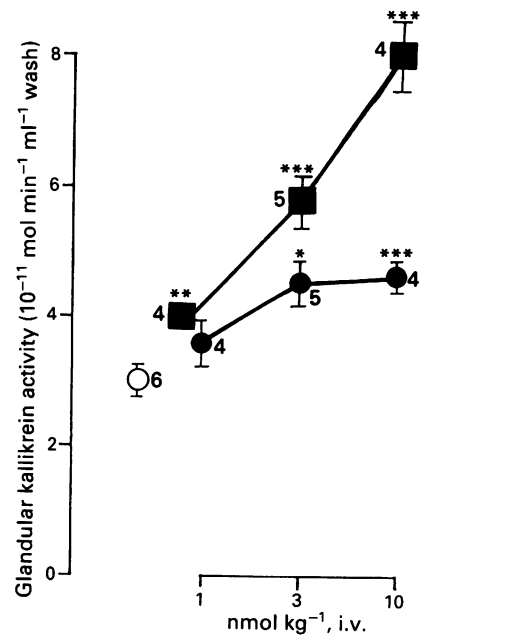

Figure 2 Dose-dependent increases in the activity of glandular-kallikrein in bronchial washings caused by leukotriene C_4 (LTC_4) and leukotriene D_4 (LTD_4) in guinea-pigs. The ordinate scale shows the levels of activity of glandular-kallikrein in bronchial washings: (■) and (●) show the levels of the activity of glandular kallikrein after administration of LTC_4 and LTD_4, respectively; (○) indicates the control levels after injection of saline only. The numbers beside each point show the numbers of experiments. Each point represents the mean value (s.e.mean shown by vertical bar) and is compared with the value of the saline control. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

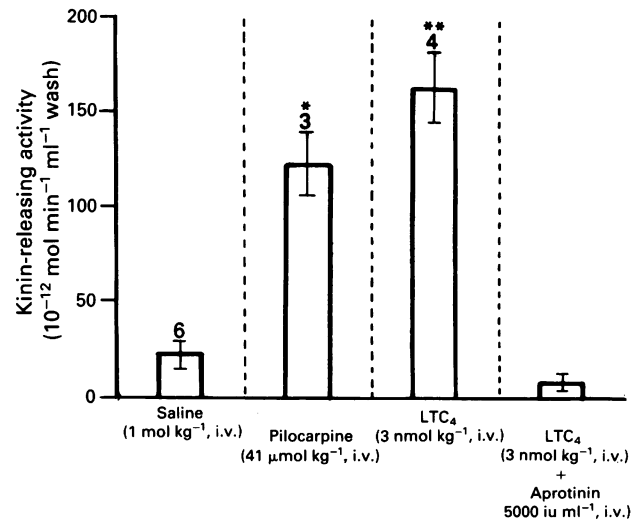

Figure 3 Kinin-generating activity in bronchial washings of guinea-pigs. The numbers beside each column show the numbers of experiments. Each column represents the mean value (s.e.mean shown by vertical bar). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

i.p.). As shown in Figure 4a, the increase in glandular-kallikrein induced by pilocarpine (41 μmol kg^{-1}, i.v.) was not inhibited by pretreatment with indomethacin.

By contrast, pretreatment with atropine (14 μmol kg^{-1}, i.v.) completely inhibited the increase in glandular-kallikrein induced by a bolus intravenous injection of STA_2 (20 pmol kg^{-1}) (Figure 4b).

Increased secretion of glandular-kallikrein into bronchial washings caused by bradykinin

As shown in Figure 5, intravenous administration of bradykinin (3, 10, 30 nmol kg^{-1}) increased the levels of glandular-kallikrein in the bronchial washings in a dose-dependent

Table 1 Inhibitory effects of drug pretreatments on leukotriene C_4-induced increases in glandular-kallikrein in bronchial washings of guinea-pigs

	Pretreatment	*Dose*	*Glandular-kallikrein activity* (10^{-11} mol min^{-1} ml^{-1} wash)	P	n
Saline	—	—	3.0 ± 0.2	—	6
	—	—	5.8 ± 0.4#	—	5
	ONO-1078	20 nmol kg^{-1}	4.1 ± 0.2	*	3
		60	3.7 ± 0.2	**	4
		200	3.1 ± 0.1	**	4
	Indomethacin	8.3 μmol kg^{-1}	5.0 ± 0.5	NS	5
		25	3.9 ± 0.1	**	5
		83	3.0 ± 0.2	**	4
LTC_4 3 nmol kg^{-1}	OKY-046	10 μmol kg^{-1}	3.5 ± 0.2	*	3
		30	2.9 ± 0.1	**	3
		100	2.9 ± 0.2	**	3
	S-1452	0.3 μmol kg^{-1}	3.9 ± 0.3	*	4
		1.0	2.7 ± 0.3	**	5
	Atropine	4.2 μmol kg^{-1}	5.0 ± 0.5	NS	4
		13	3.8 ± 0.2	*	4
		42	3.8 ± 0.1	*	4
	Scopolamine	10 μmol kg^{-1}	5.3 ± 0.4	NS	4
		30	4.1 ± 0.1	*	4
		100	3.1 ± 0.2	**	4

Each value represents the mean ± s.e.mean and is compared with the increased level # induced by leukotriene C_4 (LTC_4): * $P < 0.05$; ** $P < 0.01$, NS; not significant. *n*; numbers of animals. ONO-1078; a leukotriene antagonist. OKY-046; a thromboxane synthetase inhibitor. S-1452; a thromboxane antagonist.

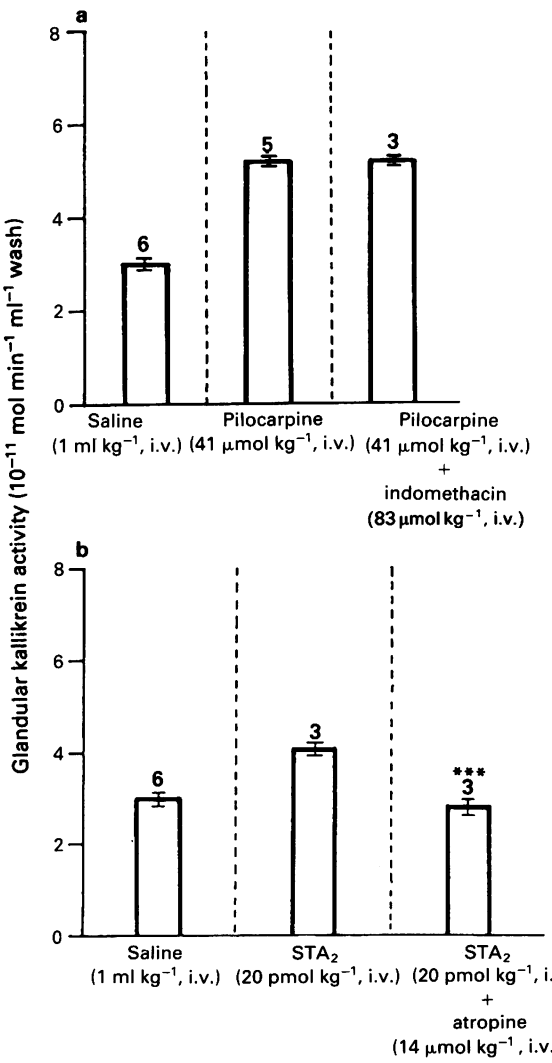

Figure 4 Effects of indomethacin (a) and atropine (b) on the glandular-kallikrein activity induced by pilocarpine (a) and STA_2 (b) in guinea-pigs. The ordinate scales show the levels of glandular-kallikrein activity in bronchial washings. The numbers beside each column show the numbers of experiments. Each column represents the mean value (s.e.mean shown by vertical bar). *** $P < 0.001$.

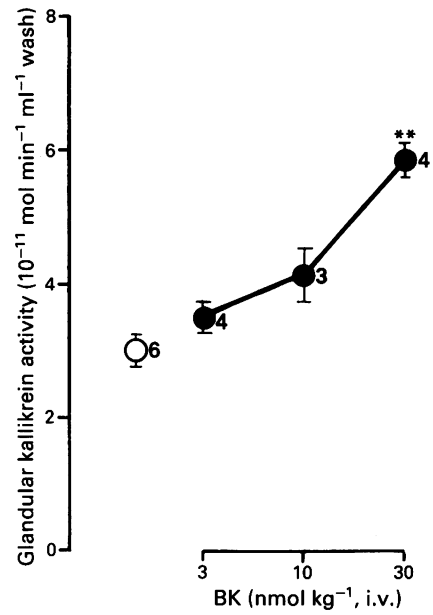

Figure 5 The increases in glandular-kallikrein activity in bronchial washings by bradykinin in guinea-pigs. The ordinate scale shows the levels of glandular-kallikrein activity in bronchial washings. (○) indicates the control level when only saline was injected. The numbers besides each point show the numbers of experiments. Each point represents the mean value (s.e.mean shown by vertical bar) and is compared with saline control (saline). ** $P < 0.01$.

manner to 118%, 133% and 198% of the levels in saline controls, respectively. Indomethacin (83 μmol kg^{-1}, i.p.) completely inhibited the increase in the levels of the glandular-kallikrein that was induced by bradykinin (30 nmol kg^{-1}). S-1452, a selective antagonist of thromboxane A_2 (0.3–1.0 μmol ml^{-1} saline kg^{-1}, i.v.) also inhibited the increase induced by bradykinin in a dose-dependent manner. Pretreatment with atropine (14 μmol kg^{-1}, i.v.), also completely inhibited this increase in the glandular-kallikrein (Figure 6).

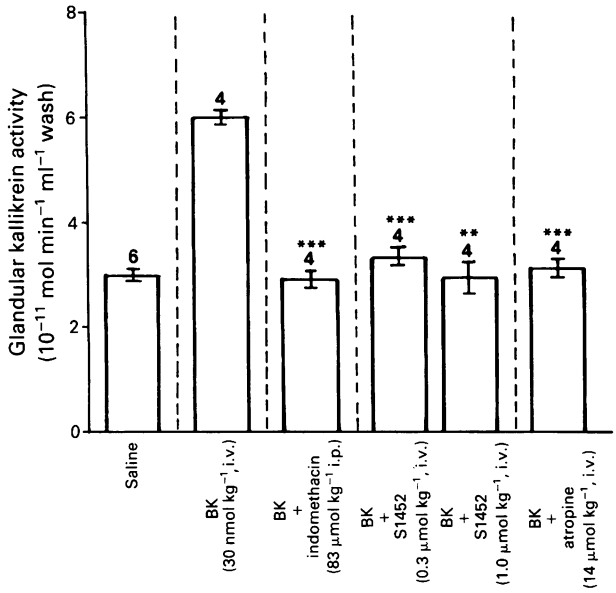

Figure 6 Inhibition of the bradykinin-induced increase in glandular-kallikrein activity by indomethacin, thromboxane antagonist (S-1452) and atropine in guinea-pigs. The ordinate scale shows the levels of glandular-kallikrein activity in bronchial washings. The numbers beside each column show the numbers of experiments. Each column represents the mean value (s.e.mean shown by vertical bar) and is compared with the increased levels induced by bradykinin (BK). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Inhibitory effects of a bradykinin antagonist on the increases in glandular-kallikrein activity induced by leukotriene C_4 and pilocarpine

As shown in Figure 7, intravenous administration of a bradykinin antagonist, Thi^{5,8}-D-Phe^{7}-bradykinin (100–1000 nmol kg^{-1}), 10 min before stimulation, caused a dose-dependent inhibition (by 55–80%) of the increases in levels of glandular-kallikrein caused by LTC_4. The increases in levels of glandular-kallikrein activity induced by pilocarpine were also inhibited by 81% by pretreatment with this bradykinin antagonist (300 nmol kg^{-1}).

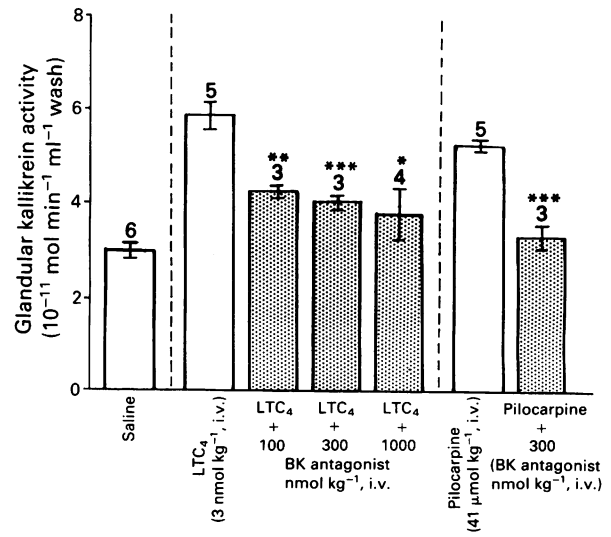

Figure 7 Inhibitory effects of bradykinin antagonist (BK antagonist) on the increases in glandular-kallikrein activity induced by leukotriene C_4 (LTC_4) and pilocarpine in guinea-pig. The ordinate scale shows the levels of the glandular-kallikrein activity in bronchial washings. The numbers beside each column show the numbers of experiments. Each column represents the mean value (s.e.mean shown by vertical bar) and is compared with the increased levels induced by leukotriene C_4 and pilocarpine, respectively. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Discussion

The kallikrein-kinin system in the body can be divided into two separate systems: the plasma kallikrein-high-molecular-weight (HMW) kininogen and glandular-kallikrein-low-molecular-weight (LMW) kininogen. Both systems operate independently *in vitro* and *in vivo* (Uchida & Katori, 1985; Katori *et al.*, 1988; 1989; Majima *et al.*, 1990). The plasma kallikrein-HMW kininogen system is activated via the activation of factor XII (Hageman factor) and activated plasma prekallikrein (plasma kallikrein) can release bradykinin from its substrate, HMW-kininogen. Glandular-kallikrein, present in the tissues of exocrine glands and their secretions, can act on its substrate, LMW kininogen, from which kallidin (Lys-bradykinin) is released (Katori *et al.*, 1988).

In the present study, intravenous injection of LTC_4 was found to induce an increase in the level of glandular-kallikrein in bronchial washings in guinea-pigs. This finding suggests that the increased activity of the glandular kallikrein-LMW kininogen system and, hence, increased formation of kinin in the airway might be involved in the pathogenesis of some kinds of airway disease, such as bronchial asthma, airway infection, stimulation by cold and dry breathing air, via the secretion of glandular-kallikrein.

Slow reacting substance of anaphylaxis (SRS-A) was originally reported to be released from lungs of sensitized guinea-pig (Kellaway & Trethewie, 1940) and it has potent bronchoconstrictor activity in this species (Adams & Lichtenstein, 1979). LTC_4, LTD_4 and LTE_4, chemical principles of SRS-A (Murphy *et al.*, 1979; Samuelsson & Hammarstrom, 1980), share this bronchoconstrictive activity (Ueno *et al.*, 1982). Furthermore, it was also reported that from guinea-pig isolated lung, SRS-A releases rabbit aorta contracting substance (RCS), which is now known to be mainly thromboxane A_2 (Piper & Vane, 1969). Similarly, an intravenous challenge with SRS-A also produces significant increases in tracheal mucus gel thickness and this increase is inhibited by pretreatment with indomethacin or imidazole (Yanni & Smith, 1986).

It is reported that in an isolated preparation of human bronchial mucosa, methacholine induced secretion both of glycoprotein from submucosal glands and goblet cells and of lysozyme, a selective indicator of the secretion from serous cells of submucosal glands, whereas LTC_4 and LTD_4 induced only the secretion of glycoprotein from submucosal glands (Coles *et al.*, 1981; 1983). An immuno-histochemical study for the localization of kallikrein in the gastrointestinal tracts indicated that goblet cells, not chief cells, in the mucosa express immunoreactive glandular-kallikrein intensely (Schachter *et al.*, 1983). Glandular-kallikrein may have an analogous location in the airway.

It is noteworthy that the enhancement of secretion by LTD_4 was much lower than that by LTC_4 in the present experiments, although the activity of LTD_4 in bronchoconstriction is three times that of LTC_4 in guinea-pigs (Ueno *et al.*, 1982). This difference may indicate a difference between the activities of the two leukotrienes in the airway.

The increased secretion of glandular-kallikrein in bronchial washings caused by LTC_4 was almost completely suppressed by either indomethacin, a thromboxane synthetase inhibitor (OKY-046) or a thromboxane antagonist (S-1452), and it resembled our previous report showing that bronchoconstriction induced by peptide leukotrienes in guinea-pigs was inhibited by indomethacin or a thromboxane synthetase inhibitor (Ueno *et al.*, 1982). Furthermore, this increased secretion by LTC_4 was also inhibited by atropine or scopolamine in the present experiment, suggesting the increased secretion of glandular kallikrein may be mediated by the release of acetylcholine from cholinergic nerves.

The cellular source of thromboxane A_2 formation is still

unclear, but the adventitial layer of the vessels may be a possible site, since constriction of the arterioles in the hamster cheek pouch by LTC_4 can be almost completely inhibited by a thromboxane synthetase inhibitor (Ueno *et al.*, 1983) and the bronchoconstriction induced by inhalation of LTD_4 is not inhibited by indomethacin, although that induced by intravenous LTD_4 is suppressed by the cyclo-oxygenase inhibitor (Hamel *et al.*, 1982). Intravenously administered STA_2 increased the secretion of kallikrein in bronchial washings. However, only small doses of STA_2 could be used, because death by thromboembolism occurs with large doses of this agonist. The observation that atropine inhibited the increased secretion of glandular kallikrein caused by STA_2 and the observation that indomethacin did not suppress that caused by pilocarpine (Figure 4) indicates that thromboxane A_2 formed in response to LTC_4 may stimulate the cholinergic nerve endings.

There are few reports in which thromboxane A_2 is described as stimulating cholinergic nerve endings. Thromboxane A_2 does, however, stimulate sensory nerves of dogs with the same potency as prostaglandin E_2, when injected into the canine splenic artery (Hori *et al.*, 1986).

Secreted glandular kallikrein may cause the release of kinin from LMW kininogen present in the surrounding interstitial space of the bronchus. We failed, however, to detect kinin in the bronchial washings, even in the presence of inhibitors of kininase I (D,L-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid, *o*-phenanthroline) and of kininase II (captopril, *o*-phenanthroline), since a major kininase in the plasma of guinea-pigs is an aminopeptidase, that is resistant to these inhibitors (data to be published).

Intravenous bradykinin itself had effects similar to those of LTC_4 (Figure 5). A more recent observation suggested that kinin increases lung mucus secretions. It was reported that when canine tracheal explants were incubated with ^{14}C-labelled D-glucosamine in the medium and labelled macromolecules were isolated from the culture medium, kinin enhanced the incorporation of the labelled material into high molecular weight molecules that may be mucin-type glycoproteins (Baker *et al.*, 1977). Thus, the observation in the present study that bradykinin increased the secretion of glandular-kallikrein into the bronchial washings via thromboxane formation and acetylcholine release (Figure 6), seems to be plausible. Furthermore, the increase in release of glandular-kallikrein induced by LTC_4 and pilocarpine could be inhibited by pretreatment with bradykinin antagonist (Figure 7). These observations suggest that kinin formation stimulated by LTC_4 results in a positive feedback loop that controls the release of the glandular-kallikrein into bronchial secretions. The activation of the glandular-kallikrein-LMW kininogen system in the airways may be involved in the pathogenesis of airway diseases via the capacity of bradykinin to induce bronchoconstriction and mucus secretion.

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Characterization of P_1-purinoceptors on rat duodenum and urinary bladder

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1 The P_1-purinoceptors mediating relaxation of the rat duodenum and inhibition of contraction of the rat urinary bladder were characterized by use of adenosine and its analogues 5'-N-ethylcarboxamidoadenosine (NECA), N^6-cyclopentyladenosine (CPA) and 2-*p*-((carboxyethyl)phenethylamino)-5'-carboxamidoadenosine (CGS 21680), as well as the A_1-selective antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX). The stable analogue of adenosine 5'-triphosphate (ATP), adenylyl 5'-(β,γ-methylene)diphosphonate (AMPPCP), was also used as previous work had indicated that it has a direct action on some P_1 receptors in addition to its P_2-purinoceptor activity.

2 In the rat duodenum, the order of potency of the adenosine agonists was NECA $\geqslant$ CPA > AMPPCP = adenosine > CGS 21680, and DPCPX antagonized CPA and AMPPCP at a concentration of 1 nM whereas equivalent antagonism of NECA and adenosine required a concentration of 1 μM. This suggests the presence of a mixture of A_1 and A_2 receptors in this tissue, with CPA and AMPPCP acting on the A_1 and NECA and adenosine acting on the A_2 receptors.

3 In the rat bladder, the order of potency of the adenosine agonists for inhibition of carbachol-induced contractions was NECA $\gg$ adenosine > CPA = CGS 21680, and a concentration of DPCPX of 1 μM was required to antagonize responses to NECA and adenosine. This suggests the presence of A_2 receptors in this tissue. ATP and AMPPCP each caused contractions which were not enhanced by DPCPX (1 μM) which suggests that in this tissue AMPPCP was acting only via P_2 receptors and had no P_1 agonist activity. That AMPPCP was active on the A_1 receptors in the duodenum but inactive on the A_2 receptors in the bladder implies that it has selectivity for the A_1 subtype.

4 That CGS 21680, which has been reported to bind selectively to the high affinity A_{2a} subclass of A_2 receptors, had a very low potency on the A_2 receptors in the duodenum and in the bladder suggests that these receptors are of the low affinity A_{2b} subclass.

Keywords: Adenosine; ATP; purinoceptors; duodenum; bladder

Introduction

Adenosine and adenine nucleotides have pharmacological actions on a variety of smooth muscle preparations, and these effects are mediated via receptors which have been classified as P_1- and P_2-purinoceptors, recognising adenosine and adenosine 5'-triphosphate (ATP) respectively (Burnstock, 1990; Kennedy, 1990). Studies with ATP are complicated by its rapid dephosphorylation by ectonucleotidases present on smooth muscle preparations, to give adenosine which has its own effects (Slakey *et al.*, 1990).

P_2-purinoceptors on smooth muscle have been subdivided into P_{2X}, usually mediating contraction, and P_{2Y}, usually mediating relaxation, and this classification is largely based on the different structure-activity relationships of ATP analogues in causing these two effects (Burnstock & Kennedy, 1985; Gordon, 1986; Kennedy, 1990; Cusack & Hourani, 1990). For P_{2X}-purinoceptors, methylene phosphonate analogues of ATP such as adenylyl 5'-(β,γ-methylene)diphosphonate (AMPPCP) and adenosine 5'-(α,β-methylene)triphosphonate (AMPCPP), which are resistant to dephosphorylation, are more potent than ATP which is equipotent with 2-substituted analogues such as 2-methylthioadenosine 5'-triphosphate (MeSATP), whereas on P_{2Y}-purinoceptors MeSATP is more potent than ATP which is more potent than AMPPCP or AMPCPP. However, these structure-activity relationships may be partly dependent on the different sensitivities to degradation of the analogues by ectonucleotidases, which appears to enhance their potency on some tissues but not on others (Welford *et al.*, 1986; 1987).

P_1-purinoceptors have been subdivided into A_1 and A_2 according to the potency of adenosine analogues and their sensitivity to A_1-selective antagonists such as 1,3-dipropyl-8-cyclopentylxanthine (DPCPX). On A_1 receptors, N^6-substituted analogues such as N^6-cyclopentyladenosine (CPA) are more potent than 5'-substituted analogues such as 5'-N-ethylcarboxamidoadenosine (NECA), and DPCPX has a dissociation constant in the nanomolar range, whereas on A_2 receptors NECA is more potent than CPA and DPCPX has a dissociation constant in the micromolar range (Bruns, 1990; Kennedy, 1990). Because the affinities of adenosine agonists are generally less for A_2 than for A_1 receptors, NECA is not selective for A_2 receptors but has roughly equal potency on each subtype, but a new analogue, 2-*p*-((carboxyethyl)phenethylamino)-5'-carboxamidoadenosine (CGS 21680) has recently been developed and reported to be highly A_2-selective (Hutchison *et al.*, 1989; Jacobson, 1990). Although some problems have been encountered in the classification of P_1-purinoceptors in smooth muscle, as compounds which have been reported to be selective in the central nervous system do not always display selectivity in the periphery, DPCPX has been shown to discriminate clearly between the A_1 and A_2 subtypes in pharmacological studies (Collis *et al.*, 1989; Collis, 1990).

Little is known about the ontogeny of purinergic systems in smooth muscle, but we have studied the development of responses to adenosine and ATP in two tissues, the rat duodenum and urinary bladder. In each tissue responses to purines are present from birth, and in some cases the potency of the purines is considerably higher in the neonate than in the adult, indicating that purines may play an important role in neonatal tissues. The rat duodenum relaxes to ATP and the rat bladder contracts, and these responses, as expected, are mediated by P_{2Y}- and P_{2X}-purinoceptors respectively. In the neonatal rat duodenum however, ATP causes contraction rather than relaxation, and this contraction is also mediated by P_{2Y}-purinoceptors and is not due to secondary release of prostaglandins (Nicholls *et al.*, 1990). Adenosine is inhibitory

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in both these tissues, but the subtype of P_1-purinoceptor which mediates its responses has not been defined so far. In the duodenum we have shown that AMPPCP acts unexpectedly via P_1 and not P_2-purinoceptors to cause relaxation, as its effects are inhibited by the P_1 antagonist 8-*p*-sulphophenyltheophylline (8-SPT), whereas the effects of ATP, MeSATP and AMPCPP are not inhibited (Hourani *et al.*, 1991). The ontogeny of responses of the duodenum to AMPPCP also mirrors that of adenosine rather than of ATP, and indeed AMPPCP is never observed to induce contraction (Nicholls *et al.*, 1990). We have also shown an anomolous P_1 effect of AMPPCP in another smooth muscle preparation, the rat colon muscularis mucosae (Bailey & Hourani, 1990), and to a lesser extent in the guinea-pig taenia caeci (Hourani *et al.*, 1991). The P_1 effect of AMPPCP in these tissues is direct and not a consequence of its breakdown to adenosine, as it is much more resistant to degradation than is ATP or MeSATP, which are not inhibited by 8-SPT (Bailey & Hourani, 1990; Hourani *et al.*, 1991). In the rat duodenum, a ten fold lower concentration of 8-SPT was required to inhibit the effects of AMPPCP than to inhibit the effects of adenosine, which could indicate that their effects were mediated by different P_1-purinoceptors as 8-SPT has been reported to be selective for A_1 receptors in the central nervous system (Bruns *et al.*, 1986), although it may not discriminate between the subtypes in smooth muscle (Collis *et al.*, 1987). We therefore decided to use the more selective antagonist DPCPX, together with the agonists NECA, CPA and CGS 21680, to characterize the P_1-purinoceptor subtypes in the rat duodenum and urinary bladder.

Methods

Adult male Wistar rats (200–250 g) were killed by cervical dislocation, and the duodenum and urinary bladder dissected out and responses quantified as described in Nicholls *et al.* (1990). Briefly, the tissues were mounted in 10 ml organ baths containing Krebs buffer of the following composition (mM): NaCl 118, KCl 4.8, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11, aerated with 95% O_2/5% CO_2 and maintained at 35°C. A resting tension of 1 g was applied to the tissues, and isometric responses were recorded with a Grass FT03 transducer and displayed on a Grass 79D polygraph. Tissues were equilibrated for 45 min before addition of drugs, concentration-response curves were obtained non-cumulatively and potency estimates (EC_{50} values) were obtained from linear regression analysis of the ascending, linear portion of the individual concentration-response curves. For the duodenum, inhibitory responses were quantified by pre-contracting the tissue with 0.1 μM carbachol before challenging with agonist, and the relaxation expressed as % inhibition of this carbachol-induced contraction. For the bladder, excitatory responses to ATP and to AMPPCP were expressed as % of the maximal contraction induced by KCl (120–180 mM). The inhibitory effects of adenosine and its analogues were expressed as % inhibition of carbachol contraction, where the purines were added 30 s before challenge with carbachol (3 μM). In each tissue, after control concentration-response curves had been obtained, the tissues were incubated for 30 min with DPCPX, and the concentration-response curves repeated in the presence of the antagonist. Dose-ratios were calculated from the ratio of the EC_{50} values in the absence and presence of antagonist, and dissociation constants (K_D values) were calculated as the molar concentration of the antagonist divided by the dose-ratio − 1. In some experiments with the duodenum, hexamethonium (10 μM) was used as a nicotinic antagonist with the same protocol as DPCPX, and dimethylphenylpiperazinium (DMPP) was used as a nicotinic agonist.

Materials

Adenosine, ATP, AMPPCP, NECA, DMPP, carbachol and hexamethonium were obtained from Sigma UK, Ltd, and DPCPX, CPA and CGS 21680 from Research Biochemicals Inc. CPA (1 mM) was dissolved in 2% ethanol and DPCPX (1 mM) in 2% aqueous dimethylsulphoxide (DMSO) containing 6 mM NaOH. After dilution corresponding to the final bath concentration of the substances used, these solvents had no effect on the responses of the tissues.

Results

Rat duodenum

Adenosine, AMPPCP, CPA, NECA and CGS 21680 all relaxed the carbachol-contracted rat duodenum, the order of potency being NECA ≥ CPA > AMPPCP = adenosine > CGS 21680, and the EC_{50} values of these compounds were 0.4 μM, 0.7 μM, 18 μM, 28.5 μM and 98 μM respectively (Figure 1). DPCPX (1 nM) caused a parallel shift to the right of the concentration-response curves to AMPPCP and CPA, giving dose-ratios of 6.7 and 5.7, corresponding to K_D values of 0.18 nM and 0.21 nM, respectively (Figures 2a,b), but had no effect on the concentration-response curves to adenosine or NECA (Figures 2c,d). At 1 μM, DPCPX did cause shifts in the concentration-response curves to adenosine and NECA, giving dose-ratios of 6.3 and 6.7, corresponding to K_D values of 0.19 μM and 0.18 μM, respectively (Figures 2e,f). DMPP (0.1–100 μM) caused relaxation of the rat duodenum, followed at high concentrations by contraction, and these effects were inhibited by hexamethonium (10 μM), which had no effect on the contractions induced by carbachol, or on the concentration-response curves to AMPPCP or to CPA (results not shown).

Rat urinary bladder

Adenosine and NECA each inhibited contractions of the rat bladder induced by carbachol (3 μM), and the order of potency was NECA ≫ adenosine, the EC_{50} values being 1.6 μM and 92 μM respectively (Figure 3a). CPA and CGS 21680 (10 μM) also weakly inhibited carbachol-induced contractions, but EC_{50} values could not be obtained due to their low potency (results not shown). DPCPX (1 μM) caused parallel shifts to the right of the concentration-response curves for NECA and adenosine, giving dose-ratios of 5.7 and 6.3, corresponding to K_D values of 0.21 μM and 0.19 μM, respectively (Figure 3a). ATP and AMPPCP each contracted the rat urinary bladder, and these contractions were not enhanced by DPCPX (1 μM) (Figure 3b).

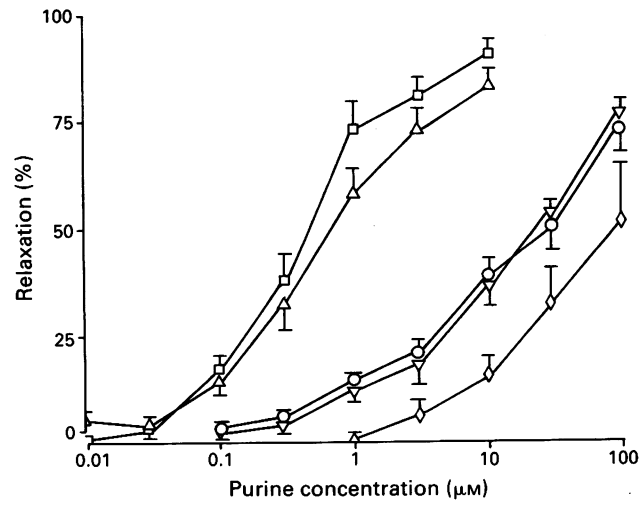

Figure 1 Relaxation of the rat duodenum induced by NECA (□), CPA (△), AMPPCP (▽), adenosine (○) and CGS 21680 (◇). Each point is the mean of at least nine determinations, and the vertical bars show s.e.mean. For abbreviations, see text.

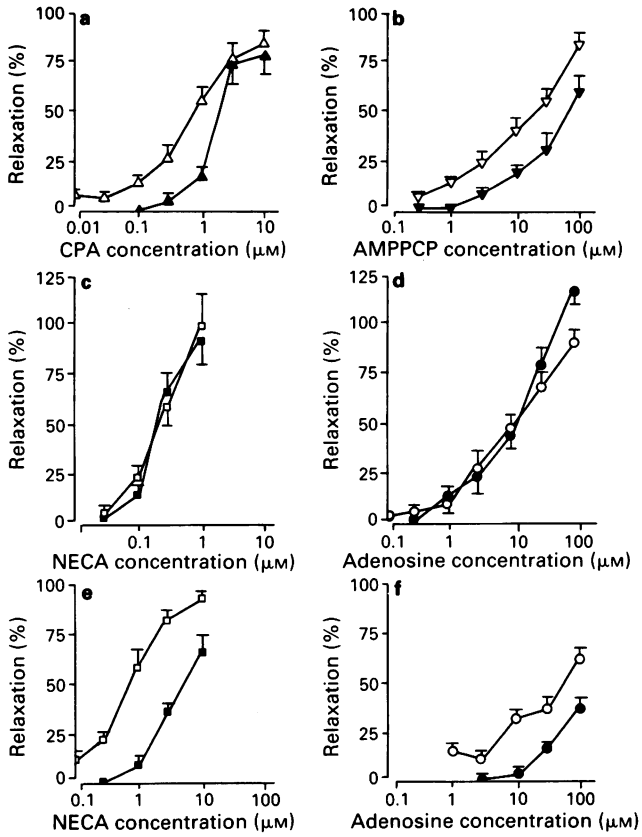

Figure 2 Relaxation of the rat duodenum induced by purines in the absence (open symbols) or presence (closed symbols) of DPCPX. (a) CPA ± DPCPX (1 nM), (b) AMPPCP ± DPCPX (1 nM), (c) NECA ± DPCPX (1 nM), (d) adenosine ± DPCPX (1 nM), (e) NECA ± DPCPX (1 μM), (f) adenosine ± DPCPX (1 μM). Each point is the mean of at least seven determinations, and the vertical bars show s.e.mean. For abbreviations, see text.

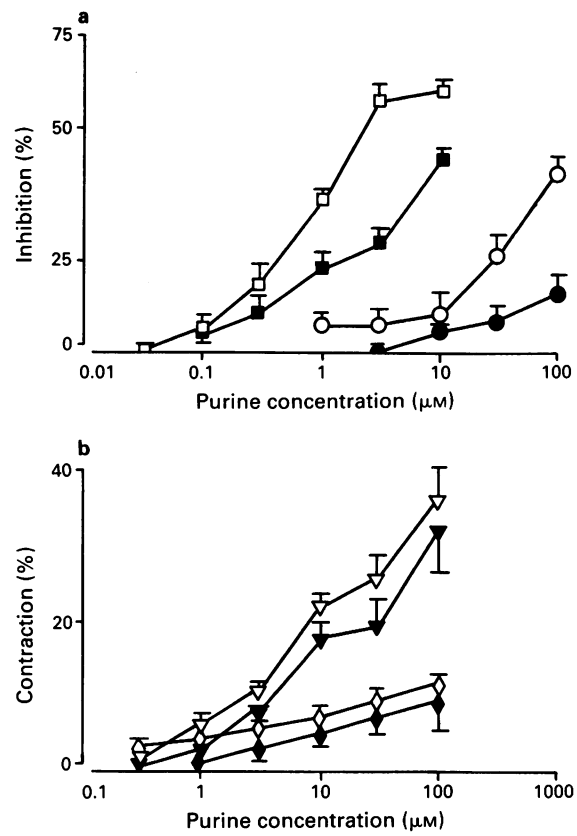

Figure 3 Effects of purines on rat urinary bladder in the absence (open symbols) or presence (closed symbols) of DPCPX (1 μM). (a) Inhibitory actions of NECA (□, ■) and adenosine (○, ●) on carbachol-induced contractions. (b) Contractile effects of AMPPCP (▽, ▼) and ATP (◇, ◆). Each point is the mean of at least five determinations, and the vertical bars show s.e.mean. For abbreviations, see text.

Discussion

These results show that in the duodenum all the adenosine receptor agonists used caused relaxation, but that the order of potency did not fall clearly into one of the two expected patterns for A_1 or A_2 receptors, as NECA and CPA were almost equipotent. Similar results have been interpreted to imply the existence of a third class of adenosine receptor, which has been called A_3 (Ribeiro & Sebastiao, 1986), but this suggestion has not gained wide acceptance. Our results with DPCPX show that whereas CPA and AMPPCP were inhibited by an A_1-selective concentration of DPCPX (1 nM), much higher concentrations (1 μM) were required to inhibit adenosine or NECA. This suggests that the duodenum contains a mixture of A_1 and A_2 receptors, with NECA and adenosine acting on the A_2 population and being inhibited by DPCPX with a dissociation constant in the micromolar range, whereas CPA and AMPPCP act on the A_1 receptors and are inhibited by DPCPX with a dissociation constant in the nanomolar range. The log concentration-response curve to CPA was somewhat steeper in the presence of 1 nM DPCPX, suggesting that at high concentrations CPA may be acting on the A_2 receptors as well. It is possible that in other tissues where NECA and CPA are equipotent, the same explanation may apply, and that a mixture of A_1 and A_2 receptors may account for the proposed A_3 subclass.

The significance of the presence of two P_1-purinoceptor subclasses both mediating an inhibitory response is unclear, as in general in smooth muscle A_2 receptors mediate relaxation whereas A_1 receptors mediate presynaptic inhibition of transmitter release or postsynaptic contractile responses (White, 1988; Kennedy, 1990). Although in several tissues, such as the guinea-pig trachea and aorta, the presence of both subclasses of P_1-purinoceptor has been reported, in each case the A_1 receptor mediates contraction while only the A_2 mediates relaxation (Farmer *et al.*, 1988; Stoggall & Shaw, 1990). For two receptor subtypes to mediate the same response in one tissue preparation is unusual, and it is not at all clear why such an apparently redundant system should have evolved. One possible explanation for the relaxant A_1 effect we have observed is that the carbachol used to precontract the tissues could have been stimulating ganglionic nicotinic receptors to cause release of acetylcholine or some other excitatory transmitter, with the A_1 receptors being situated presynaptically to oppose this release. However, the initial response to the nicotinic agonist DMPP was relaxation rather than contraction, as found by Manzini *et al.* (1985) who suggested that this is due to ganglionic stimulation of purinergic nerves. Hexamethonium at a concentration sufficient to block nicotinic receptors (10 μM) inhibited these responses to DMPP but did not inhibit the carbachol-induced contractions, suggesting that carbachol was acting directly on the muscle. In addition, hexamethonium did not affect the responses to the A_1 agonists, which again suggests that they were not acting on presynaptic receptors.

The results with the rat bladder show that the P_1-purinoceptor mediating inhibition of contraction in this tissue is of the A_2 class, as NECA was much more potent than CPA and a high concentration (1 μM) of DPCPX was required to antagonize adenosine or NECA, an A_1-selective concentration (1 nM) having no effect. The log concentration-response curve to NECA was somewhat flattened by DPCPX (1 μM), suggesting that it may not be having a solely competitive effect here. ATP and AMPPCP, which act at

P_{2X}-purinoceptors in this tissue to cause contraction, do not appear to have any action on the A_2 receptors in this tissue, as their contractions were not enhanced by blockade of P_1-purinoceptors by DPCPX. The relaxation induced by AMPPCP in the rat duodenum was however antagonized by an A_1-selective concentration of DPCPX, which confirms our previous conclusions (Hourani *et al.*, 1991) that this agonist, although it is an ATP analogue which is resistant to degradation, acts entirely and directly via P_1-purinoceptors in this tissue, and has no P_{2Y} effect. That AMPPCP has apparently no effect on the A_2 receptors in the bladder whereas it is clearly an A_1 agonist in the duodenum, suggests that it is selective for A_1 receptors and has little A_2 agonist activity. This suggestion would be compatible with its rather weak P_1 effect in the guinea-pig taenia caeci (Hourani *et al.*, 1991), which also contains A_2 receptors (Burnstock & Kennedy, 1985).

One surprising finding was the lack of potency of the A_2-selective agonist CGS 21680 in these tissues. In both the duodenum and the bladder it was much less potent than NECA, whereas it binds to rat brain A_2 receptors with an affinity roughly equal to NECA (Jarvis *et al.*, 1989), and is roughly equipotent with NECA in relaxing a number of blood vessels (Balwierczak *et al.*, 1991). A_2 receptors have been subdivided into A_{2a}, which have a high affinity and are found in the striatum, and A_{2b} which have low affinity and are found elsewhere in the brain and in fibroblasts (Bruns *et al.*, 1986; Lupica *et al.*, 1990). CGS 21680 appears to bind only to the A_{2a} sites in rat brain (Jarvis *et al.*, 1989), and one possible explanation for the lack of potency of CGS 21680 in the rat duodenum and bladder may therefore be that the A_2 receptors in these tissues are of the A_{2b} subtype. CGS 21680 has also been found to have very weak relaxant effects compared to NECA in the dog saphenous vein and guinea-pig aorta, and this has been interpreted as indicating the presence of A_{2b} receptors in these tissues (Hargreaves *et al.*, 1991), although in the absence of any selective antagonists this cannot be confirmed.

In conclusion, we have shown that the P_1-purinoceptors on the rat duodenum are a mixture of A_1 and A_2, each mediating relaxation, and that the inhibitory P_1-purinoceptors on the rat bladder are of the A_2 subtype. The stable ATP analogue, AMPPCP, appears to act directly on the A_1 receptors but not on the A_2, in addition to its potent action on the contractile P_{2X} receptors in the bladder. In each tissue the A_{2a}-selective agonist CGS 21680 has a very low potency, suggesting that the A_2 receptors may be of the low affinity A_{2b} subtype.

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Inhibition of endothelium-dependent vasorelaxation by arginine analogues: a pharmacological analysis of agonist and tissue dependence

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1 Isolated rings of rabbit external jugular vein (RbJV) and rat thoracic aorta (RA) were used to study the effect of the NO synthase inhibitor L-N^G-nitroarginine methyl ester (L-NAME) on muscarinic and 5-hydroxytryptamine (5-HT) receptor-stimulated, endothelium-dependent vascular relaxations.

2 In RbJV relaxations produced by the endothelial 5-HT receptor agonist α-methyl-5-HT were potently and non-surmountably inhibited by L-NAME (10 μM), whereas acetylcholine relaxations in this tissue were unaffected by this concentration of inhibitor. By contrast, acetylcholine relaxations in RA were virtually abolished by 10 μM L-NAME. In each case an equivalent concentration of D-NAME was without effect on agonist-induced relaxations.

3 The different effect of L-NAME on acetylcholine relaxations in RbJV and RA was not due to muscarinic receptor differences. Affinity estimates for acetylcholine ($pK_A = 6.12 \pm 0.09$; 6.09 ± 0.08 respectively) and for 4-diphenyl-acetoxy-N-methylpiperidine methobromide (4-DAMP, $pK_B = 9.01 \pm 0.12$; 9.24 ± 0.16 respectively) indicated that the receptors in both tissues belong to the same M_3 class. Tissue differences resulting from the release of a cyclo-oxygenase product or a glibenclamide-sensitive K^+-channel-linked hyperpolarizing factor were also ruled out by selective inhibition of these pathways.

4 When phenoxybenzamine was used to reduce the efficacy of acetylcholine in RbJV so that it behaved as a partial agonist in this tissue, L-NAME (10 μM) now produced non-surmountable inhibition of relaxation responses. In untreated tissues the same concentration of L-NAME also profoundly inhibited responses produced by butyrylcholine and pilocarpine, both of which behave as partial agonists at the M_3 receptor in RbJV.

5 A simple model was developed which describes the theoretical behaviour of receptor-stimulated synthesis and release of NO. The model predicts that competitive inhibition of NO formation results in parallel displacements of the agonist response curve in the case of high efficacy agonist, but right-shift with concomitant depression of the curve maximum in the case of low efficacy agonists. Simulations based on the model showed reasonable agreement with the experimental data.

6 It is concluded that analogues of L-arginine demonstrate tissue- and agonist-dependence in terms of their ability to inhibit receptor-mediated events involving the liberation of NO. This behaviour can reflect differences in agonist efficacy in the receptor systems being studied, a possibility that should be ruled out before apparent resistance to inhibition is taken as evidence for the involvement of heterogeneous endothelium-derived relaxing factors (EDRFs).

Keywords: Endothelium-derived relaxing factor (EDRF); L-N^G-nitroarginine methyl ester (L-NAME); 5-HT receptors; muscarinic receptors; vasorelaxation

Introduction

A number of pharmacological agents indirectly mediate vascular relaxation by stimulating the release from endothelial cells of a short-lived humoral substance which Furchgott & Zawadski (1980) termed endothelium-derived relaxing factor (EDRF). Numerous studies have subsequently shown that the biological effects of EDRF are consistent with it being nitric oxide (NO: Ignarro *et al.*, 1987; Kahn & Furchgott, 1987; Palmer *et al.*, 1987), possibly incorporated into one, or several different nitrosothiols (Myers *et al.*, 1990). The source of endothelial cell NO appears to be the terminal guanidino nitrogen atom(s) of the amino-acid L-arginine (Palmer *et al.*, 1988; Schmidt *et al.*, 1988) and a growing body of evidence suggests that the biochemical pathway involved in its liberation is similar in a variety of other cell types (see Moncada *et al.*, 1989). Support for this comes from the ability of a number of arginine analogues to inhibit stereoselectively the formation of NO and/or its biological effects. In this regard analogues such as L-N^G-monomethyl arginine (L-NMMA), L-N^G-nitroarginine methylester (L-NAME) and more recently L-iminoethyl-L-ornithine (L-NIO) are being used increasingly as probes with which to investigate the possible physiological and pathological roles of NO (see Rees *et al.*, 1990).

In our laboratory we have been using L-NAME to study the characteristics of receptor-stimulated, endothelium-dependent relaxations in rings of rabbit jugular vein (RbJV) and rat aorta (RA). The endothelium of RbJV possesses 5-hydroxytryptamine (5-HT) receptors effecting vascular relaxation indirectly (Leff *et al.*, 1987; Martin *et al.*, 1987) and, as shown here, endothelial muscarinic receptors are also present in this tissue. The endothelium of RA likewise possesses muscarinic receptors (Choo *et al.*, 1986) but is devoid of receptors for 5-HT (this study). To our surprise we found that in RbJV, endothelium-dependent relaxations elicited by the 5-HT receptor agonist α-methyl-5-HT (α-Me-5-HT) were effectively inhibited by L-NAME whereas the effects of acetylcholine (ACh) were not. On the other hand, ACh relaxations obtained in RA were virtually abolished in the presence of L-NAME.

In view of recent reports implying that different receptor systems stimulate the release of different EDRFs (Hoeffner *et al.*, 1989; Boulanger *et al.*, 1989) we sought to ascertain whether or not this might also be the case for endothelial

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5-HT and muscarinic receptors. This prompted a series of experiments which led us to consider and then reject the data as evidence for heterogeneous EDRFs. Instead an alternative possibility was explored, namely that the agonist- and tissue-dependence of inhibition by arginine analogues might result from differences in the efficiency of occupancy-effect coupling for each receptor system studied. The good agreement between experimental data and predictions based upon a simple theoretical model support this premise. Here we describe the results of these studies and consider their implications for the use of inhibitors in the study of biological events mediated by the receptor-stimulated release of NO.

A preliminary account of these findings was presented to the Spring meeting of the British Pharmacological Society (Giles *et al.*, 1990).

Methods

Tissue preparation

Right and left external jugular veins were removed from male New Zealand White rabbits (2.4–3.0 kg) treated with heparin (200 units, i.v.) and then killed by injecting pentobarbitone sodium (Sagatal; 120 mg kg^{-1}) into a marginal ear vein. After removal of adhering connective tissue, three ring segments 5 mm wide were prepared from each vessel. Rings of thoracic aorta (3 mm) were obtained from male Wistar rats (350–425 g) killed by a blow to the head. As above, each animal provided six ring preparations. In both cases, care was taken to preserve the endothelium.

Rings of RbJV or RA were suspended in 20 ml organ baths between tungsten wire hooks (0.5 mm), one attached to a Grass FT03C force displacement transducer and the other to a stationary support. The bathing medium was Krebs solution 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 and $CaCl_2$ 2.50. This was maintained at 37 ± 0.5°C, pH 7.4 ± 0.1 and continually aerated with 95% O_2:5% CO_2. Responses were measured as changes in tissue isometric force and recorded on Gould BS272 pen recorders.

Protocols

At the beginning of each experiment vascular rings were subject to a force of either 0.75 g (RbJV) or 1.0 g (RA) and subsequently allowed to stabilize for 60 min. During this period the organ bath buffer was replaced three times and the force reinstated. Tissues were then contracted either with phenylephrine (0.1–1 μM: RA) or the thromboxane A_2-mimetic, U-46619 (10 nM: RbJV). In both cases the concentration of spasmogen used produced 60–70% of the maximum response, the required concentration of phenylephrine being determined in each tissue by first constructing a cumulative concentration-effect (E/[A]) curve. At steady-state contracture ACh (1 μM: RbJV; 10 μM: RA) or α-Me-5-HT (1 μM: RbJV only) was added to check viability of the endothelium. Preparations which failed to relax by more than 50% of the induced tone were discarded. In tissues denuded of endothelium, ACh and α-Me-5-HT relaxations were abolished (see also Leff *et al.*, 1987).

Endothelium-dependent responses to agonists

Experiments using RbJV followed a multiple curve design (pairs or triplets) whereas in RA only a single curve per preparation was obtained. Separate experiments in RbJV demonstrated that first, second and third relaxation curves were superimposable, but data from a particular tissue were rejected if the successive contractures produced by U-46619 (10 nM) differed by more than 10%. In both types of experiment, E/[A] curves were cumulative, agonist concentrations increasing in 0.5 $\log_{10}$ increments.

Affinity estimates using irreversible receptor alkylation

Muscarinic receptor agonist affinities were determined by Furchgott's (1966) method of partial irreversible receptor occlusion. In these experiments a fraction of the total functional muscarinic receptor pool was inactivated with phenoxybenzamine (0.1–10 μM, 30 min). U-46619 was the spasmogen used in both RA (20 nM) and RbJV (10 nM). In multiple curve studies the first curve was always to ACh; second and third curves to either ACh, pilocarpine or butyrylcholine followed after complete washout of the previous agonist additions and a recovery period of 60 min. Phenoxybenzamine was added between first and second curves (ACh) or between second and third curves (pilocarpine, butyrylcholine).

Studies with L-N^G-nitroarginine methyl ester

Studies with α-Me-5-HT followed a paired curve design. The effects of 10 μM and 100 μM L-NAME (30 min) on muscarinic agonist curves were studied between first and second curves and second and third curves respectively, except when ACh curves were obtained after phenoxybenzamine treatment. In these studies phenoxybenzamine was added between first and second curves and the effects of only a single L-NAME concentration (10 μM or 100 μM) studied between second and third curves. Control experiments with ACh indicated that there was a recovery from receptor alkylation between second and third curves, consistent with a 1.98 fold increase in agonist efficacy (average increase from 8 experiments). This factor was used to correct third curves to ACh obtained in the presence of L-NAME.

Experiments with glibenclamide (10 μM, 60 min) and indomethacin (2.8 μM, 60 min) required only paired curves.

Studies with 4-diphenyl-acetoxy-N-methylpiperidine methobromide (4-DAMP)

Following washout of the first ACh challenge to establish viability of the endothelium, tissues were exposed to 4-DAMP (0.001–1.0 μM) for 60 min before recontracture and construction of an agonist cumulative E/[A] curve.

Data analysis

E/[A] curve data were routinely fitted to a logistic function of the form;

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

which provided estimates of the asymptote (α), mid-point ($p[A_{50}]$) and slope parameter (m). In paired curve experiments parameter estimates were compared using a paired t test. When, in antagonist studies, α and m were not different, $\Delta p[A_{50}]$ values at each antagonist concentration were analysed using the Schild regression (Arunlakshana & Schild, 1959). If the Schild plot slope was not different from unity it was constrained to this value in order to estimate pK_B. When antagonists were studied using a single curve per preparation design a one-way analysis of variance compared computed estimates of α and m within and between treatment groups. If the antagonist produced parallel curve displacements with common asymptotes, suggesting competitive antagonism, computed $p[A_{50}]$ values were fitted directly to the following form of the Schild equation (Black *et al.*, 1985a; Trist & Leff, 1985);

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

where $[A^C_{50}]$ is a control curve $[A_{50}]$ value, [B] is the concentration of antagonist, K_B its dissociation constant and n a value equivalent to the slope of the Schild regression. If n was not different from unity it was constrained to this value in order to estimate K_B.

Estimates of agonist affinity and efficacy were derived using direct operational model-fitting methods (Black & Leff, 1983; Leff *et al.*, 1990). Agonist E/[A] curve data obtained before and after fractional receptor inactivation using phenoxybenzamine were fitted directly to the expression;

$$E = \frac{E_m \tau^n [A]^n}{(K_A + [A])^n + \tau^n [A]^n} \quad (3)$$

in which K_A is the agonist dissociation constant, τ is the efficacy of the agonist in a particular tissue, E_m is the maximum possible effect in the receptor system and n defines the slope parameter of the occupancy-effect relation. The different statistical treatments which apply to single as opposed to multiple curve/preparation studies have been discussed in detail elsewhere (Leff *et al.*, 1990).

Drugs

Acetylcholine hydrochloride (Research Biochemicals Inc.); pilocarpine hydrochloride, butyrylcholine hydrochloride, phenylephrine hydrochloride, histamine acid phosphate, glibenclamide and N^G-nitro-L-arginine methyl ester (Sigma Chemical Co. Ltd.); 9,11-dideoxy-9α,11α-methanoepoxy-$PGF_1\alpha$ (U-46619: Cayman Chemical); glyceryl trinitrate (Wellcome Research Laboratories); 4-diphenylacetoxy-N-methylpiperidine methobromide (4-DAMP: a generous gift from Dr R.B. Barlow, University of Bristol); phenoxybenzamine hydrochloride (Smith, Kline & French). N^G-nitro-D-arginine methyl ester and (±)-α-methyl-5-hydroxytryptamine creatinine sulphate were synthesized respectively by Drs H.F. Hodson and A.D. Robertson, Medicinal Chemistry Department, Wellcome Research Laboratories.

U-46619 and phenoxybenzamine were dissolved at 2 mM in absolute ethanol and subsequently diluted in distilled water to give a final concentration in the organ bath of ≤0.05% v/v. Tissue responsiveness was unchanged by the vehicle. All other drugs were dissolved in distilled water.

Results

Effects of D- and L-N^G-nitroarginine methyl ester in rat aorta and rabbit jugular vein

Endothelium-dependent relaxations to ACh or α-Me-5-HT were obtained in vascular ring preparations contracted with a submaximally effective concentration (~$p[A_{60-70}]$) of either U-46619 (10 nM: RbJV) or phenylephrine (0.1–1 μM: RA). ACh was 20 times more potent in RbJV ($p[A_{50}] = 8.58 \pm 0.11$) than in RA ($p[A_{50}] = 7.27 \pm 0.28$) (Figure 1a,b). α-Me-5-HT also caused potent endothelium-dependent relaxations in RbJV ($p[A_{50}] = 8.18 \pm 0.18$) (Figure 1c) but was devoid of endothelium-dependent activity in RA.

Prior exposure of rat aortic rings to L-NAME resulted in a graded inhibition of endothelium-dependent responses to ACh, 10 μM of the inhibitor causing almost complete abolition of relaxations (Figure 1b). D-NAME (10 μM) was without effect on ACh responses in this tissue ($\Delta p[A_{50}] = 0.07 \pm 0.21$, $\Delta max = -4 \pm 4\%$; $n = 4$). Essentially the same results were obtained when U-46619 (20 nM) was the spasmogen (data not shown). By comparison, L-NAME was much less effective against ACh responses in RbJV. Figure 1a shows that 10 μM inhibitor was without significant effect on ACh E/[A] curves, although 100 μM L-NAME caused an eight fold displacement of the curve and depressed the maximum by 18%. In contrast, α-Me-5-HT relaxations in RbJV were effectively inhibited by L-NAME (10–100 μM) (Figure 1c) showing that the inhibitor demonstrates agonist- as well as tissue-differences. As in RA responses to both agonists were unaffected by D-NAME (100 μM) (vs ACh $\Delta p[A_{50}] = 0.03 \pm 0.04$, $\Delta max = 3 \pm 4\%$; $n = 4$: vs α-Me-5-HT $\Delta p[A_{50}] - 0.03 \pm 0.05$, $\Delta max = 3 \pm 4\%$; $n = 3$).

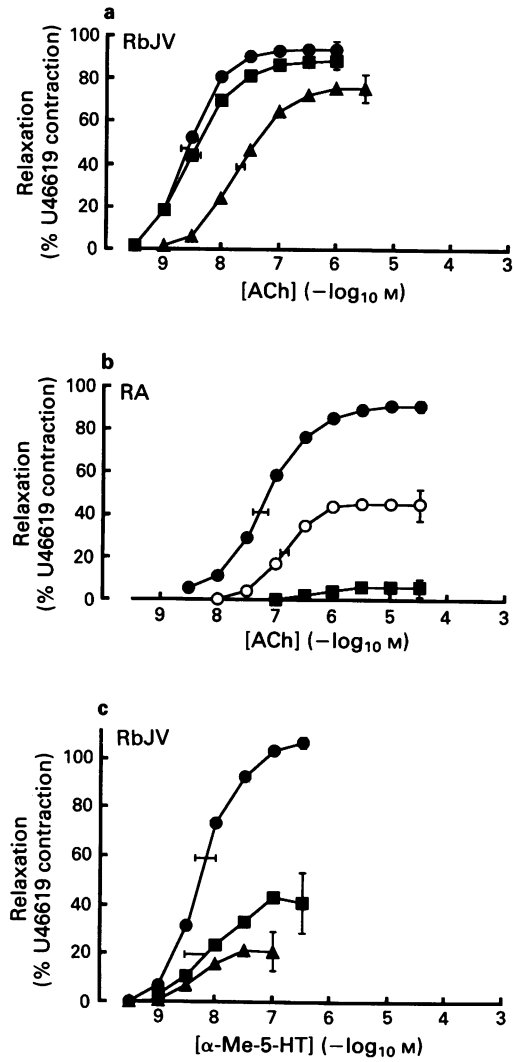

Figure 1 Differential effects of L-N^G-nitroarginine methyl ester (L-NAME, ● 0, ○ 1, ■ 10, ▲ 100 μM) on endothelium-dependent relaxations to acetylcholine (ACh) in rings of RbJV (a) and RA (b) and to α-methyl-5-hydroxytryptamine (α-Me-5-HT) in RbJV (c). Data are the averages of 3–6 E/[A] curves in each case. For clarity s.e.mean, denoted by vertical bars, are shown on maximum responses only. Horizontal bars denote s.e.mean on the average computed $p[A_{50}]$ values.

In separate paired curve experiments E/[A] curves to U-46619 in RbJV were essentially unchanged in the presence of 100 μM L-NAME ($\Delta p[A_{50}] = -0.03 \pm 0.04$, $\Delta max = 13 \pm 4\%$, $n = 6$), consistent with a previous report showing that U-46619 curves are unaffected by endothelial denudation in this tissue (Leff *et al.*, 1987). In contrast, phenylephrine curves obtained in RA ($n = 5$) were potentiated ($\Delta p[A_{50}] = -0.51 \pm 0.09$) and augmented $\Delta max = 25 \pm 6\%$) by L-NAME (10 μM), whilst responses to U-46619 were simply potentiated ($\Delta p[A_{50}] = -0.08 \pm 0.03$, $n = 9$: $P = 0.03$). These results imply that the inhibition of basal NO release by L-NAME increases the tonic state of the phenylephrine- and U-46619-contracted RA but has little impact on the contraction induced by U-46619 in RbJV.

Muscarinic receptor characterization in rat aorta and rabbit jugular vein

Affinity estimates for the muscarinic receptor antagonist 4-DAMP and for ACh itself were obtained in order to establish the identity of the endothelial muscarinic receptors in each tissue. Figure 2 (a and b) shows that in both RA and

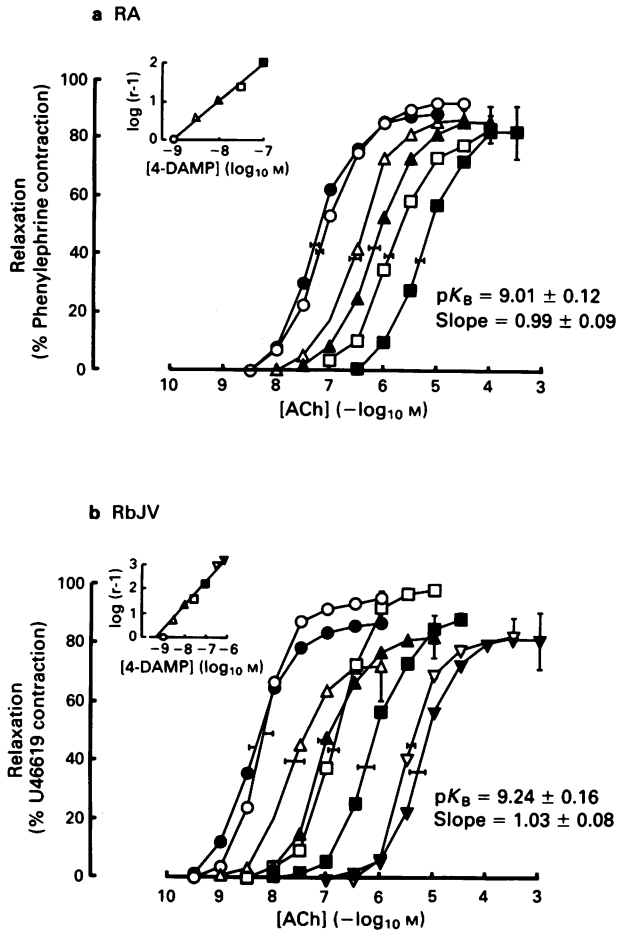

Figure 2 Antagonism of acetylcholine (ACh) relaxations by 4-diphenyl-acetoxy-N-methylpiperidine (4-DAMP, ● 0, ○ 1, △ 3, ▲ 10, □ 30, ■ 100, ▽ 300 and ▼ 1000 nM) in RA (a) and RbJV (b) respectively. Data are the averages of 3–5 E/[A] curves in each case and horizontal bars identify s.e.mean about their mean mid-point location. For clarity vertical bars denoting s.e.mean are shown on maximum values only. Inserts show Schild plots displaying the average log (r − 1) values obtained at each concentration of 4-DAMP in each tissue.

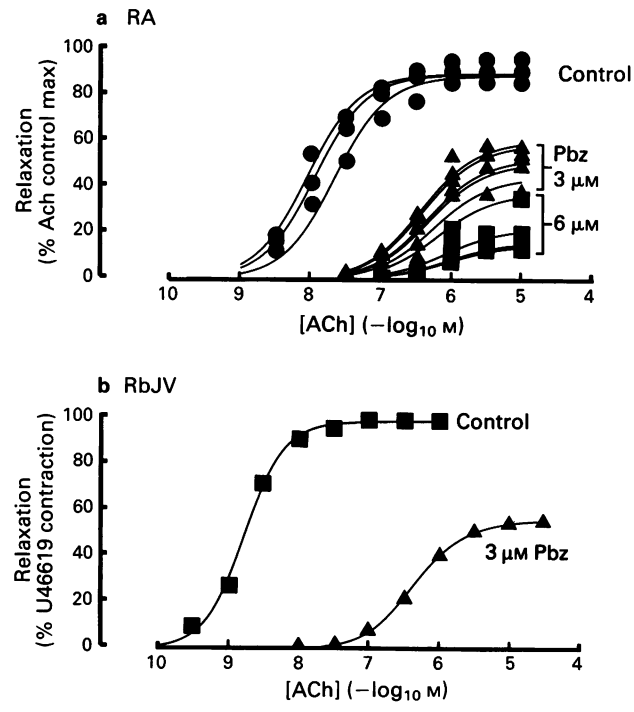

Figure 3 Estimation of acetylcholine (ACh) affinity (pK_A) at the muscarinic receptor in RA (a) and RbJV (b) by the method of fractional receptor inactivation. In (a) is shown a family of 12 ACh E/[A] curves, each obtained in a ring of RA in the absence or presence of phenoxybenzamine (Pbz); (b) shows a representative ACh curve pair (before and after Pbz treatment) from 11 such pairs obtained in separate rings of RbJV. Fitting these data to equation (3) provided the following parameter estimates in each tissue;
RA: $pK_A = 6.08 \pm 0.22$ (10 d.f.), $n = 1.19 \pm 0.25$, $E_m = 90.4 \pm 4.0$
RbJV: $pK_A = 6.12 \pm 0.09$ (10 d.f.), $n = 1.54 \pm 0.18$, $E_m = 93.0 \pm 3.0$
These parameter estimates, together with the estimates of τ (not shown), were used to produce the lines through the data.

RbJV 4-DAMP (0.003–1.0 μM) behaved as a simple competitive antagonist of ACh. Analysis of the data using equation (2) showed that the Schild plot slope was not different from unity (RA = 0.99 ± 0.09, 17 d.f.; RbJV = 1.03 ± 0.08, 29 d.f.) and that estimates of pK_B for 4-DAMP were not significantly different in RA (9.01 ± 0.12) and RbJV (9.24 ± 0.26). The concentrations of antagonist used had no effect on either U-46619 (10 nM) contractions in RbJV or on phenylephrine contractions in RA (ANOVA, $P > 0.05$).

Estimates of affinity (pK_A) for ACh were obtained following partial, irreversible receptor occlusion using phenoxybenzamine (Figure 3a,b). Concomitant incubation with 4-DAMP (0.1 μM) protected completely against the effects of phenoxybenzamine indicating that the latter simply inactivated muscarinic receptors and had no post-receptor actions. In RA a single E/[A] curve to ACh was obtained in each preparation after 30 min exposure to 0, 3 or 6 μM phenoxybenzamine and the resulting 'family' of twelve curves fitted directly to equation (3) yielding a pK_A of 6.08 ± 0.22 (10 d.f.). In RbJV, paired curves were obtained in each of eleven preparations before and after exposure to phenoxybenzamine (3 μM) so that model fitting provided six estimates of pK_A, the average of which was 6.12 ± 0.09, not different from that in RA.

These data indicate that the endothelial muscarinic receptors in RA and RbJV belong to the same class, the affinity estimates obtained being compatible with an M_3 classification. Moreover it is clear from the ratio $[A_{50}]/K_A$ that there exists a considerable 'reserve' in the system subserved by muscarinic receptors in RbJV, but not in RA.

Effects of glyceryl trinitrate, indomethacin and glibenclamide

Glyceryl trinitrate (GTN), like NO, activates the guanylyl cyclase transduction pathway in smooth muscle cells. Cumulative additions of GTN caused concentration-dependent relaxations of both RA and RbJV which completely reversed the U-46619 (10 nM) contracture in each case. Effects were expressed more potently in RbJV ($p[A_{50}] = 8.37 \pm 0.16$, $n = 6$) than in RA ($p[A_{50}] = 7.48 \pm 0.29$, $n = 6$) and in both cases were unaffected by L-NAME (100 μM).

In RbJV, the possibility that ACh responses were mediated in part either by cyclo-oxygenase products or by a K^+-channel linked hyperpolarizing factor was examined using indomethacin and glibenclamide respectively. ACh relaxations were unaltered in the presence of indomethacin (2.8 μM). Similarly treatment of tissues with 1 μM glibenclamide failed to modify either the location or maximum ($\Delta p[A_{50}] = 0.07 \pm 0.07$; Δ max = 2 ± 2%, $n = 5$) of the ACh response curve. In these experiments histamine (3 μM) was used to induce tissue contraction because glibenclamide is a competitive antagonist at thromboxane A_2 receptors (Cocks *et al.*, 1990).

Influence of agonist efficacy on inhibition by L-NAME in rabbit jugular vein

The influence of receptor-effector coupling efficiency was examined in RbJV by determining the effects of L-NAME (10 and 100 μM) on ACh relaxations after partial inactivation of the muscarinic receptor pool using phenoxybenzamine (3 μM

for 30 min). Following this treatment ACh behaved as a partial agonist attaining only about 75% of E_m. Figure 4a shows that under these conditions the inhibitory effect of L-NAME was more profound than in untreated tissues, inhibition of ACh effects being similar to that obtained when α-methyl-5-HT was the agonist (see Figure 1c).

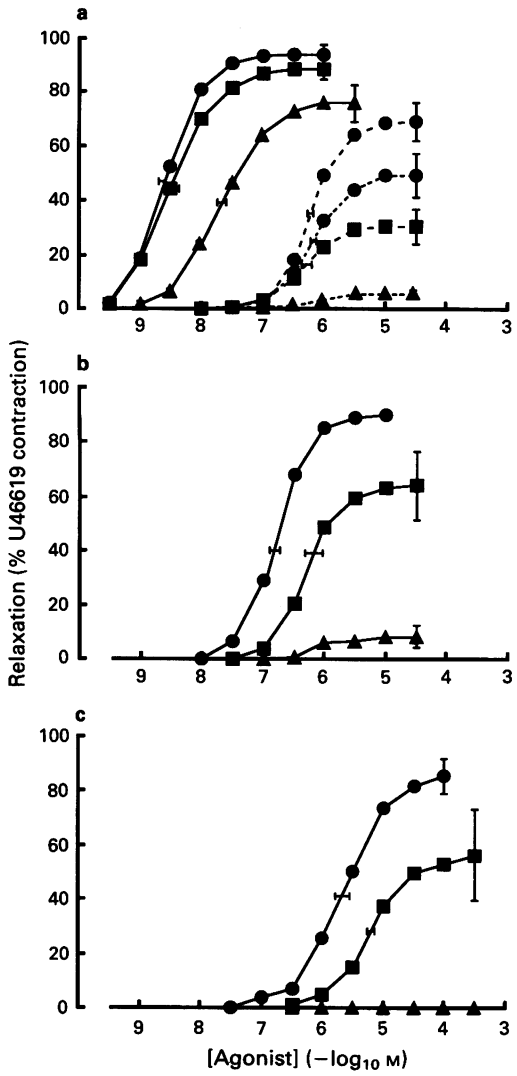

Figure 4 The effect of L-N^G-nitroarginine methyl ester (L-NAME, ● 0, ■ 10 μM, and ▲ 100 μM) on endothelium-dependent relaxations obtained with acetylcholine (ACh) (a), pilocarpine (b) and butyrylcholine (c) in rings of RbJV. Each experiment followed a triple curve design. Panel (a) is a composite of three separate experiments, one showing L-NAME effects in untreated tissues (solid lines), the other two showing inhibitor effects after treatment with phenoxybenzamine (dashed and dotted lines). In the latter case only second and third curves are shown representing respectively, a control curve after receptor inactivation (--●-- or ···●···) and the corresponding curve obtained in the presence of either 10 μM (--■--) or 100 μM (···▲···) L-NAME. Data are the averages of 4-6 E/[A] curves in each case. Vertical bars denote s.e.mean, shown for clarity on maximum values only. Horizontal bars identify the mean (±s.e.mean) computed $p[A_{50}]$ values in each case.

This phenomenon was explored further by studying the inhibition of pilocarpine and butyrylcholine relaxations. These agonists act at the same endothelial M_3 receptor type as ACh in RbJV since antagonism by 4-DAMP (10 nM) provided estimates of pA_2 (vs butyrylcholine = 9.35 ± 0.11, $n = 6$; vs pilocarpine = 9.41 ± 0.11, $n = 4$) that were not different from the estimate of pK_B obtained when ACh was the agonist (9.24 ± 0.26; see above). Furthermore, receptor inactivation experiments (phenoxybenzamine 0.3 μM for 30 min) showed that, unlike ACh, both pilocarpine and butyrylcholine behaved as partial agonists achieving on average 95% and 93% of E_m respectively. Estimates of agonist affinity and efficacy obtained by fitting these data to equation (3) are given in Table 1. L-NAME (10 and 100 μM) inhibited relaxations induced by both partial agonists producing a concentration-related right-shift and depression of E/[A] curves similar to that obtained against ACh after receptor inactivation (Figure 3b,c, cf. a).

A model describing inhibition of receptor-stimulated NO release Theory

Figure 5 illustrates a simplistic scheme for the receptor-stimulated release of NO in which agonist occupancy of receptors on endothelial cells activates an intracellular enzyme cascade responsible for generating NO from L-arginine. The subsequent liberation of NO stimulates a transduction pathway in smooth muscle cells which leads ultimately to the measured effect, vasorelaxation. Inhibitors are regarded as 'false substrates' for the enzyme NO synthase competing directly with the endogenous substrate L-arginine to block the formation of NO. Each of these steps is visualised in Figure 5 and was used as a basis to derive a simple theoretical model. At the outset the following assumptions were made; (i) equilibrium conditions pertain throughout, (ii) the intracellular concentration of L-arginine vastly exceeds that of the enzyme, NO synthase, (iii) the pool of L-arginine is not depleted by NO formation and (iv) inhibitors exhibit reversible, competitive inhibition kinetics.

Firstly, it is assumed that the equilibrium occupancy of endothelial receptors is governed by Mass Law;

$$[AR] = \frac{[R_0][A]}{K_A + [A]} \quad (4)$$

where $[R_0]$ denotes the total functional receptor pool, [A] is the concentration of agonist and K_A its equilibrium dissociation constant. Receptor occupancy then leads to activation of an intracellular enzyme (NO synthase). For simplicity the concentration of enzyme in the active form ([Ea]) is assumed to be proportional to [AR];

$$[Ea] = \gamma[AR] = \frac{\gamma[R_0][A]}{[A] + K_A} \quad (5)$$

Next, substrate (ARG) and inhibitor (B) compete for Ea such that;

$$[Ea] + [Arg] \underset{}{\overset{K_m}{\rightleftharpoons}} [EaArg] \xrightarrow{K_{cat}} [E] + [NO]$$

$$+$$

$$[B] \quad \Big\| \; K_B$$

$$[EaB]_{inactive}$$

Table 1 Parameter estimates describing the agonist effects of acetylcholine, pilocarpine and butyrylcholine at the endothelial muscarinic receptor in rabbit external jugular vein

Agonist	*No. experiments*	pK_A	τ_{test}/τ_{ACh} (×100)	n	E_{max}
Acetylcholine	11	6.12 ± 0.09	100	1.54 ± 0.18	93.0 ± 3.0
Pilocarpine	6	4.86 ± 0.09	3.6 ± 1.2	1.83 ± 0.40	92.7 ± 3.8
Butyrylcholine	5	6.02 ± 0.14	4.1 ± 1.1	1.71 ± 0.09	93.0 ± 1.5

Values are estimates provided by fitting the E/[A] curve data for each agonist to the operational model of agonism (equation 3).

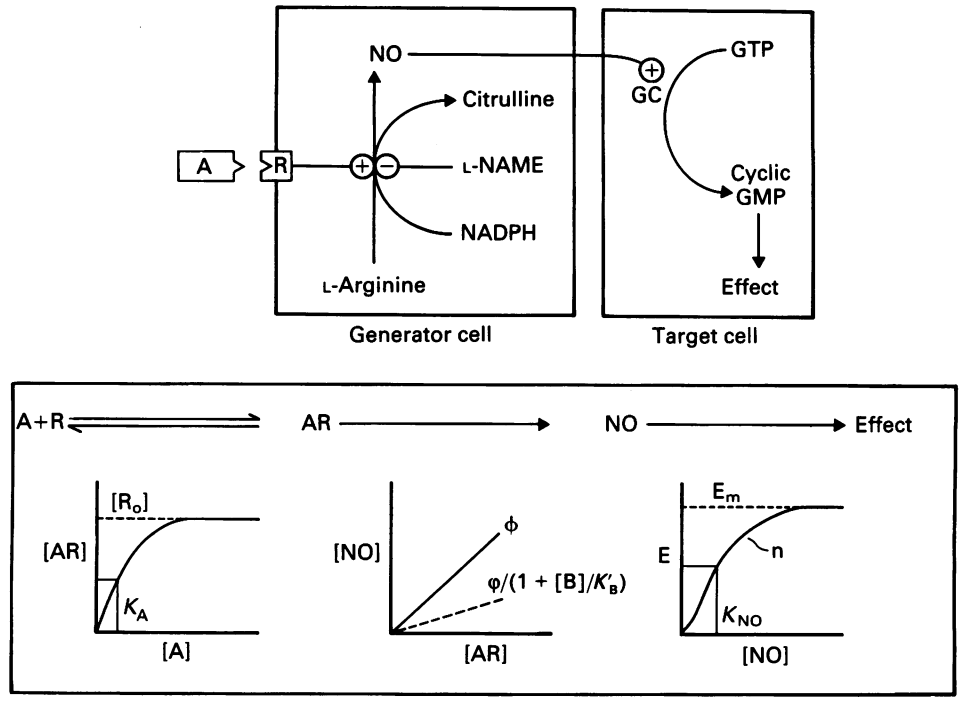

Figure 5 Representation of the biochemical cascade which results in vascular smooth muscle (target cell) relaxation following receptor-stimulated synthesis and release of NO from endothelial (generator) cells (adapted from Knowles *et al.*, 1990). In model terms the cascade can be conveniently regarded as three serial events; (i) the binding of agonist to endothelial cell receptors, (ii) the subsequent formation and liberation of NO and (iii) the interaction of NO with a transducer molecule (GC: guanylate cyclase) in the target cell resulting ultimately in the biological effect.
Key: [A] = concentration of agonist, K_A = its dissociation constant, $[R_0]$ = concentration of functional receptors, ϕ = proportionality constant for [NO]/[AR] relation, [B] = concentration of inhibitor, K'_B a parameter related to the inhibitor dissociation constant (see eq. 10), E_m = maximum effect possible, K_{NO} = value of [NO] for $0.5E_{max}$ and n = slope parameter of E/[NO] relation.

Hence the rate (V) of NO formation is;

$$V = \frac{[Arg][Ea]K_{cat}}{[Arg] + K_m\{1 + [B]/K_B\}} \quad (6)$$

It seems reasonable to suppose that following its liberation NO disappears from the site of action according to first order kinetics, hence the concentration available to initiate a response in the target cell will be proportional to its rate of formation;

$$[NO] = \beta V \quad (7)$$

Substitution of equation (5) into equation (6) now gives;

$$[NO] = \frac{\beta\gamma K_{cat}[Arg][A][R_0]}{([Arg] + K_m\{1 + [B]/K_B\})([A] + K_A)} \quad (8)$$

By rearrangement;

$$[NO] = \frac{\beta\gamma K_{cat}([Arg]/[Arg] + K_m)[A][R_0]}{(1 + [B]K_m/([Arg] + K_m)K_B)([A] + K_A)} \quad (9)$$

The assumption that [Arg] is not depleted during the formation of NO means that $[Arg]/[Arg] + K_m$ can be regarded as a tissue-dependent constant like β, γ, K_{cat} and $[R_0]$. Together these can be described by a single constant, ϕ. For convenience however, $[R_0]$ will be kept separate from ϕ. Now equation (9) can be simplified to;

$$[NO] = \frac{[A][R_0]\phi}{([A] + K_A)\{1 + [B]/K'_B\}} \quad (10)$$

where $K'_B = K_B([Arg] + K_m)/K_m$.

The final step in the pathway relates [NO] to effect, E. In the absence of evidence to the contrary, it seems sensible to assume that NO binds in a saturable way to an effector protein which initiates a cascade to produce the final observed effect. Here, in line with the reasoning of Black & Leff (1983), we have assumed that this relation corresponds to a logistic function analogous to the E/[AR] relation in their operational model of agonism;

$$E = \frac{E_m[NO]^n}{K_{NO}{}^n + [NO]^n} \quad (11)$$

where E_m denotes the maximum possible effect in the system, K_{NO} the value of [NO] for half E_m and n is the slope parameter of the E/[NO] relation. Substitution of equation (10) into equation (11) gives the overall E/[A] relation;

$$E = \frac{E_m\phi^n[R_0]^n[A]^n}{K_{NO}{}^n(K_A + [A])^n \times \{1 + [B]/K'_B\}^n + \phi^n[R_0]^n[A]^n} \quad (12)$$

This equation is equivalent to the operational model (equation 3) except, in this case, $\tau = (R_0\phi/K_{NO})$ (13) modified by the factor $1/(1 + [B]/K'_B)$.

Model predictions In model terms the constant ϕ is simply a proportionality constant determining the availability of NO at the target (smooth muscle) cell transducer following its receptor-stimulated synthesis and release from the generator (endothelial) cell (see Figure 5). Assuming that access to the target cell transducer is not limiting, a large value of ϕ implies that NO synthesis and release is efficiently coupled to the generator cell receptor system, whereas a small value implies the opposite.

However the quantity ϕ is not accessible experimentally because it is inextricably bound up with the other tissue-dependent parameters that define agonist efficacy, in this case $[R_0]$ and K_{NO}. This is clear from equation (13) which shows that ϕ is a proportionality constant on the ratio $[R_0]/K_{NO}$

where this ratio has the same operational meaning as τ (the transducer ratio $[R_0]/K_E$) described originally by Black & Leff (1983). In principle then, the operational efficacy of an agonist in an endothelial receptor system will depend upon the availability of NO to the target cell (determined by ϕ and $[R_0]$) and the efficiency of the target cell transducer machinery (determined by K_{NO}). Since in the presence of a competitive inhibitor of NO synthesis the value of ϕ is reduced by the factor $(1 + [B]/K'_B)$ (equation 10), the inhibitor effectively decreases the operational efficacy (τ) of the receptor system and in this regard has the same consequences as irreversible receptor occlusion ($[R_o]$) or decreasing the efficiency with which NO-guanylate cyclase coupling produces smooth muscle relaxation.

The simulations in Figure 6 illustrate the important features of inhibition predicted for endothelial agonist E/[A] curves that are either rectangular hyperbolic (Figure 6a) or inherently steep (Figure 6b). Model parameters were chosen to represent either full or partial agonism. Under these two conditions increasing the value of $[B]/K'_B$ produces a different pattern of curve displacement. With a full agonist the effect of the inhibitor is the same for both hyperbolic and steep curves i.e. parallel rightward displacements. However in the case of a partial agonist the inhibitor causes depression of the curves with little change in their location. Furthermore E/[A] curve shape now has an important impact upon the inhibition observed. Comparison of the hyperbolic (a) and steep (b) cases in Figure 6 shows that the same increments in inhibitor concentration produce a more profound depression of steep curves than of hyperbolic curves. This behaviour is analogous to that predicted following receptor inactivation (Black *et al.*, 1985a).

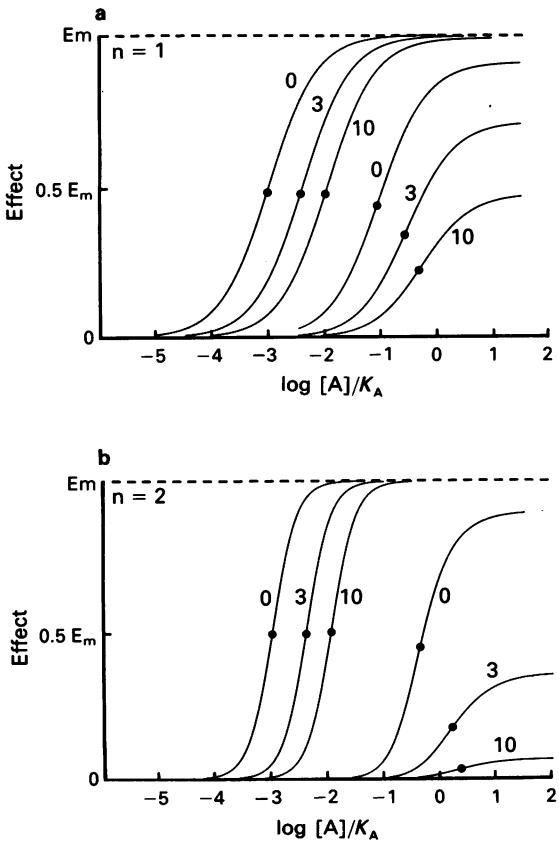

Figure 6 Simulations based on equation (12) depicting the effect of an inhibitor L-arginine analogue on full and partial agonist E/[A] curves which are rectangular hyperbolic (n = 1: a) or steep (n = 2: b) respectively. The number against each curve indicates the value of $[B]/K'_B$. Solid circles identify the mid-point of each curve. Parameter values were; $\phi = 100$, $K_{NO} = 1$, $E_m = 1$ and $[R_0] = 10$ (full agonist) and 0.03 (a) or 0.01 (b) (partial agonist).

In summary the effect of NO synthase inhibition on receptor-stimulated endothelium-dependent responses is predicted to be dependent not only on the efficacy of the agonist in the system under study, but also on the gradient of the resulting E/[A] curve.

Model simulations of experimental data Figure 7 shows model simulations predicting the effects of NO synthase inhibition superimposed on the E/[A] curve data points from Figure 4. The line depicting the control E/[A] curve in each case was obtained using the parameter estimates derived from previous receptor inactivation studies (Table 1) with τ providing a value for the term $(R_o/K_{NO})\phi$ (see eq. 13) and $[B]/K'_B$ set to zero. The lines depicting the effects of L-NAME were produced simply by increasing the value of $[B]/K'_B$. Clearly good agreement can be obtained between model simulations and the experimental data reinforcing the notion that L-NAME behaves as if it acts simply to reduce efficacy in the receptor system under study. However, quantitative differences between the predicted and observed behaviour of the inhibitor are evident. Firstly, in untreated tissues where ACh behaves as a full agonist (Figure 7a), depression of the curve maximum was observed experimentally which was not predicted by the model. Secondly, the values of $[B]/K'_B$ used to simulate the

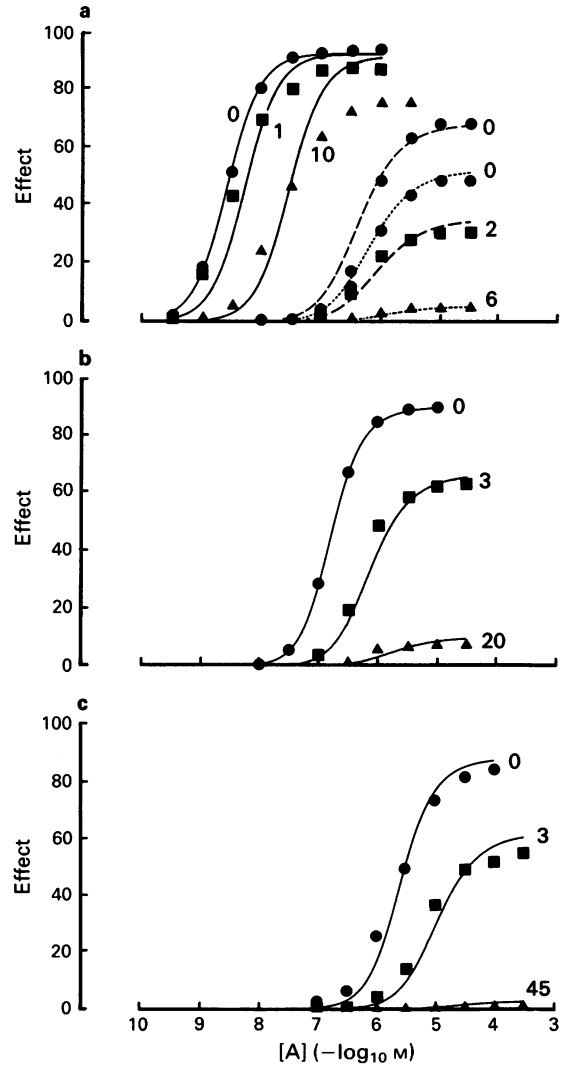

Figure 7 Simulations of L-N^G nitroarginine methyl ester (L-NAME) effects on E/[A] curves to acetylcholine (ACh) (a), pilocarpine (b) and butyrylcholine (c). The lines were obtained as described in the text and values of $[B]/K'_B$ used to simulate the effects of L-NAME are shown next to the lines. The data points shown are taken from Figure 4.

effects of 10 and 100 μM L-NAME in each case would obviously be expected to differ by ten fold, whereas agreement between the simulations and experimental data was achieved by increments of $[B]/K'_B$ that ranged between 5 and 15 fold (see legend to Figure 7). Possible reasons for these discrepancies are considered in detail in the discussion.

Discussion

L-NAME is a potent, enantiomerically specific inhibitor of NO synthase in a variety of cell types (Palacios *et al.*, 1989; Knowles *et al.*, 1990; Radomski *et al.*, 1990; McCall *et al.*, 1991) including vascular endothelial cells (Rees *et al.*, 1990). It also inhibits endothelium-dependent responses induced by ACh and bradykinin *in vivo* as well as *in vitro*, implying that NO synthesis and release accounts for the biological effects of these agents (Moore *et al.*, 1990; Rees *et al.*, 1990). The present experiments confirmed that micromolar concentrations of L-NAME virtually abolish ACh relaxations in endothelium-intact rat aorta (Rees *et al.*, 1990) but they also showed that the inhibitor demonstrates agonist- and tissue-differences. Hence in another tissue, the rabbit jugular vein, ACh responses were much less susceptible to inhibition by L-NAME whereas relaxations elicited by α-methyl-5-HT via endothelial 5-HT receptors remained sensitive to blockade. The purpose of this study was to ascertain whether or not these data could be interpreted as evidence for the existence of heterogeneous EDRFs.

The possibility that different muscarinic receptor subtypes accounted for the tissue-dependence of L-NAME effects was eliminated at the outset. In RbJV affinity estimates for the muscarinic receptor agonist 4-DAMP were independent of the agonist used and not different from the estimate obtained in RA when ACh was the agonist ($pK_B \sim 9.4$). Neither were affinity estimates for ACh itself different in the two tissues ($pK_A = 6.1$). These results indicated the involvement of only a single population of endothelial muscarinic receptors in each tissue and identified the receptor type as M_3 (see e.g. Furchgott & Cherry, 1984; Eglen & Whiting, 1986). Of particular interest was the finding from receptor inactivation experiments that in RbJV, but not RA, there was a considerable M_3 receptor reserve. Traditionally this would allow the higher potency of ACh in RbJV ($p[A_{50}] = 8.58$) compared to RA ($p[A_{50}] = 7.27$) to be explained in terms of a greater M_3 receptor density and/or more efficient occupancy-effect coupling. However, in this case the coupling process is not entirely intracellular; it involves the transfer of an intermediate mediator, NO, from one cell type to another, raising the possibility that anatomical i.e. arterial vs. venous differences may be important. For example the actions of endothelium-derived NO may be favoured in the venous tissue where there is a higher endothelial/smooth muscle cell ratio and the media provides less of a diffusion barrier (Bassenge *et al.*, 1987). Alternatively, and perhaps more likely, the greater potency of ACh in RbJV may reflect a better coupling of the NO-guanylate cyclase cascade in this tissue. This would be consistent with the ~ 30 fold greater potency of the nitrovasodilator glyceryltrinitrate in RbJV compared to RA and compatible with the generally recognised tendency for nitrates to demonstrate a preferential dilator effect on veins (Ignarro & Kadowitz, 1985).

Early experiments also excluded the possibility that resistance of ACh relaxations to inhibition by L-NAME was due to the stimulated release of other known endothelium-derived vasodilator substances. The lack of effect of indomethacin indicated that cyclo-oxygenase products such as prostacyclin (PGI_2) were not involved. Neither was there evidence for the involvement of a glibenclamide-sensitive K^+-channel-linked hyperpolarizing factor. The release of such a factor from vascular endothelial cells has been implied by electrophysiological as well as pharmacological studies (Taylor & Weston, 1988; Feletou & Vanhoutte, 1988), but in the present experiments the K^+-channel blocker, glibenclamide, failed to affect ACh responses in RbJV indicating that involvement of vascular smooth muscle K^+ channels in endothelium-dependent relaxations was unlikely. Since neither receptor differences nor the release of mediators other than NO appeared to explain the discrepant behaviour of L-NAME in the two tissues the possibility was considered that the agonist- and tissue-dependence of inhibition was in some way related to the efficiency of receptor occupancy-effect coupling. Evidence to support this notion was first obtained using phenoxybenzamine to inactivate a fraction of the muscarinic receptors in RbJV. This intervention converted ACh from a full to a partial agonist so that it attained only about 75% of E_m and in this regard resembled closely the partial agonist behaviour of α-Me-5-HT at the endothelial 5-HT receptor in this tissue (Leff *et al.*, 1987). Under these conditions L-NAME produced an impressive non-surmountable inhibition of ACh relaxations (Figure 3a) similar to that obtained when α-Me-5-HT was the agonist (Figure 1a). Subsequent studies using untreated tissues showed that L-NAME was equally effective against relaxations produced by the muscarinic receptor partial agonists, pilocarpine and butyrylcholine (Figure 4b and c) providing further evidence that the nature of inhibition obtained is dependent upon agonist efficacy. These results are reminiscent of the differential effects produced by a basal release of EDRF on contractions of isolated vascular rings elicited by high and low efficacy α-adrenoceptor agonists (Martin *et al.*, 1986). In that study, endothelial denudation increased tissue sensitivity to the full agonist phenylephrine without affecting the curve maximum, but increased profoundly the maximum response to the partial agonist clonidine. Clearly L-NAME would be expected to produce similar effects in rings with an intact endothelium.

Although agonist efficacy clearly accounts for a part of the tissue-differences in inhibitor behaviour observed here, L-NAME remained substantially more potent in RA than in RbJV (Figure 1b cf. Figure 3a) even when fractional receptor occlusion reduced the efficacy of ACh to a similar level in both tissues. This is most probably explained by the different effects of basal EDRF release on the spasmogens used. U-46619 (10 nM) was used to contract rings of RbJV and in this tissue the E/[A] curve is unaffected either by endothelial denudation (Leff *et al.*, 1987) or by L-NAME (this study). Hence the tonic state of the preparation is the same in the presence and absence of inhibitor. However, phenylephrine was the spasmogen in RA and in this tissue E/[A] curves are potentiated and augmented by L-NAME. This effect of the inhibitor was not taken into account in these experiments so that in RA, but not RbJV, L-NAME not only reduced the release of EDRF, it also increased functional opposition to ACh-induced relaxations. Its greater potency in RA compared to RbJV may therefore be more apparent than real.

Consideration of a simple theoretical model suggests a rational basis for the effects of L-NAME on endothelium-dependent relaxations. In terms of the model described here competitive inhibition of NO synthase is expected to produce a right-shift and, ultimately, depression of the agonist E/[A] curve because the inhibitor effectively decreases the efficacy of the indirectly acting agonist, ACh, by the factor $1 + [B]/K'_B$, (see Results). This is similar to agonist curve behaviour predicted for the simple competitive antagonism of a mediator after its liberation by an indirectly acting agonist, as exemplified by the antagonism of tyramine by α- and β-adrenoceptor antagonists (Fedan *et al.*, 1977; Black *et al.*, 1980) and the antagonism of pentagastrin by histamine H_2-receptor antagonists (Black, 1973; Black *et al.*, 1978; 1985b). In these latter cases the efficacy of the indirectly acting agonist is reduced by the factor $1 + [B]/K_B$ so that ratios of efficacy estimates obtained in the absence and presence of antagonist can be treated just like agonist dose-ratios to provide an estimate of K_B (see Black *et al.*, 1985b). However when the inhibitor competes with substrate to block the synthesis and/or release of the mediator, as is the case for L-NAME in the present study,

the efficacy ratios of the indirectly acting agonist are governed by K'_B which subsumes the true value of K_B (equation 10). This means that in the absence of more precise knowledge concerning the events leading to the liberation of (in this case) NO, only empirical information concerning inhibitor activity can be obtained from these experiments.

A more important prediction from the present model is evident when the dependency of E/[A] curve mid-points on agonist efficacy is considered. The midpoint location ($[A_{50}]$) for the ACh curve can be derived from equation (12) as:

$$[A_{50}] = K_A/\{(2 + (\phi[R_0]/K_{NO})^n)^{1/n} - 1\}$$

where the parameters have the same meaning as defined earlier. From this it is clear that decreasing the value of ϕ (which occurs when [L-NAME] is increased; see equation 10) produces the same result as decreasing $[R_o]$ and therefore, by analogy, the effect of L-NAME on ACh E/[A] curves allows estimation of K_A, the equilibrium dissociation constant for the indirectly acting agonist.

This predicted parity between the effects of a competitive NO synthase inhibitor and an irreversible receptor antagonist implies that the former can be used just like the latter to estimate agonist affinity constants (Furchgott, 1966). In principle this offers a means of assessing the applicability of the model. This test was applied to the data for pilocarpine and butyrylcholine, since in both cases there was a reasonable agreement between model simulations and experimental results (Figure 7). Interestingly, agonist affinities were overestimated when the E/[A] curve data obtained before and after L-NAME treatment were fitted directly to equation (3): the pK_A estimate for pilocarpine was 6.42 ± 0.08 (mean $\pm$ s.e.mean, $n = 6$) and for butyrylcholine was 5.55 ± 0.11 (mean $\pm$ s.e.mean, $n = 5$). These values are significantly ($P < 0.05$) higher than the respective estimates of 6.02 ± 0.14 and 4.86 ± 0.09 obtained following partial receptor occlusion with phenoxybenzamine (Table 1) suggesting that L-NAME produces a more rapid collapse of the agonist curves than would be expected from a simple decrease in efficacy resulting from competitive inhibition of NO synthesis and release. Although less obvious in the case of the partial agonists, such an effect is evident in the experiments with ACh (Figure 7a) where, in untreated tissues, L-NAME produced a right-shift accompanied by depression of the agonist curve maximum when progressive parallel shifts in the concentration-effect curve were predicted.

A second difference between the predicted and observed behaviour of L-NAME was that a generally smaller than ten fold increment in the value of $[B]/K'_B$ was required to simulate the experimental effects of a ten fold increase in L-NAME concentration. Indeed, given the observation above that L-NAME appears to induce a more rapid collapse of the E/[A] curve than predicted by the model, an increment in $[B]/K'_B$ of greater than ten fold might have been anticipated. Obviously these discrepancies might be explained by deficiencies in the model which is inevitably an oversimplification. Alternatively it might indicate that L-NAME does not inhibit NO production in the endothelium of RbJV in a simple competitive manner. Perhaps relevant to this, Moncada and colleagues have demonstrated significant differences in the properties of NO synthase in different tissues (Moncada *et al.*, 1989). Moreover the enzyme present in cultured vascular endothelial cells exists in two forms, one Ca^{2+}-dependent and constitutive, the other Ca^{2+}-independent and inducible (Radomski *et al.*, 1990). Whether or not these two forms of the enzyme exhibit differential sensitivity to L-NAME is unknown at present, but East & Garthwaite (1990) have shown that in rat cerebellar slices the inhibition by N^G-nitroarginine of NMDA-induced cyclic GMP formation involves two components implying an action at two receptor-activated NO synthase enzymes (see also Garthwaite *et al.*, 1989). If such multiple forms of the synthase enzyme contribute to the muscarinic receptor-stimulated production of NO in RbJV then an overall non-competitive pattern of inhibition by L-NAME would be expected and the choice of a simple linear relation between [AR] and [NO] in the model would be inadequate to describe the experimental data. Unfortunately the choice between these possibilities remains arbitrary given the present inability to determine directly the nature of the inhibitor-enzyme interaction.

In spite of these quantitative limitations the good agreement between the observed and predicted behaviour of L-NAME in these experiments implies that the model nevertheless provides a useful basis for studying the effects of inhibitors on receptor-stimulated release of NO. Importantly it indicates that tissue- and agonist-differences observed with such inhibitors can be accounted for by the factors which determine agonist efficacy in the system. This possibility should be ruled out before apparent resistance to blockade by the inhibitor is taken as evidence for the involvement of heterogeneous EDRFs.

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The influence of atropine and atenolol on the cardiac haemodynamic effects of N^G-nitro-L-arginine methyl ester in conscious, Long Evans rats

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1 In the present study, the extent to which baroreflexes contribute to the cardiac effects of N^G-nitro-L-arginine methyl ester (L-NAME) was assessed in conscious, Long Evans rats chronically instrumented with thoracic electromagnetic flow probes for the measurement of cardiac haemodynamics.

2 L-NAME (10 mg kg^{-1}, i.v.) was administered in the absence ($n = 6$) and in the presence ($n = 7$) of atropine (1 mg kg^{-1}) and atenolol (1 mg kg^{-1}).

3 L-NAME caused a marked increase in mean arterial pressure and marked reductions in total peripheral conductance, cardiac output, heart rate, stroke volume, peak thoracic flow and the maximum rate of rise of aortic flow.

4 Administration of atropine, after the maximal bradycardic effect of L-NAME was established, restored the heart rate to resting levels. Concurrently, there was a reduction in stroke volume, such that cardiac output, although transiently elevated, did not show a sustained increase. No other variables were significantly affected by atropine. Additional administration of atenolol had no effect other than to cause a slight bradycardia, such that in the presence of atropine and atenolol, heart rate was not different from that in animals receiving atropine and atenolol before L-NAME.

5 In the presence of atropine and atenolol, L-NAME had similar pressor, vasoconstrictor and cardiac haemodynamic effects to those in untreated animals, although the bradycardia was significantly attenuated. However, there was still a significant reduction in heart rate following L-NAME in the presence of atropine and atenolol.

6 These results indicate that the major component of the bradycardia following L-NAME is indirect and mediated through an increase in vagal efferent activity. However, the substantial reduction in cardiac function caused by L-NAME is not dependent on the autonomic control of the heart but rather, may depend on the increase in afterload and/or a direct effect of L-NAME on the heart and/or its vasculature.

Keywords: N^G-nitro-L-arginine methyl ester; atropine; atenolol; cardiac output; heart rate; conscious rats

Introduction

It is now well established that nitric oxide (NO) is a major endothelium-derived relaxing factor, both *in vitro* and *in vivo* (see Moncada *et al.*, 1989; 1991). The inhibition of NO synthesis, from its precursor L-arginine, can be achieved with several analogues of L-arginine, including N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine methyl ester (L-NAME) (Palmer *et al.*, 1988; Rees *et al.*, 1990; Moore *et al.*, 1990). Administration of these compounds *in vivo* leads to systemic hypertension, due to widespread vasoconstriction (Gardiner *et al.*, 1990b,c). However, in conscious rats, the rise in systemic arterial blood pressure evoked by L-NAME is accompanied by bradycardia and a marked reduction in cardiac output (Gardiner *et al.*, 1990c), which would act to buffer the pressor effects of L-NAME. It is feasible that the changes in cardiac function in the presence of L-NAME are due to baroreflex-mediated effects, and/or the marked increase in afterload, and/or impairment of myocardial perfusion due to coronary vasoconstriction. Therefore, in order to determine the extent to which baroreflexes contributed to the changes in myocardial function we assessed cardiac haemodynamic responses to L-NAME in the absence and in the presence of atropine and atenolol.

Methods

Male Long Evans rats (350–450 g) were anaesthetized with sodium methohexitone (60 mg kg^{-1}, i.p., supplemented as required) and an electromagnetic flow probe (Skalar, Delft, Netherlands) was implanted around the ascending thoracic aorta (Smith & Hutchins, 1979; Smits *et al.*, 1982; Gardiner *et al.*, 1990c). Animals were then given an intramuscular injection of ampicillin (7 mg kg^{-1}, Penbritin) and allowed at least 7 days to recover before 3 catheters were implanted for separate drug administration in the right jugular vein and an intra-arterial catheter was implanted in the distal abdominal aorta (via the ventral caudal artery) under brief anaesthesia (sodium methohexitone, 40 mg kg^{-1}). A harness was fitted to the rat and the catheters were passed through a flexible spring connected at one end to the harness and at the other to a counterbalanced, universally-jointed lever. A short interconnecting cable from the flow probe was taped to the spring to allow connections to be made to the flowmeter (Skalar, MDL 1401) without disturbing the animal. Rats were left in their home cages to recover until the following day.

During an experiment, the inputs from the thoracic aortic flow probe and arterial pressure transducer were fed into a custom-built, haemodynamics microprocessor (Departments of Pharmacology and Instrument Services, University of Limburg, Netherlands) interfaced with a Compaq 386/20e microcomputer. This system digitised the input signals and stored the data on disc as required, e.g. every 1–10 s. Off-line, the experiments were analysed and the haemodynamic processing system provided values for mean arterial blood pressure (MAP), heart rate, cardiac output, total peripheral conductance, stroke volume, peak thoracic flow, and maximum positive slope of thoracic aortic flow ($+dF/dt_{max}$), the latter two variables being taken as indices of contractility (Schoemaker, 1989).

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Experimental protocols

In pilot experiments we determined that bolus doses of atropine (1 mg kg^{-1}) and atenolol (1 mg kg^{-1}) given together abolished all reflex heart rate responses to rises or falls in MAP for at least 30 min.

Protocol 1: Effect of atropine and atenolol in the presence of L-NAME

L-NAME (10 mg kg^{-1}) was injected i.v. into 6 rats. It has previously been shown that this dose of L-NAME produces cardiovascular changes which are maximal by 10–15 min and sustained for at least 60 min subsequently (Gardiner *et al.*, 1990c). Therefore, 15 min after L-NAME, atropine (1 mg kg^{-1}) was given, followed by atenolol (1 mg kg^{-1}) at 25 min.

Protocol 2: Effect of L-NAME in the presence of atropine and atenolol

Atropine (1 mg kg^{-1}) and atenolol (1 mg kg^{-1}) were injected i.v. into 7 rats, and L-NAME (10 mg kg^{-1}, i.v.) was then injected 15 min later.

Data analysis

Four of the rats in protocol 1 were also used in protocol 2. However, 2 days were left between experimental runs and the order of the experimental protocols was randomized. The results produced were essentially identical to those obtained from animals which were involved in only one of the protocols, thus all data were included in the analysis.

The effects of atropine, atenolol and L-NAME on the cardiac haemodynamic variables were subjected to an initial analysis of variance (ANOVA) with repeated measures using a commercially available statistical package (CLR ANOVA on a Macintosh SE).

Drugs

L-NAME hydrochloride, atropine methyl nitrate and atenolol were all obtained from Sigma. All substances were dissolved in saline and injected in 0.1 ml volume flushed in with a further 0.1 ml (the dead space of the i.v. catheter).

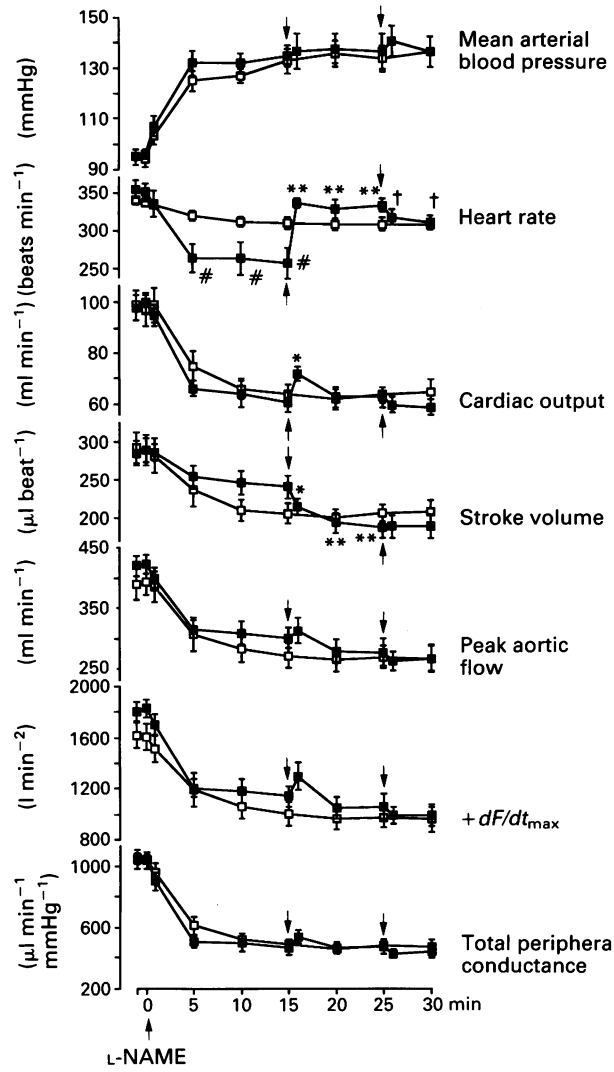

Figure 1 Effect of N^{G}-nitro-L-arginine methyl ester (L-NAME, 10 mg kg^{-1}, i.v.) alone followed by administration of atropine (1 mg kg^{-1}) and atenolol (1 mg kg^{-1}) at 15 and 25 min (arrows), respectively (■, $n = 6$, protocol 1) and the effect of L-NAME administered in the presence of atropine and atenolol (□, $n = 7$, protocol 2). All variables, except for heart rate values in protocol 1 were significantly different ($P < 0.01$, ANOVA) from their respective baselines from 5–30 min, and reference to this has been omitted for the sake of clarity. Other comparisons are as follows: * $P < 0.05$, ** $P < 0.01$ versus pre-atropine baseline (protocol 1); † $P < 0.05$ versus pre-atenolol baseline (protocol 1); # $P < 0.01$ protocol 1 versus protocol 2.

Results

The resting values for the measured and derived haemodynamic variables for animals in protocol 1 and protocol 2 (Table 1) were not significantly different.

Protocol 1

The effect of L-NAME (10 mg kg^{-1}) in the absence and in the presence of atropine and atenolol is shown in Figure 1. L-NAME given alone evoked marked hypertension and bradycardia, accompanied by reductions in cardiac output, total peripheral conductance, stroke volume, $+dF/dt_{max}$ and peak thoracic flow. The increase in MAP ($P < 0.05$) and the decreases in $+dF/dt_{max}$ ($P < 0.05$) and total peripheral con-

Table 1 Resting values for cardiac haemodynamic variables and the effect of atropine (1 mg kg^{-1}) and atenolol (1 mg kg^{-1}) (protocol 2)

	Protocol 1 ($n = 6$)	*Protocol 2* ($n = 7$)		
	Control	*Control*	*Atropine*	*Atropine + atenolol*
Mean arterial pressure (mmHg)	96 ± 2	98 ± 3	97 ± 2	94 ± 3
Heart rate (beats min^{-1})	351 ± 12	356 ± 9	411 ± 6**	338 ± 5
Cardiac output (ml min^{-1})	100 ± 4	102 ± 5	108 ± 7*	97 ± 6
Stroke volume (μl beat^{-1})	289 ± 16	286 ± 15	256 ± 20*	289 ± 20
Peak thoracic flow (ml min^{-1})	423 ± 16	408 ± 23	422 ± 24**	394 ± 24
$+dF/dt_{max}$ (l min^{-1} min^{-1})	1828 ± 69	1736 ± 103	1907 ± 120**	1607 ± 103*
Total peripheral conductance (μl min^{-1} mmHg^{-1})	1042 ± 32	1049 ± 62	1121 ± 73	1038 ± 56

Values are mean ± s.e.mean.
* $P < 0.05$; ** $P < 0.01$ versus control.

ductance ($P < 0.01$) were significant by 1 min after L-NAME, while the changes in all other variables were significant ($P < 0.01$) subsequent to this time (Figure 1).

Atropine administered 15 min after L-NAME, restored the heart rate to pre-L-NAME levels. There was only a small, transient increase in cardiac output, since the atropine-induced increase in heart rate was accompanied by a further significant reduction in stroke volume. All other variables were not significantly affected by atropine. Atenolol, administered 10 min after atropine (25 min after L-NAME) caused a small but significant bradycardia ($P < 0.05$, ANOVA) with no other changes in cardiac haemodynamic status (Figure 1).

Protocol 2

Following atropine administration, there were increases in heart rate, cardiac output, peak thoracic flow and $+dF/dt_{max}$, and a decrease in stroke volume (Table 1). However, with the subsequent administration of atenolol, all variables, except for $+dF/dt_{max}$, returned to control levels. In the presence of atropine and atenolol there was a small, but significant decrease in $+dF/dt_{max}$ ($P < 0.05$) (Table 1).

In the presence of atropine and atenolol, L-NAME produced a pressor response not significantly different from the response evoked by L-NAME given alone, and this pressor effect was accompanied by significant reductions in cardiac output, total peripheral conductance, stroke volume, $+dF/dt_{max}$ and peak thoracic flow ($P < 0.01$, ANOVA, from 5 min after L-NAME injection) (Figure 1). These changes were similar ($P > 0.05$, ANOVA) to the responses evoked by L-NAME alone. However, although under these conditions there was a small, but significant, bradycardia ($P < 0.01$, ANOVA, starting 5 min after L-NAME injection), this was significantly less than the heart rate response following administration of L-NAME alone ($P < 0.01$, ANOVA). The reduction in stroke volume caused by L-NAME was numerically greater in the presence of atropine and atenolol compared with the effect of L-NAME alone, although this did not achieve statistical significance ($P > 0.05$, ANOVA) (Figure 1).

Discussion

The two major findings from this study are that (1) an increase in vagal efferent activity is primarily responsible for the bradycardia evoked by L-NAME (at least in the steady-state) and (2) the L-NAME-induced changes in cardiac function, other than heart rate, are not dependent on efferent neural control of the heart.

Previous studies in anaesthetized rabbits (Rees *et al.*, 1989) and guinea-pigs (Aisaka *et al.*, 1989) have shown that the bradycardic effects of L-NMMA are abolished by vagotomy or atropine, respectively. These results are consistent with ours, and contrast with those of Wang & Pang (1991), who found the slope of the relation between heart rate and MAP following administration of N^G-nitro-L-arginine (L-NNA) was unaffected by atropine in conscious Sprague-Dawley rats. Wang & Pang (1991) concluded that inhibition of sympathetic nerve activity, rather than potentiation of parasympathetic nerve activity, was involved in the reflex changes in heart rate in their conscious rats. We can see no obvious explanation for the fundamental difference between our results and those of Wang & Pang (1991), but the latter are at variance with other studies showing that increased vagal efferent activity is the major factor responsible for the reflex bradycardia elicited by different pressor agents in rats (Head & McCarty, 1987).

Although it is feasible that treatment with L-NAME or L-NNA could affect the mechanisms normally contributing to the relation between heart rate and MAP, direct assessment of the slope of this relation in the presence of L-NAME has shown this is not the case (Gardiner *et al.*, 1991).

In the presence of atropine and atenolol, there was a small (-28 ± 6 beats min^{-1}) bradycardia following the administration of L-NAME, despite the use of supramaximal doses of atropine and atenolol. This bradycardia may possibly have been due to coronary vasoconstriction induced by L-NAME, as has been reported to occur after administration of N^G-nitro-L-arginine in conscious rabbits (Humphries *et al.*, 1991).

In contrast to the effects on heart rate, following administration of L-NAME the rise in MAP and reductions in cardiac output, stroke volume, peak thoracic flow, $+dF/dt_{max}$, and total peripheral conductance were not different in the absence and presence of atropine plus atenolol. Thus, while it appears the bradycardia following L-NAME is largely reflex in origin, the changes in cardiac haemodynamics are clearly not dependent on the efferent neural control of the heart. It is possible these myocardial effects were due to the increase in afterload (i.e. reduction in total peripheral conductance) and/or coronary vasoconstriction. However, if the latter were responsible for the slight bradycardia following L-NAME in the presence of atropine and atenolol (see above), it seems unlikely it alone would have caused such profound reductions in myocardial function.

Support for the proposition that the increase in afterload was responsible for the diminution in cardiac function following L-NAME comes from findings with several other vasoconstrictor agents, including angiotensin II (Webb *et al.*, 1986; Osborn *et al.*, 1987), vasopressin (Webb *et al.*, 1986; Osborn *et al.*, 1987; Veelken *et al.*, 1989) and endothelin-1 (Gardiner *et al.*, 1990a; Rohmeiss *et al.*, 1990). All produce decreases in heart rate and cardiac output in conscious rats, but only the chronotropic effect is reflexly mediated (i.e. reversible by autonomic blockade). Moreover, these agents reduce cardiac output although they have differing ability to stimulate myocardial contractility directly (Heyndrickx *et al.*, 1976; Hernandez *et al.*, 1990; Yang *et al.*, 1991). Therefore, at least in the rat, the increase in afterload evoked by L-NAME is likely to be a major determinant of the decrease in cardiac output.

In conclusion, cardiac autonomic blockade (with atropine and atenolol) revealed a dissociation between the changes in heart rate and cardiac output in response to L-NAME, such that the bradycardia, but not the reduction in cardiac output, was markedly attenuated under these conditions. The pressor, vasoconstrictor and the additional negative myocardial effects evoked by L-NAME were unaltered by cardiac autonomic blockade, and were most probably due to the increase in afterload, although a direct myocardial effect of L-NAME cannot be discounted.

Finally, in the presence of atropine and atenolol the dissociation between the changes in cardiac function and heart rate, when pressor responses to L-NAME were unaffected, makes it clear that the assessment of the baroreflex-mediated control of MAP should not be based solely on the relation between heart rate and MAP.

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α4-2β2 and other nicotinic acetylcholine receptor subtypes as targets of psychoactive and addictive drugs

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1 *Xenopus* oocytes were injected with various muscle and neuronal nicotinic acetylcholine receptor (ACh receptor, cholinoceptor) subunit RNA combinations and their pharmacological properties studied using two-electrode voltage clamp. The functional expression of one of these combinations, rat α4-2β2, has not been previously described. The α4-2 mRNA is a splicing variant transcribed from the α4 gene. In the experiments reported here, the α4-2β2 subtype was functionally indistinguishable from the α4-1β2 subtype.

2 For each subtype, the relative potency of nicotine compared with acetylcholine was obtained by estimating the relative concentration of nicotine which would elicit the same current response as 0.1 μM ACh. The ratios of these concentrations (nicotine: ACh) for the mouse muscle ACh receptor-(α1β1γδ) was 96.1:1. In contrast, the ratios for the rat neuronal subtypes were: α2β2, 1.01:1; α3β2, 2.01:1; α4β2, 0.76:1 and α4-2β2, 0.76:1. The much greater relative nicotine sensitivity of the neuronal subtypes as compared with muscle receptors illustrates their potential to mediate the psychoactive and addictive effects of nicotine. However, it does not appear that the differences in relative nicotinic sensitivity among the neuronal receptors themselves can be used as a simple discriminative tool in neuronal tissue.

3 The slopes of the log dose-log response curves at low ACh concentrations were all greater than 1 but less than 2, suggesting that at least two agonist binding sites mediate the functional response of each hetero-oligomer.

4 The response of all the neuronal subtypes to ACh could be inhibited by the psychoactive drugs mecamylamine, amitriptyline, phencyclidine, trifluoperazine and promethazine. With the exception of the very potent antagonist, mecamylamine, the degree of block of the peak current to ACh produced by 10 μM concentrations of these drugs was remarkably similar (around 50%).

5 The degree of inhibition produced when the antipsychotic drug, trifluoperazine, was co-applied with ACh increased as the duration of application increased. Such an effect was not observed with promethazine, a related phenothiazine derivative which does not have antipsychotic actions.

Keywords: Nicotinic acetylcholine receptors; voltage-clamp; ooctyes; psychoactive; nicotine; drug design; α4-2 cDNA; schizophrenia

Introduction

The psychological actions of nicotine are important reinforcers of the smoking habit (Benowitz, 1988, review). Some of the objectively confirmed effects reported by smokers include arousal, mood-elevation, improved concentration and a reduction in feelings of stress and anxiety (Wesnes & Warburton, 1983; Warburton, 1987). In contrast, the selective loss of cortical, high-affinity, nicotine binding sites from the brain in Alzheimer's disease is correlated with a loss of cognitive function (Whitehouse *et al.*, 1988). Apart from their obvious role in addiction, an additional implication of these observations is that nicotinic acetylcholine receptors (ACh receptors, cholinoceptors) are important elements in some of the neuronal circuits that mediate psychological functions.

In order to obtain a more complete understanding of the pharmacological and molecular bases of these phenomena, we decided to examine the effects of nicotine and other psychoactive drugs on a variety of nicotinic ACh receptor subtypes. cDNAs whch encode subunits of muscle and neuronal nicotinic ACh receptors have been isolated from several species and in many cases their corresponding mRNAs have been functionally expressed (Deneris *et al.*, 1991, review, Couturier *et al.*, 1990a). The cDNAs encode polypeptides which fall into two major classes. A major distinguishing feature of one class, the α-subunits, is that each subunit contains two consecutive cysteines at amino-acid positions 192,193 (*Torpedo* numbering) which are thought to be in the vicinity of the agonist binding site (Kao *et al.*, 1984). The α-subunit in muscle is called α1, and to date those found in neuronal receptors have been numbered in the historical order in which they were discovered, from α2 to α7. There are also ACh receptor subunits which do not contain these two cysteines. Members of this latter class include the mouse muscle β1, γ and δ subunits, and the rat neuronal β2 and β4 subunits. Among several demonstrations of expression, it has been shown that functional nicotinic ACh receptors can be detected following the co-injection of the mouse muscle α1β1γδ RNAs into *Xenopus* oocytes (Yoshii *et al.*, 1987; Connolly, 1989). Co-injection of any one of the rat neuronal α2, α3 or α4-1 RNAs with either β2 (Boulter *et al.*, 1987; Deneris *et al.*, 1988, Wada *et al.*, 1988b) or β4 (Duvoisin *et al.*, 1989) RNA also results in robust and reproducible functional expression of nicotinic ACh receptors.

Although the β4 gene is expressed in PC12 cells (Boulter *et al.*, 1990) and embryonic chick ganglia (Couturier *et al.*, 1990a, non-α3 in chick nomenclature), it shows very limited expression in rat brain, being mainly transcribed in the medial habenula (Duvoisin *et al.*, 1989). In contrast the β2 subunit gene is very widely expressed (Wada *et al.*, 1988a). Therefore the neuronal ACh receptor subtypes examined in this study, which was directed towards the understanding of the potential involvement of nicotinic ACh receptors in a variety of brain processes, combined neuronal α-subunits with the more ubiquitous β2 subunit.

In many brain regions, the distribution of the β2 mRNAs was found to overlap with that of α4 mRNAs (Wada *et al.*, 1988a). However, when hypothalamic cDNA libraries were first screened, two different, but equally abundant, species of α4 clones were found (Goldman *et al.*, 1987). It was thought that the two types of α4 mRNAs from which these cDNA clones were derived may have arisen by alternate splicing of the primary transcript of the α4 gene. The first species, which was full length, was called α4-1 and is the subtype we have

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used in functional expression studies of nicotinic ACh receptors to date (Boulter et al., 1987; Deneris et al., 1988; Wada et al., 1988b). The amino acid sequence of the α4-1 subunit was deduced from the cDNA clone, and the last two amino acids of the carboxy (C)-terminus were predicted to be alanine and cysteine. The α4-2 cDNA was not full length, and differed from the α4-1 cDNA at its 3' end. The deduced amino acid sequence of this cDNA was one amino acid longer at the C-terminus, and ended in the three amino acids glycine, methionine and isoleucine (GMI). The 3' untranslated region of the clone also differed from that of α4-1. For the rest of the overlapping regions of the cDNAs, the sequences were identical. If, like α4-1, the α4-2 subunit can form functional receptors with β2, then it too may be incorporated into central nicotinic ACh receptors. Therefore, in addition to examining the actions of psychoactive drugs on the ACh receptor subtypes described above, we also describe here the first functional expression of the neuronal α4-2 subunit in conjunction with the β2 subunit.

Methods

Expressible clones

A full length α4-2 cDNA was constructed from partial clones by use of conventional molecular biological techniques. The coding region and 5'-end of α4-2 mRNA is identical to that of α4-1 mRNA with the exception of the differences in the 3'-end mentioned above. The other expressible clones used in this study have all been described previously: α1 (Boulter et al., 1985); γ (Boulter et al., 1986b); β1 and δ (Heinemann et al., 1986); α2 (Wada et al., 1988b); α3 and α4-1 (Boulter et al., 1986a; 1987; Goldman et al., 1987) and β2 (Deneris et al., 1988).

In vitro *synthesis of RNA and injection into oocytes*

Plasmid DNA for each subunit constructed was linearised with restriction enzymes at the 3' end of each clone. Diguanosine triphosphate-capped RNA was then transcribed from these DNA templates by bacteriophage SP6 RNA polymerase (Melton et al., 1984).

Oocytes were prepared for injection as described previously (Boulter et al., 1987). This technique removes the follicular cell layer but leaves the vitelline membrane intact. A total of 3–5 ng of RNA was injected into each oocyte, in a volume of 50 nl of H_2O. The mouse muscle ACh receptor (cholinoceptor) subunits (α, β, γ and δ) were injected into the oocytes in the ratio 1:1:1:1. Neuronal ACh receptor subunit combinations were also injected in a 1:1 ratio.

Drug solutions

The agonists (−)-nicotine hydrogen tartrate and acetylcholine chloride and antagonists, phenycyclidine (PCP) hydrochloride, promethazine hydrochloride, amitriptyline hydrochloride, mecamylamine hydrochloride and trifluoperazine hydrochloride, were all obtained from Sigma Co., St. Louis, U.S.A.

Recording procedures

Two-electrode voltage-clamp recordings of the responses of oocytes to various agonists and antagonists were made with a Dagan 8500 voltage-clamp unit. The voltage micropipettes contained 3 M KCl (resistance 2–4 MΩ) and the current micropipettes were filled with 0.25 M CsCl, 0.25 M CsF, 100 mM EGTA, pH 7.2 (resistance 0.5–1 MΩ). The control solution (in which drugs were also dissolved), contained 115 mM NaCl, 1.8 mM CaCl, 2.5 mM KCl, 10 mM HEPES (pH 7.2) and 1 μM atropine.

Individual oocytes were placed in a groove in the base of a narrow perspex chamber. A system of switches close to the bath inlet allowed either drug or control solutions to be perfused at a rate of approximately 30 ml min^{-1} through a bath volume of 0.5 ml. Under these conditions, the current response reached 90% of its initial peak value within 2–3 s (see Figures 1, 4 and 5). Washing out of the drug took slightly longer, perhaps because some of the drug is trapped between the vitelline and cell membranes. With this rate of perfusion, care must be taken to ensure that the oocytes are stably positioned and do not move about the recording pipettes while the solutions are being changed, as this can give rise to small, artefactual inward currents.

Potential interference by endogenous subunits

Spurious results due to the incorporation of endogenous oocyte ACh receptor subunits into receptors expressed after injection of mouse muscle of rat neuronal subunit RNAs does not seem likely. We have found previously, using voltage recordings, that with the exception of a very weak and inconsistent response from α4-1, rat neuronal α and β subunit RNAs injected singly will not form functional receptors (Boulter et al., 1987; Deneris et al., 1988; Wada et al., 1988b, Duvoisin et al., 1989). Similarly, functional expression has also not been detected for singly injected chick α4 and non-α1 (most homologous to rat β2) subunit RNAs (Bertrand et al., 1990). Thus we have no positive evidence that endogenous *Xenopus* subunits which can combine with the neuronal subunits we have injected are present. Nor have we seen nicotinic responses in oocytes following mouse muscle α1 RNA, water, sham or mouse muscle β1γδ RNA injections (Deneris et al., 1991; Connolly, 1989). Similar results have been obtained with *Torpedo* receptor subunits (Kurosaki et al., 1987). In their experiments, Leutje & Patrick (1991) have also found that injections of mouse muscle β1γδ and rat β2 RNAs do not lead to functional expression. Hence if endogenous, compatible, muscle type subunits were present in oocytes used in these experiments, they were expressed at an undetectably low level.

However, there has been a report of functional expression when only mouse muscle β1γδ subunits were injected, but this current was only just detectable at mM concentrations of ACh (Buller & White, 1990). Also, a low level of endogenous alpha-subunit RNA has been reported in oocytes (Hartmann & Claudio, 1990), but the amount per oocyte is less than 0.5 picograms, 10,000 fold less than the nanogram amount of synthesized RNA injected here. If there is endogenous subunit RNA expression at this level in some of the oocytes we have used, then at the concentrations applied here, the above results suggest that such expression would not make a significant contribution to the current responses we observed.

Collection of data for dose-response curve

Currents greater than 2μA could not be reliably clamped under our recording conditions and currents smaller than 1–2 nA were difficult to measure accurately. The steepness of the dose-response curves meant that it was not possible to record the responses to a wide range of concentrations in an individual cell within these current limits. Therefore, to obtain the data depicted in Figures 2 and 3, several trials were made at a standard concentration of ACh during the course of each experiment. The standard concentrations were 1 μM ACh for the α2β2 and α3β2 combinations and 0.1 μM ACh for the α1β1γδ, α4-1β2 and α4-2β2 combinations, all voltage-clamped at −70 mV. The current produced by this standard dose was defined as 100% and the responses of that oocyte to other concentrations of ACh and all concentrations of nicotine, were expressed as a % of it.

For nicotine and ACh concentrations of 1 μM and below, at least duplicate trials for each agonist were performed in each oocyte at each concentration and the responses averaged. At agonist concentrations greater than 1 μM, the responses were not always duplicated, since full recovery from desensitization could take more than 30 min. At these high agonist concentra-

tions, considerable desensitization may have occurred before the peak response was attained. Unfortunately, the large size of oocytes (>1 mm) and the slowing of solution exchange by the vitelline membrane prohibit rapid and simultaneous perfusion of the entire cell surface. This prevents estimation of fast rates of desensitization in this preparation. Additional problems at high agonist concentrations are the probability of channel block by the agonists and the difficulty of comparison with a convenient standard, non-desensitizing, response. Because of these considerations, the dose-response curves were not extended beyond agonist concentrations of 10 μM.

Estimation of relative potencies and low-concentration slopes

Unlike the muscle receptor dose-response curves at low agonist concentrations, the nicotine and ACh dose-response curves plots are not parallel for the neuronal receptors, possibly because the rates of desensitization of neuronal nicotinic ACh receptors are different for ACh and nicotine. For this reason, the potency ratios are compared at concentrations where the curves are closest to parallel. The relative potency of ACh compared to nicotine was then calculated as the antilog of the difference between the logs of the concentrations of nicotine and ACh that gave a particular current response.

In order to estimate these concentrations for a particular subtype, straight lines were fitted by linear regression to the three or four lowest concentration points of the ACh and nicotine plots. Calculated log (current) values corresponding to either 0.1 μM ACh or 0.316 μM ACh were then obtained from the new lines. These log (current) values were then applied to the calculated nicotine curve to obtain the corresponding nicotine concentrations. The antilog of the difference between the logs of these two agonist concentrations provides the ratio shown in Table 1. The low concentration slopes were calculated from the straight lines fitted by linear regression to the lowest 3 concentration points on the log [ACh]-log (current) plots. It was noticed that some batches of oocytes would express receptors with lower mean slopes than others, but data from several batches were pooled to give the results shown here in Figures 2 and 3. Where appropriate, Student's t tests for paired and independent samples were used to test for significance.

Results

Functional expression of the α4-2β2 combination

Figure 1 shows the current responses of an oocyte injected with the α4-2β2 RNA combination to 0.316 μM applications of ACh and nicotine. The strong expression clearly demonstrates that the combination is functional. The dose-response curves for the α4-1β2 and α4-2β2 combinations shown in Figure 3c and 3d are almost superimposable. Analysis of their responses to ACh and nicotine does not reveal any significant difference, the relative potency being the same for each (Table 1).

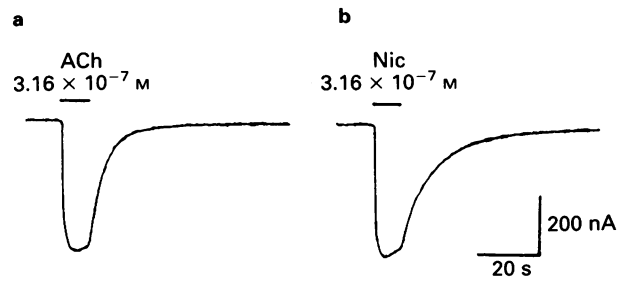

Figure 1 Functional expression in oocytes injected with the α4-2β2 RNA combination. Current responses to 0.316 μM acetylcholine (ACh) (a) and 0.316 μM nicotine (Nic) (b) illustrate typical rates for the application and removal of drug solutions.

Table 1 Summary of characteristics of dose-response plots

Injected	*Relative potency 0.1 μM ACh*	*Relative potency 0.316 μM ACh*	*Slope* (mean ± s.e.)
α1β1γδ	96.1	—	1.71 ± 0.04
α2β2	1.01	1.27	1.22 ± 0.02
α3β2	2.01	2.20	1.49 ± 0.11
α4-1β2	0.76	1.03	1.08 ± 0.12
α4-2β2	0.76	1.02	1.37 ± 0.03

The relative potency indicates the ratio of the concentrations of nicotine and acetylcholine which give the same magnitude of current response. The [nicotine]/[acetylcholine] ratio is shown for two current values, which correspond to the currents elicited by the application of acetylcholine (ACh) at concentrations of 0.1 μM and 0.316 μM. The slope values (± s.e.) are those of lines fitted by linear regression to the lowest three concentration points of the averaged ACh dose-response plots shown in Figures 2 (muscle) and 3 (neuronal). Details of the procedures used to calculate these values are described in the Methods section.

Nicotinic acetylcholine receptor subtypes show differential sensitivity to (−)-nicotine

It has been long appreciated that among native nicotinic ACh receptors, there is a clear differentiation between the relative sensitivity of the muscle and neuronal nicotinic ACh receptors to nicotine, with muscle receptors being much less sensitive to nicotine (Hill, 1909; Curtis & Eccles, 1958; Lukas, 1989). Such differences in the nicotine sensitivities of receptors formed from cloned subunits might also be expected from earlier voltage (Boulter *et al.*, 1987; Duvoisin *et al.*, 1989) and voltage-clamp (Bertrand *et al.*, 1990; Couturier *et al.*, 1990a) studies.

This marked difference is confirmed here with the receptor subtypes we have introduced into *Xenopus* oocytes. The dose-response curves in Figure 2a were obtained from oocytes injected with the RNA combination α1β1γδ. As the summary in Table 1 indicates, it would require 9.6 μM (−)-nicotine to produce the same current response as that produced by 0.1 μM ACh, approximately 2 orders of magnitude difference in the respective agonist concentrations. However, the graphs in Figure 3 demonstrate that the relative sensitivity of the neuronal receptor subtypes to nicotine is much greater. Table 1

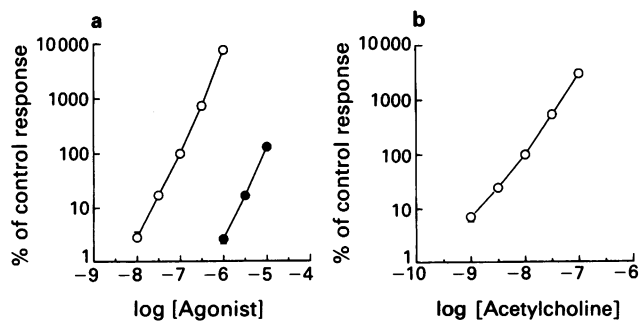

Figure 2 (a) Log-log plots of the agonist dose-current response relationships of acetylcholine (○) and nicotine (●) with oocytes injected with mouse muscle α1β1γ and δ subunit RNAs. Each point represents averaged, normalised (to the 0.1 μM acetylcholine response) data pooled from a minimum of 4 oocytes (see Methods). The current value (± s.e.) corresponding to 100% was 59 ± 12.1 nA (n = 20 oocytes). The oocytes were voltage clamped at −70 mV. Vertical bars, where visible, represent the standard errors at each point. (b) Log-log plot of the acetylcholine dose-current response relationship of oocytes injected with the α4-1β2 combination and voltage clamped at −100 mV. Responses were normalised to the response to 0.01 μM acetylcholine and each point represents the data collected from at least 4 cells. The current value corresponding to the 100% response was 50.1 ± 2.1 nA (n = 5 oocytes). The maximum slope, fitted by linear regression to the points included in the range 10 nM to 100 nM, was 1.47 (± 0.02, s.e.).

shows that the nicotine/ACh potency ratio for neuronal receptors is, at most, approximately 2:1. Even more remarkable is that for the $\alpha 4\beta 2$ combinations, at low concentrations, nicotine is more potent than ACh, the natural transmitter. Thus, when compared with their sensitivity to ACh, neuronal receptors are at least 50 times more sensitive to nicotine than muscle ACh receptors.

Although muscle and neuronal receptors are easily distinguished on the basis of their relative sensitivity, the differences among the neuronal receptors themselves are more subtle. In many of the experiments upon which Figure 2 is based, an identical concentration of both ACh and nicotine was applied to the same oocyte. To test if these more subtle differences were significant, the current response of an oocyte to a given concentration of nicotine was expressed as a % of this response to the same concentration of ACh. The mean of the % nicotine responses from several oocytes was obtained. This was done for those concentrations (0.1, 0.32 and 1 μM) which were common to the neuronal dose-response curves (see Table 2). At all three agonist concentrations, the means of the nicotine responses of the $\alpha 4$-$1\beta 2$ and $\alpha 4$-$2\beta 2$ subtypes were not significantly different from each other. In contrast, the responses of these two combinations were significantly different from the less sensitive $\alpha 2\beta 2$ subtype ($P < 0.05$) and even more easily distinguished from the $\alpha 3\beta 2$ combination ($P < 0.002$). The means of the % nicotine responses of the $\alpha 3\beta 2$ and $\alpha 2\beta 2$ combinations were also different from each other ($P < 0.02$). Thus there are agonist concentrations at which these particular subtypes can be distinguished upon the basis of their relative nicotine sensitivity.

Table 1 also shows the value of the slope of the log dose–log mean current response curve for the muscle receptor at low ACh concentrations. At these low concentrations the slope value will approximate to the Hill coefficient. The value of 1.71 suggests that there are at least 2 agonist binding sites in each mouse muscle receptor hetero-oligomer that we have expressed. The values of the slopes of lines fitted through the lowest three concentration points of each of the neuronal ACh receptor dose-response plots shown in Figure 3 are also listed in Table 1. However the slopes of the neuronal dose-response plots were all lower than that of the muscle ACh receptors (Figure 3, Table 1). The lowest value was obtained for the $\alpha 4$-$1\beta 2$ combination. This combination was re-examined, and to minimize desensitization and increase the sensitivity of measurement of the smaller currents, nanomolar concentrations of acetylcholine and a holding potential of -100 mV were used (Figure 2b). The maximum slope obtained, between 10 nM and 100 nM ACh, was still only 1.47 ± 0.02 ($n = 4$ oocytes). A higher value of 1.83 has been reported for the chick $\alpha 4$ non-$\alpha 1$ combination (Ballivet *et al.*, 1988). The reasons for the low slope values of rat neuronal ACh receptor dose-response curves are not clear, but it may be a consequence of very rapid desensitization, even at these low agonist concentrations. Recently, Cooper and colleagues (1991) have deduced that for the chick $\alpha 4$ non-$\alpha 1$ combination, the stoichiometry is two alpha subunits and three non-α subunits. It would therefore seem most probable that our receptors also contain two α-subunits and three β subunits.

Table 2 Variation in the relative nicotine responses for different neuronal ACh receptor subtypes

	0.1 μM (% ± s.e.)	n	*0.32 μM* (% ± s.e.)	n	*1.0 μM* (% ± s.e.)	n
$\alpha 2\beta 2$	83.1 ± 3.2	6	64.4 ± 7.5	5	54.2 ± 4.0	8
$\alpha 3\beta 2$	47.2 ± 5.1	5	38.1 ± 0.9	4	30.0 ± 3.7	10
$\alpha 4$-$1\beta 2$	126 ± 12.8	8	92.8 ± 4.4	4	106.1 ± 1.5	3
$\alpha 4$-$2\beta 2$	132 ± 10.3	4	99.8 ± 10.7	3	—	—

The current responses to nicotine were expressed as a percentage of the current responses to an equal concentration of acetylcholine (ACh) applied to the same oocyte. Each row shows the mean of these responses for oocytes injected with a particular mRNA combination. The % means (± s.e.) are given at three agonist concentrations. These results have been included among the more extensive data shown graphically in Figure 2 (n = the number of oocytes from which paired responses to nicotine and ACh were obtained).

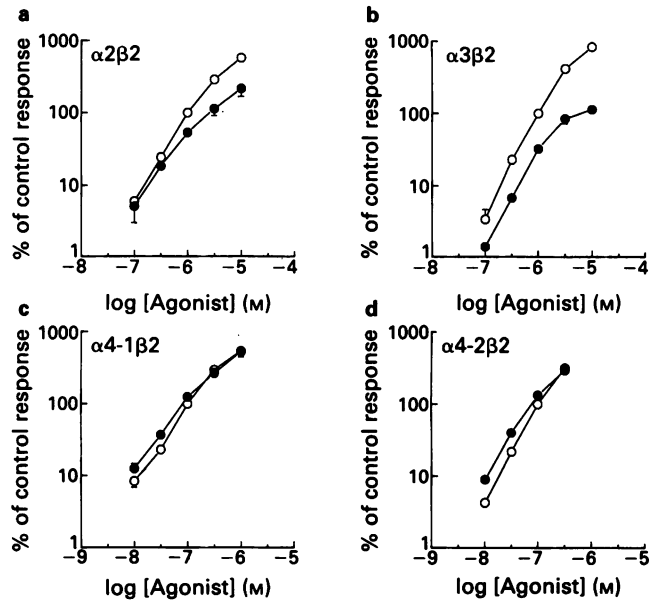

Figure 3 Log-log plots of the normalised, agonist concentration-current response relationships of oocytes injected with (a) $\alpha 2\beta 2$, (b) $\alpha 3\beta 2$, (c) $\alpha 4$-$1\beta 2$ and (d) $\alpha 4$-$2\beta 2$ RNAs. Each data point for acetylcholine (○) or nicotine (●) represents the results averaged from a minimum of 4 oocytes, except for (d) where the minimum is 3. The oocytes were voltage-clamped at -70 mV. Vertical bars, where visible outside the plot symbols, show standard errors. The current values (± s.e.) corresponding to the 100% (control) response were (a) 101.9 ± 16.2 nA, $n = 11$ oocytes; (b) 126.6 ± 28 nA, $n = 16$ oocytes; (c) 84.5 ± 18.5 nA, $n = 19$ oocytes and (d) 170.8 ± 66.6 nA, $n = 6$ oocytes. A summary of these neuronal ACh receptor dose-response characteristics is shown in Table 1.

Interaction of psychoactive drugs with neuronal nicotinic acetylcholine receptors

We took advantage of the accessibility of neuronal ACh receptors expressed in oocytes to screen the actions of several psychoactive drugs on the four neuronal subtypes described above. Representative responses shown in Figures 4 and 5, and their means are summarized in Table 3. Apart from known nicotinic antagonists, we examined a selection of clinically important drugs which, although targeted at other receptor systems, also exhibit 'anti-cholinoceptor' side effects.

Figure 4a shows the blocking of the $\alpha 2\beta 2$ receptor by mecamylamine. This drug is a potent antagonist of nicotinic ACh receptors in ganglia (Ascher *et al.*, 1979), but has limited clinical application since it crosses the blood-brain barrier and causes psychotic-like side effects. The chick $\alpha 4$ nonα-1 receptor has also been shown to be blocked by mecamylamine in a voltage independent manner (Bertrand *et al.*, 1990).

Figure 4b shows the blocking action of phencyclidine (PCP) on the $\alpha 3\beta 2$ subtype. Its non-competitive blocking action on the muscle type nicotinic ACh receptors in C2 myotubes (Changeux *et al.*, 1986) and BC3H1 cells (Albuquerque *et al.*, 1980; Papke & Oswald, 1989) has also been documented. Our results show that it also blocks central neuronal nicotinic ACh receptors (Table 3).

The major therapeutic disadvantage of amitriptyline (Ami) lies in its strong anticholinoceptor side effects. We found it was able to block neuronal nicotinic ACh receptors (Figure 4c, Table 3). At 10 μM Ami, the peak of the inhibited response is 48% of the corresponding control current value. However, if the degree of block is examined at a later time point in the

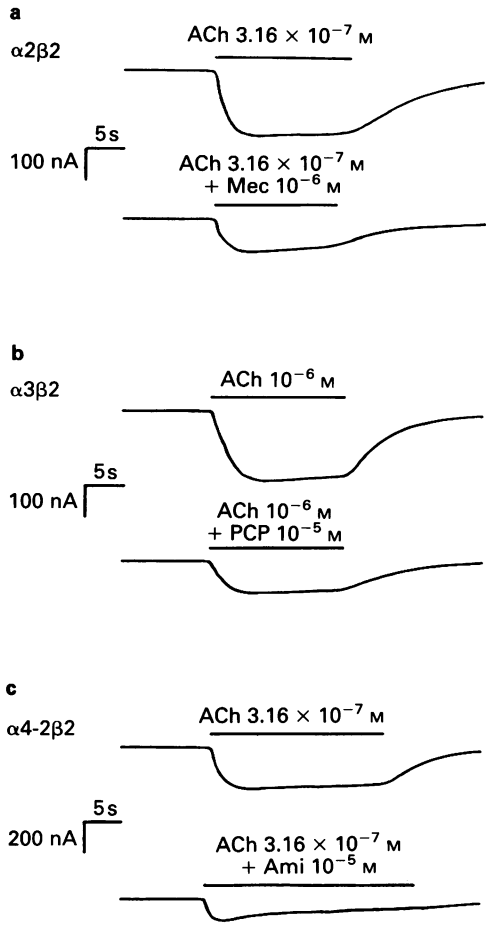

Figure 4 (a) The upper trace shows the current response to acetylcholine (ACh) of oocytes injected with α2 + β2 RNAs. The response is inhibited when both mecamylamine (Mec) and acetylcholine are applied (lower trace). (b) The control response to ACh (upper trace) of oocytes injected with α3 + β2 DNAs is reduced in the presence of phencyclidine (PCP). (c) Amitriptyline (Ami) inhibits the α4-2β2 response to acetylcholine. Note the increase in the degree of block with duration of application of the drug. All antagonists were preincubated with the oocytes for 1 min before application of the acetylcholine/antagonist mixture. The cells were voltage clamped at −70 mV. A summary of these results is shown in Table 3.

trace, then the current has diminished further (only about 32% of the control response is remaining 10 s after the start of drug application). The mechanism for this increased reduction in response is not clear, although it has been shown that the related antidepressant desipramine is an open-channel blocker of NMDA receptors and has a similar IC_{50} (Sernagor *et al.*, 1989). In those experiments shown in Table 3 where identical concentrations of agonist and antagonist were used, there was no significant difference between the subtypes at the 15% level. Therefore these antagonists do not readily discriminate between the subtypes. Also, the degree of inhibition of the peak response is remarkably similar for all the antagonists applied at a concentration of 10 μM.

Comparison of the actions of trifluoperazine and promethazine on acetylcholine receptors

The two antipsychotic drugs, trifluoperazine (TFP) and its related phenothiazine, chlorpromazine, have long been known to have an anti-nicotinic effect on *Torpedo* (Heidemann *et al.*, 1986) and peripheral ACh receptors (Changeux *et al.*, 1986). Giraudat *et al.* (1986, 1987) and Revah *et al.* (1990) have additionally found that chlorpromazine binds to amino acids in the M2 domain of the β, γ and δ subunits, a region that is thought to line the wall of the ion channel (Imoto *et al.*, 1988). The chlorpromazine binding is PCP-sensitive. Similar amino

Table 3 Inhibition of nicotinic ACh receptor response to acetylcholine by centrally active antagonists

cRNAs injected	*Antagonist*	*[ACh]* (μM)	*[Antag.]* (μM)	*% control response* (± s.e.)	n
α4-2β2	Amitriptyline	0.316	10.0	48.5 ± 7.1	3
	Promethazine	0.032	10.0	45.4 ± 0.7	3
	Mecamylamine	0.316	1.0	45.2 ± 2.3	3
	Trifluoperazine	0.316	10.0	40.0 ± 4.1	3
	Phencyclidine	0.316	10.0	34.8 ± 2.0	3
α3β2	Amitriptyline	1.0	10.0	51.0 ± 2.2	3
	Promethazine	1.0	10.0	59.9 ± 1.5	3
	Mecamylamine	1.0	1.0	62.5 ± 5.3	3
	Trifluoperazine	1.0	10.0	52.2 ± 1.6	3
	Phencyclidine	1.0	10.0	48.3 ± 2.2	3
α2β2	Amitriptyline	0.316	10.0	40.6 ± 0.6	3
	Promethazine	0.316	10.0	53.7 ± 2.0	4
	Mecamylamine	0.316	1.0	50.5 ± 0.7	3
	Trifluoperazine	0.316	10.0	47.8 ± 3.8	3
	Phencyclidine	0.316	10.0	44.6 ± 4.1	4

Initially a reproducible peak current response was obtained to the application of a control concentration of acetylcholine (ACh) (left column). Then, following a preincubation of 1 min with the antagonist, the oocytes were perfused with a mixture containing the control ACh concentration and the indicated antagonist concentration. The peak response in the presence of antagonist was divided by the control peak response and multiplied by 100 to give the values in the 5th column. The oocytes were voltage-clamped at −70 mV (n = number of oocytes in which experiments were performed).

acid residues are present in homologous positions in all the cloned neuronal ACh receptor subunits used here. We therefore tested whether receptors formed from cloned subunits of central nicotinic ACh receptors would be blocked by this TFP to see if the anti-nicotinic actions of this drug might be correlated with its clinical effects.

As a partial control for this study, the drug promethazine (Prometh) was chosen. Like TFP, the chemical structure of Prometh is also based on the phenothiazine nucleus. However Prometh lacks antipsychotic activity. If the degree of nicotinic block produced by Prometh is the same as the degree of block produced by TFP, then it would not be probable that nicotinic ACh receptors contributed to the antipsychotic effect of TFP or its motor side effects. The correlation between anti-nicotinic activity and antipsychotic activity would be negated by such a result.

The degree of blocking of the response of the α4-1β2 combination to 0.1 μM ACh by TFP and Prometh was measured over a concentration range 0.316 μM to 10 μM for these antagonists. The % of the response remaining in the presence of antagonist was assessed at two time points, firstly at the initial peak of the response, and secondly 15 s after the ACh/antagonist mixture was applied. If Figure 5a is compared with Figure 5b, then it is clear that Prometh does not show the same kind of time-dependent block as TFP. This interpretation is born out by the plots in 5C and 5D from which it is clear that the degree of block by TFP is much greater than that seen with Prometh at the 15 s time point. Similar results were also obtained with the α2β2 and α3β2 subtypes. For Prometh, the % blocks of the responses to ACh after 15 s drug application were 55.0 ± 3.0% and 60.1 ± 1.5% respectively. This is not significantly greater than the inhibition observed at the initial peaks of the responses (see Table 3). However TFP does show an increased block for the α2β2 and α3β2 combinations at the 15 s time point (34.5 ± 3.7%, 33.9 ± 3.4% respectively). In bovine chromaffin cells there is also a time-dependent increase in the degree of block by TFP (Clapham & Neher, 1984). However, unlike the latter study, the current responses obtained here in oocytes showed complete recovery, although the time course of recovery of the peak response to its initial value before antagonist application was very slow

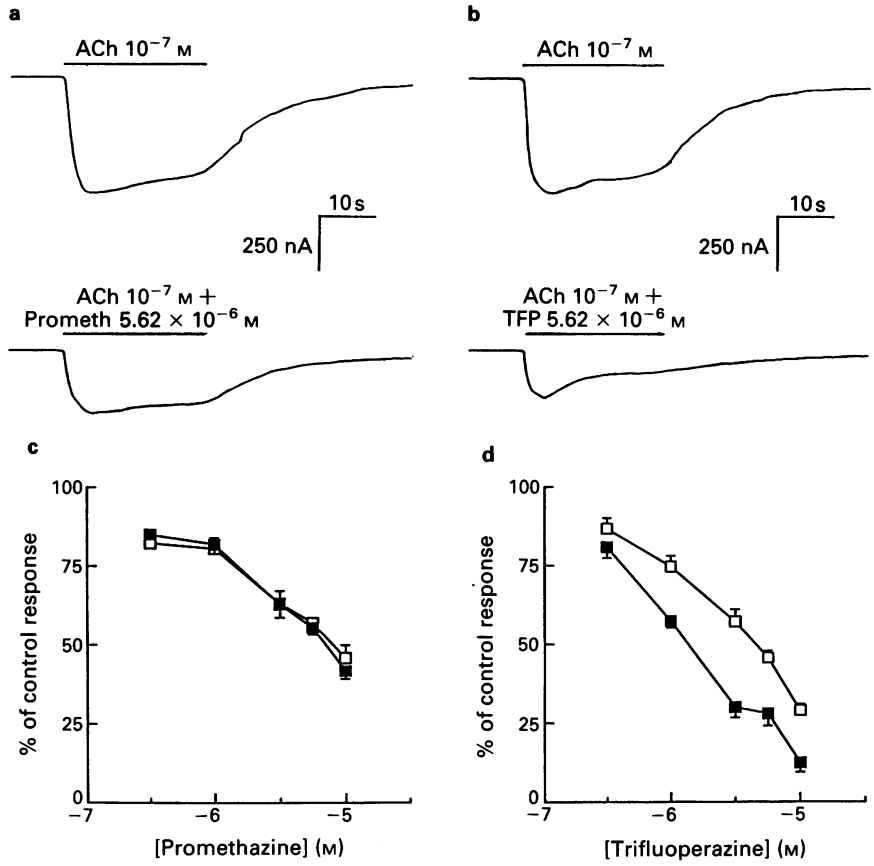

Figure 5 (a and b) The control responses of oocytes injected with the α4-1β2 RNAs to acetylcholine (ACh) are shown in the upper traces. Below them in the lower traces are the inhibited responses obtained in the presence of the antagonists promethazine (Prometh) (a) and trifluoperazine (TFP) (b). The oocytes were preincubated with the antagonists for 1 min before the application of the ACh/antagonist mixture. Note that with TFP in (b), there is a time-dependent increase in the degree of block when it is compared with the control response. (c and d) The concentration-dependent increase in the degree of block is plotted for promethazine in (c) and trifluoperazine in (d). The response remaining in the presence of antagonist is expressed as a % of the control response to 0.1 μM ACh. The degrees of inhibition at the peak of the inhibited response (□) and at a time point 15 s after the start of the response (■) were plotted. Each point represents the mean of data obtained from at least 4 oocytes. Vertical bars represent standard errors. The oocytes were voltage-clamped at −70 mV.

(longer than 20 min). Since the degree of block produced by TFP is much greater than that of Prometh, these results are consistent with the idea that the anti-nicotinic actions of TFP may contribute to its central effects.

Discussion

Functional expression of the α4-2β2 neuronal nicotinic acetylcholine receptors

Here we report the functional expression of the rat α4-2 subunit in conjunction with the β2 subunit. The experiments we have performed do not reveal any significant functional distinction between the α4-1β2 and α4-2β2 subtypes. This might be expected, given the position and size of the altered amino acid sequence involved. In rat brain, α4-2 mRNAs are slightly more abundant than α4-1 RNAs (Goldman *et al.*, 1987), although *in situ* hybridization studies show that they share an almost identical pattern of distribution (Wada *et al.*, 1988a). However, these results do not rule out the possibility that the different C-termini are related to some other property of the receptors such as recognition motif for incoming nerve fibres. It is interesting to note that the rat α2 subunit also ends in the sequence GMI, although there is no evidence of an additional splicing variant of α2 in rat. The GMI sequence is also found at the C-terminal of the chick α2 and α4 subunits, although neither subunit has splicing variants in this species (Nef *et al.*, 1988).

The *in situ* hybridisation study described above additionally showed that the distribution patterns of the α4 transcripts overlap with that of the β2 transcripts and also coincide with many of the brain regions previously associated with high-affinity nicotine binding (Clarke *et al.*, 1985). N-terminal sequencing of immuno-affinity purified, neuronal ACh receptors reveals that the predominant subunit ACh receptor species in brain are α4 and β2 subunits (Whiting *et al.*, 1987a; Schoepfer *et al.*, 1988). It was further found that the α4 subunit binds nicotine with nanomolar affinity (Whiting *et al.*, 1987b). However, since the N-termini of the rat α4-1 and α4-2 subunits are identical, it is not possible to determine the relative proportions of the α4-1 and α4-2 subunit proteins by this method. An antibody directed against β2 subunits has been shown to bind extensively throughout the brain and in many presynaptic locations (Swanson *et al.*, 1987). This additional evidence, coupled to the present demonstration that the α4-2β2 combination is functional, makes it probable both α4-1β2 and α4-2β2 are subunit combinations present in nicotinic ACh receptors in mammalian brain, possibly in presynaptic locations. It is also possible that both the α4-1 and α4-2 subunits might be found in the same brain receptor.

Differential sensitivity of nicotinic acetylcholine receptors to nicotine

There is a clear difference between muscle and neuronal receptors in their relative sensitivity to nicotine as compared to ACh. Muscle receptors are at least 50 times less sensitive than neuronal receptors at low agonist concentrations. There are also differences among the neuronal receptors. The α4-1β2 and α4-2β2 combinations are the most sensitive to nicotine. A

similar degree of sensitivity to nicotine has been seen for the chick combination of $\alpha4\beta2$ (Bertrand *et al.*, 1990), for which a nicotine EC_{50} of 0.77 μM was reported. The rat $\alpha2\beta2$ combination was less sensitive than these, while the $\alpha3\beta2$ combination was the least sensitive of the neuronal receptors. Overall, the relative nicotine sensitivities of ACh receptor subtypes studied in this present work (compared with their response to 0.1 μM ACh and presented in the order of decreasing sensitivity) were: $\alpha4\text{-}1\beta2$, (126) = $\alpha4\text{-}2\beta2$, (126) > $\alpha2\text{-}2\beta2$, (95.1) > $\alpha3\beta2$, (47.8) > $\alpha1\beta1\gamma\delta$, (1).

Recently, Leutje & Patrick (1991) have also examined the relative nicotine sensitivity of the cloned receptors and have observed a similar difference in potency between muscle and neuronal nicotinic ACh receptors. There is broad agreement between their results for neuronal receptors and those described here, except in the case of the $\alpha2\beta2$ combination. They report a nicotine: ACh current response ratio greater than 5:1 for agonist concentrations of 3 μM, whereas our results give an estimated ratio of 0.4:1 at that concentration. The time interval required between agonist applications for recovery of the peak current response suggests that there is considerable desensitization of these receptors at μM concentrations (see Methods). For muscle receptors, the time constant for this desensitization is less than 100 ms (Dilger & Brett, 1990; Franke *et al.*, 1991). The rate of drug application was slightly slower in the Leutje & Patrick study than that used here, and intracellular Ca^{2+} concentrations were not buffered. Therefore one possible explanation for the discrepancy between the two sets of observations is that the different conditions could give rise to different degrees of desensitization at the observed peak responses if the rates of desensitization associated with the two drugs are different. None the less, both sets of studies do illustrate the great sensitivity of the neuronal receptors to nicotine.

Despite this sensitivity, the results described here and for the chick $\alpha4\beta2$ combination are not consistent wtih K_d estimates from binding studies of 1–10 nM for the high affinity (−)-nicotine binding site in rat brain (Wonnacott *et al.*, 1990, review). However, the equilibrium conditions under which binding studies are carried out often involve prolonged exposure of the receptors to high concentrations of agonist. This favours desensitization, in which state the receptor may be expected to have a higher affinity for nicotine than during the less desensitizing conditions of electrophysiological assays.

The above results suggest that the subunits we have expressed may mediate many of the effects of nicotine. However, it should be noted that the recently cloned chick $\alpha7$ subunit is also very sensitive to nicotine (Couturier *et al.*, 1990b). In addition, studies in *Xenopus* oocytes suggest that neuronal ACh receptors incorporating the rat $\beta4$ subunit will have different properties, including enhanced nicotine sensitivity, when compared with receptors in which $\beta2$ is the only non-α subunit (Duvoisin *et al.*, 1989; Leutje & Patrick, 1991; Papke & Heinemann, 1991). This is also true for the equivalent subunits in chick (Couturier *et al.*, 1990a). Therefore the total response of the brain to nicotine will involve more subunits than those examined here. These considerations, desensitization, and the possible involvement of uncloned subunits, make nicotine sensitivity difficult to use, at present, as a test to identify and discriminate between subtypes in the nervous system.

Sensitivity of neuronal acetylcholine receptors to psychoactive drugs

It is interesting to note that despite their structural diversity, all the psychoactive antagonists tested were effective blockers of neuronal nicotinic ACh receptors. The most potent of these antagonists was mecamylamine. The estimated equilibrium constant of mecamylamine for ACh receptors in ganglia is 50 nM (Ascher *et al.*, 1979) and an IC_{50} of 200 nM has been observed in PC12 cells (Lukas, 1989). This is somewhat lower than the value of approximately 1 μM suggested by the results obtained here (Table 3). The difference may arise because we have not fully reproduced the pharmacological properties of native ganglionic receptors with the combinations we have introduced into oocytes. However, for combinations which are representative of native receptors, the pharmacological properties of these receptors are expected to be essentially identical to those of equivalent receptors found in man. This is because human muscle (Luther *et al.*, 1989) and neuronal ($\alpha3$: Fornasari *et al.*, 1990; $\beta2$: Anand & Lindstrom, 1990) nicotinic ACh receptor clones encode proteins with a very high sequence identity with the equivalent rat subunits. Studies such as these may therefore be useful in addressing drug design problems.

The potent inhibitory action of the tricyclic antidepressant, amitryptiline, on the nicotinic receptors expressed here illustrates this point. Amitriptyline has a tendency to induce weight gain (Vendsborg *et al.*, 1976) whereas nicotine addition tends to inhibit it (Wack & Rodin, 1982). It therefore seems possible that an anti-nicotinic action of amitriptyline could be responsible for its weight gain side-effect. However, as noted above, the related antidepressant desipramine blocks NMDA receptors (Sernagor *et al.*, 1989).

NMDA receptors are also blocked by mecamylamine (O'Dell & Christensen, 1988) and PCP (MacDonald *et al.*, 1987). The observation that (+)-tubocurarine and MK-801 block both NMDA receptors (MacDonald *et al.*, 1987; Halliwell *et al.*, 1989) and nicotinic ACh receptors (Ascher *et al.*, 1979; Kavanaugh *et al.*, 1989; Amador & Dani, 1991) adds to the pharmacological similarity of these ion channels. Interestingly, (+)-tubocurarine, cocaine and ondansetron have also been shown to block both 5-HT_3 receptors (Higashi & Nishi, 1982; Richardson *et al.*, 1985; Yakel & Jackson, 1988; Peters *et al.*, 1990) and nicotinic ACh receptors (Bertrand *et al.*, 1990; Vanner & Suprenant, 1990). However, ondansetron is 100–1000 times more potent a blocker of 5-HT_3 receptors than of nicotinic ACh receptors and 5-HT_3 receptors are relatively insensitive to the ganglionic nicotinic antagonists, hexamethonium (Vanner & Suprenant, 1990) and trimetaphan (Peters *et al.*, 1990), and voltage recordings suggest that the block of these receptors by (+)-tubocurarine is voltage-independent (Vanner & Suprenant, 1990). Despite these differences between the receptor classes, the pharmacological, similarity is still strong enough to add to the difficulty of interpreting electrophysiological and binding studies in neuronal tissue. However, perhaps the most remarkable aspect of the observations described here is the similarity in the degree of block produced by these varied compounds.

Trifluoperazine is a potent blocker of central neuronal nicotinic acetylcholine receptors

Pharmacological studies of TFP and other drugs used to relieve the symptoms of schizophrenia have correlated their anti-psycholtic potency with their anti-dopaminergic potency (Creese *et al.*, 1976; Seeman *et al.*, 1976). Unlike TFP, Prometh does not have antipsychotic activity and does not inhibit vasopressin release (Dyball *et al.*, 1968) (a hormone whose circulating levels are increased by nicotine administration, Seyler *et al.*, 1986). These results suggest that Prometh may be less anti-nicotinic than TFP.

We have seen above that 3.16 μM TFP can diminish the ACh response of neuronal nicotinic ACh receptors to about 25% of its control value. Prometh will only inhibit ACh receptors to about 60% of their control responses, and does not show a time-dependent increase in block. Therefore, our results suggest that in the brain there could be considerable, selective inhibition of central nicotinic ACh receptors at therapeutic concentrations of TFP (0.5–5.0 μM). The existence of excitatory presynaptic nicotinic receptors, which can elicit dopamine release, has been shown in several studies (Connelly & Littleton, 1983; Takano *et al.*, 1983; Schulz & Zigmond, 1989; Rapier *et al.*, 1990; Brazell *et al.*, 1990). It therefore

seems possible that TFP could block presynaptic nicotinic ACh receptors and diminish their contribution to dopamine release. This would help limit the number of postsynaptic dopamine receptors that are activated, augmenting the drug's postsynaptic blocking action associated with its antipsychotic activity. Alternatively, nicotinic receptor blockade by antipsychotic drugs may be more important in the development of side effects such as weight gain, inappropriate vasopressin release syndrome and tardive dyskinesia. It has been noted that smokers have a reduced risk of suffering from Parkinson's disease (Baron, 1986) and a protective effect for nicotine has been suggested by Moss *et al.* (1989) who have found it to be an effective adjunct to neuroleptic treatment for the reduction of motor tics in Tourettes Syndrome.

The present results show the potential usefulness of pharmacological studies on defined subunit combinations for identifying novel targets for therapeutic intervention and improving the understanding of the mechanisms by which illnesses produce their symptoms. In addition, the present study demonstrates that it is feasible that neuronal nicotinic ACh receptors make a significant contribution to the therapeutic and adverse actions of psychoactive drugs in man.

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Characterization of histamine-H_3 receptors controlling non-adrenergic non-cholinergic contractions of the guinea-pig isolated ileum

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1 In the presence of atropine, mepyramine and ranitidine, electric field stimulation of the guinea-pig isolated ileum longitudinal muscle-myenteric plexus preparation resulted in a two component non-adrenergic non-cholinergic contraction. The initial contraction had a duration of approximately 1 s whereas the second contraction lasted approximately 10 s. The second contraction was completely inhibited by tetrodotoxin (0.2×10^{-6} M) with minimal effect on the initial contraction. Phentolamine (3×10^{-6} M), propranolol (3×10^{-6} M) and hexamethonium (10^{-4} M), did not significantly reduce either component of the contractile response.

2 The neurokinin NK_1 receptor antagonists, GR82334 and GR71251, produced concentration-related (EC_{50} = 564 and 173 nM respectively) inhibitions of the second contraction with no effect on the initial contraction. The neurokinin NK_2 receptor antagonists MEN 10207 and Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH_2 (R 396), 1×10^{-9}–10^{-5} M, were without effect on either component of the contractile response.

3 Concentration-related inhibitions of the second contraction, with no effect on the initial contraction, were observed after inclusion of the histamine H_3 receptor agonists (**R**)-α-methylhistamine (pD_2 = 7.6), N^{α}-methylhistamine (pD_2 = 7.7) and N^{α},N^{α}-dimethylhistamine (pD_2 = 6.3). Histamine also inhibited the second contraction (pD_2 = 6.2) in a concentration-related manner but produced a lower maximum inhibitory effect than the other agonists tested.

4 Inclusion of the H_3 receptor antagonists, thioperamide, burimamide, impromidine and phenylbutanoylhistamine, caused parallel concentration-related rightward shifts in the concentration-response curve to (**R**)-α-methylhistamine. In each case, Schild analysis of these data gave slopes not significantly different from unity. Antagonist affinity values for thioperamide (pA_2 = 8.2), burimamide (pA_2 = 7.0) and impromidine (pA_2 = 7.0) were consistent with values obtained in other assays of the H_3 receptor. However, phenylbutanoylhistamine (pA_2 = 5.8) and betahistine ($pK_B \leq 4$) had affinities more than ten fold lower than values obtained in other assays of the H_3 receptor.

5 Exposure of the tissues to N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (10^{-6} M) for 7–30 min followed by extensive washing, had no effect on basal contractions, but produced a rightward shift in the concentration-response curves to (**R**)-α-methylhistamine, N^{α}-methylhistamine, N^{α},N^{α}-dimethylhistamine and histamine. This treatment also resulted in a decrease in the maximum inhibitory response obtainable. Apparent agonist affinity (pK_D) values of 7.01, 7.06, 6.09 and 6.13 were estimated for (**R**)-α-methylhistamine, N^{α}-methylhistamine, N^{α},N^{α}-dimethylhistamine and histamine respectively.

6 In conclusion, pharmacological analysis has revealed that histamine H_3 receptors in the guinea-pig ileum modulate the release of non-adrenergic non-cholinergic neurotransmitters, one of which is probably substance P. In addition we have identified N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline as an irreversible antagonist at H_3 receptors and have used this compound to estimate apparent affinity values of agonists at H_3 receptors in this preparation.

Keywords: Guinea-pig ileum; histamine H_3 receptors; non-adrenergic non-cholinergic contractions

Introduction

The effects of histamine are thought to be mediated through H_1, H_2, and H_3 receptors. The most recently discovered of these, the H_3 receptor, was first described by Arrang *et al.* (1983) as a presynaptic receptor, regulating neuronal release and synthesis of histamine in slices of rat cortex. The most potent and selective H_3 receptor agonist described to date is the chiral compound, (**R**)-α-methylhistamine (RAMH) (Arrang *et al.*, 1987); other H_3 receptor agonists include histamine itself and a number of other methylated derivatives of histamine, notably N^{α}-methylhistamine (NAMH) and N^{α},N^{α}-dimethylhistamine (NADMH) (Arrang *et al.*, 1983). Antagonists at H_3 receptors include the highly selective and potent compound, thioperamide (Arrang *et al.*, 1987), the H_2 receptor antagonist burimamide (Arrang *et al.*, 1983), the H_2 receptor agonist impromidine (Arrang *et al.*, 1983), phenylbutanoylhistamine (Lipp *et al.*, 1988) and betahistine (Arrang *et al.*, 1985).

Since their discovery, it has substantially been shown that presynaptic H_3 receptors can regulate release of neurotransmitters other than histamine in both the central and peripheral nervous systems. Thus, in the brain, activation of H_3 receptors has been shown to inhibit release of noradrenaline (Schlicker *et al.*, 1989), and 5-hydroxytryptamine (Schlicker *et al.*, 1988), whilst in the periphery, H_3 receptor activation inhibits release of acetylcholine (Ichinose *et al.*, 1989) and neurotransmitters of the non-adrenergic non-cholinergic (NANC) type (Ichinose & Barnes, 1989).

Trzeciakowski (1987) and Hew *et al.* (1990) demonstrated that activation of H_3 receptors in the guinea-pig isolated ileum inhibited contractions of the smooth muscle mediated by electrically-stimulated release of acetylcholine. Prior to this, Ambache & Zar (1970) described an inhibitory action of histamine on NANC contractions within this preparation but

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only in the presence of H_1 receptor antagonists. Furthermore, they found that the inhibitory action of histamine could be blocked by burimamide at concentrations much lower than that needed for effective H_2 receptor blockade (Ambache *et al.*, 1973) and close to those now known to be required to block the H_3 receptor. Thus, in addition to inhibiting cholinergically-mediated contractions of the guinea-pig isolated ileum, it would appear that activation of H_3 receptors in this preparation also inhibits contractions mediated by NANC neurotransmitter(s). The main aim of this study was to explore this possibility and, by use of the known H_3 receptor agonists and antagonists described earlier, to characterize the H_3 receptor mediating inhibition of NANC contractions of the guinea-pig isolated ileum.

Additional aims of this study were to examine the effects of neurokinin receptor antagonists on the NANC contractions and also to investigate the effect of the alkylating agent N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) on H_3 receptor agonist-mediated inhibitions of the NANC contractions.

Methods

Tissue preparation

Male Dunkin Hartley guinea-pigs (*Porcellus*) weighing 300–350 g were humanely killed by cervical dislocation. A 10 cm section of ileum containing mesentery was immediately removed 10 cm from the ileo-caecal junction and placed in Krebs-Henseleit solution aerated with 95% O_2: 5% CO_2 (Krebs Henseleit solution composition (mM): NaCl 118, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.18, $MgSO_4 \cdot 7H_2O$ 1.18, $CaCl_2$ 2.5 and glucose 11). Longitudinal muscle strips containing the myenteric plexus were prepared from 3 cm portions of the ileum by the method of Rang (1964). The strips of muscle were transferred to 5 ml organ baths and bathed in Krebs-Henseleit solution containing atropine sulphate 1×10^{-7} M, mepyramine maleate 1×10^{-6} M and ranitidine hydrochloride 1×10^{-5} M, aerated with 95% O_2: 5% CO_2 and maintained at 37 ± 1°C. The tissues were placed under a resting tension of 0.5 g and contractions were recorded isometrically. Once mounted in the organ baths, the muscle strips were subjected to an electric field stimulation delivered via electrodes above and beneath the preparation. The tissues were stimulated every minute by a train of 20 pulses of 100 Hz frequency, 0.5 ms pulse duration and supramaximal intensity of 30 V. Maximal consistent contractions were obtained routinely within 1 h, and during this time the Krebs-Henseleit solution in the organ baths was replaced every 10 min.

Drug applications

Addition of phentolamine, propranolol, tetrodotoxin, hexamethonium, neurokinin receptor antagonists and H_3 receptor agonists The effects of phentolamine (3×10^{-6} M), propranolol (3×10^{-6} M), tetrodotoxin (TTX, 0.2×10^{-6} M), hexamethonium (1×10^{-4} M), the neurokinin NK_1 receptor antagonists GR82334 (1×10^{-10}–3×10^{-6} M) and GR71251 (1×10^{-10}–10^{-5} M), the neurokinin NK_2 receptor antagonists MEN 10207 (1×10^{-8}–10^{-5} M) and Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH_2 (R 396) (1×10^{-9}–10^{-5} M), and the H_3 receptor agonists RAMH, NAMH, NADMH (1×10^{-9}–10^{-5} M) and histamine (1×10^{-9}–10^{-4} M) on the electrically-mediated NANC contractions were investigated only when a regular pattern of reproducible contractile responses was observed. All compounds were added directly to the bath in a 50 µl volume and where a range of drug concentrations was tested, the drug was added in a cumulative fashion, such that the total volume added did not exceed 7% of the bath volume. The effect of each drug concentration (except tetrodotoxin) was examined for 3 min before either washing the tissue or applying the next concentration of drug: the effect of tetrodotoxin was examined for 10 min.

Addition of H_3 receptor antagonists and EEDQ The actions of the H_3 receptor antagonists thioperamide (1×10^{-8}–10^{-5} M), burimamide (1×10^{-7}–10^{-5} M), impromidine (3×10^{-7}–3×10^{-6} M), phenylbutanoylhistamine (1×10^{-5}–10^{-4} M) and betahistine (1×10^{-6}–10^{-4} M) were investigated with RAMH used as the agonist. In each case a concentration-effect curve to RAMH was produced, the tissues washed and contractions allowed to recover. To investigate the action of the known H_3 receptor antagonists, the compounds were allowed to equilibrate with the tissue for 30 min before repeating the RAMH concentration-effect curve in the presence of antagonist. The effect of the H_3 receptor antagonists was investigated at a minimum of three different concentrations.

The effects of EEDQ were investigated with RAMH, NAMH, NADMH and histamine as agonists. In experiments where NAMH, NADMH and histamine were used as agonists the concentration of mepyramine in the Krebs-Henseleit solution was increased to 1×10^{-5} M. For each agonist, a concentration-effect curve to the particular agonist was produced, the tissues washed and contractions allowed to recover. The effect of EEDQ was investigated by exposing the tissues to this compound for various times, followed by extensive washing and repetition of the agonist concentration-effect curve.

Chemicals

Atropine sulphate, histamine dihydrochloride, (S)-(−)-propranolol hydrochloride, tetrodotoxin and hexamethonium bromide were purchased from Sigma Chemical Company Ltd (St. Louis, U.S.A.), phentolamine mesylate from CIBA Laboratories Ltd (Sussex), betahistine dihydrochloride and EEDQ from Aldrich Chemical Company Ltd. (U.K.), and N^{α}-methylhistamine from Calbiochem Corporation (U.S.A.). Gifts of N^{α},N^{α}-dimethylhistamine, burimamide and impromidine trihydrochloride (SmithKline Beecham) and mepyramine maleate (Rhone Poulenc) are gratefully acknowledged. Thioperamide, (R)-α-methylhistamine hydrochloride, ranitidine hydrochloride, phenylbutanoylhistamine, GR71251 ([D-Pro^9[Spiro-γ-Lactam]Leu^{10},Trp^{11}]substance P (1-11)), GR82334 ([D-Pro^9[Sprio-γ-Lactam]Leu^{10},Trp^{11}]physalaemin (1-11)), Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH_2 (R 396) and Asp-Tyr-Trp-Val-Trp-Arg-NH_2 (MEN 10207) were synthesized by the Chemistry Research Department, Glaxo Group Research.

Analysis of data

The effects of all the drugs used in this study were determined by analysing chart recorder tracings of tissue contractile responses. The effect of all drugs was assessed by comparing the average height of three control NANC contractions (taken immediately prior to drug application) with the average height of the three NANC contractions of the tissue in the presence of the drug. The effect of the drug was expressed as a % inhibition of the NANC contractions (where 100% represented complete inhibition).

For H_3 receptor agonists and neurokinin receptor antagonists, EC_{50} values and maxima of concentration-effect curves were estimated with the curve-fitting programme 'Allfit' (De Lean *et al.*, 1977). Apparent agonist affinity (pK_D) values were estimated by the null method of Furchgott using a weighted non-linear curve-fitting programme (McPherson *et al.*, 1983).

H_3 receptor antagonist-induced parallel displacement of RAMH concentration-effect curves was quantified as the ratio

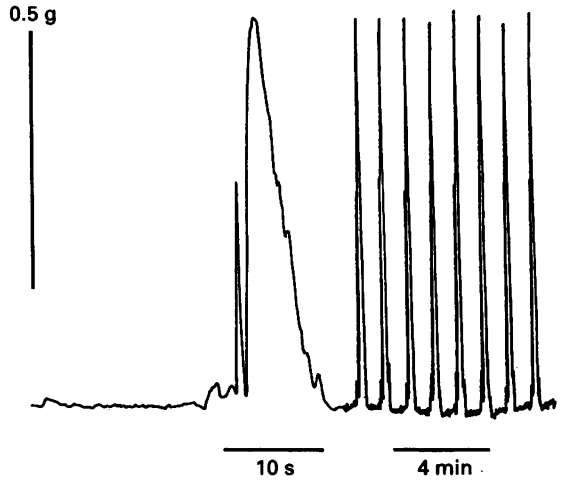

Figure 1 Electrically-mediated non-cholinergic contractions of the guinea-pig isolated ileum longitudinal muscle-myenteric plexus preparation. Once mounted in organ baths, the tissues were subjected to an electric field stimulation delivered via electrodes above and beneath the preparation. Tissues were stimulated every minute by a train of 20 pulses of 100 Hz frequency, 0.5 ms pulse duration and supramaximal intensity of 30 V.

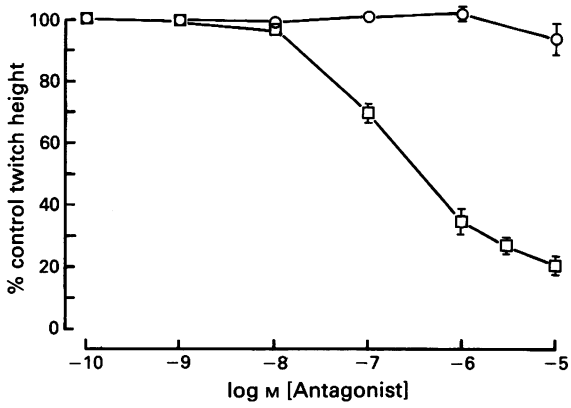

Figure 2 Effect of a neurokinin NK_1 and NK_2 receptor antagonist on NANC contractions of the guinea-pig ileum longitudinal muscle-myenteric plexus preparation. The neurokinin NK_1 receptor antagonist, GR71251 (□), and the neurokinin NK_2 receptor antagonist, MEN 10207 (○) were added to the Krebs-Henseleit solution bathing the tissues in a cumulative fashion such that the total volume added did not exceed 7% of the bath volume. Each point is the mean, with vertical lines indicating the s.e.mean, of determinations in at least four separate preparations, each obtained from a different animal.

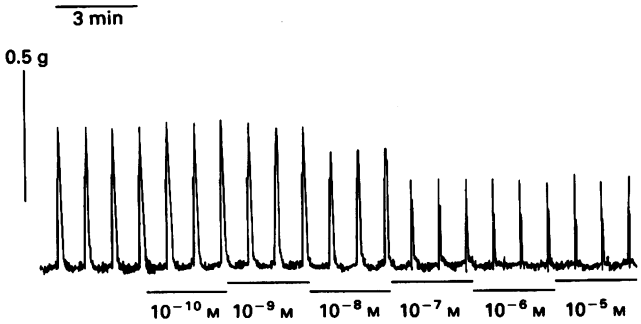

Figure 3 Effect of the H_3 receptor agonist (R)-α-methylhistamine on NANC contractions of the guinea-pig ileum longitudinal muscle-myenteric plexus preparation. Each concentration of (R)-α-methylhistamine was added in a volume of 50 μl directly to the Krebs-Henseleit solution bathing the tissues.

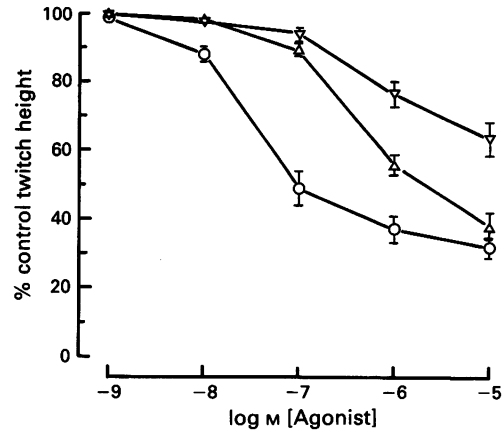

Figure 4 Effect of the H_3 receptor agonists (R)-α-methylhistamine (○), N^{α},N^{α}-dimethylhistamine (△) and histamine (▽) on the NANC contractions of the guinea-pig ileum longitudinal muscle-myenteric plexus preparation. Each point is the mean, with vertical lines indicating the s.e.mean, of determinations in at least five separate preparations each obtained from a different animal.

of equi-active molar concentrations. These values were estimated graphically at the level of the half-maximal response. Antagonist affinity (pA_2 and pK_B) values were estimated with the Schild analysis of these data (Arunlakshana & Schild, 1959). Tissue preparations from at least four different animals (unless indicated otherwise) were used for each drug treatment.

Results

Electrically-mediated contractile responses

In the presence of atropine, the electric field stimulation parameters employed in this study produced contractile responses of the longitudinal muscle preparations which reached maximal tension after 1–1.5 h. Once established, a regular pattern of reproducible contractile responses could be maintained for over 5 h. Individual contractile responses consisted of two discrete contractions: an initial contraction (tension = 0.41 ± 0.04 g; mean ± s.e.mean, $n = 38$) which was rapid in both onset and offset, and a second contraction (tension = 0.58 ± 0.05 g; mean ± s.e.mean, $n = 38$) which lasted for approximately 10 s (Figure 1). The contraction phase of this second contractile component was smooth and progressive whilst the relaxation phase was more complex usually having two or more 'shoulders' (Figure 1).

Effects of phentolamine, propranolol, tetrodotoxin, hexamethonium and neurokinin receptor antagonists

Inclusion of phentolamine (3×10^{-6} M) and propranolol (3×10^{-6} M) in the Krebs-Henseleit solution bathing the tissues resulted, respectively, in a small increase (19 ± 2%) and decrease (8 ± 2%) in the height of the second component of the contractile response with no effect on the initial component. Inclusion of TTX (0.2×10^{-6} M) in the Krebs-Henseleit solution resulted in complete inhibition of the second component of the contractile response within 3 min. The initial component of the contractile response was inhibited maximally by only 20 ± 3%. Inclusion of hexamethonium (1×10^{-4} M) in the Krebs-Henseleit solution had no significant effect on either component of the contractile response.

The effect of inclusion of the neurokinin receptor antagonists in the Krebs-Henseleit solution is shown in Figure 2. Inclusion of either of the neurokinin NK_1 receptor antagonists, GR82334 or GR71251, resulted in a concentration-related inhibition of the second component of the contractile response with no effect on the initial component (Figure 2). Analysis of these data revealed EC_{50} values for GR82334 and GR71251 of 564 ± 75 nM and 173 ± 34 nM respectively, and maximum inhibitory effects of 83 ± 4% and 79 ± 3% respectively. Maximal contractions of the tissues were recovered after two washes with fresh Krebs-Henseleit solution.

Inclusion of the neurokinin NK_2 receptor antagonists, MEN 10207 (1×10^{-8}–10^{-5} M) and R 396 (1×10^{-9}–10^{-5} M) in the Krebs-Henseleit solution had no significant effect on either component of the contractile response (Figure 2).

Effects of H_3 receptor agonists and antagonists

Cumulative additions to the organ bath of increasing concentrations of RAMH resulted in inhibition of the second component of the contractile response with no effect on the initial component (Figures 3 and 4). Similar concentration-related inhibition of the secondary contraction was observed with NAMH, NADMH and histamine, although the apparent maximum inhibitory effect produced by histamine at 1×10^{-5} M was markedly lower than the other H_3 receptor agonists (Figure 4; Table 1): a higher concentration of histamine (1×10^{-4} M) was seen to increase the baseline signal significantly and to cause an increase in the height of the second contraction (results not shown). Agonist potency (pD_2) values and maximal inhibitory effects of the H_3 receptor agonists are shown in Table 1. No change in the maximum inhibitory effect or potency value was apparent on repeated challenge with each agonist (results not shown).

Inclusion of thioperamide (Figure 5a), burimamide (Figure 5b) impromidine and phenylbutanoyl-histamine in the organ baths resulted in concentration-related parallel rightward shifts of the RAMH concentration-response curve, with no effect on the maximal inhibitory effect obtainable. Schild plots constructed with these data showed, in each case, a slope not significantly different from unity. The calculated affinity (pA_2 and pK_B) values for these compounds are shown in Table 2. Inclusion of betahistine in the organ bath had no effect on the RAMH concentration-response curve at any concentration tested (1×10^{-6}–10^{-4} M). A Schild plot could not be constructed from these data and so an affinity (pK_B) value ≤ 4 was estimated for this compound ($n = 12$).

Exposure of the tissues to EEDQ (1×10^{-6} M) for various times (7–30 min), followed by extensive washing, had no effect on either component of the contractile response. However,

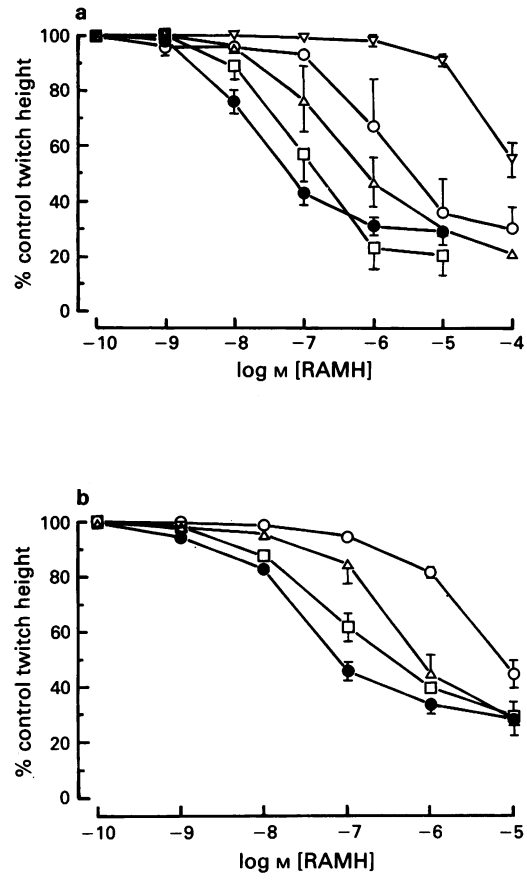

Figure 5 Effect of (a) thioperamide and (b) burimamide on (R)-α-methylhistamine (RAMH)-mediated inhibition of NANC contractions of the guinea-pig ileum longitudinal muscle-myenteric plexus preparation. The 'control' concentration-response curve (●) in each graph was constructed from cumulative additions of RAMH; each preparation was exposed to only one concentration of antagonist. In all graphs each point is the mean, with vertical lines indicating the s.e.mean, of single determinations in at least four separate preparations, each obtained from a different animal. Antagonist concentrations are as follows: thioperamide (□ 10^{-8} M, △ 10^{-7} M, ○ 10^{-6} M, ▽ 10^{-5} M), burimamide (□ 10^{-6} M, △ 10^{-5} M, ○ 3×10^{-5} M).

Table 1 Mean pD_2 values and maximal inhibitions of agonists to inhibit electrically-evoked NANC contractions of the guinea-pig isolated ileum longitudinal muscle-myenteric plexus preparation

Compound	*pD_2*	*Maximum inhibition of NANC contraction obtainable* (%)
NAMH	7.70 ± 0.05	63 ± 1
RAMH	7.64 ± 0.06	72 ± 1
NADMH	6.31 ± 0.05	65 ± 2
Histamine	6.16 ± 0.09	40 ± 3

Each value is the mean (± s.e.mean) of determinations in at least five separate preparations each obtained from a different animal.
Abbreviations: NAMH: N^{α}-methylhistamine; RAMH: (R)-α-methylhistamine; NADMH: N^{α},N^{α}-dimethylhistamine.

Table 2 Apparent affinity values of compounds used to inhibit (R)-α-methylhistamine-mediated inhibition of NANC contractions of the guinea-pig isolated ileum longitudinal muscle-myenteric plexus preparation

Compound	*pA_2*	*Schild slope*	*pK_B*	n
Thioperamide	8.22 ± 0.22	0.95 ± 0.10	8.11 ± 0.11	17
Burimamide	6.98 ± 0.16	0.93 ± 0.08	6.85 ± 0.04	14
Impromidine	6.98 ± 0.38	0.63 ± 0.22	6.60 ± 0.09	12
PBH	5.80 ± 0.21	0.88 ± 0.13	5.64 ± 0.06	12

Each value is the mean (± s.e.mean) of determinations in n separate preparations each obtained from a different animal. At least three different concentrations of each compound were tested. pA_2 values were calculated by use of the equation of Arunlakshana & Schild (1959) and pK_B values were calculated by constraining the slope of this analysis to unity. Abbreviations: PBH; phenylbutanoylhistamine

this treatment had profound actions on the concentration-effect curves to all the agonists tested (Figure 6) (Table 3). For each agonist, increasing the exposure time of the tissues to EEDQ resulted in an increase in the EC_{50} of that agonist and a decrease in the maximum inhibitory effect obtainable. However, exposure of the tissues to EEDQ for 7 min produced an increase in the EC_{50} value of RAMH with no change in the maximum inhibitory effect obtainable. By use of a weighted non-linear curve fitting programme (McPherson *et al.*, 1983) agonist affinity (pK_B) values of 7.01, 7.06, 6.09 and 6.13 were estimated for RAMH, NAMH, NADMH and histamine respectively.

Discussion

The contractile effect of histamine on the guinea-pig isolated ileum preparation has been well characterized (Arunlakshana & Schild, 1959; Ash & Schild, 1966; Leurs *et al.*, 1991) and as such has provided a valuable tool for assaying H_1 receptor activity. However, histamine receptors which mediate inhibition of both cholinergic (Fjalland, 1979) and NANC (Ambache *et al.*, 1973) contractions within this preparation have also been suggested: on the basis of pharmacological data it was proposed that these effects were mediated by a

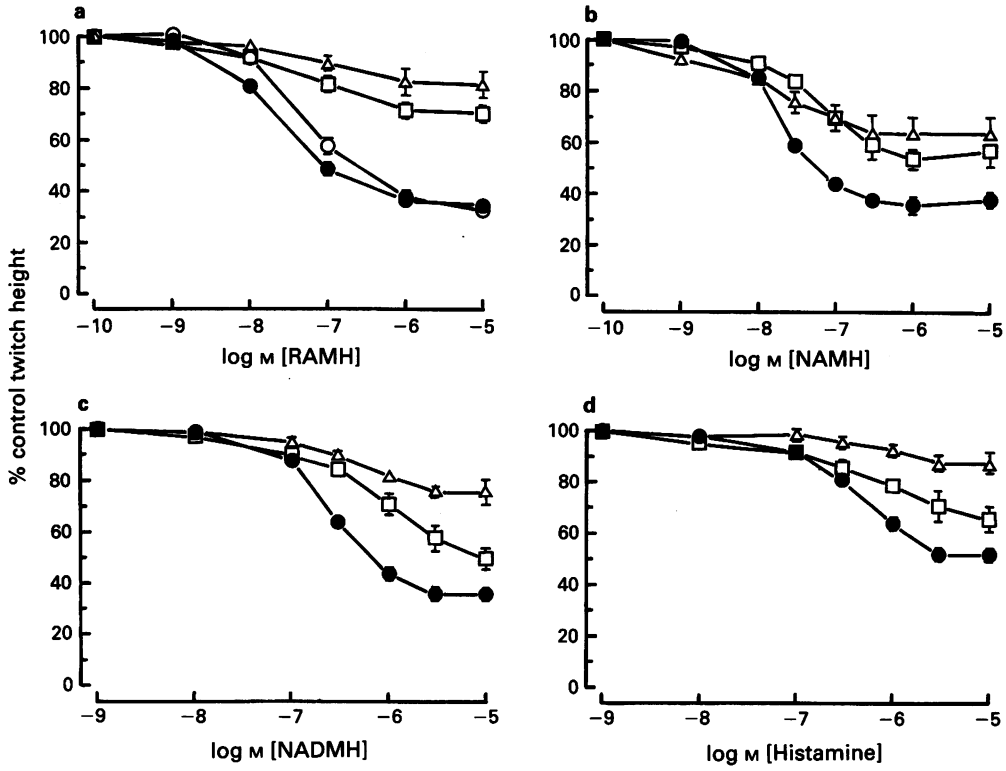

Figure 6 Effect of exposure of tissues to EEDQ (10^{-6} M) on (a) (R)-α-methylhistamine (RAMH) (b) N^{α}-methylhistamine (NAMH), (c) N^{α},N^{α}-dimethylhistamine (NADMH) and histamine-mediated inhibitions (d) of NANC contractions of the guinea-pig ileum longitudinal muscle-myenteric plexus preparation. Each point is the mean, with vertical lines indicating the s.e.mean, of determinations in at least four separate preparations, each obtained from a different animal. EEDQ exposure times are as follows: ● 0 min (control), ○ 7 min, □ 15 min and △ 30 min.

Table 3 Effect of increasing the time of exposure of the tissues to N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ, 1×10^{-6} M) on maximum inhibitory effect and potency of H_3 receptor agonists

Agonist	*Exposure time* (min)	*Maximum inhibition of NANC contraction obtainable* (%)	EC_{50} (nM)	pD_2	K_D (nM)	pK_D (average)	n
RAMH	Control	61.8 ± 1.1	28 ± 3	7.55 ± 0.004	—		18
	7	66.5 ± 0.9	59 ± 4	7.23 ± 0.03	—		6
	15	26.4 ± 1.3	61 ± 15	7.21 ± 0.09	90	7.01	6
	30	16.9 ± 1.9	87 ± 47	7.06 ± 0.19	110		6
NAMH	Control	66.4 ± 1.9	23 ± 3	7.64 ± 0.05	—		10
	15	46.1 ± 1.8	49 ± 9	7.31 ± 0.07	130	7.06	6
	30	37.0 ± 2.1	42 ± 11	7.38 ± 0.10	60		4
NADMH	Control	69.0 ± 1.5	288 ± 26	6.54 ± 0.04	—		13
	15	30.3 ± 1.8	911 ± 205	6.04 ± 0.09	1470	6.09	6
	30	27.0 ± 1.7	400 ± 105	6.40 ± 0.10	460		7
Hist	Control	52.1 ± 2.0	518 ± 75	6.29 ± 0.06	—		12
	15	33.4 ± 2.4	504 ± 146	6.30 ± 0.11	600	6.13	6
	30	13.6 ± 1.8	842 ± 413	6.07 ± 0.17	940		6

Each value is the mean (± s.e.mean) of determinations in *n* separate preparations each obtained in a different animal. Data analysis was carried out by use of a weighted non-linear curve-fitting programme (McPherson *et al.*, 1983). Abbrevations: RAMH; (R)-α-methylhistamine: NAMH; N^{α}-methylhistamine: NADMH; N^{α},N^{α}-dimethylhistamine: Hist; histamine.

subtype of the H_2 receptor. However, doubt was cast on this proposal by the subsequent discovery of the H_3 receptor, and the realization that some H_1 and H_2 receptor ligands are in fact potent antagonists at these receptors. Indeed in 1987, Trzeciakowski provided strong evidence to suggest that the inhibitory action of histamine on cholinergic contractions of the guinea-pig ileum was mediated via H_3 receptors. In this study we have examined NANC contractions within this preparation and have provided evidence to suggest that inhibition of these contractions can also be mediated by activation of H_3 receptors. Indeed, during the preparation of this manuscript, a paper supporting this suggestion was published (Menkveld & Timmerman, 1990).

The atropine-resistant response evoked by electric field stimulation of the guinea-pig isolated ileum preparation in this study comprised an initial fast contraction and a second slow contraction. Neither component of this contractile response was affected to any major degree by the presence of phentolamine and propranolol at concentrations which would have almost completely blocked α- and β-adrenoceptors. These observations suggest that the contractile responses observed could be mediated by direct electrical stimulation of the smooth muscle and/or a non-adrenergic non-cholinergic (NANC) neurotransmitter(s) released by electric field stimulation. Whilst the second component of the contractile response could be completely abolished by TTX, the initial fast component was only inhibited maximally by approximately 20%. These observations suggest that the second contraction is mediated by neuronal release of a NANC neurotransmitter(s) whereas the initial contraction is due, in most part, to direct smooth muscle stimulation. Such a proposal is in good agreement with the recent findings of Menkveld & Timmerman (1990). Furthermore, the lack of effect of hexamethonium on the NANC contraction suggests that release of the NANC neurotransmitter(s) does not occur from preganglionic neurones.

No conclusive evidence has yet been offered as to the nature of the neurotransmitter(s) and receptor(s) involved in the NANC contraction evoked by electric field stimulation of the guinea-pig isolated ileum. However, it has been established that the neurotransmitter is not histamine, 5-hydroxytryptamine or a prostaglandin (Ambache & Freeman, 1968). In this study we have demonstrated that GR71251 and GR82334, two selective neurokinin NK_1 receptor antagonists (Hagan *et al.*, 1989; 1990) inhibited the NANC contractions in a concentration-related manner. In contrast, no inhibition of the NANC contractions was observed using the selective neurokinin NK_2 receptor antagonists MEN 10207 (Maggi *et al.*, 1990) and R 396 (Maggi *et al.*, 1990). The possible function of neurokinin NK_3 receptors in the NANC contraction could not be investigated since no selective neurokinin NK_3 receptor antagonist has yet been reported. Nevertheless, the results suggest that neurokinin NK_1, but not neurokinin NK_2 receptors have an important role in the mediation of the NANC contraction of the guinea-pig isolated ileum. This observation provides some clues albeit indirect as to the nature of the neurotransmitter mediating the NANC contraction: of the neurokinins, substance P is known to be the most potent at neurokinin NK_1 receptors (Ireland *et al.*, 1988) and so it would seem likely that this neurotransmitter is one of the hitherto unidentified NANC neurotransmitters in this tissue preparation.

We have demonstrated that all of the H_3 receptor agonists used in this study could inhibit, in a concentration-dependent fashion, the secondary component of the contractile response (the NANC contraction) with no effect on the initial fast component. The lower maximum inhibitory effect observed for histamine is probably due to H_1 or H_2 receptor-mediated contraction of the longitudinal muscle, caused by histamine overcoming the blocking actions of mepyramine and ranitidine. This idea is supported by the observation that a higher concentration of histamine (1×10^{-4} M) significantly increased the baseline signal and actually caused a decrease in the inhibitory effect seen (results not presented). In the light of the earlier findings of this study, the mechanism of action of the H_3 receptor agonists is presumably to activate H_3 receptors located on nerve terminals containing substance P thereby inhibiting its release. The potency values for the agonists tested in this preparation are consistent with the values obtained for these compounds to inhibit [^{3}H]-histamine release from rat cortical slices (Arrang *et al.*, 1987) and the rank order of potency is identical to that reported by Menkveld & Timmerman (1990).

Conclusive evidence that inhibition of the NANC contractions was mediated via H_3 receptor activation comes from the observations made with known H_3 receptor antagonists. The inhibitory response mediated by RAMH was blocked by thioperamide, burimamide, impromidine and phenylbutanoylhistamine but not, to any significant extent, by betahistine. The antagonistic actions of thioperamide, burimamide, impromidine and phenylbutanoylhistamine appeared to be competitive because Schild analysis of the effects of these compounds revealed slopes not significantly different from unity (although the apparent slope for impromidine was low). The affinity values obtained for thioperamide, burimamide and impromidine in other assays of the H_3 receptor (Arrang *et al.*, 1987; Kilpatrick & Michel, 1991) are in good agreement with the affinity values obtained for these compounds in this preparation. However, the affinity value of phenylbutanoylhistamine for H_3 receptors in this preparation ($pA_2 = 5.8$) was more than ten fold lower than the value obtained for this compound in a receptor binding ($pK_i = 6.8$; Kilpatrick & Michel, 1991) and functional ($pK_i = 7.1$; see Timmerman, 1990) assay of the H_3 receptor. Such a discrepancy was even more marked for betahistine.

The reasons for the discrepancies in antagonist affinity are currently under investigation in our laboratory. However, a simple species-difference in H_3 receptors can be discounted in this case because both phenylbutanoylhistamine and betahistine have been shown to be equipotent at inhibiting [^{3}H]-RAMH binding to H_3 receptors in homogenates of guinea-pig and rat cortex (Kilpatrick & Michel, 1991). Possible explanations might therefore include a reduction in the effective concentration of the compounds at H_3 receptors, caused by poor accessibility and/or breakdown of the compounds within the organ bath, or, sub-types of the H_3 receptor.

EEDQ has previously been reported to act as an irreversible antagonist at various mono-amine receptors in peripheral tissues and brain. EEDQ irreversibly blocks binding to α-adrenoceptors in peripheral tissues (Belleau *et al.*, 1968) and blocks binding to D_1 and D_2 dopamine receptors (Hamblin & Creese, 1983) and 5-HT_2 receptors (Battaglia *et al.*, 1986) in brain after peripheral administration. EEDQ has also been reported to act as an irreversible antagonist at muscarinic receptors in peripheral tissues *in vitro* (Chang *et al.*, 1970). The mechanism of action of EEDQ is thought to involve the activation of peptide carboxyl groups so creating a highly reactive mixed carbonic anhydride (Belleau *et al.*, 1969). In a receptor protein this reactive group may then interact with suitable nucleophilic groups, such as adjacent α-amino groups, thus forming an irreversible covalent crosslinking of peptide chains within the receptor (Belleau *et al.*, 1969).

In this study we have demonstrated that exposure of the tissues to EEDQ resulted in a rightward shift in the agonist concentration-effect curve, with a reduction in the maximal inhibitory effect obtainable. This action of EEDQ increased as the exposure time of the tissues to EEDQ was increased. For each of the agonists tested, the apparent dissociation constants (K_D) estimated at the different exposure times were found to be consistent. The effects of EEDQ (i.e. increased EC_{50}, reduced maximal effect obtainable and an identical dissociation constant) are characteristic of a compound that acts as an irreversible antagonist. As such, these results strongly indicate that EEDQ acts as an irreversible antagonist at H_3 receptors within this preparation. It is noteworthy that exposure of the tissues to EEDQ for up to 45 min had no effect on

basal NANC contractions. This observation would perhaps indicate that EEDQ does not act as an irreversible antagonist at neurokinin NK_1 receptors, although it is possible that if the concentration of EEDQ and/or exposure time had been increased, a reduction in the height of the NANC contractions would then have been apparent.

The relative affinity values of the H_3 receptor agonists is very similar to the relative potency of these compounds in this preparation. As the potency of an agonist is the product of its affinity and efficacy, this observation reveals that the H_3 receptor agonists used in this study have approximately the same efficacy at H_3 receptors in this preparation. However, one should be cautious in interpreting the results using EEDQ as it is clear that inaccuracies can occur when estimating agonist affinity values (see Leff, 1988; Leff *et al.*, 1990).

The apparent affinity (pK_D) values calculated for RAMH, NAMH, NADMH and histamine at H_3 receptors in this preparation, are significantly lower than the affinity (pK_i) values obtained for these compounds to inhibit [^{3}H]-RAMH binding to homogenates of rat cortex (Arrang *et al.*, 1987; West *et al.*, 1990; Kilpatrick & Michel, 1991). However, such a difference is not unexpected when making a comparison of agonist affinities between a functional and a receptor binding assay because many receptors are thought to exist in two agonist affinity states: a functionally important low affinity state and a high affinity state. A radioactive agonist ligand is thought to label selectively the high affinity state of the receptor and so higher agonist affinity values would be expected in a receptor binding assay than a functional assay.

In summary, this study demonstrates that activation of H_3 receptors within the guinea-pig ileum, can inhibit NANC contractions elicited by electric field stimulation: the NANC contraction is probably mediated by substance P acting at neurokinin NK_1 receptors. In addition, we have identified an irreversible antagonist at H_3 receptors within this preparation and as such this compound should become a useful tool for both *in vivo* and *in vitro* study of H_3 receptors. Finally, we feel that this preparation provides a rapid and simple functional assay for investigating the H_3 receptor, and for determining agonist and antagonist activity at this receptor.

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Inhibitory effect of morphine on yawning induced by cholinoceptor and dopamine D_2 receptor activation in rats

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1 Bromocriptine (2, 4 and 8 mg kg^{-1}, i.p.), physostigmine (0.05, 0.1 and 0.2 mg kg^{-1}, i.p.) and pilocarpine (1, 3 and 5 mg kg^{-1}, i.p.) induced dose-dependent yawning in rats.

2 These responses were reduced in a dose-dependent manner by pretreatment with morphine.

3 The inhibitory effect of morphine was reversed by naloxone.

4 Naloxone alone induced slight but significant yawning.

5 The present results suggest that morphine inhibits yawning in rats at an opiate receptor downstream from the sites at which cholinoceptor and dopamine D_2 activation induce yawning. The anatomical location of these sites remains to be established.

Keywords: Morphine; pilocarpine; physostigmine; bromocriptine; yawning; dopamine receptors; cholinoceptors

Introduction

Yawning is a physiological response which is associated with fatigue and recovery from stress (Barbizet, 1958; Stoessl *et al.*, 1987). A number of dopamine agonists including apomorphine and bromocriptine elicited yawning behaviour in rats (Mogilnicka & Klimek, 1977; Baggio & Ferrari, 1983; Zarrindast & Poursoltan, 1989). It has been proposed that this behaviour is mediated via a D_2 dopamine autoreceptor (Yamada & Furukawa, 1980; Protais *et al.*, 1983; Baggio & Ferrari, 1983; Zarrindast & Poursoltan, 1989). Central cholinoceptor activation by physostigmine or pilocarpine also induces yawning (Urba-Holmgren *et al.*, 1977; Zarrindast & Poursoltan, 1989). The yawning induced by dopamine agonists and agents acting on cholinoceptors are inhibited by muscarinic antagonists, such as scopolamine and atropine (Mogilnicka *et al.*, 1984; Zarrindast & Poursoltan, 1989). These studies suggest that dopamine receptor agonists induce yawning by activation of the cholinergic system (Carlsson, 1975; Di-Chiara *et al.*, 1976). Links between acetylcholine and dopaminergic neurones have been described in the septo-hippocampal system and the striatum (Guyenet *et al.*, 1974; Agid *et al.*, 1975; Storm-Mathisen, 1977) and it has been shown that stimulation and lesion of these structures affect yawning (Jakobartl & Huston, 1977; Dourish & Huston, 1985; Yamada *et al.*, 1986; Stoessl *et al.*, 1987; Laping & Ramirez, 1988). It has also been suggested that dopamine agonists elicit yawning by stimulating dopamine D_2 receptors within these structures (Yamada *et al.*, 1986).

Opiate interactions with both dopaminergic and cholinergic systems have been demonstrated. There appears to be a close association between opiate receptors and dopaminergic cell bodies and nerve endings in the substantia nigra (Llorens-Cortes *et al.*, 1979) and striatum (Pollard *et al.*, 1977). Morphine, methadone and other opiates modulate dopaminergic neurotransmission in mesolimbic (Wood, 1983; Iyenger *et al.*, 1987), mesocortical (Kim *et al.*, 1986) and nigrostriatal (Ahtee & Kaariainen, 1973; Gauchy *et al.*, 1973; Costa *et al.*, 1975; Wood *et al.*, 1980) projections. Opiate agonists also inhibit the release of acetylcholine in the central nervous system, an effect which is antagonized by naloxone (Jhamandas *et al.*, 1975).

In the present study, we have evaluated the effect of morphine on yawning induced by dopaminergic and cholinergic activation.

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Methods

Male albino rats weighing 200–250 g, bred in our animal house, were used. The animals were housed in groups of five per cage at a controlled temperature with a 12 h light/dark cycle. Standard laboratory food and tap water were freely available except during the time of the experiments. Prior to the experiments the animals were weighed and placed in individual cages to habituate for at least 30 min.

Statistical analysis

Yawning responses were expressed as the mean value. ANOVA followed by Student's *t* test were used for analysis of the data. Differences between means were considered statistically significant if $P < 0.05$.

Drugs

The drugs used were bromocriptine mesylate (Sandoz, Switzerland), morphine hydrochloride (MacFarlan, Smith Ltd, England), naloxone hydrochloride (Dupont, Germany), physostigmine salicylate (Sigma, England) and pilocarpine nitrate (Merck, Germany).

All drugs were dissolved in water except for bromocriptine which was dissolved in saline with crystalline tartaric acid and a few drops of alcohol. The drugs were prepared immediately before use and were injected in a volume of 1 ml kg^{-1}.

Results

The D_2 receptor agonist bromocriptine (2, 4 and 8 mg kg^{-1}, i.p.) induced yawning in rats (Figure 1). Morphine (1, 2, and 3 mg kg^{-1}, s.c., 5 min) pretreatment decreased the yawning induced by bromocriptine (8 mg kg^{-1}) in a dose-dependent manner (Figure 2). The inhibitory effect of morphine was decreased by naloxone (1.5 mg kg^{-1}, s.c., Table 1). Physostigmine (0.05, 0.1 and 0.2 mg kg^{-1}, i.p.) induced yawning in rats (Figure 3). Morphine (0.5, 1 and 2 mg kg^{-1}, s.c.) administered 5 min before physostigmine (0.2 mg kg^{-1}, i.p.) dose-dependently inhibited physostigmine-induced yawning (Figure 4). The inhibitory effect of morphine on physostigmine-induced yawning was decreased in naloxone (1.5 mg kg^{-1}) pretreatment (Table 2). Pilocarpine (1, 3 and 5 mg kg^{-1}, i.p.) also induced yawning behaviour with a maximum response at 3 mg kg^{-1} (Figure 5). Morphine (0.5, 1 and 2 mg kg^{-1}, s.c.) dose-dependently decreased the pilocarpine-induced yawning

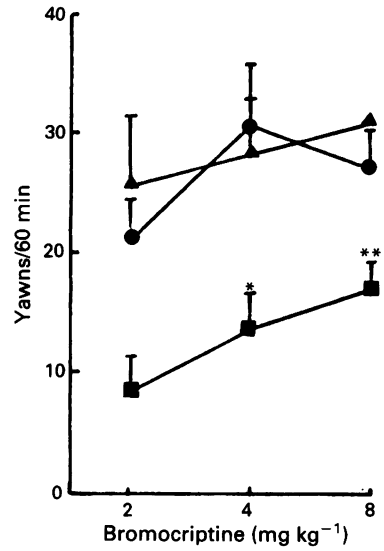

Figure 1 Yawning induced by bromocriptine in the presence or absence of morphine. Rats were injected with bromocriptine (●) and either saline (▲, 1 ml kg^{-1}, s.c.) or morphine (■, 2 mg kg^{-1}, s.c.) 5 min before bromocriptine. The number of yawns was recorded for 60 min. Each point is the mean for a minimum of eight animals; vertical bars show s.e.mean.

Significant differences between the morphine pretreated group and the bromocriptine and bromocriptine plus saline groups are denoted by * $P < 0.05$; ** $P < 0.01$.

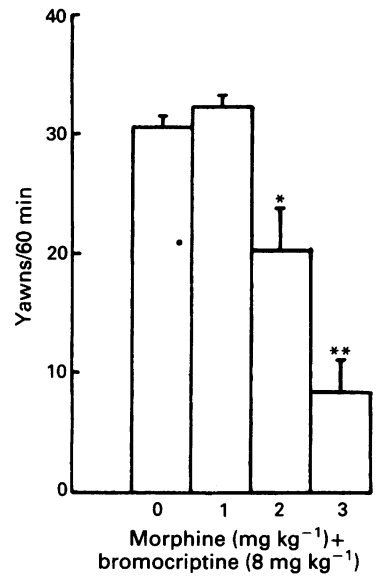

Figure 2 The effect of morphine (1–3 mg kg^{-1}, s.c.) on yawning induced by bromocriptine (8 mg kg^{-1}, i.p.). Results are shown for total yawns induced in 60 min after bromocriptine injection. Each column is the mean for a minimum of eight animals; vertical bars show s.e.mean. Significant differences from the bromocriptine-treated group are indicated by * $P < 0.01$; ** $P < 0.001$.

Table 1 Frequency of yawns induced by bromocriptine (8 mg kg^{-1}) in rats in the presence or absence of morphine or naloxone

Drugs	(mg kg^{-1})	*Yawns/60 min* (mean ± s.e.)	n	P < *vs saline*
Saline	1 ml	30.6 ± 0.6	10	—
Morphine	2	17.0 ± 2.6	10	0.01
Naloxone + morphine	1.5 2	26.0 ± 1.2 24.5 ± 1.6*	7 9	NS NS

Saline, naloxone or morphine was administered s.c. 5, 10 or 5 min respectively before bromocriptine.
* $P < 0.02$: different from morphine-treated control group.
NS = not significant.

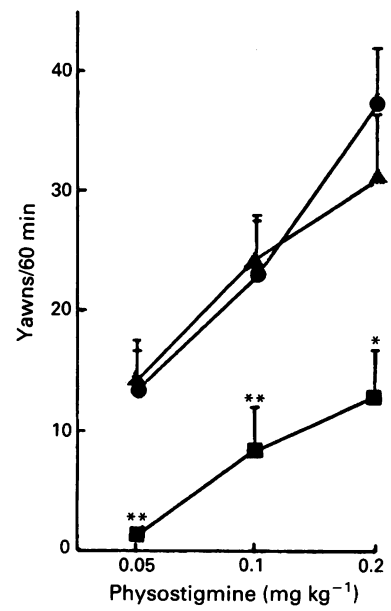

Figure 3 Yawning induced by physostigmine in the presence or absence of morphine. Rats were injected intraperitoneally with physostigmine (●) and either saline (▲, 1 ml kg^{-1}, s.c.) or morphine (■, 1 mg kg^{-1}, s.c.) 5 min before physostigmine injection. The number of yawns was recorded for 60 min. Each point is the mean for a minimum of 7 animals; vertical bars show s.e.mean. Significant differences between the morphine pretreated group and the bromocriptine and bromocriptine plus saline groups are denoted by * $P < 0.05$; ** $P < 0.02$.

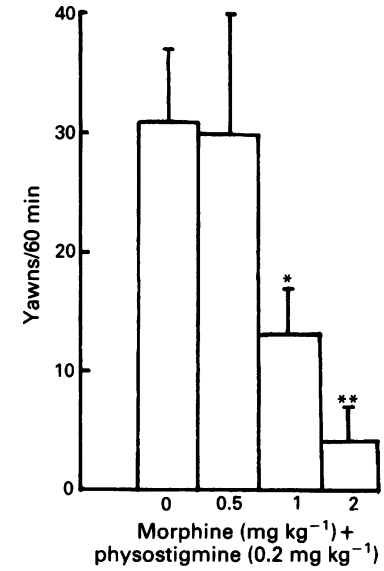

Figure 4 The effect of morphine (0.5–2 mg kg^{-1}) on yawning induced by physostigmine (0.2 mg kg^{-1}, i.p.). Results are shown for total yawns induced in 60 min after physostigmine injection. Each column is the mean for a minimum of 7 animals; vertical bars show s.e.mean. Signficant differences from the physostigmine group are indicated by * $P < 0.05$; ** $P < 0.01$.

(Figure 6). The inhibitory effect of morphine was decreased by naloxone (1.5 mg kg^{-1}, s.c.) pretreatment (Table 3).

Naloxone did not alter the effect of bromocriptine (Table 1), physostigmine (Table 2) or pilocarpine (Table 3).

Naloxone (0.75, 1.5 and 3 mg kg^{-1}, s.c.) alone induced a dose-dependent yawning compared with saline-treated animals (Figure 7). Morphine (0.5, 1 and 2 mg kg^{-1}, s.c.) (not shown) or saline (1 ml kg^{-1}, s.c.) did not induce yawning (Figure 7).

Table 2 Frequency of yawns induced by physostigmine (0.2 mg kg^{-1}) in rats in the presence or absence of morphine or naloxone

Drugs	(mg kg^{-1})	*Yawns/60 min* (mean ± s.e.)	n	P < *vs saline*
Saline	1 ml	30.8 ± 6.2	7	—
Morphine	2	4.3 ± 2.6	7	0.01
Naloxone	1.5	24.0 ± 8.6	7	NS
Naloxone + morphine	2	19.4 ± 3.2*	14	NS

Saline, naloxone or morphine was administered s.c. 5, 10 or 5 min respectively before physostigmine.
* $P < 0.001$: different from morphine-treated control animals.
NS = not significant.

Table 3 Frequency of yawns induced by pilocarpine (5 mg kg^{-1}) in rats in the presence or absence of morphine or naloxone

Drugs	(mg kg^{-1})	*Yawns/60 min* (mean ± s.e)	n	P < *vs saline*
Saline	1 ml	16.5 ± 2.5	7	—
Morphine	2	1.7 ± 0.4	7	0.001
Naloxone	1.5	11.0 ± 3.0	7	NS
Naloxone + morphine	1.5 2	12.0 ± 2.8*	7	NS

Saline, naloxone or morphine was administered s.c. 5, 10 or 5 min respectively before pilocarpine.
* $P < 0.001$: different from morphine-treated control group.
NS = not significant.

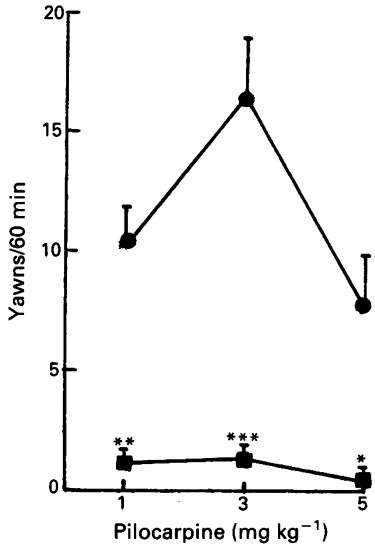

Figure 5 Yawning induced by pilocarpine in the presence or absence of morphine. Rats were injected intraperitoneally with pilocarpine (●) or with morphine (■), (2 mg kg^{-1}, s.c.) 5 min before pilocarpine injection. Each point represents the mean of yawns/60 min ($n = 7$); vertical bars show s.e.mean. Significant differences between the morphine pretreated and pilocarpine alone are indicated by * $P < 0.05$; ** $P < 0.02$; *** $P < 0.001$.

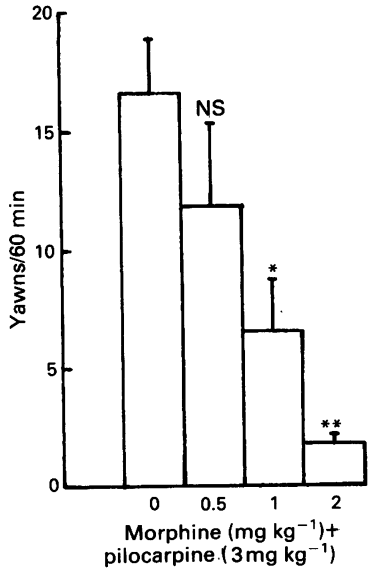

Figure 6 The effect of morphine (0.5–2 mg kg^{-1}) on yawning induced by pilocarpine (3 mg kg^{-1}, i.p.). Results are shown for total yawns induced during 60 min after pilocarpine injection. Each column is the mean for minimum of 7 animals; vertical bars show s.e.mean. Significant differences from pilocarpine-treated group are indicated by * $P < 0.1$; ** $P < 0.001$.

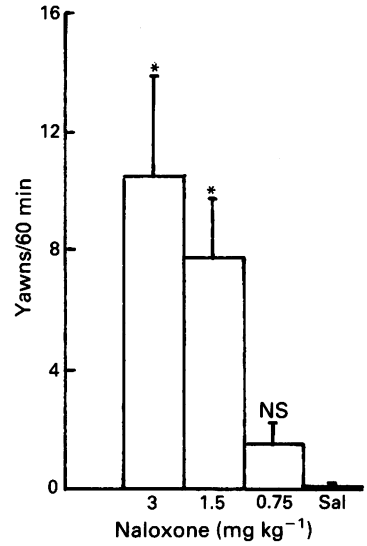

Figure 7 Yawning induced by naloxone (0.75–3 mg kg^{-1}, s.c.). The number of yawns was counted immediately after drug administration for 60 min. Each column is the mean for minimum of 7 animals; vertical bars show s.e.mean. Significant differences from saline control group are indicated by * $P < 0.001$.

Discussion

It has been proposed that yawning induced by low doses of dopamine receptor agonists is due to activation of D_2 receptors (Yamada *et al.*, 1986; Zarrindast & Poursoltan, 1989), although D_1 receptors may be involved in yawning induced by apomorphine (Morelli *et al.*, 1986). The yawning induced by dopamine agonists appears to be mediated via a cholinergic mechanism, since it is inhibited by muscarinic receptor blockade (Mogilnicka *et al.*, 1984; Yamada *et al.*, 1986; Zarrindast & Poursoltan, 1989).

Although dopaminergic and cholinergic interactions have been described at various anatomical sites within the brain (see Introduction), the locus of the interaction with respect to yawning behaviour has not been established, although it has been demonstrated that intact nigrostriatal projections are required for drugs acting on dopamine receptors to induce yawning (Stoessl *et al.*, 1987). In the present study bromocryptine-induced yawning was inhibited in a naloxone-sensitive manner by morphine. Although the nigro-striatal pathway has been shown to be regulated by μ- and δ-opioid receptors in both the striatum and the substantia nigra (Biggio *et al.*, 1978; Wood *et al.*, 1980; Yonehara & Clouet, 1984; Wood & Richard, 1982), such a mechanism is unlikely to explain the present results since morphine in a similar dose-range also inhibited cholinoceptor-mediated yawning.

Although morphine is able to inhibit the release of acetyl-

choline in the central nervous system in a naloxone-sensitive manner (Jhamandas *et al.*, 1975) such a mechanism is unlikely to explain the present results since morphine was able to inhibit the yawning induced by the direct acting cholinoceptor agonist, as well as that induced by physostigmine.

We have previously demonstrated that yawning induced by bromocriptine and physostigmine are decreased by theophylline (Zarrindast & Poursoltan, 1989). Theophylline is an adenosine receptor antagonist (Stiles, 1986) and a phosphodiesterase inhibitor which increases intracellular adenosine 3',5'-cyclic monophosphate (cyclic AMP) concentrations (Butcher & Sutherland, 1962), and may also release dopamine (Govoni *et al.*, 1984). However, a similar mechanism is unlikely to explain the present effects since morphine has been reported to stimulate adenosine release (Sawynok *et al.*, 1989).

The most likely explanation of the present findings is that morphine inhibits yawning induced by dopamine agonists and drugs acting at cholinoceptors at an opioid site downstream from both the dopamine receptors and cholinoceptors. The weak but dose-dependent induction of yawning by naloxone suggests that endogenous opioids may mediate a tonic inhibitory effect on yawning behaviour.

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Sustained decreases in weight and serum insulin, glucose, triacylglycerol and cholesterol in JCR:LA-corpulent rats treated with D-fenfluramine

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1 The effects of D-fenfluramine were studied in the JCR:LA-corpulent rat that is grossly obese, hyperphagic, hyperlipidaemic, hyperinsulinaemic and atherosclerosis-prone.

2 Daily doses of 1, 2.5 and 5 mg kg^{-1} of D-fenfluramine produced sustained decreases in body weight and food intake over a period of 30 days in 6 month old female rats fed *ad libitum*. This was accompanied by decreases in the circulating concentrations of glucose, triacylglycerol, free cholesterol and insulin.

3 Food restriction imposed by meal feeding also decreased circulating glucose, triacylglycerols, cholesterol and insulin and diminished the effect of D-fenfluramine on these parameters in male and female rats.

4 Addition of D-fenfluramine to drinking water to give a dose of about 0.25 mg kg^{-1} daily produced a sustained decrease in body weight and food intake of male and female rats over a nine week period.

5 The results show that the JCR:LA-corpulent rat is very sensitive to the pharmacological effects of D-fenfluramine. These rats should provide an appropriate animal model for determining the mechanisms of action of this anti-obesity agent and whether apparently beneficial changes in metabolism translate into long-term protection against premature atherosclerosis.

Keywords: Adipose tissue; anorectics; atherosclerosis; diabetes; glucocorticoids; hypercholesterolaemia; hypertriglyceridaemia; insulin; lipogenesis; obesity

Introduction

The JCR:LA-corpulent rat, if homozygous for the cp gene (cp/cp), is grossly obese, hyperphagic, insulin-insensitive and hyperlipidaemic (Russell *et al.*, 1987; Dolphin *et al.*, 1987; 1990). The hyperlipidaemia is mainly caused by high concentrations of very low density lipoprotein (VLDL) caused by the oversecretion of this lipoprotein which is more pronounced in the females (Russell *et al.*, 1989a; Vance & Russell, 1990). The corpulent male rats spontaneously develop both atherosclerotic and myocardial lesions. By contrast the female cp/cp rats do not develop cardiovascular lesions even though they are more hypertriglycerdemic (Russell & Amy, 1986a,b; Amy *et al.*, 1988; Russell *et al.*, 1990a; Dolphin *et al.*, 1990). These characteristics make the JCR:LA-cp rat an ideal experimental model to investigate the relationship between sex, obesity, diabetes, hyperlipidaemias and the progression to cardiovascular disease.

In the present work, the corpulent rats were treated chronically with D-fenfluramine which is used clinically to manage obesity. This compound has also been reported to improve insulin sensitivity and to have hypoglycaemic and hypolipidaemic effects (Dannenberg, 1983; Brindley, 1983; Arnaud & Nathan, 1990). The main purpose of the work was therefore to determine whether D-fenfluramine would correct the metabolic derangements of the JCR:LA-corpulent rat. These animals were treated in three different ways and also with different concentrations of D-fenfluramine to establish dose-response relationships. This was determined in terms of body weight changes, adipose tissue mass, food intake, the concentrations of circulating hormones and metabolites, and the capacity of the adipose tissue to synthesize lipids. The results demonstrate that relatively low concentrations of D-fenfluramine were effective in partially reversing the metabolic abnormalities present in the JCR:LA-corpulent rat. A preliminary abstract of some of these results has been presented (Brindley *et al.*, 1989).

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Methods

Male and female JCR:LA rats that were cp/cp were raised in the established colony at the University of Alberta as previously described (Russell & Amy, 1986a,b). Rats were killed by guillotine and blood was collected from the necks and the serum obtained after centrifugation. The entire fat depots from the subcutaneous, inguinal, mesenteric and perirenal regions were dissected and weighed.

Series 1

In the first series of experiments, 5–6 month old rats were transferred to a room that was lit from 08 h 00 min to 20 h 00 min and which was maintained at 22°C. Rats were housed in groups of two or three in grid-bottomed cages. They were fed *ad libitum*, on Wayne Lab Blox (Continental Grain Co., Chicago, IL, U.S.A.) which had an energy content of 1240 kJ g^{-1}. The D-fenfluramine was injected i.p. in 0.16 M NaCl at 1, 2.5 or 5 mg kg^{-1} between 08 h 00 min and 10 h 00 min each day. Control rats were injected with the equivalent volume of 0.16 M NaCl. The weights of the rats and the food intake were recorded daily.

Series 2

In the second series of experiments, which were concerned with pair-feeding, 6 month old rats were put on a reversed light cycle so that the room was lit from 22 h 00 min to 10 h 00 min. There were two rats per grid-bottomed cage. They were then allowed 5 days to become accustomed to this change before restricting the period of feeding to 6 h from 10 h

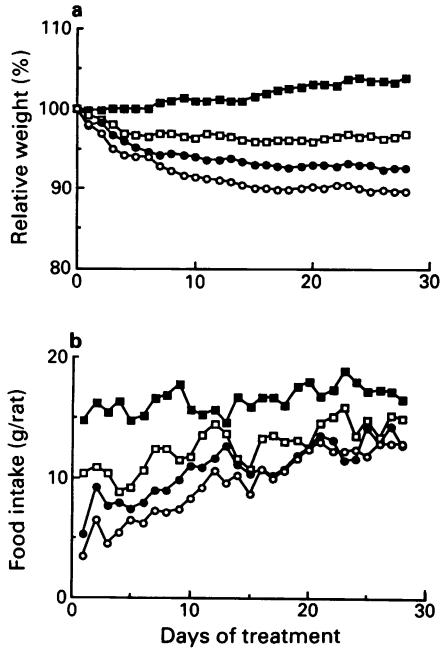

Figure 1 Effect of different doses of D-fenfluramine on the body weights and food intakes of female JCR:LA-corpulent rats. Female corpulent rats were fed *ad libitum* and injected daily for 30 days with 0.16 M NaCl, alone (controls, ■), or with 0.16 M NaCl containing a dose of 1 mg kg^{-1} (□), 2.5 mg kg^{-1} (●), or 5 mg kg^{-1} (○) of D-fenfluramine (Series 1). The initial body weights of these rats were 464 ± 17 g (9), 462 ± 30 g (9), 456 ± 31 g (8), and 474 ± 18 g (9) respectively. The mean weight change relative to the weight of each rat at the beginning of the treatment is given in (a). The mean daily food consumption for three cages of rats is given in (b). The s.d. values have been omitted from the figure for the sake of clarity.

00 min to 16 h 00 min. After a further 18 days the rats were divided into three groups: control, D-fenfluramine-treated and a group pair fed to the treated group. The treated rats were injected i.p. with D-fenfluramine 2.5 mg kg^{-1}, whereas other rats were injected with the equivalent volume of 0.16 M NaCl. The pair-fed animals were fed the equivalent weight of food consumed the day before by the paired rat which most closely matched their weight on day 18. An additional quantity of food equivalent to that wasted by the pair-fed rats on the previous day was added to the food allowance. The treatment of six rats from the D-fenfluramine group was discontinued at day 33 and these rats were then allowed free access to food during the 6 h period. All rats were killed on day 47, 2 h after the last injection. Access to food was not allowed during this 2 h period. The reversal of the light cycle facilitated the injection of the D-fenfluramine just before the feeding period.

Series 3

In the third series of experiments male and female corpulent rats were given D-fenfluramine in drinking water at 6 weeks of age. The concentration was adjusted so that the rats consumed 0.25 mg kg^{-1} body weight and this had no effect on water consumption. Treatment continued up to 15 weeks of age; the rats were caged individually on wood chip bedding in polycarbonate cages. Control rats were provided with tap water and food was available *ad libitum*.

Analytical methods

Most of the methods and the suppliers of reagents have been described previously (Brindley *et al.*, 1988; Al-Sieni *et al.*, 1989). The rates of lipid synthesis were measured in samples of subcutaneous, or inguinal adipose tissue by determining the incorporation of [^{14}C]-glucose into the fatty acid and glycerol moieties of triacylglycerol (Al Sieni *et al.*, 1989). Phospholipids, triacylglycerol and fatty acids in blood serum were determined enzymically with kits purchased from Wako Chemicals Inc. Richmond, VA, U.S.A. The assays followed the manufacturer's instructions, but they were miniaturized and absorbancies were measured with a microtitration plate reader. Triacylglycerol concentrations were corrected by subtracting the concentration of free glycerol from the total glycerol concentration determined with the Wako kit. Glycerol concentrations were determined on 10 μl of serum in a reaction mixture which had a final volume of 250 μl. This contained: triethanolamine 200 mM buffered to pH 7.5 with HCl, diaphorase 0.18 units ml^{-1}; glycerol 3-phosphate dehydrogenase 1.69 units ml^{-1}; 1-(4,5-dimethylthiazole-2-yl)-3,5-diphenyltetrazolium bromide 0.4 mg ml^{-1}, ATP 1.23 mM, NAD 1.6 mM, $MgCl_2$ 12.4 mM and Triton X-100 2 μl ml^{-1}. The reaction was started with glycerol kinase 0.63 units ml^{-1}. The NADH-dependent formation of formazan was determined at the completion of the coupled reaction with a microtitration plate reader at 600 nm. Results were calculated by use of a standard curve with glycerol concentrations from 0.1–1.0 mM prepared in dialysed rat serum.

Statistical evaluation

Results are expressed as means ± s.d. Statistical differences between the groups were established by ANOVA and differences between groups were determined with an unpaired *t* test, $P < 0.05$ in a two-tailed test being considered significant.

Table 1 Effects of D-fenfluramine on adipose tissue mass

		Dose of D-fenfluramine		
Adipose site	*Control* (9)	1 mg kg^{-1} (9)	2.5 mg kg^{-1} (8)	5 mg kg^{-1} (9)
Subcutaneous (g)	80.3 ± 11.6	67.8 ± 12.1 $P < 0.025$	58.6 ± 7.1 $P < 0.0005$	60.6 ± 8.4 $P < 0.005$
Inguinal (g)	14.3 ± 1.3	13.5 ± 2.1	13.9 ± 1.9	12.2 ± 1.3 $P < 0.005$
Mesenteric (g)	13.3 ± 1.5	11.4 ± 1.6 $P < 0.025$	11.4 ± 1.6 $P < 0.025$	10.4 ± 2.1 $P < 0.005$
Peri-renal (g)	25.5 ± 3.9	20.4 ± 3.9 $P < 0.01$	20.4 ± 2.3 $P < 0.005$	19.8 ± 3.5 $P < 0.005$

JCR:LA-corpulent female rats (Series 1) were fed *ad libitum* and treated daily for 30 days with the dose of D-fenfluramine indicated in Figure 1. Adipose tissue from the various sites was then obtained by dissection. The weights are expressed as means ± s.d. for the number of rats indicated in parentheses. The *P* values indicate the significance of the difference between the treated group and the untreated control rats.

Table 2 Effects of D-fenfluramine treatment of JCR:LA-corpulent female rats on the rate of fatty acid synthesis from glucose by subcutaneous adipose tissue

	Glucose (mmol incorporated per depot) *Dose of D-fenfluramine*			
Insulin ($ng\,ml^{-1}$)	*Control* $n = 9$	$1\,mg\,kg^{-1}$ $n = 9$	$2.5\,mg\,kg^{-1}$ $n = 8$	$5\,mg\,kg^{-1}$ $n = 9$
0	3.62 ± 1.55	2.28 ± 0.59 $P < 0.025$	2.03 ± 0.83 $P < 0.025$	2.42 ± 1.35
0.1	3.63 ± 1.41	2.04 ± 0.49 $P < 0.005$	1.91 ± 0.74 $P < 0.01$	2.62 ± 1.40
1	3.80 ± 1.30	2.65 ± 0.60 $P < 0.025$	2.18 ± 0.78 $P < 0.025$	2.64 ± 1.67
10	5.01 ± 0.94	3.27 ± 0.92 $P < 0.005$	2.52 ± 0.56 $P < 0.005$	3.07 ± 1.52 $P < 0.01$
100	6.88 ± 2.77	4.00 ± 1.09 $P < 0.01$	2.96 ± 0.70 $P < 0.005$	3.64 ± 2.37 $P < 0.025$

Subcutaneous adipose tissue was taken from the rats described in Table 1 and the rate of incorporation of [U-^{14}C]-glucose into fatty acids was measured over a 2 h period in the presence of various concentrations of insulin. Results are means ± s.d. for the number of rats indicated. The *P* values indicate the significance of the difference between tissue from untreated (control) rats and the D-fenfluramine-treated rats at the equivalent concentration of insulin.

Results

Effects of D-fenfluramine treatment on rats fed ad libitum

Treatment of the 6 month old cp/cp female rats with D-fenfluramine (Series 1) produced a sustained and dose-dependent loss in relative body weight over a 28 day period compared to the control rats (injected with NaCl above) that slowly increased their body weights (Figure 1a). There was a decrease in the absolute body weight relative to the controls over this period of 32 g ($P < 0.025$), 52 g ($P < 0.0005$) and 67 g ($P < 0.0005$) respectively at the 1, 2.5 and $5\,mg\,kg^{-1}$ doses. These decreases were largely accounted for by decreases in the size of adipose tissue depots (Table 1). The subcutaneous depot was the largest and it showed the greatest decrease.

D-Fenfluramine treatment also produced a sustained decrease in food intake. This was more pronounced at the beginning, but persisted ($P < 0.0005$) through the treatment period (Figure 1b). During the last 7 days of treatment the average daily food intake for the control rats was 17.6 ± 2.4 g which was higher ($P < 0.005$) than that for the rats treated with 1, 2.5 and $5.0\,mg\,kg^{-1}$ of D-fenfluramine which consumed 14.8 ± 1.9 g, 12.9 ± 2.3 g and 12.6 ± 1.5 g of food per day respectively.

Samples of subcutaneous and inguinal adipose tissue were incubated with 5.5 mM glucose to determine whether metabolism was altered by D-fenfluramine treatment. Subcutaneous adipose tissue from the rats treated with D-fenfluramine at all doses had lower rates of fatty acid synthesis from [U-^{14}C]-glucose when this was calculated for the whole adipose tissue depot (Table 2) by using the weights given in Table 1. These values also remained statistically significant when the results were calculated per g of tissue for the group treated with $1\,mg\,kg^{-1}$ of D-fenfluramine at all insulin concentrations, and for the $2.5\,mg\,kg^{-1}$ group at 10 and $100\,ng\,ml^{-1}$ of insulin. The greater variability in the group treated with

Table 3 Effects of D-fenfluramine treatment of JCR:LA-corpulent female rats, on the concentrations and some metabolites and hormones in the blood serum

		Dose of D-fenfluramine		
	Control $n = 9$	$1.0\,mg\,kg^{-1}$ $n = 9$	$2.5\,mg\,kg^{-1}$ $n = 8$	$5.0\,mg\,kg^{-1}$ $n = 9$
Glucose (mM)	6.90 ± 0.53	6.40 ± 0.53 $P < 0.05$	6.27 ± 0.48 $P < 0.025$	6.56 ± 0.77
Lactic acid (mM)	3.25 ± 0.88	3.36 ± 0.94	2.63 ± 0.42 $P < 0.05$	3.23 ± 1.18
Glycerol (mM)	0.647 ± 0.263	0.793 ± 0.212	0.623 ± 0.194	0.718 ± 0.299
Fatty acid (mM)	0.743 ± 0.414	0.991 ± 0.331	0.705 ± 0.249	0.642 ± 0.383
Triacylglycerol (mM)	8.28 ± 3.36	6.60 ± 2.82	4.99 ± 2.72 $P < 0.025$	5.10 ± 2.84 $P < 0.025$
Total cholesterol (mM)	3.20 ± 0.99	2.75 ± 0.52	2.52 ± 0.70	2.86 ± 0.49
Free cholesterol (mM)	1.88 ± 0.81	1.60 ± 0.64	1.07 ± 0.39 $P < 0.025$	1.28 ± 0.50
Esterified cholesterol (mM)	1.37 ± 0.50	1.15 ± 0.31	1.45 ± 0.45	1.59 ± 0.54
Phospholipids (mM)	4.40 ± 1.061	3.94 ± 0.919	3.16 ± 0.66 $P < 0.01$	3.56 ± 0.636 $P < 0.05$
Insulin ($mu\,l^{-1}$)	260 ± 161	139 ± 90 $P < 0.05$	131 ± 57 $P < 0.025$	104 ± 50 $P < 0.01$
Corticosterone (nM)	502 ± 473	611 ± 754	946 ± 388 $P < 0.025$	1273 ± 1493

The rats were fed *ad libitum* (Series 1) and treated with the daily dose of D-fenfluramine indicated in Figure 1. The results are expressed as means ± s.d. for the number of animals indicated. The *P* values show the significance of the difference between the treated group and the untreated (control) rats.

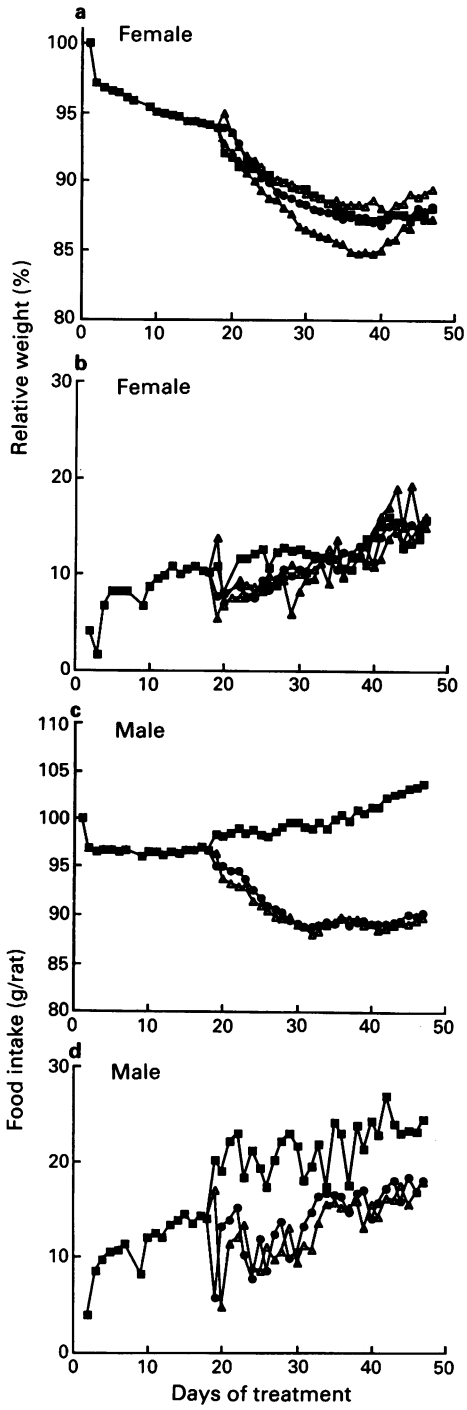

Figure 2 Effects of food restriction and D-fenfluramine on food intake and body weights in male and female corpulent rats. Female (a and b) and male (c and d) corpulent rats were restricted to a feeding period of 6 h/day (controls, ■) as described for Series 2. After 18 days 12 female and 6 male rats were injected daily with 2.5 mg kg^{-1} of D-fenfluramine (●). Six female and 4 male rats were pair-fed (△) with the treated female and male rats respectively. At day 33, six of the female rats were withdrawn from D-fenfluramine treatment (▲) and all rats were killed on day 48. Results for relative weights and food intakes are the means for the groups; s.d. are not shown for the sake of clarity.

5 mg kg^{-1} prevented the results expressed per g of adipose tissue being significantly different from the control group. The incorporation of glucose into glyceride-glycerol was about 3.5 fold higher than that for fatty acid synthesis. This incorporation was not significantly changed by D-fenfluramine treatment when expressed as per depot or per g of tissue. There were also no consistent changes in the total uptake of glucose, or output of lactate by the subcutaneous adipose tissue (results not shown).

Similar measurements were made on the inguinal adipose tissue. D-Fenfluramine treatment produced no consistent changes in glucose uptake, lactate output, or in the synthesis of glyceride-glycerol or fatty acids independently of whether this was expressed as per depot, or per g of tissue.

The concentrations of insulin, corticosterone and some metabolites were also determined in blood samples from the female rats. Treatment of the female rats with 1 mg kg^{-1} of D-fenfluramine decreased insulin concentrations by about 47% and the effect appeared to increase at the higher doses (Table 3). The concentration of corticosterone was only increased significantly at the 2.5 mg kg^{-1} dose. However, this was not accompanied by an increased stress reaction as indicated by the unaltered concentrations of fatty acids and glycerol.

Glucose concentrations were decreased significantly at the 1 and 2.5 mg kg^{-1} doses whereas circulating triacylglycerol was only decreased significantly at the 2.5 and 5 mg kg^{-1} doses. Total circulating phospholipids were also decreased at these two higher doses. Cholesterol concentrations showed little change except for a significant decrease in unesterified cholesterol at the 2.5 mg kg^{-1} dose.

Effect of food restriction on the actions of D-fenfluramine (Series 2)

A major pharmacological property of D-fenfluramine is its anorectic effect. Experiments were therefore performed to investigate the extent to which the metabolic changes produced in Table 3 by D-fenfluramine might be simply a reflection of diminished food intake. Pair-fed rats, with decreased food intake, consume their food rapidly. It was therefore considered more appropriate to limit the time during which both treated and pair-fed control rats consumed their food rather than to permit feeding throughout the day. Restriction of the female corpulent rats to a feeding schedule of 6 h per day caused decrease of about 3% in body weight on the first day (Figure 2a), and this was accompanied by a substantial decrease in food intake (Figure 2b). The female rats were able to increase food intake during the first 18 days (Figure 2b), but not sufficiently to maintain a normal increase in body weight (Figure 2a). On day 18, some of the rats were then treated with 2.5 mg kg^{-1} of D-fenfluramine. An anorectic effect of D-fenfluramine was seen during the next 7 days of treatment ($P < 0.0005$) such that the mean daily food intake for the treated female rats was 8.2 ± 1.2 g per rat compared with 11.1 ± 1.9 g for the control rats. From day 30 to day 47, there was no significant difference between the control and D-fenfluramine-treated rats and this is reflected in their weights (Figure 2a and b). After 16 days of administering D-fenfluramine to some of the female rats the treatment was discontinued. During the last 7 days of the experimental period 16.9 ± 2.2 g of food per day per rat was consumed by these animals which was higher ($P < 0.0005$) than the 14.5 ± 1.4 g eaten by the controls. The relative body weights of the four groups of rats at the end of the treatment period were similar (Figure 2a).

The results of the analysis of metabolites and hormones are given in Table 4. No significant differences were detected among the female groups with respect to the concentrations of glucose, lactate, fatty acids, triacylglycerol and corticosterone. Glycerol concentrations were decreased by D-fenfluramine treatment. The concentration of free cholesterol was raised in groups that were treated with D-fenfluramine for the full period, those that were only treated for 16 days and in those that were pair-fed compared to the controls. Insulin concentrations were decreased in the D-fenfluramine-treated rats compared to untreated rats that were allowed free access to food.

Table 4 Effects of food restriction on the concentrations of some metabolites, insulin and corticosterone in male and female corpulent rats

	Females				*Males*		
	Controls (6)	*Pair-fed* (6)	*Fenfluramine treated* (6)	*Fenfluramine treatment withdrawn* (6)	*Controls* (4)	*Pair-fed* (4)	*Fenfluramine treated* (6)
Glucose (mM)	6.56 ± 0.34	6.37 ± 0.37	6.55 ± 0.44	6.61 ± 0.36	5.78 ± 0.52	6.30 ± 0.5	6.22 ± 0.89
Lactate (mM)	3.46 ± 0.73	3.20 ± 0.78	3.59 ± 0.69	3.75 ± 0.63	4.38 ± 0.94	4.04 ± 0.89	4.45 ± 0.77
Glycerol (mM)	1.01 ± 0.14	0.98 ± 0.15	0.66 ± 0.21 $P < 0.005$	0.78 ± 0.35	1.16 ± 0.16	0.66 ± 0.27 $P < 0.01$	1.07 ± 0.10
Fatty acid (mM)	1.07 ± 0.29	1.09 ± 0.11	1.03 ± 0.22	1.18 ± 0.35	0.99 ± 0.03	1.12 ± 0.12 $P < 0.05$	1.03 ± 0.16
Triacylglycerol (mM)	2.31 ± 1.22	2.18 ± 0.77	3.18 ± 0.61	3.03 ± 1.95	1.27 ± 0.33	1.21 ± 0.55	0.63 ± 0.57
Total cholesterol (mM)	2.58 ± 0.42	3.55 ± 0.64 $P < 0.01$	3.05 ± 0.69	3.09 ± 0.43 $P < 0.05$	3.23 ± 0.44	2.77 ± 0.13 $P < 0.05$	2.76 ± 0.36
Free cholesterol (mM)	1.08 ± 0.11	1.43 ± 0.24 $P < 0.005$	1.32 ± 0.26 $P < 0.05$	1.29 ± 0.21 $P < 0.05$	1.22 ± 0.14	1.09 ± 0.07	1.01 ± 0.07 $P < 0.025$
Esterified cholesterol (mM)	1.5 ± 0.33	2.12 ± 0.43 $P < 0.01$	1.72 ± 0.45	1.80 ± 0.31	2.01 ± 0.33	1.69 ± 0.07	1.76 ± 0.3
Phospholipids (mM)	3.12 ± 0.35	3.67 ± 0.42 $P < 0.025$	3.54 ± 0.55	3.66 ± 0.47 $P < 0.05$	3.33 ± 0.38	2.94 ± 0.19	2.81 ± 0.24 $P < 0.05$
Insulin ($mu l^{-1}$)	78 ± 31	55 ± 22	45 ± 24 $P < 0.05$	68 ± 40	205 ± 90	120 ± 85	108 ± 46
Corticosterone (nM)	1155 ± 537	816 ± 282	1167 ± 744	1042 ± 425	649 ± 156	562 ± 155	533 ± 180

Rats were treated as described in the text (Series 2) and Figure 2 and the results are expressed as means ± s.d. for the number of rats indicated. The *P* values show the significance of the difference between the appropriate group and the untreated (control) rats.

The same experimental protocol was applied to male corpulent rats (Figure 2c and d). There was again a decrease in body weight and food intake on the first day while the rats became accustomed to the restricted period of feeding. Body weights then remained relatively constant from days 1 to 18. The rats treated with D-fenfluramine at day 18 showed an anorectic effect which prevented the further increase in food intake (Figure 2d) and weight gain (Figure 2c) that was seen with the control rats fed *ad libitum* for 6 h. The treated rats lost weight as did the rats that were pair-fed with them (Figure 2c).

The differences in the metabolite and hormone concentrations for the male rats were not marked. Treatment with D-fenfluramine appears to decrease the concentrations of blood lipids as seen for phospholipids, free cholesterol and triacylglycerol, although the latter value did not reach statistical significance. Insulin concentrations appeared to decrease by 42 and 48% with pair-feeding and D-fenfluramine-treatment, but the results were not statistically significant with the number of animals used. This was because of the large inter-animal variations which are normal with this rat model. However, other work has shown that food restriction is effective in decreasing circulating insulin in the corpulent rat (Russell *et al.*, 1990b). There were no statistically significant differences in metabolites or hormones on day 42 (when the rats were killed) between the rats treated with D-fenfluramine and those that were pair-fed.

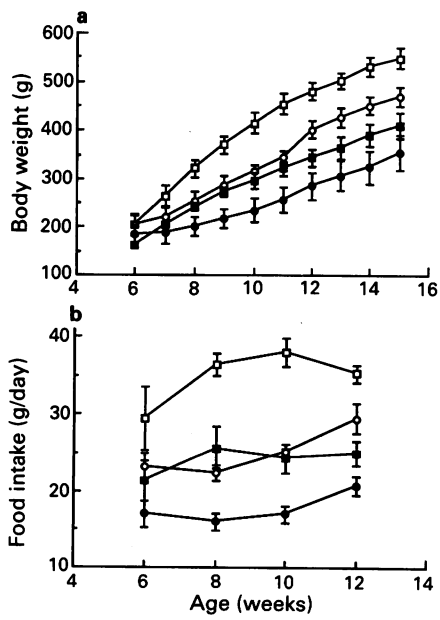

Figure 3 Effects of adding D-fenfluramine to the drinking water of male and female JCR:LA corpulent rats. D-Fenfluramine was added to the drinking water of 6 male (○) and 6 female (●) rats (Series 3) and the concentration was continually adjusted to produce a daily dose of about 0.25 mg kg^{-1} of D-fenfluramine, 6 male (□) and 6 female (■) rats received untreated water (controls). The results are means ± s.d. Food intake is given as the mean daily intake per rat and the rats were housed individually.

Effects of prolonged treatment of young JCR:LA corpulent rats with D-fenfluramine (Series 3)

The third experimental protocol was to treat 6 week old corpulent rats with D-fenfluramine in drinking water. Both male and female rats gained substantially less weight ($P < 0.001$ for males and $P < 0.01$ for females) when treated with 0.25 mg of D-fenfluramine kg^{-1} daily (Figure 3a). The mean decreases in body weight were equivalent to 54 g and 80 g respectively for the male and female rats after the 9 weeks of treatment. The decreased weight was associated with a sustained decrease in food intake (Figure 3b).

Discussion

The response curve with D-fenfluramine demonstrates that daily injections of 1 mg kg^{-1} of D-fenfluramine produced significant decreases in body weight, adipose tissue mass and food intake that were maintained throughout the 30 day treatment period of the female rats (Figure 1). This was accompanied by a decreased ability of the subcutaneous adipose tissue to synthesize fatty acids from glucose (Table 2). Decreases in fatty acid synthesis after D-fenfluramine-treatment have also been observed in lean male Wistar rats (Al-Sieni *et al.*, 1989) and in old female Wistar rats (Brindley *et al.*, 1989). However, we did not observe a diversion of glucose to lactate in the corpulent rats as was observed with male Wistar rats (Al-Sieni *et al.*, 1989).

JCR:LA-corpulent rats and old female Wistar rats (Brindley *et al.*, 1990) were much more sensitive to the weight reducing effects of D-fenfluramine than were lean male Wistar rats that were actively growing (Brindley *et al.*, 1985; Al-Sieni

et al., 1989). Treatment of male and female corpulent rats from 6 weeks of age with 0.25 mg kg^{-1} daily of D-fenfluramine substantially decreased body weight (Figure 3). To our knowledge this is the lowest dosage that has been reported to have such a profound effect (Arnaud & Nathan, 1990). However, this treatment did not completely prevent the appearance of obesity; neither did restricting the food intake of male rats to 12 g day^{-1} (Russell et al., 1990b) or making the rats run for 6–8 km day^{-1} (Russell et al., 1989b).

Although the body weights of the adult corpulent rats tended to stabilize at a lower level after about 15 days of treatment, D-fenfluramine did prevent the normal increase in weight that occurred in the untreated rats (Figure 1a). This pattern of response is reminiscent of the clinical experience in using D-fenfluramine to treat obesity (Rowland & Carlton, 1986). The failure to continue to lose weight should not be taken as an indication of pharmacological tolerance. It has been proposed that fenfluramine persistently lowers body weight set-point, and that anorexia is secondary to the weight suppressing effects (Levitsky et al., 1981; Stunkard, 1982). This conclusion is supported by the observation that fenfluramine does not have an anorectic effect in rats that had previously had their weights decreased by food deprivation (Levitsky et al., 1987; Stunkard, 1982). Similarly, the effects of D-fenfluramine in the female rats were not so prolonged when they were allowed to feed for only 6 h per day (Figure 2b). These female corpulent rats were unable to consume enough food to maintain and increase their body weights as they would have done had they been fed *ad libitum* (Figure 1). This finding was unexpected since in previous work we used a less extreme form of obesity, the aged adult female Wistar rat. These rats were able to maintain their body weights when fed during the same 6 h feeding period, and D-fenfluramine at 1, 2.5, 5 and 10 mg kg^{-1} decreased their body weight by 2, 4, 7 and 8% respectively in 8 days (Brindley et al., 1989). The male corpulent rats were also able to gain weight in the 6 h feeding period and D-fenfluramine produced a marked reduction in body weight (Figure 2c).

The stabilization of body weight after treatment for about 15 days with D-fenfluramine (Figure 1b) does not mean that there were no further beneficial metabolic effects. The decrease in circulating insulin reached 60% after 29 days of treatment at the 5 mg kg^{-1} dose in female corpulent rats (Table 3). This indicates a marked improvement in insulin sensitivity and was reflected in the decrease in circulating glucose. D-Fenfluramine did not increase lactate concentrations in the corpulent rats as do some biguanides.

D-Fenfluramine treatment also produces decreases of up to 40% in circulating triacylglycerol with smaller effects on cholesterol and phospholipids. These changes are compatible with a suppression of the high rates of VLDL secretion in the corpulent rats (Russell et al., 1991). Insulin resistance is often associated with hypertriglyceridaemia, which could be caused by the high insulin concentrations. Alternatively, the relatively low effectiveness of insulin together with increased glucocorticoid action could be responsible (Brindley & Rolland, 1989). Glucocorticoids stimulate the secretion of lipids (Mangiapane & Brindley, 1986; Bartlett & Gibbons, 1988) and apolipoproteins (Martin-Sanz et al., 1990) in VLDL. Furthermore excessive glucocorticoid action may be an important factor in the development of obesity (Bray & York, 1979; Berdanier, 1989; Devenport et al., 1989). Treatments that decrease glucocorticoid secretion (Bray & York, 1979), or effectiveness (Baulieu 1989; Langley & York, 1990) can partially reverse the obesity; and D-fenfluramine may produce some of its metabolic effects by preventing the excessive secretion of glucocorticoids (Brindley, 1983; Brindley et al., 1988). However, decreases in circulating triacylglycerols, or the weight of the corpulent rats were not associated with decreased corticosterone concentrations (Tables 3 and 4).

The anorectic effect of D-fenfluramine could in itself have the effect of raising corticosterone concentrations, which in this condition could promote catabolism rather than anabolism (Dallman et al., 1989). This catabolic phase is also observed in the first few days when lean rats were treated with fenfluramine and it is associated with anorexia and a marked stimulation of lipolysis (Dannenberg, 1983; Brindley, 1983). Furthermore, fenfluramine when administered acutely can cause the release of corticosterone (Schettini et al., 1979; Fuller et al., 1981). Glycerol and fatty acid concentrations were raised in the food-restricted rats as expected, but there was no evidence for a specific effect of D-fenfluramine in promoting lipolysis in this chronic treatment (Table 4), or in the rats fed *ad libitum* (Table 3). This conclusion was also made in other work where lean Wistar rats were treated chronically with D-fenfluramine (Brindley et al., 1985).

Food restriction also played a major part in decreasing the hypertriglyceridaemia of the female rats from 8.3 mM to 2.3 mM (Tables 3 and 4). In our male corpulent rats, serum triacylglycerol concentrations are normally about 2 mM when the rats are fed *ad libitum*. This was decreased to 1.27 mM when the rats were fed for only 6 h per day (Table 4). There was little evidence for a pronounced hypolipidaemic action of D-fenfluramine after food restriction. However, D-fenfluramine was still able to decrease insulin concentrations in the females and possibly in the males, although the latter effect was not statistically significant (Table 4). Other work with D-fenfluramine has demonstrated improvements in insulin sensitivity and hypolipidaemic effects in animals and human beings that are unrelated to anorexia and weight loss (Brindley et al., 1985; Arnaud & Nathan, 1990). However, it is concluded that the anorectic action of D-fenfluramine is an important component in producing the hypolipidaemic effects and some of the improvement in insulin sensitivity in the JCR:LA-corpulent rat.

Similar metabolic effects to those observed with D-fenfluramine were obtained by treatment of the corpulent rat with a related drug, benfluorex at 25 mg kg^{-1}. This produced decreases in food intake and body weight similar to that seen with 5 mg kg^{-1} of D-fenfluramine in the female rats (Figure 1). However, benfluorex appeared to have a greater effect in decreasing the concentration of circulating lipids (Brindley et al., 1991). Benfluorex and D-fenfluramine depress feeding through their central 5-hydroxytryptaminergic actions (Garratini, 1983; Samanin, 1983) which could also account for the beneficial changes in hormonal balance resulting in hypoglycaemic and hypolipidaemic effects (Brindley et al., 1985; 1988).

The present work establishes that the JCR:LA-corpulent rat is very sensitive to the actions of D-fenfluramine. Very low doses of this compound produce a sustained depression of food intake, weight loss, together with marked decreases in the concentrations of circulating insulin and triacylglycerol. These effects are all in the direction that should decrease the risk of developing premature atherosclerosis. The corpulent rats appear to provide an ideal model for the combined obese-diabetic-hyperlipidaemic syndrome that is associated with an increased incidence of coronary heart disease in human beings. The animals not only develop mature cardiovascular diseases spontaneously, but this disease expression can be modified by metabolic manipulation or pharmacological treatment (Russell et al., 1989b,c; 1990c). The present work provides the foundation for future studies on the mode of action of D-fenfluramine in this experimental model. Most significantly this model can be used to investigate if prolonged treatment with D-fenfluramine can decrease the incidence of cardiovascular lesions. Such knowledge will be extremely important in deciding whether to continue treating patients with drugs such as D-fenfluramine even after the initial loss of body weight has been achieved.

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Pharmacological characterization of angiotensin-induced depolarizations of rat superior cervical ganglion *in vitro*

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1 The depolarizing responses to angiotensin II and angiotensin III of the rat superior cervical ganglion have been characterized *in vitro*, by the use of peptidase inhibitors, peptide and non-peptide antagonists and dithiothreitol (DTT).

2 Angiotensin II and III depolarized the ganglion in a concentration-related manner. Angiotensin II was approximately 30 fold more potent than angiotensin III.

3 The endopeptidase inhibitor, bacitracin, increased the potency of angiotensin II and III by approximately 4 and 20 fold respectively. The aminopeptidase inhibitor, amastatin, further increased the potency of angiotensin III (but not angiotensin II) by approximately 4 fold. In the presence of bacitracin and amastatin, angiotensin II and III were equipotent.

4 The peptide antagonist [Ile^7]angiotensin III (0.01–0.3 μM) produced a non-parallel rightward displacement of the angiotensin II concentration-response curve, with a suppression of the maximum response. The potency of [Ile^7]angiotensin III was increased by bacitracin and amastatin.

5 The AT_1-selective non-peptide antagonist losartan (DuP 753; 0.03 and 0.1 μM) produced a parallel rightward displacement of the angiotensin II concentration-response curve, with an apparent pK_B of 8.3 ± 0.1. A higher concentration of losartan (0.3 μM) depressed the maximum agonist response by $32 \pm 6.5\%$, possibly reflecting non-competitive behaviour of the antagonist. The potency of losartan was not influenced by bacitracin.

6 The AT_2-selective non-peptide antagonist, PD123177 (3 μM) failed to antagonize the angiotensin II-induced depolarizations.

7 DTT (1 mM) produced a 22% reduction of the maximum response to angiotensin II.

8 We conclude that the angiotensin II-induced depolarizations of the rat superior cervical ganglion are mediated by angiotensin II receptors of the AT_1 subclass. The ability of peptidase inhibitors to modify the potency of peptide agonists and antagonists highlights the difficulties associated with the use of peptide agents to characterize angiotensin II receptors in this preparation.

Keywords: Angiotensin II; superior cervical ganglion; depolarization; peptide antagonists; non-peptide antagonists; angiotensin II receptor subtypes

Introduction

Angiotensin II is a potent stimulant of sympathetic ganglia, exerting its action directly on postganglionic cells (Reit, 1972; Dun *et al.*, 1978). The excitatory response to angiotensin II is associated with a slow depolarization, similar to that produced by muscarinic agonists (Brown *et al.*, 1980). Although Brown *et al.* (1980) have shown that this angiotensin II-induced depolarization can be antagonized by the peptide angiotensin II antagonist, [Ile^7]angiotensin III, no further pharmacology of the effects of angiotensin II on ganglia has been reported.

Over the past years, there has been much speculation about the existence of multiple receptor subtypes for angiotensin II. Some of this evidence is based on differences in the relative potencies of angiotensin II and its heptapeptide metabolite, angiotensin III. In some tissues, angiotensin III is considerably weaker than angiotensin II (Moore *et al.*, 1976; Scanlon & Moore, 1988), while in other target organs, angiotensin III is equipotent to, or even more potent than its precursor peptide (Peach & Chiu, 1974; Jackson *et al.*, 1988; Carrithers *et al.*, 1990). In view of these observations, angiotensin receptors in different tissues have been separated according to agonist orders of potency (Peach, 1977). In addition, a number of peptide antagonist analogues of angiotensin II or III, such as [Sar^1, Ile^8]angiotensin II and [Ile^7]angiotensin III have been found to be more potent as antagonists of angiotensin II or III responses in some tissues than in others (Sarstedt *et al.*, 1975; Spielman *et al.*, 1976). However, recent studies with non-peptide angiotensin II antagonists have provided more definitive evidence for the existence of angiotensin II receptor subtypes. Angiotensin II receptors have been divided into two subtypes, referred to as AT_1 and AT_2 (Bumpus *et al.*, 1991). Losartan (DuP 753) is a selective antagonist of the AT_1 subtype (alternatively known as type 1, AII-1, AII-B or AIIα; Chiu *et al.*, 1989a; Chang & Lotti, 1989; Whitebread *et al.*, 1989; Speth & Kim, 1990). PD123177, CGP42112A and [*p*-aminophenylalanine^6]angiotensin II are selective for the AT_2 subtype (type 2, AII-2, AII-A or AIIβ) (Chiu *et al.*, 1989a; Whitebread *et al.*, 1989; Speth & Kim, 1990).

Additional support for this angiotensin II receptor classification has also been provided by the specific ability of the disulphide reducing agent, dithiothreitol (DTT) to inhibit the binding of radiolabelled ligands to AT_1 sites, while enhancing the binding to AT_2 sites (Whitebread *et al.*, 1989; Chiu *et al.*, 1989b). DTT also abolishes the contractile effects of angiotensin II in rabbit isolated aorta (Fleisch *et al.*, 1973), a tissue that expresses only AT_1 sites (de Gasparo *et al.*, 1990).

The purpose of the present experiments was to perform a detailed pharmacological study of the effects of angiotensin II and III on the isolated preparation of the rat superior cervical ganglion. We have investigated the effects of peptidase inhibitors, peptide and non-peptide antagonists as well as the disulphide reducing agent DTT on the agonist-induced depolarization responses.

Methods

Male Lister-Hooded rats, weighing 300–350 g were stunned by a blow to the head, decapitated and the superior cervical

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ganglia were dissected from the surrounding tissue. Each ganglion was desheathed under a dissecting microscope and arranged in a two-compartment chamber such that the ganglion cell body was isolated from the postganglionic trunk by a notched perspex barrier coated in silicone grease. Each ganglion rested on filter paper and was perfused with Krebs-Henseleit solution, pre-equilibrated with 5% CO_2 in O_2 and maintained at 37°C. The composition of the solution was (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.6, $NaHCO_3$ 24.9 and glucose 5.6. Each compartment was perfused separately at a rate of 0.5 ml min^{-1}. Agonist drugs were added to the ganglion cell body compartment and potential differences across the two compartments were recorded with Ag/AgCl electrodes, one positioned in contact with each compartment. Up to six ganglion preparations were used in each experiment. For full details of tissue preparation and recording, see Ireland (1987).

To prevent tachyphylaxis, angiotensin II and angiotensin III were applied serially using an inter-dose interval of 20 min, each concentration remaining in contact with the tissue for 5 min. Only two concentration-response curves were determined on each preparation, the first curve always serving as a control for the second. Peptidase inhibitors or antagonists were perfused over the tissues for 30 min before construction of the second concentration-response curve. In each experiment, one preparation was perfused for the duration of the experiment without inhibitors or antagonists and served as a control. On this tissue, the first and second curves were always superimposable. To investigate the effect of antagonists in the presence of inhibitors, the inhibitors were applied before the first curve. Preliminary experiments indicated that tachyphylaxis develops rapidly to high doses of angiotensin II or III. As a result, it was not always possible to achieve the maximum of the agonist concentration-response curve. Thus, to measure agonist potency, the concentration of agonist required to produce a depolarization of 300 μV was estimated by eye from individual concentration-response curves. This value is referred to as EC_{300}. A response of 300 μV always lay on the linear part of the concentration-response curve. To measure antagonist potency, lateral displacements of the concentration-response curves were estimated by eye at the control EC_{300} level, to give 'dose-ratios' between drug-treated and control curves. Dose-ratios, estimated from individual ganglion preparations were used to calculate the negative logarithm of the apparent dissociation constant (pK_B) for the antagonists using the equation, $pK_B = \log(\text{dose-ratio} - 1) - \log(\text{antagonist concentration})$. Results of antagonist experiments are shown graphically, normalised against the response achieved by the highest control agonist concentration used. This response was not necessarily the greatest that could be achieved. Statistical analyses used paired or unpaired Student's t test where appropriate, using a criterion for statistical significance of $P < 0.05$.

The drugs used were supplied from the following sources. Angiotensin II, angiotensin III, [Ile^7]angiotensin III (Peninsula), bacitracin, amastatin, dithiothreitol (Sigma), losartan potassium (Du Pont Merck Pharmaceutical Co.) and PD123177 (1-(4-amino-3-methylphenyl) methyl-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazol[4,5-C]pyridine-6-carboxylic acid; synthesized in our laboratories).

Results

Angiotensin II and angiotensin III evoked concentration-dependent depolarization of the rat isolated superior cervical ganglion. The depolarization reached peak height within 4–5 min after the start of agonist perfusion and recovery to baseline was seen within 15 min of perfusing drug-free solution. The application of higher doses of angiotensin II or III was characterized by a rapid waning of the response as the depolarization approached its maximum. The potencies ($-\log EC_{300}$) of angiotensin II and III, were 7.2 ± 0.1 and 5.6 ± 0.2 respectively. Although depolarizing responses to angiotensin II and angiotensin III were not compared on any one tissue, angiotensin III was approximately 40 fold weaker than angiotensin II.

Effect of bacitracin and amastatin on agonist responses

The angiotensin II- and angiotensin III-induced depolarizing responses of the ganglia were potentiated by the application of bacitracin (1 mM). Bacitracin displaced the angiotensin II and III concentration-response curves to the left of the corresponding control curves (Figure 1a,b). The potencies of angiotensin II and angiotensin III in the presence of bacitracin were increased 3.6 fold and 24.1 fold respectively.

In the presence of bacitracin, the additional preincubation with amastatin (10 μM) produced differential effects on the depolarizing responses to angiotensin II and angiotensin III. The potency of angiotensin II was not influenced by amastatin (Figure 2a), although the potency of angiotensin III was increased 4.6 fold (Figure 2b). In the presence of both bacitracin and amastatin, angiotensin II and angiotensin III were equipotent, with $-\log EC_{300}$ estimates of 8.2 ± 0.2 and 8.1 ± 0.1 respectively.

Effect of antagonists

To characterize the receptor mediating the ganglion depolarization produced by angiotensin II, we investigated the effects of [Ile^7]angiotensin III, losartan, PD123177 and DTT on angiotensin II-induced depolarizations of the ganglia. Unless otherwise stated, all these studies were conducted in the presence of bacitracin (1 mM), to prevent the degradation of angiotensin II.

[Ile^7]angiotensin III

Angiotensin II-induced depolarization of the ganglion was antagonized by [Ile^7]angiotensin III. At concentrations of

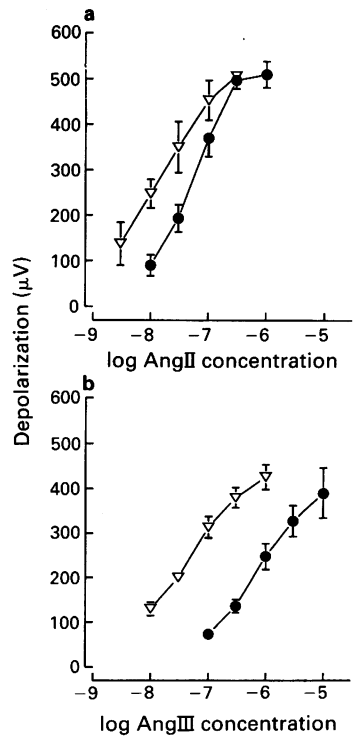

Figure 1 Effect of bacitracin on depolarizing responses to angiotensin II (Ang II) (a) and angiotensin III (Ang III) (b). The symbols represent mean depolarizations (s.e.mean shown by vertical bars) in the absence of bacitracin (●) or during perfusion with 1 mM bacitracin (▽). $n = 3$ preparations.

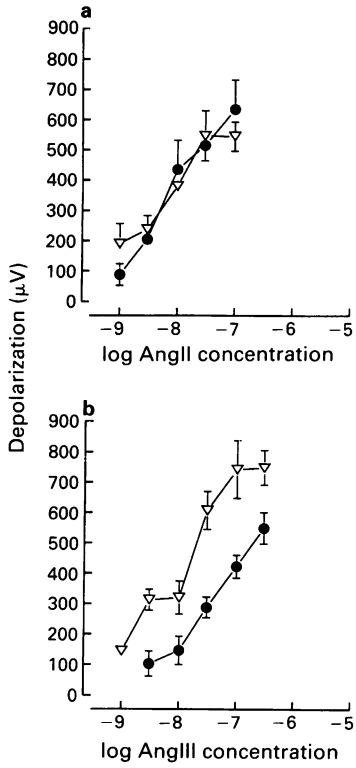

Figure 2 Effect of amastatin on depolarizing responses to angiotensin II (Ang II) (a) and angiotensin III (Ang III) (b). The symbols represent mean depolarizations (s.e.mean shown by vertical bars) in the absence of amastatin (●) or during perfusion with 10 μM amastatin (▽). $n = 4$ preparations.

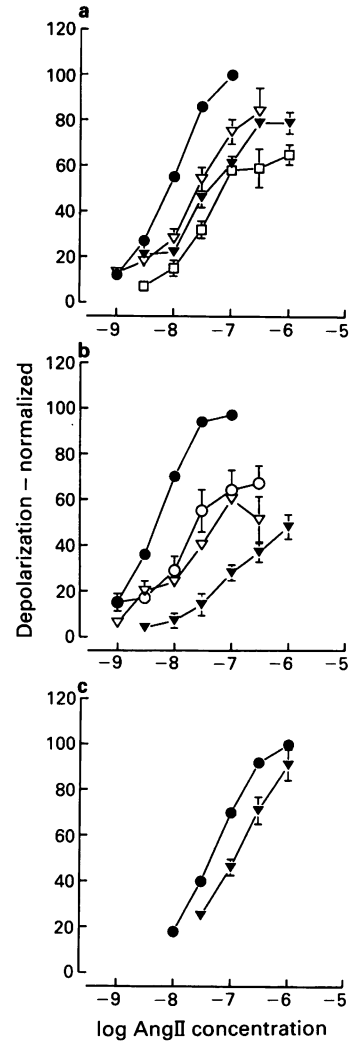

Figure 3 Effect of [Ile^7]angiotensin III on the concentration-response curve to angiotensin II (Ang II) on the rat isolated superior cervical ganglion. (a) During perfusion of bacitracin (1 mM). (b) During perfusion of bacitracin plus amastatin (10 μM). (c) No peptidase inhibitors. Control (●); [Ile^7]angiotensin III 0.01 μM (○), 0.03 μM (▽), 0.1 μM (▼), 0.3 μM (□). Depolarizing responses are normalised around the close-to-maximum control agonist response (see Methods). $n = 3$–6 preparations.

0.03–0.3 μM, the antagonist produced a non-parallel rightward displacement of the angiotensin II concentration-response curve, with suppression of the maximum response (Figure 3a). In view of these observations, the antagonist potency of [Ile^7]angiotensin III was not measured quantitatively. The antagonist effect of [Ile^7]angiotensin III was influenced by peptidase inhibitors. Thus, during the continued perfusion of amastatin (10 μM), in addition to bacitracin, [Ile^7]angiotensin III (0.01–0.1 μM) produced greater rightward displacement of the agonist concentration-response curves than during the perfusion of bacitracin alone (Figure 3b). The suppression of the maximum response was also greater. Thus, in the presence of bacitracin (1 mM) alone, 0.1 μM [Ile^7]angiotensin III reduced the agonist maximum response to 78.4 ± 7.0%, whilst in the presence of bacitracin plus amastatin (10 μM), 0.1 μM [Ile^7]angiotensin III reduced the agonist maximum response to 47.6 ± 6.1% ($P < 0.05$ versus bacitracin alone). Furthermore, in the absence of either bacitracin or amastatin, 0.1 μM [Ile^7]angiotensin III failed to reduce significantly the maximum response to angiotensin II (Figure 3c).

Non-peptide antagonists

Angiotensin II-induced ganglion depolarization was also antagonized by the non-peptide antagonist losartan. Losartan (0.03 μM and 0.1 μM) produced an apparent rightward displacement of the agonist concentration-response curve (Figure 4a), although the maximum agonist response was reduced by 32 ± 6.5% in the presence of 0.3 μM losartan. The apparent dose-ratios for the two losartan concentrations (0.03 μM and 0.1 μM) producing parallel shifts of agonist concentration-response curves were 6 ± 1.5 and 23 ± 4.2 respectively, which yielded an apparent pK_B value of 8.3 ± 0.1 from the Gaddum equation (see above). In contrast to the results obtained with [Ile^7]angiotensin III, the antagonist potency of losartan was not significantly influenced by peptidase inhibitors. Thus, the apparent pK_B of losartan in the absence of bacitracin was 8.6 ± 0.1 (Figure 4b).

Preincubation of the ganglia with PD123177 failed to antagonize the angiotensin II-induced depolarization, at an antagonist concentration of 3 μM (data not shown).

Dithiothreitol

The disulphide reducing agent DTT produced complex effects on the angiotensin II-induced depolarization. The responses to lower concentrations of angiotensin II (3–10 nM) were not influenced by DTT (1 mM) although there was some suppression of the responses produced by the higher doses of angiotensin II. Maximum responses to angiotensin II were reduced by 22 ± 5.9% (data not shown).

Discussion

The most important finding of this study is that the angiotensin II-induced depolarization of the rat superior cervical ganglion can be antagonized by the AT_1 selective non-peptide angiotensin II antagonist, losartan. The antagonist effects of

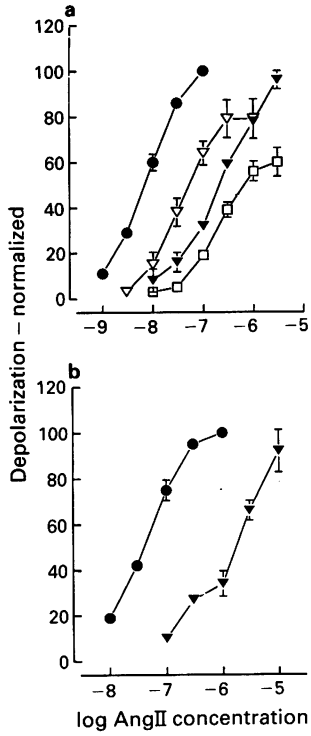

Figure 4 Effect of losartan on the concentration-response curve to angiotensin II (Ang II) on the rat isolated superior cervical ganglion. (a) During perfusion of bacitracin (1 mM), (b) no bacitracin. Control (●); losartan 0.03 μM (▽), 0.1 μM (▼), 0.3 μM (□). Depolarization responses are normalised around the close-to-maximum control agonist response (see Methods). n = 3–5 preparations.

losartan were concentration-dependent and appeared to be compatible with a competitive mode of action at low concentrations (0.03 μM and 0.1 μM), although maximum agonist responses were depressed at higher concentrations of losartan. This suggests other, non-competitive inhibitory effects of losartan. The pK_B estimate determined for losartan against the depolarization to angiotensin II is similar to that previously reported in other *in vitro* functional preparations (Wong *et al.*, 1990) and is also close to its log IC_{50} for displacement of angiotensin II binding from AT_1 binding sites in different tissues (Chiu *et al.*, 1989a; Criscione *et al.*, 1990; Michel & Barnes, 1991). These data indicate that the angiotensin II-induced depolarization of ganglion neurones is mediated by angiotensin II receptors of the AT_1 subclass. This is supported by the observation that the AT_2 selective antagonist, PD123177, failed to antagonize the depolarization, even at a concentration of 3 μM, which is at least 30 fold higher than its IC_{50} for displacement of radiolabelled angiotensin II binding from AT_2 sites (Chiu *et al.*, 1989a; Michel & Barnes, 1991).

A further important observation from this study is the influence of peptidase inhibitors on the responses to angiotensin II and III and on the antagonist effects of [Ile^7]angiotensin III. Our findings show that firstly, bacitracin increased the potency of both angiotensin II, angiotensin III and [Ile^7]angiotensin III and secondly, the potencies of angiotensin III and [Ile^7]angiotensin III, but not angiotensin II were further increased by amastatin. The metabolism of angiotensin II is known to occur in part by the action of proline endopeptidase, which cleaves phenylalanine from the carboxyterminal (Daly *et al.*, 1985). The actions of proline endopeptidase are inhibited by bacitracin. Thus, in the present study increased potency of angiotensin II, angiotensin III and [Ile^7]angiotensin III in the presence of bacitracin is not surprising, as all three peptides possess identical carboxyterminal amino acids. Sequential cleavage of amino acids from the aminoterminal end of angiotensin II also contributes to the inactivation of this peptide. Aminopeptidase-A cleaves aspartate from angiotensin II to yield angiotensin III, which can be subsequently cleaved by aminopeptidase-M to inactive fragments (Kugler, 1982). At the concentration used in the present study, amastatin is a potent inhibitor of both aminopeptidase-A and aminopeptidase-M (Powers & Harper, 1988). The potentiating effect of amastatin on angiotensin III and its antagonist analogue [Ile^7]angiotensin III in the ganglion is likely to be due to the inhibition of aminopeptidase-M. It is unlikely that aminopeptidase-A plays a role in the metabolism of angiotensin II in this tissue, as amastatin failed to increase the potency of angiotensin II.

The observations with the peptidase inhibitors highlight the problems associated with the use of peptide agents in this tissue. The ability of bacitracin and amastatin to influence differentially the potencies of angiotensin II and angiotensin III highlights the difficulties of sub-classifying angiotensin II receptors by relative potencies of the naturally occurring peptides in different tissues (see Peach, 1977). In addition, variable degradation of peptide antagonists could explain the differences in the ability of peptide angiotensin II antagonists to inhibit various angiotensin II-mediated effects in different tissues (see Introduction). As expected, the non-peptide antagonists appear much more stable in this respect. This is highlighted by the lack of effect of bacitracin on the antagonist potency of losartan.

The disulphide reducing agent DTT failed to abolish the angiotensin II-induced depolarization of the ganglion. Although DTT inhibits the binding of radioligands to AT_1 sites, its ability to inhibit AT_1 receptor-mediated responses in functional studies is less consistent. DTT has been shown to abolish functional responses produced by AT_1 receptor activation in rabbit aorta and rat liver (Fleisch *et al.*, 1973; Gunther, 1984), although the effect of DTT in the present study is less apparent. Furthermore, Barnes *et al.* (1991) found that DTT did not inhibit AT_1 receptor-mediated contractions of rat uterus. It is possible that this apparent functional selectivity of DTT reflects differences in the AT_1 receptors. Alternatively, differential accessibility of DTT to AT_1 receptors in different tissues is a possible explanation.

In conclusion, we have provided pharmacological evidence to show that depolarization of rat superior cervical ganglion by angiotensin II is mediated through angiotensin II receptors of the AT_1 subclass. As such, this preparation provides a useful assay for the investigation of the actions of angiotensin II in neuronal tissue.

We thank Du Pont Merck Pharmaceutical Co. for the supply of losartan.

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Release of vasoactive intestinal polypeptide from the rat gastric fundus

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1 Auxotonic responses and release of vasoactive intestinal polypeptide-like immunoreactivity (VIP-LI) induced by electrical field stimulation (EFS) were studied in longitudinal muscle strips from the gastric fundus of reserpinized rats suspended between parallel platinum electrodes in Krebs solution containing atropine (1 μM), 5-hydroxytryptamine (3 μM) and bovine serum albumin (50 mg l^{-1}).

2 EFS (supramaximal voltage, 1 ms, 0.25–32.0 Hz, trains of 2 min) induced frequency-dependent relaxations.

3 EFS at frequencies $\geqslant$8 Hz also produced significant increases in VIP-LI release.

4 VIP-LI release induced by EFS at 16 Hz no longer occurred in the presence of tetrodotoxin (1 μM) or a Ca^{2+}-free medium.

5 Detection of VIP-LI upon activation of inhibitory non-adrenergic, non-cholinergic neurones indicates that VIP meets the 'detectable release' criterion for an inhibitory neurotransmitter in the rat gastric fundus.

Keywords: Stomach (rat); non-adrenergic non-cholinergic (NANC) neurones; relaxation; vasoactive intestinal polypeptide (VIP); peptide histidine isoleucinamide (PHI)

Introduction

According to Orrego (1979), the following basic criteria must be met by any substance that is to be considered a neurotransmitter: 'detectable release' via a Ca^{2+}-dependent mechanism in response to neural stimulation and 'identity of action' between the effects of exogenous application of the putative neurotransmitter and those of neuronal stimulation. In many species, electrically induced relaxation of the smooth muscles of the gastric fundus has a non-adrenergic non-cholinergic (NANC) component but the identity of the neurotransmitter(s) that mediates this effect has not been definitively established. Vasoactive intestinal polypeptide (VIP) seems responsible for at least part of this relaxant response in the rat. Exogenously applied VIP has already been shown to mimic the effects of NANC neurone activation in this species (Lefebvre, 1986). In addition, both trypsin (De Beurme & Lefebvre, 1978) and specific anti-VIP antisera (De Beurme & Lefebvre, 1988; D'Amato *et al.*, 1990; Li & Rand, 1990), currently the most effective methods of antagonizing VIP-induced relaxation, partially reduce the inhibitory responses to NANC stimulation at concentrations that selectively prevent responses to VIP itself. Very recently, similarities have also been found between the mechanical, electrophysiological and cyclic nucleotide responses to VIP and those elicited by inhibitory NANC neurone stimulation (Ito *et al.*, 1990).

The aim of the present study was to determine whether VIP also meets the 'detectable release' criterion as an inhibitory NANC neurotransmitter in the gastric fundus of the rat.

Methods

Following intraperitoneal injection of reserpine (5 mg kg^{-1}) to deplete noradrenergic stocks (Lefebvre, 1986), male and female rats weighing 150–300 g were fasted, with free access to water, for 24 h. The animals were then killed and their stomachs were removed. Longitudinal muscle strips (3 mm × 20 mm) from the gastric fundi were prepared as described by Vane (1957). One strip was prepared from each fundus. Some of the strips were used for functional studies and others were used for release experiments (see below). The basic bath solution used for both types of studies was a Krebs solution containing (mM): NaCl 118.5, KCl 4.8, $CaCl_2$ 1.9, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 10.1. The bath solution was maintained at 37°C and bubbled with a 95:5 O_2/CO_2 mixture.

Functional experiments

The strips were mounted under a 1 g load between two parallel platinum electrodes (20 mm long, 4 mm wide, 5 mm apart) placed in 5 ml organ baths. The bath solution for these experiments also contained atropine 1 μM, 5-hydroxytryptamine (5-HT) 3 μM and bovine serum albumin (BSA) 50 mg l^{-1}. The tissues were allowed to equilibrate for 1 h during which the bath solution was changed every 10 min. Changes in strip length, magnified 5–10 times, were recorded auxotonically (Harvard smooth muscle transducers) with Rikadenky R-01 single-pen recorders. After the equilibration period the bath solution was renewed every 5 min between drug applications and/or periods of electrical field stimulation (EFS).

Rectangular, bipolar pulses or constant current output (120 mA) were delivered with a Palmer Bioscience 6012 Stimulator linked in series with a Basile Biological Research Apparatus constant-current unit. Throughout the period of EFS, output was controlled on a Hewlett Packard 141B oscilloscope. Stimulation voltage (supramaximal), duration (1 ms) and pulse train duration (2 min) were kept constant; frequency was varied as indicated below. At the end of all experiments, a supramaximal concentration (35 μM) of sodium nitroprusside (SNP) was added to the bath and left in contact with the strip until maximal relaxation had occurred.

Release experiments

Strips were fitted with a pair of platinum electrodes (20 mm long, 3.5 mm wide, 3 mm apart) and mounted under a load of 1 g inside 4 ml plastic tubes (Sarstedt No 55478, Numbrecht, Germany). The tubes contained 2 ml of the basic Krebs solution with atropine (1 μM), BSA (50 mg l^{-1}), bacitracin (30 mg l^{-1}) and aprotinin (100,000 iu l^{-1}). The bath solution was bubbled with a 95:5 O_2/CO_2 mixture and maintained at 37°C. The strips were placed in different tubes every 10 min

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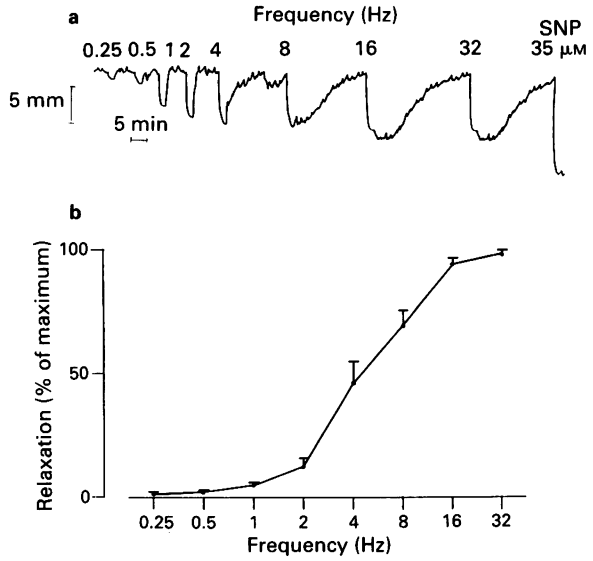

Figure 1 Relaxation of longitudinal muscle strips from the rat gastric fundus induced by electrical field stimulation (0.25–32.0 Hz, supramaximal voltage, 1 ms, pulse trains of 2 min). (a) Representative tracing from one muscle strip SNP = sodium nitroprusside. (b) Mean results ± s.e.mean (vertical bars) based on responses of 6 strips. Relaxations were measured as area under the curve and expressed as percentages of the maximal relaxation induced in each strip (32 Hz in 5 out of 6 strips).

during the 60 min equilibration period. Thereafter each strip was incubated for 2 min in different tubes before, during and after EFS delivered as described above.

At the end of each 2 min incubation period, the tube solution was collected on ice, divided into 1 ml aliquots and stored at −20°C until assayed. The wet weight of the strips was recorded at the end of all experiments.

A specific radioimmunoassay (RIA) was used to measure VIP-like immunoreactivity (VIP-LI). On the day of assay, the frozen aliquots were thawed, extracted in absolute ethanol (3/4 v/v), centrifuged at 3000 r.p.m. for 20 min at 4°C and evaporated in a Speedvac Evaporator linked with a Savant refrigerated condensation trap and vacuum pump. They were then reconstituted in assay buffer (NaCl 154 mM, phosphate 10 mM buffer, ethylene diaminetetraacetic acid (EDTA) 25 mM, thimerosal 0.01%, BSA 0.5%, pH 7.2) to a 4 fold concentration of the original.

Duplicates of each sample were assayed with an antiserum produced in white New Zealand rabbits as described elsewhere (D'Amato *et al.*, 1990). The serum showed cross-reactivity of less than 0.01% with synthetic porcine or rat peptide histidine isoleucinamide (PHI), extracted C-terminal extended forms of rat PHI, peptide histidine valine and peptide histidine glycine, secretin, corticotrophin releasing factor, human growth hormone releasing factor, sauvagine, helodermin, helospectin, gastric inhibitory polypeptide and glucagon in concentrations up to 1 μM.

For each assay, 0.1 ml of antiserum diluted 1:25,000 was mixed with 0.1 ml of the sample being tested or of VIP standard solution in disposable plastic tubes and incubated for 72 h at 4°C (with the exception of those used to assay non-specific binding). After incubation, 0.05 ml of an [^{125}I]-VIP solution (2000 Ci mmol^{-1}) containing approximately 5000 c.p.m. was added to each sample. After a second incubation period (48 h) under the same conditions, free VIP was separated from that bound to antibody by adding 0.5 ml of a 1% charcoal suspension in 0.2% BSA. The tubes were placed on a vortex mixer briefly and then centrifuged for 10 min at 3000 r.p.m. at 4°C. The supernatants were decanted into plastic tubes, and free and antibody-bound fractions of [^{125}I]-VIP were measured in a gamma counter.

Data were processed with the aid of a computer programmed to correct for non-specific binding. The IC_{50} of VIP standard curves averaged 12.9 (range 7.9–17.4) fmol per tube. The range of concentrations encountered in our samples was 9.3–94.6 fmol per tube.

Since the VIP-LI levels were influenced by the wet weight of the tissue (range: 120–210 mg), the results were expressed as fmol per fraction g^{-1} wet weight. The overall evoked VIP-LI release was calculated by subtracting the basal value from each value observed during and after the stimulus and adding them.

Experimental protocols

Frequency-response curves to electrical field stimulation In a first group of experiments the motor effect and the VIP-LI release induced by isolated EFS at increasing frequencies (0.25–32 Hz) were studied. In the functional experiments the strips were allowed to recover basal tone after each individual frequency of stimulation. All relaxations were expressed as areas under the curve, estimated by means of the trapezoidal rule for planar area. The results are expressed as percentages of the maximal response obtained in each strip. In the release experiments stimuli were delivered 30 min apart.

Effects of tetrodotoxin and of removal of extracellular Ca^{2+} The effect of tetrodotoxin (TTX) was studied in both functional and release experiments. The response to 16 Hz EFS was measured before and after 15 min incubation with TTX 1 μM. Sixty minutes elapsed between the two measurements. In the functional experiments, results are expressed as percentage of the relaxation induced before addition of the neurotoxin.

The effect of a Ca^{2+}-free medium was studied in release experiments only, because removal of Ca^{2+} reduced strip tone, making accurate quantification of the relaxant response impossible. Measurement of VIP-LI release induced by 16 Hz EFS was made before and 30 min after $CaCl_2$ had been removed from the Krebs solution and replaced with equimolar $MgCl_2$. Sixty minutes elapsed between the two determinations.

Drugs used

Aprotinin (Lepetit, Milan, Italy), atropine sulphate (Sigma, St Louis, U.S.A.), bacitracin (Lusofarmaco, Milan), bovine serum albumin (BSA, Sigma), ethylene diaminetetraacetic acid (EDTA, BDH, Poole, U.K.), 5-hydroxytryptamine creatinine sulphate (Sigma), isopropylnoradrenaline sulphate (Boehringer Ingelheim, Florence, Italy), nicotine (Merck, Munchen, Germany), reserpine (Sigma), sodium nitroprusside (Sigma), tetrodotoxin (Sigma), thimerosal (Sigma), highly purified synthetic vasoactive intestinal polypeptide (Bachem), [^{125}I]-VIP (Amersham, U.K.).

Drugs were dissolved in or diluted with distilled water. A stock solution of reserpine was prepared from powder (5 mg ml^{-1} dissolved in 10% ascorbic acid).

Drugs were kept on ice during each experiment and were added to the bath in a maximum volume of 0.1 ml. Atropine, 5-hydroxytryptamine and BSA (and bacitracin and aprotinin for the release experiments) were added directly to the reservoir containing Krebs solution.

Statistical analysis

Data are given as means ± s.e.mean. Responses were compared by means of the paired Student's *t* test.

Results

Frequency-response curves to electrical field stimulation

An actual tracing and the mean results from the functional experiments are shown in Figure 1. EFS (0.25–32.0 Hz)

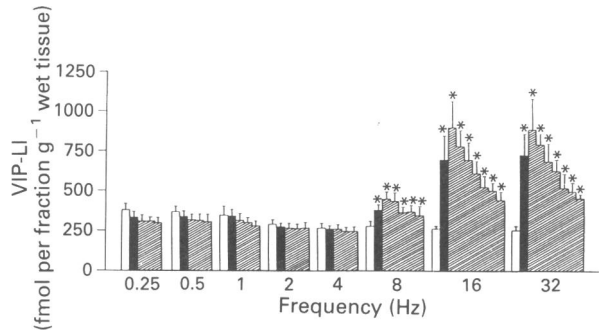

Figure 2 Release of vasoactive intestinal polypeptide-like immunoreactivity (VIP-LI) from longitudinal muscle strips of rat gastric fundus induced by electrical field stimulation (0.25–32.0 Hz, supramaximal voltage, 1 ms, pulse trains of 2 min). Each column represents mean VIP-LI (s.e.mean shown by vertical lines) found in 2 min collection fractions from 6 strips: open columns show baseline release just before electrical stimulation; closed columns show release during stimulation; hatched columns refer to post-stimulation fractions. *Values significantly different ($P < 0.05$) from baseline values.

induced frequency-dependent NANC relaxation that started almost immediately after stimulation was begun and quickly increased. Relaxation amplitude remained constant or increased slightly during the stimulation period. This pattern was more evident with higher frequencies. Recovery of basal tone after the end of stimulation was also slower when higher frequencies had been used.

Significant increases over basal levels were consistently seen in VIP-LI released in response to EFS at frequencies of 8 Hz or more (Figure 2). VIP-LI release at 16 Hz was significantly higher than that seen at 8 Hz, but no further increase was seen at 32 Hz. The VIP-LI released per unit mass of tissue during the 2 min EFS at 16 and 32 Hz was 693.7 ± 155.0 and 709.0 ± 132.0 fmol per fraction g^{-1} wet weight, respectively. VIP-LI release from the tissue continued to increase in the first fraction after the stimulation was stopped. Values returned to baseline levels within 14 min after stimulation at 8 Hz as opposed to 20 min or more after stimulation at 16 or 32 Hz (data not shown). The overall evoked VIP-LI release in response to EFS at 16 and 32 Hz was 3062.0 ± 672.8 and 3148.5 ± 545.6 fmol per fraction g^{-1} wet weight, respectively.

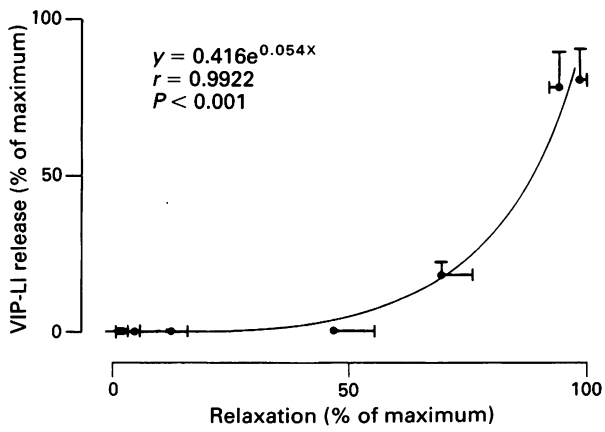

Figure 3 Relationship between relaxation (expressed as area under the curve) and overall evoked vasoactive intestinal polypeptide-like immunoreactivity (VIP-LI) release (calculated by subtracting the basal value from the value observed during and after the stimulus and adding them). Data are expressed as percentage of maximal value and given as means from 6 strips; s.e.mean shown by bars. Electrical field stimulation at 32 Hz produced maximal relaxation in 5 strips and maximal VIP-LI release in 3 strips; in the remaining strips, maximal values for both relaxation and VIP-LI release were observed at 16 Hz.

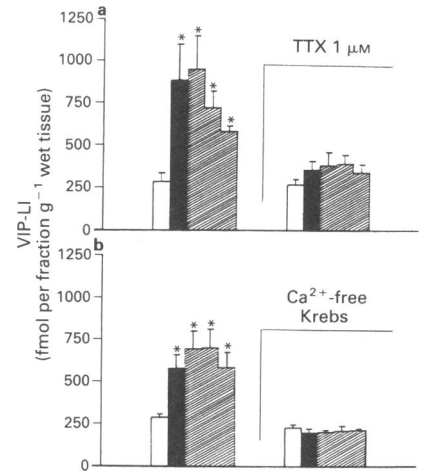

Figure 4 Release of vasoactive intestinal polypeptide-like immunoreactivity (VIP-LI) from longitudinal muscle strips of rat gastric fundus induced by electrical field stimulation (16 Hz, supramaximal voltage, 1 ms, pulse trains of 2 min) before and after addition of 1 μM tetrodotoxin (a) and before and after removal of extracellular Ca^{2+} (b). Each column represents mean VIP-LI (s.e.mean shown by vertical lines) found in 2 min collection fractions from 6 strips. Open, closed and hatched columns, as in Figure 2, refer to fractions collected before, during and after electrical stimulation. *Values significantly different ($P < 0.05$) from baseline values.

The relationship between the overall evoked VIP-LI release and muscle relaxation is illustrated in Figure 3.

Effects of tetrodotoxin and of removal of extracellular Ca^{2+}

Preliminary experiments had shown that 1 μM TTX was capable of selectively preventing relaxation induced by up to 1 μM of nicotine without affecting that induced by submaximal concentrations of VIP (3 nM), isopropylnoradrenaline (10 nM) or sodium nitroprusside (SNP, 300 nM) (data not shown). At this concentration, TTX did not affect the tone of the strips at all during the 15 min contact period. However, NANC relaxation induced by 16 Hz EFS in the presence of TTX was only 1.53 ± 0.68% ($n = 6$) of that observed in its absence. VIP-LI released in response to control periods of EFS at a single frequency of 16 Hz in this series of experiments (Figure 4) was comparable to that seen at 16 Hz in the experiments performed with increasing frequencies. Neither TTX nor the Ca^{2+}-free medium influenced baseline release of VIP, but each completely abolished the electrically stimulated release of this peptide.

Discussion

The major criteria that must be met by a neurotransmitter are 'identity of action' and 'detectable release' (Orrego, 1979). NANC inhibitory neurones of the rat gastric fundus mediate the gastric receptive relaxation during food intake (Abrahamsson, 1986) but the neurotransmitter(s) released from these neurones has (have) not been definitively established. In this study, we provide further evidence that VIP contributes to the NANC relaxation in the rat gastric fundus.

The responses to EFS that we observed were clearly NANC in nature: noradrenergic and cholinergic effects had been eliminated by reserpinization of the rats and addition of atropine to the organ baths. Details of the noradrenergic depletion that is induced with reserpine pretreatment have been described in a previous paper (Lefebvre, 1986).

In the functional experiments, muscle strip tone was increased with 5-HT. The objective here was to render the

relaxant responses more evident. The frequency-dependent relaxation that we observed was similar to that seen in previous studies (Lefebvre, 1986; D'Amato et al., 1990). Also when the tone of the rat gastric fundus strips is raised with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) NANC relaxant responses can be obtained (D'Amato & Currò, 1990; Belai et al., 1991). Preliminary experiments conducted prior to the present study showed that neither 5-HT nor $PGF_{2\alpha}$ influenced the release (basal or EFS-stimulated) of VIP-like immunoreactivity. Therefore, 5-HT was not used in our release experiments.

There is already a good deal of evidence to suggest that VIP contributes to the inhibitory NANC neurotransmission in the gastric fundus of the rat. It causes relaxation that closely mimics that induced by activation of inhibitory NANC neurones (Lefebvre, 1986). In addition, NANC relaxation is partially antagonized by VIP antiserum (De Beurme & Lefebvre, 1988; D'Amato et al., 1990; Li & Rand, 1990). Ito et al. (1990) have shown that both VIP and NANC neurone stimulation elicit increases in adenosine 3':5'-cyclic monophosphate (cyclic AMP) and guanosine 3':5'-cyclic monophosphate (cyclic GMP) and induce similar electrophysiological changes in the gastric fundas muscles of the rat.

In the present study, we have demonstrated that electrical stimulation of the inhibitory NANC neurones in longitudinal muscle strips from the gastric fundus of the rat elicits release of a VIP-like immunoreactive substance through a tetrodotoxin-sensitive, Ca^{2+}-dependent mechanism. These findings corroborate other recent findings of TTX-sensitive release of VIP-LI from a sheet of rat gastric fundus induced by EFS at 16 Hz (Belai et al., 1991) and from circular muscle strips of the rat gastric antrum with 20 Hz stimulation (Ito et al., 1990).

The maximal release of VIP-LI per unit mass of tissue during EFS occurred at 16 and 32 Hz and was approximately 700 fmol per fraction g^{-1} wet weight. Considering the size of the strips and the volume of the bath medium, this translates into a molar concentration of approximately 0.05 nM per tube. We have found that approximately 10 nM of exogenously applied VIP is necessary to produce the degree of relaxation induced by 16 Hz EFS (D'Amato et al., 1990). The amount we measured in the RIA described here is thus 200 times less than the amount of exogenous VIP required to produce the same biological effect. However, as Furness & Costa (1982) have pointed out, experiments with exogenously applied agents do not necessarily reflect the manner in which the same agent might act if it were released from axons within an organ. Neurally released transmitters may act at sites that are sequestered within the tissue and therefore less available to exogenous agents. In addition, although there is no way to calculate the concentration of VIP achieved at the synaptic level with neural stimulation, we can reasonably assume it to be higher than that detected into the bath medium, since the neuromuscular junction represents only a small fraction of the extracellular space, which is estimated in about 10% of wet tissue weight (Grider & Makhlouf, 1987).

The release of VIP-LI was observed only with stimulation frequencies of 8 Hz or more although a mechanical response could be detected with frequencies as low as 0.25 Hz. This finding conflicts with Grider & Makhlouf's (1987) observation of correlation between VIP-LI release and mechanical responses of taenia coli and gastric fundus strips from guinea-pigs at all stimulation frequencies tested. However, it has also been shown that peptide release requires higher stimulation frequencies than those needed for release of classical neurotransmitters (Bartfai et al., 1988; Agoston & Lisziewicz, 1989).

Our data suggest that the contribution of VIP to NANC relaxation is confined to that induced by higher frequency stimulation. Figure 1a clearly shows that recovery of tone after stimulation at frequencies of 8 or more Hz was slower than that observed at lower stimulation frequencies. Even when exogenously applied VIP was removed from the bath solution, recovery of baseline tone was still slow (results not shown). Thus, the mechanical response correlates with the VIP release measured, at least at higher frequencies. At lower stimulation frequencies, relaxation may be mediated by nitric oxide (NO). The L-arginine analogues, which inhibit the formation of NO from L-arginine, have been found to antagonize electrically-induced NANC relaxation of the gastric fundus of the rat, especially at lower frequencies (Li & Rand, 1990). EFS of the rat gastric fundus elicits release of a vasorelaxation agent with biological properties similar to those of NO; the substance has a short half-life and relaxes rabbit aortic rings with the epithelium removed (Boeckxstaens et al., 1991).

However, Li & Rand (1990) have recently shown that relaxation induced by EFS at 5 Hz is not completely blocked by a combination of VIP-antiserum and the L-arginine analogue L-N^G-monomethyl arginine. Other substances might, thus, be involved and PHI and ATP are two of the most likely candidates. It has been found that NANC relaxation of the rat gastric fundus is to some extent reduced by a specific PHI-antiserum (D'Amato et al., 1990). When rat gastric fundus strips were desensitized to ATP by α,β-methylene ATP, NANC relaxation decreased and rat gastric fundus sheets have also been found to release ATP upon EFS (Belai et al., 1991).

In conclusion, our finding of VIP-LI release elicited during electrical field stimulation of longitudinal muscle strips of the rat gastric fundus through a TTX-sensitive and Ca^{2+}-dependent mechanism indicates that VIP meets the 'detectable release' criterion for an inhibitory neurotransmitter in the rat gastric fundus.

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Positive inotropic and negative chronotropic effects of (−)-*cis*-diltiazem in rat isolated atria

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1 The cardiovascular effects of (−)-*cis*-diltiazem, an optical isomer of diltiazem, were studied in the isolated atrium and aortic strip. (−)-*cis*-Diltiazem (30 μM or more) increased the developed tension of the rat left atrium, while (+)-*cis*-diltiazem (1 μM or more) decreased it.

2 (−)-*cis*-Diltiazem (1 to 100 μM) decreased the rate of spontaneous beating in the right atrium as did (+)-*cis*-diltiazem.

3 The potency of the positive inotropic action of (−)-*cis*-diltiazem was almost the same as that of ouabain in the rat left atrium, but in the guinea-pig left atrium it was considerably weaker than that of ouabain.

4 In both endothelium-intact and endothelium-denuded aortic strips, (−)-*cis*-diltiazem relaxed the Ca^{2+}-induced contraction. In the endothelium-intact rat aortic strip depolarized by 15 mM KCl, Bay K 8644, a calcium channel agonist, increased the contractile force, whereas (−)-*cis*-diltiazem did not.

5 These results indicate that (−)-*cis*-diltiazem has a positive inotropic action in isolated atria in rats and guinea-pigs, but the mode of positive inotropic action of (−)-*cis*-diltiazem is different from that of ouabain or Bay K 8644.

Keywords: (−)-*cis*-Diltiazem; (+)-*cis*-diltiazem; positive inotropic action; negative chronotropic action; atrium; aorta

Introduction

Diltiazem, a 1,4-benzothiazepine derivative having L-type calcium channel blocking action, has four isomers; (+)-*cis*-, (−)-*cis*-, (+)-*trans*-, and (−)-*trans*-diltiazem. The pharmacological action of *trans*-isomers of the derivative, however, are markedly weaker than that of *cis*-isomers; according to Nagao *et al.* (1972), the (±)-*trans*-isomer is 0.03 times as potent as the (+)-*cis*-isomer in dilating coronary vessels. The pharmacologically active compond is the (+)-*cis* form ((+)-*cis*-diltiazem, i.e. diltiazem), which has been widely used for treatment of hypertension and angina pectoris. (−)-*cis*-Diltiazem, one of the optical isomers of diltiazem, is less potent in blocking the calcium channel than (+)-*cis*-diltiazem, especially in vascular smooth muscles (Nagao *et al.*, 1982). The chemical structures of (+)-*cis*- and (−)-*cis*-diltiazem are shown in Figure 1. If the effect of (−)-*cis*-diltiazem on various tissues is invariably due to the calcium channel blockade, it would be weaker than that of (+)-*cis*-diltiazem without exception. Nevertheless, there are some effects of (−)-*cis*-diltiazem which are similar or superior to those of (+)-*cis*-diltiazem in potency. For example, (−)-*cis*-diltiazem inhibits platelet aggregation and microviscosity of both erythrocyte ghosts and liposomes to a similar degree to (+)-*cis*-diltiazem (Kiyomoto *et al.*, 1983; Sasaki *et al.*, 1984). This suggests that either (+)-*cis*- or (−)-*cis*-diltiazem has an action, which may not be related to its calcium channel blocking action. There is another example; (−)-*cis*-diltiazem inhibits guanosine 3′:5′-cyclic monophosphate (cyclic GMP)-induced Ca^{2+} release more effectively than does (+)-*cis*-diltiazem in the bovine rod outer segment (Koch & Kaupp, 1985). Therefore, it seems that there are actions of (+)-*cis*- and (−)-*cis*-diltiazem, which cannot be interpreted only by calcium channel blockade.

In the present study, we investigated the effects of (−)-*cis*-diltiazem on myocardial contractile force in the isolated left atrium and on the rate of spontaneous beating in the isolated right atrium of the rat.

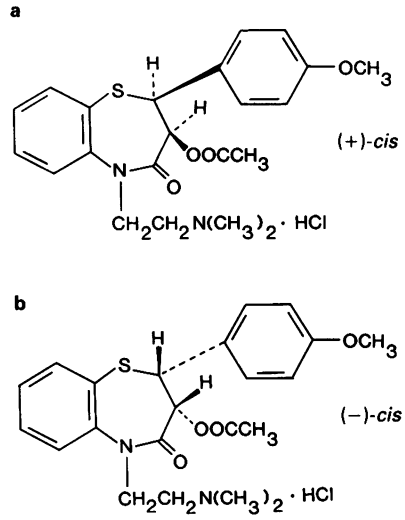

Figure 1 Chemical structures of (+)-*cis*-diltiazem (a) and (−)-*cis*-diltiazem (b).

Methods

Animals

Male Sprague-Dawley rats (270–350 g body weight) and male Hartley guinea-pigs (450–550 g body weight) were used for all the experiments.

Atrial muscle preparation

Rats were anaesthetized with an i.p. injection of sodium pentobarbitone (50 mg kg^{-1}), and the heart was quickly removed. Guinea-pigs were killed by cervical dislocation and the heart was rapidly removed. Whole left atrium and whole right atrium were cut perpendicularly to the axis of the heart,

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and each atrium was suspended in an organ bath (20 ml) containing Krebs-bicarbonate solution (KBS) having the following composition (in millimolar); NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, and glucose 10.0. The organ bath was maintained at 37°C and equilibrated continuously with a gas mixture of 95% O_2 and 5% CO_2. The resting tension of each atrium was adjusted to 500 mg and the tissues were equilibrated for at least 60 min before starting the experiments. The resting tension was re-adjusted before addition of drugs. The exchange of solution was accomplished by allowing solutions to drain from the bottom of the chamber. At 30 min intervals the bath medium was exchanged with fresh KBS. The left atrium was driven electrically by rectangular pulses with a frequency of 1 Hz, a duration of 3 ms and a voltage of 1.5 fold threshold through two parallel platinum electrodes. The isometric tension was measured and recorded by a force-displacement transducer (SB-1T-H, Nihhon Koden, Tokyo, Japan), an amplifier (Strain Amplifier, CARRI-6, San-ei, Tokyo, Japan), and a pen recorder (Recti-Horiz-8K, San-ei, Tokyo, Japan).

In some experiments, the rate of spontaneous beating of the right atria was measured, being defined as heart rate, in order to assess the chronotropic action of (−)-*cis*-diltiazem and (+)-*cis*-diltiazem. Cumulative concentration-response curves to (−)-*cis*-diltiazem, (+)-*cis*-diltiazem and ouabain were constructed by stepwise addition of these drugs. The concentration of the drugs was increased only after the previous addition of the drug had produced the maximal response.

In some experiments with rat left atria, 3×10^{-5} M (−)-*cis*-diltiazem was added first, and then 3×10^{-5} M ouabain was added and finally 3×10^{-6} M (+)-*cis*-diltiazem was added; or 3×10^{-5} M ouabain was added first, and then 3×10^{-5} M (−)-*cis*-diltiazem was added, and finally 3×10^{-6} M (+)-*cis*-diltiazem was added. In another series of experiments with rat left atria, 5×10^{-5} M (−)-*cis*-diltiazem was added, and then atenolol, bunazosin, (+)-*cis*-diltiazem or (±)-verapamil was added to the bath medium, in order to study the effects of these drugs on the (−)-*cis*-diltiazem-induced response.

Aortic smooth muscle preparation

Rats (270–350 g) were killed by cervical dislocation and exsanguination. The thoracic aorta was removed quickly and cleaned of connective tissues in ice-cold KBS. The aorta was cut into a spiral strip 3 to 4 mm wide and 20 to 25 mm length. The vascular endothelium was denuded intentionally, unless otherwise noted, by gently rubbing the intimal surface of the artery with a wooden stick. Where appropriate, endothelial integrity was assessed pharmacologically as described by Furchgott & Zawadzki (1980); the presence of a functional endothelium was confirmed by the ability of acetylcholine (1×10^{-7} to 1×10^{-5} M) to produce concentration-dependent relaxation of tissues precontracted with noradrenaline (1×10^{-6} M). Aortic strips were suspended in a 50 ml organ bath containing KBS at 37°C equilibrated continuously with a gas mixture of 95% O_2 and 5% CO_2, and were equilibrated for 60 min under the resting tension of 1 g. The isometric tension of the aortic strips was measured during the whole course of the study. Following the equilibration period, the aortic strips were re-adjusted so that the resting tension was 1 g, and then each aortic strip was initially contracted by the replacement of normal KBS by 80 mM KCl-KBS, in which KCl was substituted for NaCl so as to maintain osmolarity. After the maximum contraction was observed, the tissue was washed with normal KBS repeatedly and then experiments were started.

In the first series of experiments, we examined the effects of (−)-*cis*-diltiazem and (+)-*cis*-diltiazem on the calcium-induced contraction in 40 mM KCl-depolarized, endothelium-intact or endothelium-denuded aortic strips. The protocol involved incubation of tissue in the calcium-free KBS containing EDTA-2Na (0.5 mM) for 60 min before the addition of calcium-free 40 mM KCl-KBS. During the incubation period in the calcium-free KBS, tissues were washed several times with the calcium-free KBS. Then, the tissues were exposed to the calcium-free 40 mM KCl-KBS and the transient contraction was produced. After a plateau of contraction was obtained, the resting tension was re-adjusted to 1 g. Subsequently the tissues were incubated with the calcium-free 40 mM KCl-KBS for 10 min, and then calcium 2.5 mM (final concentration) was added to the tissues to produce the calcium-induced contraction. After the plateau of the contraction was obtained (usually 25–30 min after the addition of calcium), (−)-*cis*-diltiazem or (+)-*cis*-diltiazem was added to the tissues cumulatively in order to examine their ability to modify the calcium-induced contraction. The concentration of (−)-*cis*-diltiazem or (+)-*cis*-diltiazem was increased stepwise only after previous addition of the drug produced a steady-state response. In the second series of experiments, in which the effects of (−)-*cis*-diltiazem and Bay K 8644 were compared with those of (−)-*cis*-diltiazem, the endothelium-intact or endothelium-denuded aortic strips partially depolarized by 15 mM KCl, were used. After tissues were exposed to 15 mM KCl-KBS, (−)-*cis*-diltiazem, (+)-*cis*-diltiazem or Bay K 8644 was added to the bath medium. The vehicle for Bay K 8644 (ethanol, 0.2 mM) did not affect the 15 mM KCl-induced response in either endothelium-intact or endothelium-denuded aortic strips (data not shown).

Drugs

(−)-*cis*-Diltiazem and (+)-*cis*-diltiazem were a kind gift from Tanabe Pharmaceutical Co. Ltd. (Osaka, Japan). Ouabain (Merck, U.S.A.), acetylcholine chloride (Ovisort for Injection, Daiichi-Seiyaku Co. Ltd., Osaka, Japan), (±)-noradrenaline bitartarate (Tokyo Kasei Co. Ltd., Tokyo, Japan), (±)-verapamil hydrochloride (Eisai Co. Ltd., Tokyo, Japan), atenolol hydrochloride (ICI-Pharma. Co. Ltd., Osaka, Japan), bunazosin hydrochloride (Eisai Co. Ltd. Japan), and Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate, Funakoshi Yakuhin, Osaka, Japan) were used in the present study. The stock solution of Bay K 8644 (1×10^{-3} M in absolute ethanol) was diluted with distilled water immediately before use. Other drugs were dissolved in distilled water.

Statistical analysis

All data are expressed as mean ± s.e.mean. Difference between values was analyzed for significance by Student's paired *t* test. A *P* value of 0.05 or less was considered significant.

Results

Atrial muscle experiments

Inotropic and chronotropic effects of (−)-cis- *and* (+)-cis-*diltiazem* The inotropic effect of (−)-*cis*-diltiazem was studied in the electrically stimulated left atrium in rats and guinea-pigs, and the chronotropic effect was studied in the spontaneously beating right atrium in rats. After equilibration of the atrium for 60 min, the developed tension in the electrically stimulated rat left atrium was about 200 mg, and heart rate was about 310 beats min^{-1} in the right atrium. Addition of (−)-*cis*-diltiazem at a concentration of 3×10^{-5} M increased the developed tension gradually without affecting the resting tension, indicating that (−)-*cis*-diltiazem produced a positive inotropic effect, which reached maximum within 5 to 6 min after the addition and remained unchanged for at least 30 min in the rat left atrium (Figure 2). (−)-*cis*-Diltiazem produced a positive inotropic effect depending on the concentration of the drug (Figure 3a). On the other hand, (+)-*cis*-diltiazem in concentrations ranging from 1×10^{-6} M to 1×10^{-4} M produced only a negative inotropic effect, concentration-dependently (Figure 3a). Nevertheless, both

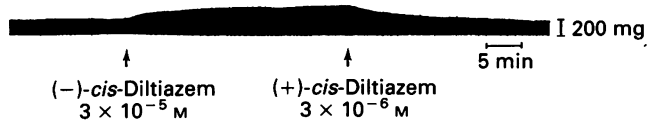

Figure 2 A representative tracing showing the positive inotropic action of (−)-*cis*-diltiazem (3×10^{-5} M) and the negative inotropic action of (+)-*cis*-diltiazem (3×10^{-6} M) in the electrically stimulated rat left atrium.

(−)-*cis*-diltiazem (3×10^{-6} to 1×10^{-4} M) and (+)-*cis*-diltiazem (3×10^{-7} to 1×10^{-5} M) had a negative chronotropic effect in the rat right atrium, although the concentration of (−)-*cis*-diltiazem required to produce a similar degree of negative chronotropic effect was higher than that of (+)-*cis*-diltiazem (Figure 3b). These results clearly indicate that (−)-*cis*-diltiazem, in contrast to (+)-*cis*-diltiazem, has a positive inotropic action, although both (+)-*cis*- and (−)-*cis*-diltiazem have negative chronotropic actions.

The positive inotropic action of (−)-*cis*-diltiazem was then compared with that of ouabain in rat left atria and guinea-pig left atria. In the rat left atrium, ouabain in concentrations ranging from 1×10^{-5} M to 1×10^{-4} M produced a positive inotropic effect, the potency of the positive inotropic action being essentially the same as that of (−)-*cis*-diltiazem (Figure 4a). It is generally accepted that the positive inotropic response to ouabain of the rat heart is less sensitive than that of the guinea-pig heart (Akera & Brody, 1989), and therefore, we carried out the same experiment with ouabain, (−)-*cis*-diltiazem and (+)-*cis*-diltiazem in the guinea-pig left atrium.

(−)-*cis*-Diltiazem produced a positive inotropic effect in the guinea-pig left atrium as well, the degree of the positive inotropic effect being similar to that in the rat left atrium. The positive inotropic response to ouabain at a concentration of 3×10^{-7} M in the guinea-pig left atrium, however, was greater than that in the rat left atrium (Figure 4b). (+)-*cis*-Diltiazem produced a negative inotropic action in both guinea-pig and rat left atria, although the negative inotropic action of (+)-*cis*-diltiazem in the guinea-pig left atrium was slightly greater than that in the rat left atrium (Figure 4).

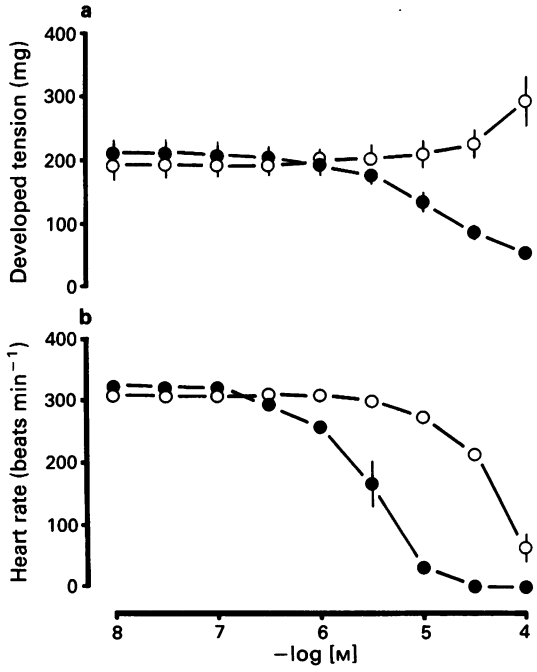

Figure 3 Effects of (−)-*cis*-diltiazem (○) and (+)-*cis*-diltiazem (●) on developed tension in the rat left atrium (a) and on heart rate in the spontaneously beating rat right atrium (b).

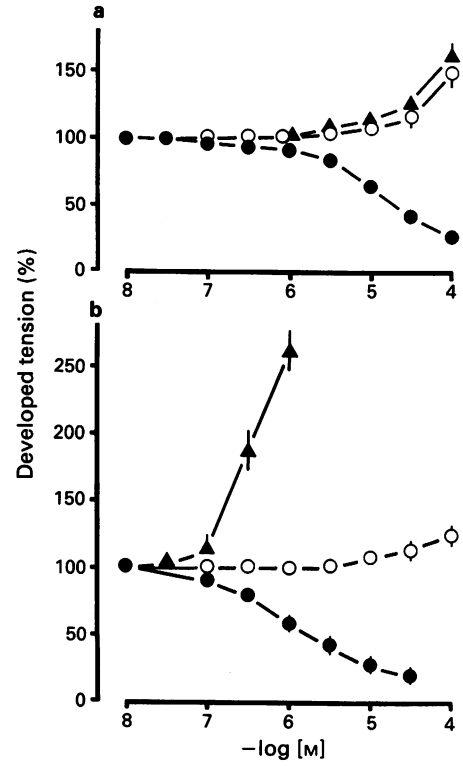

Figure 4 Effects of (−)-*cis*-diltiazem (○), (+)-*cis*-diltiazem (●) and ouabain (▲) on the developed tension in the rat left atrium (a) and in the guinea-pig left atrium (b).

(−)-cis-*Diltiazem, ouabain and* (+)-cis-*diltiazem interactions in the rat left atrium* In other experiments using rat atria, we investigated whether the (−)-*cis*-diltiazem-induced positive inotropic response was modified by treatment with ouabain or (+)-*cis*-diltiazem. The positive inotropic response to ouabain (3×10^{-5} M) was not modified by pretreatment with (−)-*cis*-diltiazem (3×10^{-5} M), but the (−)-*cis*-diltiazem- plus ouabain-induced positive inotropic response was completely abolished by the addition of (+)-*cis*-diltiazem, 1×10^{-5} M (Figure 5a). The positive inotropic response to (−)-*cis*-diltiazem was not influenced by pretreatment with ouabain, but the ouabain- plus (−)-*cis*-diltiazem-induced positive inotropic response was completely abolished by (+)-*cis*-diltiazem (Figure 5b).

Effects of atenolol, bunazosin, verapamil and (+)-cis-*diltiazem on the positive inotropic response to* (−)-cis-*diltiazem* The positive inotropic response to (−)-*cis*-diltiazem (5×10^{-5} M) of the rat left atrium was not modified by the addition of atenolol (1×10^{-5} to 1×10^{-4} M) or bunazosin (1×10^{-5} to 1×10^{-4} M) (Figure 6). In contrast, the (−)-*cis*-diltiazem-induced positive inotropic response was inhibited by the addition of (+)-*cis*-diltiazem 1×10^{-5} M or (±)-verapamil 1×10^{-5} M (Figure 6). In rat left atria, the addition of (+)-*cis*-diltiazem 3×10^{-6} M for 10 min reduced the developed tension (by 26%), and the concentration-response curve to (−)-*cis*-diltiazem in the presence of (+)-*cis*-diltiazem was not greatly different from that in the absence of (+)-*cis*-diltiazem (control) except for depression of the maximum response (Figure 7).

Aortic smooth muscle experiments

Effects of (−)-cis- *and* (+)-cis-*diltiazem on Ca-induced contraction* The contraction of vascular smooth muscle induced by high concentrations of KCl is thought to be evoked by influx of calcium during activation of the voltage-dependent calcium channel and by subsequent release of

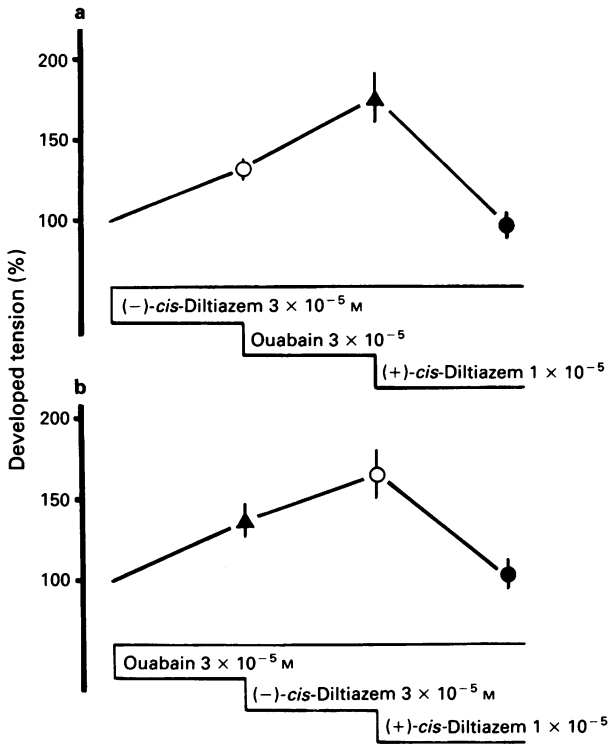

Figure 5 Effects of (−)-*cis*-diltiazem (3×10^{-5} M), ouabain (3×10^{-5} M) and (+)-*cis*-diltiazem (1×10^{-5} M) on the developed tension in the rat left atrium. In (a), (−)-*cis*-diltiazem was added first, and then ouabain, and finally (+)-*cis*-diltiazem. In (b), ouabain was added first, and then (−)-*cis*-diltiazem followed by (+)-*cis*-diltiazem.

calcium from the sarcoplasmic reticulum. In fact, the contraction induced by high concentrations of KCl of vascular smooth muscle is sensitive to organic calcium channel blockers. In the first series of experiments, we examined the

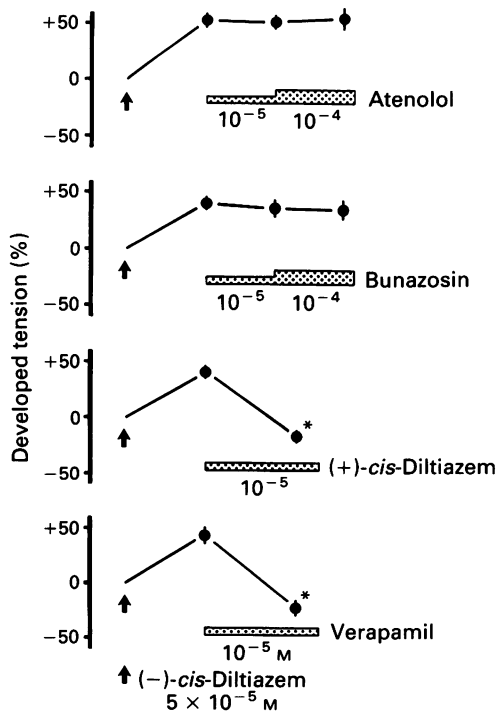

Figure 6 Effects of atenolol, bunazosin, (+)-*cis*-diltiazem and verapamil on the positive inotropic response to (−)-*cis*-diltiazem (5×10^{-5} M) in the rat left atrium. * Significantly different when compared to the value before addition of atenolol, bunazosin, (−)-*cis*-diltiazem or verapamil.

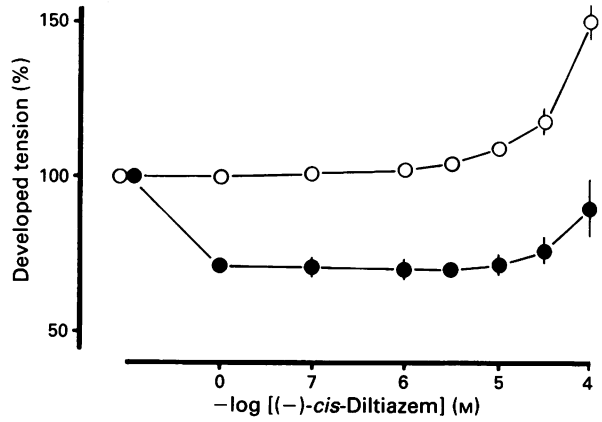

Figure 7 Effect of pretreatment with (+)-*cis*-diltiazem (3×10^{-6} M) on the positive inotropic response to (−)-*cis*-diltiazem in the rat left atrium. The inotropic response to (−)-*cis*-diltiazem in the absence (○) or presence (●) of (+)-*cis*-diltiazem.

effect of (−)-*cis*- and (+)-*cis*-diltiazem on the calcium-induced contraction in 40 mM KCl-depolarized rat aorta. Cumulative administration of (−)-*cis*-diltiazem or (+)-*cis*-diltiazem to aortic strips that had been depolarized by 40 mM KCl and contracted by calcium (2.5 mM), exerted a concentration-dependent relaxation (Figure 8). The relaxation produced by (−)-*cis*-diltiazem was independent of the presence of the vascular endothelium. The $-\log [EC_{50}]$ values of (−)-*cis*-diltiazem for relaxation of the calcium-induced contraction were 4.81 and 4.79 for endothelium-intact and endothelium-denuded rat aorta, respectively, these EC_{50} values being about 20 times the EC_{50} values for (+)-*cis*-diltiazem (Table 1).

Effects of (−)-cis-*diltiazem*, (+)-cis-*diltiazem and Bay K 8644 on 15 mM KCl depolarized aortic strips* In the second series of experiments, the effect of (−)-*cis*-diltiazem on the aortic strips depolarized by 15 mM KCl was compared with that of Bay K 8644 or (+)-*cis*-diltiazem. Exposure of the endothelium-intact aortic strip to 15 mM KCl-KBS produced a slight contraction of the strip (an increase of 5 to 20 mg in the developed tension), and the addition of (−)-*cis*-diltiazem (1×10^{-5} to

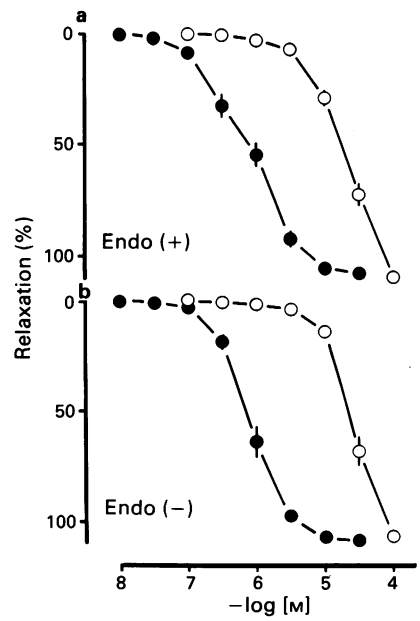

Figure 8 Concentration-response (relaxation) curves to (−)-*cis*-diltiazem (○) and (+)-*cis*-diltiazem (●) in the endothelium-intact (a) and endothelium-denuded (b) rat aortic strips that had been depolarized by 40 mM KCl and contracted by $CaCl_2$.

Table 1 $-\log EC_{50}$ values for (−)-*cis*-diltiazem and (+)-*cis*-diltiazem to relax (2.5 mM) $CaCl_2$-induced contraction in (40 mM) KCl-depolarized rat aortic strips

	Endothelium	
	intact	*denuded*
(−)-*cis*-Diltiazem	4.81 ± 0.09 (5)	4.79 ± 0.11 (5)
(+)-*cis*-Diltiazem	6.18 ± 0.10 (4)	6.25 ± 0.12 (4)

All data are expressed as mean ± s.e.mean. Number of experiments in parentheses.

3×10^{-5} M) to the tissues produced relaxation instead of contraction (Figure 9a). In the endothelium-denuded aortic strip, exposure to 15 mM KCl-KBS resulted in a considerable degree of contraction of the strip (60–70% of the contraction induced by 80 mM KCl). The addition of (−)-*cis*-diltiazem to the endothelium-denuded aortic strip, which had been contracted by 15 mM KCl-KBS, produced relaxation of the strip (Figure 9b). The difference in the contractile response to 15 mM KCl between the endothelium-intact and endothelium-denuded tissues can be explained in part if in the former, there is basal release of endothelium-derived relaxing factor from the intact endothelial cells (Furchgott, 1983; 1984) and also release of the endothelium-derived hyperpolarizing factor from the endothelium in response to KCl (Komori & Suzuki, 1987; Chen *et al.*, 1988; Vanhoutte & Katusic, 1988). (+)-*cis*-Diltiazem at a concentration of 1×10^{-6} M relaxed completely the endothelium-denuded aortic strip that had been contracted by 15 mM KCl-KBS. The relaxation induced by (+)-*cis*-diltiazem was partially reversed by the subsequent addition of calcium (Figure 9c). In contrast to (−)-*cis*- or (+)-*cis*-diltiazem, Bay K 8644 at a concentration of 2×10^{-6} M produced a marked contractile response in the endothelium-intact aortic strip depolarized by 15 mM KCl. The degree of the contraction induced by Bay K 8644 (2×10^{-6} M) was almost the same as that induced by 80 mM KCl in the endothelium-intact aortic strip. Furthermore, the addition of (−)-*cis*-diltiazem (1×10^{-5} to 3×10^{-5} M) after the addition of Bay K 8644 exerted relaxation of the aortic strip, and the (−)-*cis*-diltiazem-induced relaxation was reversed by the addition of calcium (Figure 9d).

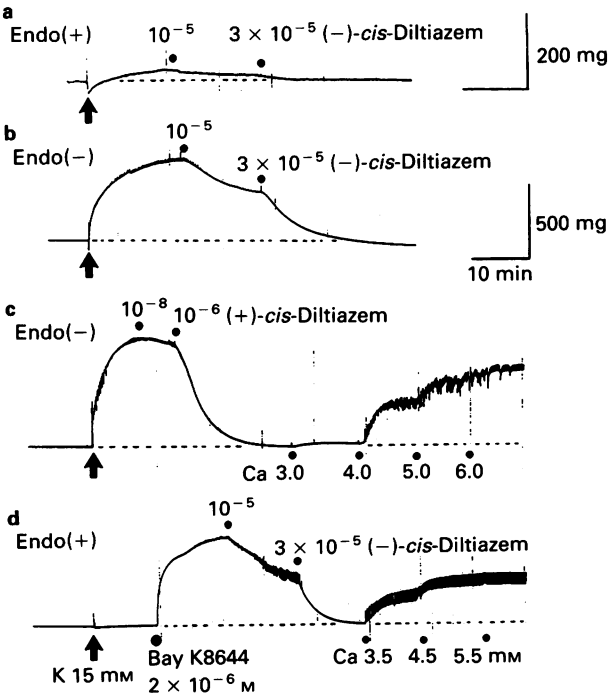

Figure 9 Effects of (−)-*cis*-diltiazem, (+)-*cis*-diltiazem and Bay K 8644 on the 15 mM KCl-depolarized, endothelium-intact (a and d) and endothelium-denuded (b and c) rat aortic strips.

Discussion

The results show that (−)-*cis*-diltiazem but not (+)-*cis*-diltiazem had a positive inotropic effect in the electrically stimulated left atrial preparation, and that it also exerted a negative chronotropic effect in the isolated, spontaneously beating rat right atrium at the same concentration-range as that producing the positive inotropic effect. On the other hand, (+)-*cis*-diltiazem had both negative inotropic and negative chronotropic actions in the rat left and the rat right atrium, respectively. These findings are supported by our previous results showing that (−)-*cis*-diltiazem elevated peak aortic pressure at the same concentrations used in the present experiments (1×10^{-5} to 3×10^{-5} M) in the rat isolated, working heart (Nasa *et al.*, 1990). (−)-*cis*-Diltiazem reduced heart rate in the rat perfused, working heart (Nasa *et al.*, 1990) as demonstrated in the rat isolated right atrium in the present study.

It has been shown that calcium channel blockers have a weak positive inotropic action in lower concentrations and a negative inotropic action in higher concentrations. In fact, low concentrations of (+)-*cis*-diltiazem increased the developed tension, while high concentrations of the drug decreased it in the blood-perfused canine papillary muscle (Himori *et al.*, 1976). The weak positive inotropic effect of (+)-*cis*-diltiazem is considered to be due to an action that is unrelated to calcium channel blockade. It is possible, therefore, that the positive inotropic effect of (−)-*cis*-diltiazem is also due to an action unrelated to calcium channel blockade. Nevertheless, the positive inotropic effect of (−)-*cis*-diltiazem may not be due to an action unrelated to calcium channel blockade for the following reasons: (1) (−)-*cis*-Diltiazem exerted a negative chronotropic response at the same concentration used in the present study in the rat right atrium, and the negative chronotropic responses to both (+)-*cis*-diltiazem and (−)-*cis*-diltiazem were closely related to their calcium channel blocking action in the rat aortic strip. (2) The potency of the positive inotropic action of (−)-*cis*-diltiazem was similar to that of ouabain in the rat left atrium. It is suggested, therefore, that the concentration of (−)-*cis*-diltiazem which exerted the positive inotropic response was sufficient to produce calcium channel blocking action in the right atrium and the aortic strip, and probably in the left atrium. Calcium channel blockade resulted in the negative inotropic and negative chronotropic effects as seen in the effect of (+)-*cis*-diltiazem. It is curious, therefore, that (−)-*cis*-diltiazem exerted the positive inotropic action in the left atrium.

There are recent publications (Van Amsterdam *et al.*, 1987; Punt *et al.*, 1988a), which have shown that calcium channel inhibitors (such as nifedipine, felodipine, verapamil and (+)-*cis*-diltiazem) produced a positive inotropic effect in isolated, perfused hearts. According to Van Amsterdam *et al.* (1987) and Punt *et al.* (1988a), however, the positive inotropic effect of nifedipine, verapamil and (+)-*cis*-diltiazem is due to an indirect effect through increased systolic ventricular pressure caused by vasodilatation, and the mechanism of the positive inotropic effects of these compounds can be explained by the 'garden-hose-effect' (Arnold *et al.*, 1968; Feigl, 1983). According to Punt *et al.* (1988a), coronary vasodilatation caused by calcium channel blockers results in an increased fibre tension of the myocardium and, by the Frank-Starling mechanism, in an increase of the myocardial contractile strength. The direct effect of calcium channel blockers on myocardial contractile force, therefore, should be examined in myocardial strip preparations stimulated electrically at a fixed rate. Pettersson *et al.* (1987) and Drake-Holland *et al.* (1987) have demonstrated that low concentrations of dihydropyridine derivatives produce calcium antagonistic effects in vascular smooth muscle and also calcium agonistic effects in the heart. Punt *et al.* (1988b) have suggested that there are two binding sites for

(+)-*cis*- and(−)-*cis*-diltiazem; one is responsible for calcium agonism and the other for calcium antagonism, although only (+)-*cis*-diltiazem has the calcium channel blocking action. In fact, (−)-*cis*-diltiazem does not possess the calcium channel blocking property in concentrations up to 1×10^{-5} M in guinea-pig papillary muscle cells (Punt *et al.*, 1988a). (−)-*cis*-Diltiazem may not have the calcium agonistic action as shown in Figure 8. The fact that high concentrations of (−)-*cis*-diltiazem were necessary to evoke the positive inotropic effect in rat left atria, suggests that there is one more specific binding site for (−)-*cis*-diltiazem that may be responsible for the positive inotropic effect. The results also indicate that the positive inotropic response to (−)-*cis*-diltiazem is due to stimulation of neither α_1- nor β_1-adrenoceptors in atria, since bunazosin and atenolol, specific antagonists of α_1- and β_1-adrenoceptors, were ineffective in modifying the positive inotropic effect of (−)-*cis*-diltiazem.

(+)-*cis*-Diltiazem has an inhibitory action on Na^+-K^+ ATPase in erythrocyte ghosts (Sasaki *et al.*, 1984), this action being the action of (+)-*cis*-diltiazem unrelated to calcium channel blockade. Because the positive inotropic action of cardiac glycosides has been considered to be due to inhibition of the sarcolemmal Na^+-K^+ ATPase, we attempted to compare the positive inotropic effect of (−)-*cis*-diltiazem with that of ouabain. The magnitude of the positive inotropic action of (−)-*cis*-diltiazem was similar with that of ouabain in the rat left atrium, and was not modified by ouabain. In guinea-pig left atria, however, the positive inotropic action of ouabain was greater than that of (−)-*cis*-diltiazem. The difference in the sensitivity to ouabain between rat and guinea-pig atria is considered to be due to a difference in the affinity of the receptor for cardiac glycosides between rat and guinea-pig myocardial cells; Tobin & Brody (1972) have demonstrated that the sarcolemmal membrane of the rat heart has only a small fraction of the Na^+-K^+ ATPase that has high affinity for cardiac glycoside. Therefore, these findings and considerations lead to a view that the underlying mechanism of positive inotropic action of (−)-*cis*-diltiazem is not due to ouabain-like inhibition of the sarcolemmal Na^+-K^+ ATPase.

It is also possible to assume that (−)-*cis*-diltiazem binds to the site responsible for calcium agonism to produce an action as a calcium agonist. The present study, however, did not support this assumption, because (−)-*cis*-diltiazem, unlike Bay K 8644 which is a dihydropyridine calcium agonist, did not produce the calcium agonistic action in the rat aortic strip (Figure 8). Bay K 8644 binds to the calcium channel that is open and increases the calcium influx (Bechem & Schramm, 1987), and therefore it produces contraction of the vascular smooth muscle and increases contractility of the heart without affecting cyclic AMP content (Skomedal *et al.*, 1988). Nevertheless, Bay K 8644 produces only a weak positive inotropic action in isolated rat left atria (Finet *et al.*, 1985), while in the partially depolarized rat aortic strip it produced a large contractile response as shown in the present study. The negative chronotropic action induced by (−)-*cis*-diltiazem and (+)-*cis*-diltiazem (Figure 3b) was parallel with the relaxing action of the drugs on the calcium-induced contraction in the aortic smooth muscle depolarized by 40 mM KCl (Figure 8). Therefore, the negative chronotropic action of (−)-*cis*-diltiazem may be related to the weak calcium channel blocking action of the drug.

According to Koch & Kaupp (1985), release of endogenous calcium caused by cyclic GMP in the outer segments of rod in the bovine retina is inhibited by organic calcium channel blockers, and (−)-*cis*-diltiazem is more effective than (+)-*cis*-diltiazem in this respect. Furthermore, the light-regulated current in the rod photoreceptors is activated by cyclic GMP, and this activation is inhibited by (−)-*cis*-diltiazem but not by (+)-*cis*-diltiazem (Stern *et al.*, 1986). They suggested the existence of a binding site which is specific for (−)-*cis*-diltiazem in rod cells (Stern *et al.*, 1986). The site specific for (−)-*cis*-diltiazem in the myocardial cells (if it exists) may not be the calcium channel but another site, which is responsible for the positive inotropic action of (−)-*cis*-diltiazem.

Calcium channel blockers, which include drugs having chemically diverse structures, are potent modulators of functional activity of cardiac and vascular smooth muscle. These drugs reduce vascular resistance by blocking Ca^{2+} influx via calcium channels, resulting in decreased systemic blood pressure and decreased myocardial contractility, the latter action limiting their therapeutic use in patients with congestive heart failure. Our previous results demonstrated that (−)-*cis*-diltiazem as well as (+)-*cis*-diltiazem exerted a cardioprotective effect in the ischaemic-reperfused, rat isolated heart (Nasa *et al.*, 1990). Therefore, a calcium channel blocker having cardiotonic activity, like (−)-*cis*-diltiazem, provides a more effective method for treating patients with heart failure.

In summary, (−)-*cis*-diltiazem exhibits positive inotropic and negative chronotropic effects in atrial preparations. The precise mechanism of the positive inotropic action of (−)-*cis*-diltiazem is unclear, but it is certainly different from that of ouabain or Bay K 8644.

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Dopamine release and metabolism in the rat frontal cortex, nucleus accumbens, and striatum: a comparison of acute clozapine and haloperidol

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1 The effects of the typical and typical neuroleptic agents clozapine (CLZ) (2.5–20 mg kg^{-1}, i.p.) and haloperidol (Hal) (0.05–1.0 mg kg^{-1}), were compared on dopamine release and metabolism in the rat prefrontal cortex (PFC), nucleus accumbens (ACC) and striatum (ST). Dopamine release was estimated by measuring the steady-state concentration of 3-methoxytyramine (3-MT) and the level of 3-MT 10 min after pargyline (3-MT accumulation); dopamine metabolism was evaluated from the steady-state concentrations of its acidic metabolites.

2 Both drugs increased 3-MT accumulation in the PFC in a dose-dependent manner. In contrast to Hal, CLZ failed to increase 3-MT accumulation in the ACC or ST. The ST was the region most sensitive to Hal in terms of 3-MT accumulation and, by inference, dopamine release.

3 Both CLZ and Hal dose-dependently elevated the concentrations of 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in all 3 brain regions studied. The ACC appears to be the region most sensitive to these drugs in terms of changes in the levels of HVA.

4 The result of the present investigations suggest measurements of 3-MT production in the 3 brain regions analysed might be a useful and simple pharmacological tool in the search for atypical neuroleptic drugs with a selectivity of action for the cortical systems.

Keywords: Dopamine release and metabolism; neuroleptics; haloperidol; clozapine; cortex; nucleus accumbens; striatum

Introduction

The recent demonstration (Kane *et al.*, 1988) that clozapine (CLZ) helps a significant percentage of schizophrenic patients who have responded poorly to the typical neuroleptics has generated a great deal of interest. Clozapine has been called an 'atypical' neuroleptic because it produces few extrapyramidal side effects and does not appear to cause tardive dyskinesia (Berzewski *et al.*, 1969; Angst *et al.*, 1971; Honigfeld *et al.*, 1984; Meltzer, 1989; Farde *et al.*, 1989). Other properties that distinguish CLZ from typical neuroleptics such as haloperidol (Hal) is the failure to produce long-lasting elevation of plasma prolactin levels, failure to induce dopamine D_2 receptor supersensitivity in the striatum after chronic treatment, and inability to inhibit markedly apomorphine-induced circling behaviours in rats with unilateral lesions of their substantia nigra (Coward *et al.*, 1989).

Several hypotheses have been put forward as to why CLZ is capable of producing clinical improvement when other neuroleptics have failed. These notions involve the effects of CLZ on 5-hydroxytryptaminergic (Meltzer, 1989), cholinergic (Stille *et al.*, 1971; Snyder *et al.*, 1974) and adrenergic systems (Bartholini *et al.*, 1972; McMillan & Shore, 1978; Chiodo & Bunney, 1985) in the brain, especially in the limbic dopamine systems (Anden & Stock, 1973; Chiodo & Bunney, 1985). However, none of these systems apparently play key roles in mediating the behavioural effects of atypical neuroleptics in the experimental animal (Coward *et al.*, 1989). There is however, a number of recent reports that implicate 5-HT_3 receptors in animal models of psychosis (Costall *et al.*, 1988) and show a relatively high affinity of CLZ for these receptors (Barnes *et al.*, 1990). It is therefore conceivable that CLZ mediates some of its antipsychotic effects via 5-HT_3 receptors.

To examine how biochemically dopaminergic neuronal function is affected by neuroleptics, we have compared the effects of CLZ with those of a representative typical neuroleptic, Hal, on indices of dopamine release and metabolism in three brain regions implicated in mediating some of the antipsychotic and side effects of the drugs. These regions include the striatum (ST), nucleus accumbens (ACC) and prefrontal cortex (PFC). Three-methoxytyramine (3-MT) accumulation was used as a measure of dopamine release (Kehr, 1981; Waldmeier *et al.*, 1981; Wood & Altar, 1988) and dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were used as indices of dopamine metabolism and turnover (Westerlink, 1985). Since changes in 3-MT steady-state concentration generally follow changes in 3-MT accumulation (but not *vice versa*) (Wood & Altar, 1988), and since 3-MT steady-state is simpler to assay and perhaps more representative of the natural state of dopamine release (Kehr, 1981; Waldmeier *et al.*, 1981; Wood & Altar, 1988), we have also measured the concentration of 3-MT in the brain after Hal and CLZ.

Methods

Male, Sprague-Dawley rats (Zivic-Miller, Allison Park, PA, U.S.A.) weighing about 200 g were used. CLZ was donated by Sandoz Research Institute (East Hanover, N.J., U.S.A.), and Hal was obtained from McNeil Pharmaceuticals (Spring House, PA, U.S.A.). For the assessment of DOPAC and HVA dose-response to varying doses of the neuroleptics, rats were injected i.p. with the neuroleptic and killed by decapitation 60 min later. 3-MT accumulation was measured 10 min after i.p. injection of pargyline, 75 mg kg^{-1}. Details of the doses and time that the rats were killed are given in the appropriate tables. Rats were decapitated for the assay of dopamine, DOPAC and HVA, and killed by microwave irradiation for the assay of 3-MT. The microwave instrument (Radio Japan NJE 2603, Great Plains Laboratories, Norman, Oklahoma, U.S.A.) was focused on the head at an output power of 7 kW for 1.3 s. Brains were quickly removed, cooled on ice, and the prefrontal cortex (PFC), the nucleus accumbens (ACC), and the striatum (ST) dissected out as previously reported (Karoum *et al.*, 1990). Dissected brain tissue was frozen on

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Table 1. 3-Methoxytyramine (concentrations) accumulation in rat brain following single doses of clozapine and haloperidol

Treatment	*Dose* [*n*]	*Frontal cortex*	*Nucleus accumbens*	*Striatum*
Saline	— [5]	1.18 (0.13)	4.40 (1.00)	6.7 (0.60)
Clozapine	2.5 [5]	1.23 (0.08)	4.46 (0.80)	5.2 (0.85)
	5.0 [5]	1.23 (0.08)	3.12 (0.28)	7.8 (0.47)
	10.0 [5]	1.79 (0.20)**	5.20 (0.53)	7.8 (0.47)
	20.0 [5]	2.93 (0.54)**	5.97 (1.15)	5.3 (1.17)
Saline	— [5]	1.26 (0.14)	4.40 (1.00)	6.6 (2.16)
Haloperidol	0.05 [5]	1.46 (0.09)	—	21.7 (3.87)**
	0.10 [10]	1.76 (0.06)**	7.80 (1.60)	13.7 (1.35)**
	0.20 [5]	1.92 (0.30)*	8.7 (1.49)*	14.7 (5.51)**
	0.40 [10]	3.88 (1.11)**	9.61 (0.87)**	11.7 (1.62)**
	1.00 [10]	1.76 (0.24)*†	8.16 (1.39)*	15.2 (1.18)**

Results are expressed as pmol mg^{-1} protein $10\,min^{-1}$; mean (s.e.mean). Treatments started at 0 time and pargyline was administered after 50 min. Rats were killed by microwave irradiation at 60 min.
* $P < 0.05$; ** $P < 0.02$ compared with saline-treated rats by unpaired t tests.
† $P < 0.05$ when compared with 0.40 haloperidol.

solid CO_2 and homogenized in 0.5 ml of 1 N HCl containing 50 ng of the deuterated 3-MT ([$^2H_3$]-3-MT) or 50 mg each of the following: dopamine ([$^2H_4$]-dopamine); DOPAC ([$^2H_5$]-DOPAC); and HVA ([$^2H_5$]-HVA). Five μl of the homogenate was removed for protein determination (Lowry *et al.*, 1951), the remaining part was centrifuged at 12,000 *g* and the clear supernatant separated and stored at −16°C until analysed.

The supernatants from the tissue homogenates were prepared for the mass fragmentography of DOPAC and HVA as previously described (Karoum, 1983). For the assay of 3-MT, 0.1 ml of the clear supernatant obtained from the tissue homogenate after centrifugation, was mixed into 0.1 ml of distilled water in a 2 ml polypropylene tube followed by 1.0 ml of ethylacetate and 0.3 ml of phosphate buffer (prepared by mixing 3 parts 0.5 M Na_3PO_4 and one part 0.5 M Na_2HPO_4). The mixture was vortex-mixed for 1 min, centrifuged at 12,000 *g* for 3 min, and about 0.8 ml of the ethylacetate transferred into a Microflex tube (Kontes, Vineland, N.J., U.S.A.). The volume of this extract was reduced to about 0.1 ml under a gentle steam of N_2. The extraction was repeated and 1.0 ml of ethylacetate transferred and combined with the first extract. The ethylacetate extract was then evaporated to dryness and the 3-MT converted to its pentafluoropropionate derivative as previously described (Karoum, 1983).

A model 4500 Finnigan gas chromatograph quadrupole mass spectrometer (Finnigan MAT, San Jose, CA, U.S.A.) was used for the assay of dopamine and its metabolites. Gas chromatograph separation was achieved on a 30 m fused silica capillary column bond coated with methyl:vinyl:silicone gum (SPB-5), (Supelco Inc., Bellefonte, PA, U.S.A.). Injection port pressure was maintained at 15 psi and a split ratio of 1:20 was used. The oven temperature was kept isothermally at 190°C. Negative chemical ionization was used for the mass fragmentography of 3-MT employing ions with mass-to-charge ratio (m/z) 311 and 314, respectively, for 3-MT and its deuterated isomer. For the assay of dopamine, DOPAC and HVA, electron impact ionization was employed and the ions detected were (compound/protonated ion/deuterated ion) as follows: (dopamine/428/431); (DOPAC/488/493); (HVA/356/361).

Results

3-Methoxytyramine concentration and rate of formation in the brain

The rates of accumulation of 3-MT in various brain regions following doses of Hal and CLZ are summarized in Table 1. Both Hal and CLZ significantly increased 3-MT accumulation in the PFC. The minimum effective doses for these drugs (doses that elevated 3-MT accumulations by other 30% in all 3 brain regions) were 0.1 and 10 mg kg^{-1}, respectively. However, 0.2 mg kg^{-1} of Hal appears to be the minimum dose that significantly elevates 3-MT levels in all 3 brain regions. The peak Hal effect on PFC 3-MT accumulation appeared to occur around 0.4 mg kg^{-1}; the accumulation at 1.0 mg kg^{-1} was significantly lower. The inverted 'U' response curve for Hal was observed in a total of 4 separate experiments (results not shown). The responses of the ACC and PFC to Hal were similar but not so clear. The ST was more responsive to Hal than were the other brain areas. At 0.05 mg kg^{-1}, 3-MT accumulation increased by over 3 fold in the ST while it remained unchanged in the PFC.

In contrast to the effect of Hal, CLZ did not increase 3-MT accumulation in the ACC or the ST (Table 1). This experiment was repeated on 5 other occasions. In 3 of these experiments, 10 mg kg^{-1} CLZ produced a slight but significant reduction in striatal 3-MT. In one experiment CLZ produced a marked and highly significant ($P < 0.01$) reduction in 3-MT accumulation at a dose of 20 mg kg^{-1}. 3-MT accumulation in the ACC was never affected. It thus appeared that CLZ selectively enhances dopamine release in the PFC but not in the ACC and ST.

As shown in Table 2, the changes in the steady-state concentration of 3-MT following 10 mg kg^{-1} CLZ or 0.4 mg kg^{-1} Hal are similar to those in 3-MT accumulation, except for the

Table 2 Steady-state concentrations of 3-methoxytyramine in rat brain, 60 min after intraperitoneal injections of clozapine (10 mg kg^{-1}) and haloperidol (0.4 mg kg^{-1})

Treatment	[*n*]	*Frontal cortex*	*Nucleus accumbens*	*Striatum*
Saline	[15]	0.39 (0.09)	1.24 (0.09)	1.29 (0.09)
Clozapine	[8]	0.43 (0.10)	1.09 (0.23)	1.25 (0.10)
Haloperidol	[5]	0.38 (0.04)	2.13 (0.25)**	2.31 (0.35)*

Results are expressed in pmol mg^{-1} protein; mean (s.e.mean).
* $P < 0.05$, ** $P < 0.02$; comparison with saline by unpaired t tests.

Table 3 3,4-Dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) concentrations in rat brain following various doses of clozapine and haloperidol

		Frontal cortex		*Nucleus accumbens*		*Striatum*	
Treatment	*Dose* (mg kg^{-1})	*DOPAC*	*HVA*	*DOPAC*	*HVA*	*DOPAC*	*HVA*
Saline	—	1.3 (0.1)	3.5 (0.32)	51.2 (4.8)	29.0 (0.5)	65.4 (4.7)	49.3 (5.3)
Clozapine	2.5	1.1 (0.1)	3.7 (0.46)	51.5 (2.7)	34.5 (1.6)**	79.1 (6.5)	63.3 (7.4)
	5.0	1.4 (0.1)	3.6 (0.46)	60.3 (4.1)	37.5 (1.7)**	90.4 (3.9)	72.0 (3.0)**
	10.0	1.8 (0.2)*	6.1 (1.3)*	81.2 (10.0)**	63.0 (6.7)**	155.3 (5.2)**	129.3 (9.5)**
	20.0	2.9 (0.3)**	7.9 (1.0)**	108.0 (7.7)**	82.9 (5.4)**	182.3 (11.4)**	142.8 (8.7)**
Saline	—	1.0 (0.2)	2.5 (0.2)	50.2 (2.8)	25.4 (1.7)	74.6 (8.1)	53.6 (4.1)
Haloperidol	0.025	1.1 (0.1)	3.1 (0.4)	64.9 (5.2)*	34.0 (3.0)*	88.2 (7.8)	57.0 (4.6)
	0.05	1.1 (0.1)	3.2 (0.4)	67.0 (6.9)*	29.6 (7.4)	106.3 (9.3)**	57.2 (4.6)
	0.1	1.35 (0.1)*	3.9 (0.3)**	77.4 (3.2)**	38.5 (5.7)*	195.2 (8.2)**	79.6 (7.3)**
	0.2	2.31 (0.2)**	6.4 (0.3)**	147.2 (8.1)**	60.8 (7.8)**	232.6 (19.8)**	112.9 (7.1)**
	0.4	3.1 (0.2)	6.2 (0.9)**	137.6 (12.8)**	61.6 (4.5)**	212.2 (33.6)**	106.5 (17.2)**

Results are in pmol mg^{-1} protein; mean (s.e.mean).
Rats were killed by decapitation 60 min after treatment.
* $P < 0.05$, ** $P < 0.02$; comparisons with saline by unpaired t tests.
Five rats were included in each experiment.

PFC where both Hal and CLZ had no effect on 3-MT steady-state content.

The effects of haloperidol and clozapine on the levels of brain DOPAC and HVA

The effects of low doses of Hal and CLZ on brain concentrations of DOPAC and HVA are summarized in Table 3. As shown, DOPAC and HVA content were elevated by both drugs in all 3 brain regions analysed. The minimum i.p. doses of CLZ and Hal that consistently elevated both DOPAC and HVA in all three brain regions were respectively 10.0 and 0.2 mg kg^{-1}. These minimal doses are similar to those observed for 3-MT accumulation (see Table 1). Furthermore, the ACC appears to be more sensitive than the ST, especially in terms of changes in HVA levels.

Discussion

Dopamine release can be measured directly by a variety of approaches: *in vivo* microdialysis (Zetterstrom *et al.*, 1985; Imperato & Di Chiara, 1984; 1985), push-pull perfusion (Besson *et al.*, 1971; Mora & Myers, 1977), and *in vivo* voltammetry (Gonon *et al.*, 1984; Gonon & Buda, 1985; Lane & Blaha, 1986). However, it has been shown through pharmacological (Di Giulio *et al.*, 1978; Wood, 1982a,b; 1983; Altar *et al.*, 1987) and lesion studies (Groppetti *et al.*, 1978; Wood, 1982a,b; Wood *et al.*, 1987a,b) that the steady-state concentration and accumulation of 3-MT are reliable indices of dopamine release from neurones (Kehr, 1981; Waldmeier *et al.*, 1981; Wood & Altar, 1988). Further, although the concentrations of DOPAC and HVA or its rate of synthesis have been employed for the assessment of dopamine release and impulse flow (Demarest & Moore, 1979; Roth *et al.*, 1976), these metabolites are generally regarded as indices of dopamine synthesis and dopamine turnover (Westerink, 1985; Nissbrandt *et al.*, 1989). In the discussion that follows changes in 3-MT (both steady-state changes and changes in accumulation) are used to indicate relative changes in dopamine release, whereas changes in DOPAC and HVA are used as indices of dopamine rate of utilization and turnover.

The most important observation in the present study is the existence of selective regional differences in the effects of Hal and CLZ on brain dopamine release. Both drugs increased 3-MT accumulation in the PFC, but it was elevated only by Hal in the ACC and the ST. In contrast, dopamine metabolism was equally increased by Hal and CLZ in all three brain regions examined. Results similar to the acute effects of Hal and CLZ on 3-MT accumulation were recently also observed after chronic (28 days) CLZ and Hal treatment (Egan *et al.*, 1991), suggesting that the therapeutic action of typical and atypical neuroleptics may be associated with their persistent potentiation of dopamine release in the PFC and perhaps other cortical regions (Wood & Altar, 1988). This notion is partly in line with other data implicating the mesocortical and meso-accumbens dopamine systems in the antipsychotic effects of neuroleptics (Stevens, 1973; Snyder *et al.*, 1974; Chiodo & Bunney, 1983; White & Wang, 1983; Thierry *et al.*, 1984).

The effect of Hal on 3-MT steady-state and accumulation in the ST are consistent with those found by other workers in the ST (Saller & Salama 1986; Nissbrandt *et al.*, 1989) and several cortical areas (Wood *et al.*, 1987a). The effects of Hal on 3-MT accumulation in the ACC and of CLZ on 3-MT steady-state and accumulation in the ACC and PFC have not previously been described. Further, as demonstrated here for CLZ (compare the effects of CLZ in Tables 2 and 3) and previously reported by others (Groppetti *et al.*, 1978; Moleman *et al.*, 1978; Zetterstrom *et al.*, 1985; Nissbrandt *et al.*, 1989), dopamine release and metabolism are clearly dissociable and hence may well represent different neuronal activities.

Persistent blockade of dopamine receptors, as manifested biochemically by sustained elevations in brain dopamine release and metabolism (Hernandez & Hoebel, 1989; Invernizzi *et al.*, 1990) have been linked in various ways, to the therapeutic benefits of neuroleptics and to the development of tardive dyskinesia. As can be deduced from Table 3, dopamine metabolism does not differentiate between the acute effects of typical from atypical neuroleptics. Therefore tardive dyskinesia is more likely to be associated with persistent stimulation of dopamine release in the ST than is stimulation of dopamine metabolism (Egan *et al.*, 1991). Further, if the ACC is at all involved in mediating the antipsychotic effect of neuroleptics, this effect does not seem to involve stimulation of dopamine release.

Except for one study (Saller & Salama 1986), there appears to be a consensus that CLZ and other atypical neuroleptics produce no or little change in 3-MT production in the ST (Groppetti *et al.*, 1978; Ponzio *et al.*, 1981; Wood *et al.*, 1983; Altar *et al.*, 1986). For example, the putative atypical neuroleptic, CGS 10746B, was shown to reduce 3-MT concentration in the ST (Alter *et al.*, 1986). Similarly, the selective dopamine D_1-receptor antagonist, SCH 23390 (Setler *et al.*, 1978; Huff & Molinoff, 1982; Hytell, 1983), which shares many pharmacological features with CLZ (Lorio *et al.*, 1983; Coward *et al.*, 1989; Farde *et al.*, 1989), has no effect on ST 3-MT accumulation (Boyar & Altar, 1987; Saller & Salama, 1986). The discordance between our findings and those of Saller & Salama, 1986, may result from methodological differences, e.g. greater microwave fixation power (7 versus 1.3 kW) and a considerably shorter period of 3-MT accumulation in our study (10 versus 30 min). Longer accumulation time might increase shunting of dopamine metabolism through *O*-methylation, thus producing higher apparent rates of 3-MT accumulation. This latter may become even more prominent

after treatment with CLZ which mainly stimulates dopamine acidic metabolite production.

While the ST (followed by the PFC) appeared to be the area most sensitive to Hal in terms of 3-MT production (Table 3) the ACC appears to be most responsive to CLZ and Hal in terms of changes in HVA steady-state content. Differential effects of Hal and CLZ on DOPAC and HVA in the ACC and ST have previously been described (Anden & Stock, 1973; Wilk *et al.*, 1975; Westerink & Korf, 1976). However, since Hal is known to inhibit the efflux of DOPAC and HVA from the brain (Moleman *et al.*, 1978; Westerink *et al.*, 1984), the elevations produced by Hal and CLZ may overestimate dopamine turnover or rate of utilization.

Since CLZ and other atypical neuroleptics (Farde *et al.*, 1989) preferentially bind to D_1 dopamine receptors (Rupniak *et al.*, 1985; Fuxe *et al.*, 1989; Invernizzi *et al.*, 1990), the failure of CLZ to increase 3-MT accumulation (and by implication, dopamine release) in the ST and ACC may be related to its relatively low affinity for release-modulating D_2-dopamine autoreceptors (Coward *et al.*, 1989; Farde *et al.*, 1989). Recent microdialysis studies have shown that the infusion of a number of dopamine D_1-specific compounds into the nigra did not release dopamine from either the nigra or the ST (Santiago & Westerink, 1991). Hence, unlike Hal which blocks both D_1 and D_2 receptors, CLZ effects on dopamine release are apparently primarily linked to its ability to interact with postsynaptic dopamine D_1 receptors. If we assume blockade of the dopamine postsynaptic receptor to be a general property of CLZ, then stimulation of dopamine release and metabolism in the PFC is probably also linked to blockade of postsynaptic dopamine D_1 receptors. This view does not agree with the concept that dopamine release (Altar *et al.*, 1987) and synthesis (Demarest & Moore, 1979) in the PFC are regulated by autoreceptors.

Evidence supporting the presence of dopamine autoreceptors in the cortex were in most cases extrapolated from studies related to experiments that used the so-called presynaptic doses of dopamine agonists (Chiodo *et al.*, 1984; Talmaciu *et al.*, 1986; Altar *et al.*, 1987). Inherent in these studies is the assumption that the doses of dopamine receptor agonists that were employed do not effectively stimulate dopamine postsynaptic receptors in the PFC. It can be argued, however that the so called presynaptic doses of dopamine agonists may stimulate postsynaptically non-dopaminergic neurones in the PFC. This stimulation might be responsible for the reduction in dopamine release via a direct negative feed back mechanism. Data generated here on the effect of CLZ on dopamine release in the PFC are compatible with this hypothesis.

In conclusion, comparison of the effects of single administrations of Hal and CLZ on dopamine release (as reflected by 3-MT production) and metabolism (as reflected by DOPAC and HVA contents) in the PFC, ACC, and ST, revealed differences that are apparently not clearly detected by either microdialysis measurement of dopamine release or changes in the dopamine acidic metabolites. The difference between typical and atypical neuroleptics as reflected in 3-MT production may offer an important and a convenient approach to the search for new agents for the treatment of schizophrenia.

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Participation of endothelium-derived nitric oxide but not prostacyclin in the gastric mucosal hyperaemia due to acid back-diffusion

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1 The possible participation of prostacyclin and nitric oxide (NO) in the gastric mucosal hyperaemic response to acid back-diffusion through a disrupted gastric mucosal barrier was examined. The experiments were carried out on anaesthetized rats in which acid back-diffusion was elicited by gastric perfusion with dilute ethanol in 0.15 M HCl and gastric mucosal blood flow (MBF) was measured by the hydrogen gas clearance technique.

2 Indomethacin (28 μmol kg^{-1}, s.c.), an inhibitor of the formation of cyclo-oxygenase products including prostacyclin, failed to alter mean arterial blood pressure (MAP), basal MBF and the hyperaemic response to acid back-diffusion in urethane-anaesthetized rats.

3 N^G-nitro-L-arginine methyl ester (L-NAME; 13 and 43 μmol kg^{-1}, i.v.), an inhibitor of endothelium-derived NO formation, increased MAP in a dose-dependent manner. Whilst basal MBF in urethane-anaesthetized rats was not changed, the increase in MBF caused by gastric perfusion with dilute ethanol in acid was dose-dependently depressed by L-NAME. The loss of H^+ ions from the gastric lumen, an indirect measure of acid back-diffusion, was significantly enhanced by 43 μmol kg^{-1} L-NAME. In contrast, D-NAME (13 and 43 μmol kg^{-1}) was without effect on MAP, basal and stimulated MBF, and acid back-diffusion.

4 Unlike in urethane-anaesthetized rats, L-NAME led to a significant reduction of basal MBF in phenobarbitone-anaesthetized rats. MAP in the phenobarbitone-anaesthetized rats was significantly higher than in urethane-anaesthetized rats, and the hypertensive effect of L-NAME under phenobarbitone anaesthesia was significantly smaller than under urethane anaesthesia.

5 The rise in MBF brought about by acid back-diffusion was blocked by L-NAME administered to phenobarbitone-anaesthetized rats. Infusion of L-arginine (120 μmol kg^{-1} min^{-1}, i.v.) led to a partial, but significant, reversal of the effects of L-NAME on MAP and the hyperaemia due to acid back-diffusion.

6 These findings indicate that endothelium-derived NO plays an important mediator role in the gastric mucosal vasodilatation caused by back-diffusion whilst vasodilator prostanoids such as prostacyclin are not involved.

Keywords: Gastric mucosal blood flow; acid back-diffusion; vasoactive mediators; endothelium-derived relaxing factor; prostanoids; indomethacin; N^G-nitro-L-arginine methyl ester; L-arginine

Introduction

Acid back-diffusion through a disrupted gastric mucosal barrier leads to an increase in gastric mucosal blood flow (MBF) (Ritchie, 1975; Whittle, 1977; Bruggeman *et al.*, 1979). This hyperaemia, which is thought to limit acid injury to the mucosa, is mediated by a neural reflex since it is blocked by local administration of tetrodotoxin to the rat stomach and by capsaicin-induced defunctionalization of afferent neurones (Holzer *et al.*, 1991a). However, the mediators of the vasodilator response have not yet been explored. Since acetylcholine and histamine are not involved (Holzer *et al.*, 1991a), it has been postulated that hyperaemia results from local 'axon reflexes' between collaterals of afferent nerve fibres within the gastric wall (Holzer *et al.*, 1991a). This assumption is consistent with the findings that capsaicin-sensitive afferent neurones in the rat stomach contain calcitonin gene-related peptide (Green & Dockray, 1988) which potently augments MBF (Holzer & Guth, 1991) and that capsaicin-evoked stimulation of afferent nerve endings releases this peptide into the gastric circulation (Holzer *et al.*, 1990) and increases MBF (Lippe *et al.*, 1989; Holzer *et al.*, 1991b).

In addition to a direct action on vascular smooth muscle, transmitter substances can regulate tissue blood flow via release of vasodilator mediators from endothelial cells (Furchgott, 1984). The major endothelium-derived relaxing factor is nitric oxide (NO) (Ignarro *et al.*, 1987; Palmer *et al.*, 1987) which is synthesized from L-arginine (Palmer *et al.*, 1988). Inhibition of NO synthesis by L-arginine analogues such as N^G-monomethyl-L-arginine increases blood pressure (Rees *et al.*, 1989; Whittle *et al.*, 1989) and reduces MBF in ketamine-anaesthetized rats (Pique *et al.*, 1989). In rats anaesthetized with inactin, however, N^G-monomethyl-L-arginine is without effect on basal MBF but blocks the rise of MBF elicited by pentagastrin (Walder *et al.*, 1990).

The objective of the present study was to investigate the possible involvement of endothelium-derived NO and cyclo-oxygenase products such as prostacyclin in the gastric mucosal hyperaemia due to acid back-diffusion. For this purpose, the cyclo-oxygenase inhibitor indomethacin (Whittle, 1986; Walder *et al.*, 1990) and the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) (Gardiner *et al.*, 1990; Moore *et al.*, 1990; Rees *et al.*, 1990) were examined for their effects on mean arterial blood pressure (MAP), basal MBF and MBF stimulated by acid back-diffusion. Since in the course of these studies it was found that inhibition of NO synthesis failed to alter significantly basal MBF in urethane-anaesthetized rats (unlike results in ketamine-anaesthetized rats, Pique *et al.*, 1989) the effects of L-NAME were also evaluated on phenobarbitone-anaesthetized animals.

Methods

Animal preparation

All experiments of this study were approved by the Ministry of Science and Research of the Republic of Austria. Sprague-

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Dawley rats of either sex, weighing 230–250 g, were fasted for 20 h but allowed free access to water. After the induction of anaesthesia with urethane (14 mmol kg^{-1}, s.c.) or phenobarbitone sodium (0.92 mmol kg^{-1}, i.p.) the rats were fitted with a tracheal cannula to facilitate spontaneous respiration and allow for the administration of hydrogen. Mean arterial blood pressure (MAP) was recorded from a cannula in the left carotid artery by means of an ISOTEC pressure transducer (HSE, March, Germany) and displayed on a chart recorder (SERVOGOR 464, ABB GOERZ, Vienna, Austria). The blood pressure signal was fed into a MINO heart ratemeter with digital readout (HSE, March, Germany). Saline (0.9% NaCl, wt/wt) was continuously infused into a jugular vein at a rate of 1.5 ml h^{-1} to avoid dehydration. The stomach, exposed by a midline laparotomy, was fitted with an inflow cannula placed in the forestomach and an outflow cannula inserted through the pylorus, and was continuously perfused with saline (room temperature) at a rate of 0.7–0.8 ml min^{-1}. The body temperature of the animals was kept at 36–37°C by means of a water-perfused heating pad.

Measurement of gastric mucosal blood flow (MBF) by the hydrogen gas clearance technique

The clearance of hydrogen gas was measured by a needle-type platinum electrode inserted from the serosa into the basal portion of the gastric mucosa and positioned at the submucosal border of the muscularis mucosae (Holzer *et al.*, 1991a,b). To ensure monoexponential desaturation curves, the electrode was placed in such a way that it was not in direct contact with any visible gastric artery (Holzer & Guth, 1991; Holzer *et al.*, 1991a,b). The construction of the electrode has been described previously by Holzer *et al.* (1991a,b). A reference electrode was placed inside the peritoneal cavity, and the current signal was amplified and recorded on a chart recorder in such a way that the amplitude of the desaturation curves amounted to 15–20 cm. The desaturation curves were computerized by use of digitizing tablet and the software SigmaScan (Jandel Scientific, Corte Madera, California, U.S.A.). The time needed for 50% desaturation was determined by linear regression analysis of the plot log current versus time, and MBF was calculated as described previously (Leung *et al.*, 1984). MBF was expressed as ml min^{-1} 100 g^{-1}.

Determination of the loss of H^+ ions from the gastric perfusate as a measure of acid back-diffusion

The gastric perfusate was collected in 15 min fractions, and their acid content was determined by titration of the samples to pH 7 with 0.1 M NaOH (Holzer *et al.*, 1991a). Blank samples of the perfusion media were treated in the same manner as the gastric perfusate fractions, and the loss of H^+ ions was determined by subtracting the acidity of the perfusate fractions from the acidity of the respective blank samples.

Experimental protocol

The experimental protocol involved alternating 15 min periods of saturation, and desaturation, of the tissue with hydrogen gas (3% in air, vol/vol). Thus, MBF was determined during the periods of 45–60 min, 75–90 min and 105–120 min after the start of the experiments. During the first 90 min the stomach was perfused with 0.15 M hydrochloric acid (HCl) whilst during the period of 90–120 min dilute ethanol (15 or 30%, wt/wt) in 0.15 M HCl was perfused through the stomach to disrupt the gastric mucosal barrier and induce acid back-diffusion (Holzer *et al.*, 1991a). The increase in MBF was expressed as MBF increment (stimulated minus baseline values).

Four sets of experiments were carried out. In the first set, urethane-anaesthetized rats were treated with indomethacin (28 μmol kg^{-1}, s.c.) or its vehicle 90 min before gastric perfusion with ethanol (15%) in 0.15 M HCl. The second set of experiments was performed with urethane-anaesthetized rats which received an i.v. injection of either saline (2 ml kg^{-1}), D-NAME or L-NAME (13 or 43 μmol kg^{-1}). Either enantiomer was administered 30 min before the gastric perfusion with ethanol (15%) in 0.15 M HCl began. In these experiments, the loss of H^+ ions from the gastric perfusate was also determined. In the third set of experiments arterial blood pressure, basal MBF and the hyperaemic response to acid back-diffusion in urethane-anaesthetized rats were compared with those in animals anaesthetized with phenobarbitone sodium. In urethane-anaesthetized rats, acid back-diffusion was elicited by gastric perfusion with 15% ethanol in 0.15 M HCl (Holzer *et al.*, 1991a) whereas both 15 and 30% ethanol in 0.15 M HCl were tested in animals anaesthetized with phenobarbitone sodium. The fourth set of experiments, carried out on phenobarbitone-anaesthetized rats, was to determine whether L-arginine would be able to antagonize the effects of L-NAME on blood pressure and MBF. L-NAME (43 μmol kg^{-1}) was given by i.v. injection 30 min before gastric perfusion with 30% ethanol in 0.15 M HCl. L-Arginine (30 μmol kg^{-1} min^{-1}, 25 μl min^{-1}) was infused i.v. throughout the experiment except that during the period between 15 min before and 15 min after the injection of L-NAME the rate of L-arginine infusion was raised to 120 μmol kg^{-1} min^{-1}.

Substances and solutions

Indomethacin (Sigma, Deisenhofen, Germany) was dissolved (0.11 M) in 2% (wt/wt) Na_2CO_3 and diluted with saline to give a concentration of 14 mM, the pH of the solution being adjusted to neutrality with HCl. This solution was administered s.c. at a volume of 2 ml kg^{-1}. Control rats received the same volume of the vehicle. L-NAME, D-NAME and L-arginine were obtained from Bachem (Bubendorf, Switzerland). L-NAME and D-NAME were dissolved in saline to give solutions of 21.5 mM which were injected i.v. at a volume of 2 ml kg^{-1}. L-Arginine (0.3 M) was dissolved in water. Phenobarbitone sodium (Sigma, Deisenhofen, Germany) was dissolved in saline to give a concentration of 0.13 M. This solution was made up fresh every day and injected i.p. at a volume of 7 ml kg^{-1} to induce surgical anaesthesia. Urethane was obtained from Fluka (Buchs, Switzerland), dissolved in water (25%, wt/wt) and injected s.c. at a volume of 5 ml kg^{-1}.

Statistics

All data are presented as mean ± s.e.mean. Statistical evaluation of the results was performed by use of the Mann-Whitney U test or the Wilcoxon test for pair differences. Probability values $P < 0.05$ were regarded as significant.

Results

Effect of indomethacin in urethane-anaesthetized rats

Indomethacin (28 μmol kg^{-1}, s.c.) had no significant effect on mean arterial blood pressure (MAP), basal MBF, and the increase in MBF caused by acid back-diffusion when compared with rats treated with the vehicle ($n = 6$). In vehicle-treated rats MAP was 93 ± 4 mmHg, basal MBF was 57 ± 7 ml min^{-1} 100 g^{-1}, and the increase in MBF was 73 ± 23 ml min^{-1} 100 g^{-1}. The respective values in indomethacin-treated rats were 92 ± 2 mmHg, 62 ± 14 ml min^{-1} 100 g^{-1} and 63 ± 12 ml min^{-1} 100 g^{-1}. The heart rate was not measured in these experiments.

Effect of D-NAME and L-NAME in urethane-anaesthetized rats

D-NAME (13 and 43 μmol kg^{-1}, i.v.) had no effect on MAP, heart rate, basal MBF and the loss of H^+ ions from the

gastric perfusate when compared with the respective values measured before drug injection or with the values measured after injection of saline ($n = 7$–8 for each dose). The values measured before and after injection of saline and 43 μmol kg^{-1} D-NAME are given in Table 1. Although this dose of D-NAME failed to raise MAP significantly during the first 30 min after its injection (Table 1), an increase in MAP by up to 20 mmHg was discernible 60 min after its injection in some experiments. D-NAME (13 and 43 μmol kg^{-1}) did not alter the ethanol-induced rise in the loss of H^+ ions from the gastric perfusate and the concomitant increase in MBF as compared with the respective values in saline-treated rats (Figure 1c).

L-NAME (13 and 43 μmol kg^{-1}) did not influence basal MBF and the loss of H^+ ions from the gastric perfusate when compared with the respective values measured before drug injection or with the values measured after injection of saline or D-NAME ($n = 7$–9 for each dose). In contrast, L-NAME led, within 10 min, to a dose-related increase in MAP as compared with the values before L-NAME injection (Table 1) and with the values measured in rats treated with saline or D-NAME (Figure 1a). The hypertensive effect of L-NAME was sustained, and during the subsequent period of 60 min no appreciable recovery of MAP was observed. In addition, the heart rate tended to fall but this effect did not reach statistical significance even with 43 μmol kg^{-1} L-NAME (Table 1). The mucosal hyperaemia caused by gastric mucosal barrier disruption in the presence of luminal acid was blocked by L-NAME in a dose-dependent manner (Figure 1b). Conversely, the ethanol-induced rise in the loss of H^+ ions from the gastric perfusate remained unaltered by 13 μmol kg^{-1} L-NAME but was significantly augmented by 43 μmol kg^{-1} L-NAME (Figure 1c).

Comparison of MAP, heart rate and gastric MBF under urethane and phenobarbitone anaesthesia

MAP in phenobarbitone-anaesthetized rats was on average 120 mmHg or higher (Table 2; Figure 3) and thus differed significantly ($P < 0.01$, U test) from MAP in urethane-anaesthetized animals (90–110 mmHg; Table 1; Figure 1). This difference in MAP was paralleled by a disparity in the heart rate which under phenobarbitone anaesthesia (Table 2) was significantly ($P < 0.01$, U test) lower than under urethane anaesthesia (Table 1).

L-NAME (43 μmol kg^{-1}) increased MAP of phenobarbitone-anaesthetized rats by 33 mmHg (Table 2), a value that was significantly ($P < 0.01$, U test) smaller than the increase in MAP (+56 mmHg; Table 1) induced by L-NAME in urethane-anaesthetized animals. As a consequence, MAP reached after administration of L-NAME did not differ in urethane- and phenobarbitone-anaesthetized rats (compare Tables 1 and 2).

The magnitude of basal MBF was very similar in urethane- and phenobarbitone-anaesthetized animals (Figure 2). However, gastric perfusion with 15% ethanol in 0.15 M HCl increased MBF in phenobarbitone-anaesthetized rats to an extent that was significantly smaller than in urethane-

Table 1 Effect of saline (2 ml kg^{-1} i.v), N^G-nitro-D-arginine methyl ester (D-NAME, 43 μmol kg^{-1}, i.v.), and L-NAME (43 μmol kg^{-1}, i.v.) on mean arterial blood pressure (MAP, mmHg), heart rate (HR, beats min^{-1}), basal gastric MBF (ml min^{-1} min^{-1}), and the loss of H^+ ions from the gastric lumen (μmol 15 min^{-1}) in urethane-anaesthetized rats

	Before saline	*After saline*	*Change* (Δ)	P
MAP	95 ± 4	94 ± 2	−1 ± 2	NS
HR	412 ± 14	416 ± 12	+4 ± 9	NS
MBF	48 ± 5	47 ± 4	−1 ± 3	NS
H^+ loss	28 ± 9	22 ± 8	−6 ± 4	NS
	Before D-NAME	*After D-NAME*	*Change* (Δ)	P
MAP	88 ± 4	92 ± 5	+4 ± 2	NS
HR	400 ± 13	415 ± 12	+15 ± 8	NS
MBF	51 ± 4	46 ± 3	−5 ± 3	NS
H^+ loss	32 ± 8	16 ± 8	−16 ± 9	NS
	Before L-NAME	*After L-NAME*	*Change* (Δ)	P
MAP	92 ± 3	149 ± 3	+56 ± 3	<0.01
HR	410 ± 17	386 ± 13	−24 ± 11	NS
MBF	48 ± 6	56 ± 8	+8 ± 7	NS
H^+ loss	30 ± 8	34 ± 10	+4 ± 7	NS

Values are mean ± s.e.mean; $n = 8$–9; NS = not significant (Wilcoxon test for pair differences).

Table 2 Effect of N^G-nitro-L-arginine methyl ester (L-NAME, 43 μmol kg^{-1} i.v.) on mean arterial blood pressure (MAP, mmHg), heart rate (HR, beats min^{-1}), and basal gastric MBF (ml min^{-1} min^{-1}) in phenobarbitone-anaesthetized rats under i.v. infusion of saline (0.1 ml min^{-1}) or L-arginine (120 μmol kg^{-1} min^{-1})

		Infusion of saline		
	Before L-NAME	*After L-NAME*	*Change* (Δ)	P
MAP	124 ± 2	158 ± 2	+33 ± 3	<0.01
HR	349 ± 8	313 ± 10	−36 ± 9	<0.01
MBF	64 ± 8	50 ± 4	−14 ± 5	<0.025
		Infusion of L-arginine		
	Before L-NAME	*After L-NAME*	*Change* (Δ)	P
MAP	122 ± 2	148 ± 2	+26 ± 3	<0.05
HR	356 ± 12	356 ± 12	0 ± 8	NS
MBF	65 ± 8	51 ± 5	−14 ± 6	<0.05

Values are mean ± s.e.mean; $n = 8$; NS = not significant (Wilcoxon test for pair differences). L-Arginine (30 μmol kg^{-1} min^{-1}) or saline (25 μl min^{-1}) was infused i.v. throughout the experiment except that during the period between 15 min before and 15 min after the injection of L-NAME the rate of infusion was raised to 120 μmol kg^{-1} min^{-1}.

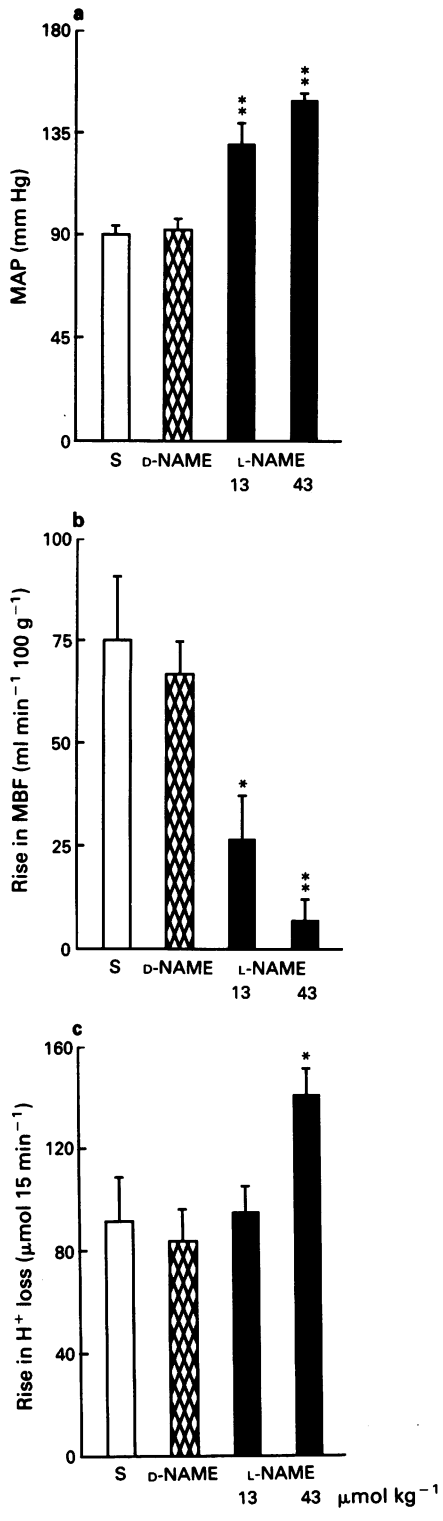

Figure 1 Effects of N^G-nitro-L-arginine methyl ester (L-NAME, 13 and 43 μmol kg^{-1}) on mean arterial blood pressure (MAP, a), the rise in gastric mucosal blood flow (MBF; stimulated minus basal MBF) caused by gastric perfusion with 15% ethanol in 0.15 M HCl (b) and the associated rise in H^+ loss from the gastric lumen (c) in urethane-anaesthetized rats, as compared with the respective values measured after injection of saline (S) and D-NAME. Two doses of D-NAME (13 and 43 μmol kg^{-1}, i.v.) were used but, because neither dose had any effect relative to saline, the values obtained with the two doses are presented in combined form. Saline (2 ml kg^{-1}, $n = 8$), D-NAME ($n = 16$) and L-NAME ($n = 7$–9) were injected i.v. 30 min before gastric perfusion of dilute ethanol in HCl. Results are mean with s.e.mean shown by vertical bars. * $P < 0.05$, ** $P < 0.01$ versus saline and D-NAME (U test).

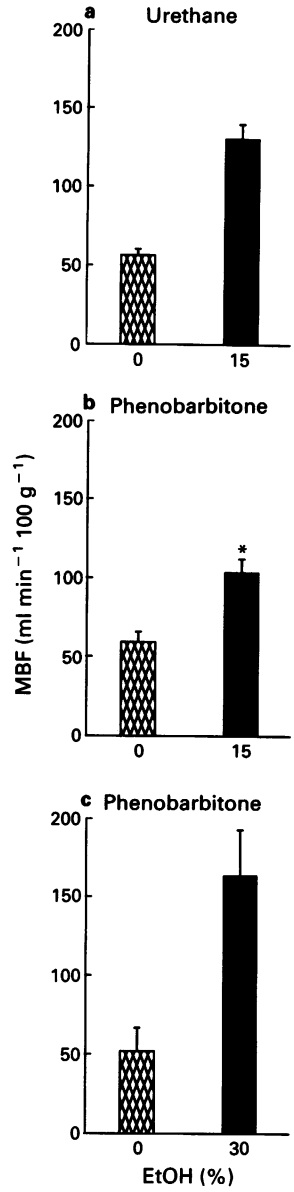

Figure 2 Effect of gastric perfusion with ethanol (concentrations in % as indicated) in the presence of 0.15 M HCl on gastric mucosal blood flow (MBF) in urethane- and phenobarbitone-anaesthetized rats. Results are mean with s.e.mean shown by vertical bars; $n = 6$–8. * $P < 0.05$ versus respective value obtained under urethane anaesthesia (U test).

anaesthetized animals (compare Figure 2a and 2b). This difference was not evident when the stomachs of pheno- barbitone-anaesthetized rats were perfused with 30% ethanol in 0.15 M HCl (compare Figure 2a and 2c).

Effect of L-NAME in phenobarbitone-anaesthetized rats in the absence and presence of L-arginine

L-NAME (43 μmol kg^{-1}) significantly enhanced MAP and reduced heart rate and basal MBF when compared with the respective pre-drug values (Table 2). The hypertension induced by L-NAME was also evident when rats treated with the drug were compared with rats treated with the saline vehicle whereas basal MBF did not significantly differ between these two groups of rats (Figure 3a and c). The rise in MBF caused by gastric perfusion with 30% ethanol in 0.15 M HCl was blocked by more than 80% after injection of L-NAME when compared with the hyperaemia seen in saline-treated rats (Figure 3b).

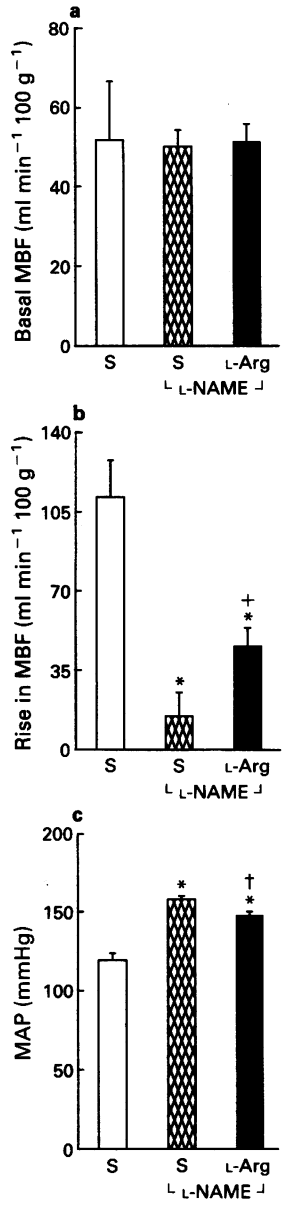

Figure 3 Interaction between L-arginine and N^G-nitro-L-arginine methyl ester (L-NAME). The graph shows gastric mucosal blood flow (MBF, a), rise in MBF (stimulated minus basal MBF) caused by gastric perfusion with 30% ethanol in 0.15 M HCl (b), and mean arterial blood pressure (MAP, c) in 3 groups in 0.15 M HCl (b), and mean arterial blood pressure (MAP, c) in 3 groups of phenobarbitone-anaesthetized rats. Open columns: rats receiving an i.v. infusion of saline (S); cross-hatched columns: rats receiving an i.v. infusion of saline and an i.v. injection of L-NAME; solid columns: rats receiving an i.v. infusion of L-arginine (L-Arg) and an i.v. injection of L-NAME. L-NAME was injected 30 min before gastric perfusion of dilute ethanol in HCl. L-Arg was infused at a rate of 30 μmol kg^{-1} min^{-1} throughout the experiment except that during the period between 15 min before and 15 min after the injection of L-NAME the rate of infusion was raised to 120 μmol kg^{-1} min^{-1}. The respective infusion rates of saline were 25 μl min^{-1} and 100 μl min^{-1}. Results are mean with s.e.mean shown by vertical bars, $n = 8$. * $P < 0.05$ versus saline only; † $P < 0.05$ versus saline plus L-NAME (U test).

Infusion of L-arginine (120 μmol kg^{-1} min^{-1}) did not change the values of basal MBF, MAP and heart rate, measured before i.v. injection of L-NAME (Table 2). However, L-arginine attenuated the hypertensive effect of L-NAME (43 μmol kg^{-1}) (Table 2; Figure 3c) and abolished the bradycardia due to L-NAME (Table 2). In contrast, the decrease in basal MBF brought about by L-NAME was not antagonized by L-arginine (Table 2), and basal MBF after administration of L-NAME did not differ between rats receiving saline and those receiving L-arginine (Figure 3a). L-Arginine, though, was able to attenuate significantly the inhibitory effect of L-NAME on the hyperaemia due to acid back-diffusion (Figure 3b). Higher doses of L-arginine were not used because preliminary experiments had shown that a dose of L-arginine (240 μmol kg^{-1} min^{-1}) induced hypotension and MAP instability ($n = 3$).

Discussion

When the stomachs of urethane-anaesthetized rats are perfused with ethanol in 0.15 M HCl, gastric MBF increases concomitantly with a rise in acid loss from the gastric lumen. It thus appears that hyperaemia is a response to acid back-diffusion into the mucosa (Holzer *et al.*, 1991a). Dilute ethanol was used to break the gastric mucosal barrier because its adverse effects on MBF are confined to the superficial mucosa (Pihan *et al.*, 1986; Yonei & Guth, 1988) whereas MBF at the base of the mucosa is not altered by ethanol in the presence of luminal acid (Holzer *et al.*, 1991a,b). As the hyperaemic response to acid back-diffusion takes place without a change in MAP it probably results from vasodilatation in the gastric mucosa and submucosa (Holzer *et al.*, 1991a). The present data corroborate these observations and show that hyperaemia in response to acid back-diffusion also occurs in rats anaesthetized with phenobarbitone.

There were, however, several differences between urethane- and phenobarbitone-anaesthetized rats. Under phenobarbitone anaesthesia higher concentrations of ethanol were necessary to induce the same degree of hyperaemia as seen under urethane anaesthesia, which probably reflects different degrees of resistance of the gastric mucosal barrier to the disruptive effect of intraluminal ethanol. It has previously been found that the vulnerability of the rat gastric mucosa to ethanol is significantly enhanced in urethane-anaesthetized animals as compared to conscious rats (Holzer & Lippe, 1988). Urethane also depresses gastric acid secretion (Barrett *et al.*, 1966) and thus appears to have profound effects on gastric mucosal physiology. In addition, phenobarbitone-anaesthetized rats have a significantly higher MAP and lower heart rate than urethane-anaesthetized animals. Although basal gastric MBF did not differ, L-NAME failed to decrease basal MBF in urethane-anaesthetized rats whilst inhibition of NO synthesis by N^G-monomethyl-L-arginine reduces MBF in ketamine-anaesthetized rats (Pique *et al.*, 1989). L-NAME, though, diminished MBF in phenobarbitone-anaesthetized rats, and it appears as if the effect of NO synthesis inhibition on basal MBF depends critically on the level of MAP. Thus, blockade of NO synthesis lowers basal MBF only when MAP is $\geqslant$ 120 mmHg as is the case under ketamine (Pique *et al.*, 1989) or phenobarbitone (present study) anaesthesia but fails to change basal MBF in inactin- (Walder *et al.*, 1990) and urethane-anaesthetized (present study) rats in which MAP is less than 100 mmHg. It may be reasoned that the pronounced rise of MAP caused by L-NAME in urethane-anaesthetized rats is able to balance the local vasoconstrictor action of L-NAME in the stomach so that MBF does not change (see also Walder *et al.*, 1990).

The most important result of the current study was that in both urethane- and phenobarbitone-anaesthetized rats, hypertensive doses of L-NAME blocked the rise in gastric MBF caused by gastric mucosal barrier disruption in the presence of luminal acid. This inhibitory effect cannot be explained by inhibition of acid back-diffusion, because L-NAME did not attenuate but amplified the loss of H^+ ions from the gastric lumen, an action that is consistent with enhanced susceptibility of the gastric mucosa to acid injury after inhibition of NO synthesis (Whittle *et al.*, 1990). The actions of L-NAME on gastric mucosal hyperaemia, MAP and heart rate were enantiomer-specific and significantly attenuated by concomitant administration of L-arginine. The inability of L-arginine to reverse completely the effects of L-NAME on MAP and

MBF might be explained by the conjecture that tolerable doses of L-arginine are insufficient to overcome the effect of L-NAME (see also Gardiner *et al.*, 1990; Rees *et al.*, 1990). Enantiomer specificity and L-arginine reversibility of the actions of L-NAME on the cardiovascular system are taken to indicate that the drug acts by inhibition of the L-arginine: NO pathway (Gardiner *et al.*, 1990; Rees *et al.*, 1990). It can be inferred, therefore, that formation of endothelium-derived nitric oxide plays an essential role in the mucosal vasodilatation that ensues when the gastric mucosal barrier is disrupted and hydrogen ions diffuse into the tissue. Since the hyperaemic response to acid back-diffusion is brought about by a neural reflex (Holzer *et al.*, 1991a) it would appear that the neurotransmitters that give rise to submucosal and mucosal vasodilatation in the stomach act via stimulation of the L-arginine: NO pathway. This vasodilator mechanism is likely to be of importance in the future identification of the neurotransmitters that are involved in the vasodilatation due to acid back-diffusion.

It is worth mentioning that basal gastric MBF was less altered by L-NAME than MBF stimulated by acid back-diffusion. This finding may indicate that endothelium-derived NO is an essential mediator of active vasodilatation in the gastric mucosa but is comparatively less important for the regulation of basal vascular tone. Further support for this argument comes from the finding that inhibition of NO synthesis by N^G-monomethyl-L-arginine greatly reduces the hyperaemic response to pentagastrin while leaving basal MBF unchanged in inactin-anaesthetized rats (Walder *et al.*, 1990). This reasoning does not negate that basal vascular resistance in the gastric mucosa does rise after blockade of NO synthesis (Walder *et al.*, 1990), an effect that has to be inferred from the findings that in spite of marked hypertension basal gastric MBF remains unchanged or even decreases slightly.

Another vasodilator mediator that can be released from the endothelium and which is known to enhance gastric MBF is prostacyclin (Whittle & Vane, 1987). However, neither basal MBF nor MBF stimulated by acid back-diffusion was altered in rats that had been treated with a dose of indomethacin ($28\,\mu mol\,kg^{-1}$) known to effectively inhibit the formation of cyclo-oxygenase products including prostacyclin in the gastric mucosa (Whittle & Vane, 1987). It is deduced from this result that vasodilator prostanoids, such as endothelium-derived prostacyclin, are not involved in the hyperaemic response under study. This inference does not necessarily conflict with the finding that indomethacin can reduce the gastric clearance of aniline before and after taurocholate-induced back-diffusion of acid (Whittle, 1977), because the clearance of aniline is only an indirect measure of gastric MBF that can critically be influenced by transepithelial movements of hydrogen ions (see Guth *et al.*, 1989).

In conclusion, the current study has provided evidence that endothelium-derived NO is an essential mediator of neurogenic vasodilatation taking place in the gastric mucosa following back-diffusion of acid. As this increase in MBF is thought to limit acid injury to the mucosa (Whittle, 1977; Guth *et al.*, 1989; Oates, 1990; Holzer *et al.*, 1991a), NO appears to play a pivotal role in the physiological and pathophysiological control of gastric mucosal circulation (see Pique *et al.*, 1989; Walder *et al.*, 1990; Whittle *et al.*, 1990).

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Effects of pinacidil on guinea-pig isolated perfused heart with particular reference to the proarrhythmic effect

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1 The effects of pinacidil (10, 30, 50 μM) on contractility ($+dP/dt_{max}$), coronary perfusion pressure (cP), and ECG intervals (PR, QRS, QT) have been studied on constant-flow perfused guinea-pig hearts, driven at four frequencies (2.5, 3, 3.5, 4 Hz).

2 Pinacidil decreased $+dP/dt_{max}$, cP and the QT interval in a dose-dependent manner, whereas the PR interval was increased. QRS duration was not modified. All these effects were independent of driving frequency. Pinacidil decreased the interval from Q-wave to T-wave peak (QTpeak) to a greater extent than the QT interval, thus decreasing the QTpeak/QT ratio. This effect, unlike that on QT interval, was more evident at the highest frequency of stimulation.

3 In 4 out of 20 hearts treated with pinacidil sustained ventricular fibrillation (VF) occurred following a short run of premature ventricular beats (R on T phenomenon).

4 In separate experiments, an attempt to induce VF electrically was made at drug concentrations ranging from 10 μM to 100 μM (8 experiments for each concentration). In control conditions and at the lowest concentration of pinacidil tested (10 μM) VF could never be induced; in the presence of 30 μM pinacidil VF was induced in 5 out of 8 experiments. Drug concentrations higher that 50 μM permitted the induction of VF in every case.

5 Although the concentrations of pinacidil producing ventricular fibrillation are 30–40 times higher than those found in patients under long term treatment with this agent, it is suggested that caution should be used in prescribing this drug, at least in patients suffering from myocardial ischaemia.

Keywords: Pinacidil; heart contractility; coronary tone; ECG; ventricular fibrillation

Introduction

As a typical 'K channel opener' (KCO), pinacidil has been shown to relax various types of smooth muscle by increasing K conductance and hyperpolarizing the cell membrane (Weston, 1989). These properties account for the successful clinical application of pinacidil in antihypertensive therapy (Goldberg, 1988) and for the therapeutic potential of KCOs in the treatment of bronchial asthma, irritable bladder syndrome and angina pectoris (Cook, 1988). At a cardiac level, pinacidil shortens the action potential (AP) duration of ventricular and Purkinje cells (Smallwood & Steinberg, 1988); decreases the automaticity of Purkinje fibres (Steinberg *et al.*, 1988); decreases contractility and sinus rate (at least in isolated preparations) (Longman *et al.*, 1988; Steinberg *et al.*, 1988; Nielsen *et al.*, 1989); and increases coronary flow (Kawashima & Liang, 1985). Increasing amounts of experimental data have become available on the influence of pinacidil on heart rhythmicity. This aspect of the action of the drug, while of great clinical importance, is somewhat puzzling in that pinacidil, on the one hand, can favour re-entrant type arrhythmias (acute ischaemia models; Wollenberg *et al.*, 1989; Chi *et al.*, 1990) and, on the other, it exhibits a definite antiarrhythmic action on arrhythmias produced by increased automaticity (subacute ischaemia, barium-induced arrhythmias, Kerr *et al.*, Steinberg *et al.*, 1988).

The present work describes the pharmacodynamic profile of pinacidil in Langendorff-perfused guinea-pig hearts driven at different stimulation rates and, specifically, focuses on the influence of the drug on heart rhythmicity in normoxic conditions.

Methods

The preparation used was the constant flow-perfused guinea-pig heart (Langendorff preparation). An extensive description of the procedure followed can be found elsewhere (Bova *et al.*, 1989). In brief, guinea-pigs of either sex (weight: 250–350 g) were killed by a blow to the neck and the heart was rapidly removed and retrogradely perfused within 45 s of removal. The perfusion medium was a modified Krebs-Henseleit solution (mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaCO_3$ 25, KH_2PO_4 1.2, glucose 11.1 and pyruvate 2) bubbled with an O_2:CO_2 gas mixture (95%:5%) and maintained at 37°C (pH 7.4 ± 0.01). The flow of perfusate was kept constant at about 10 ml g^{-1} tissue min^{-1} with a Gilson peristaltic micropump. After excising the sinus node the heart was driven at various frequencies (see below) with a S88 Grass stimulator through platinum electrodes placed on the left atrium.

Intraventricular pressure was measured by means of a rubber balloon inserted into the left ventricle and connected to a mechano-electrical transducer (MARB P82). The maximal positive derivative of the intraventricular pressure ($+dP/dt_{max}$) was taken as an index of contractility.

Surface ECG was recorded by means of two electrodes, one placed on the crux cordis and the other on the left ventricle free wall. The signal, amplified with a P16 Grass amplifier, was monitored with a Tektronics oscilloscope and simultaneously recorded on magnetic tape. PR, QRS and QT intervals were measured on magnified photographs of the tracings 'frozen' on the oscilloscope screen. In addition to QT duration the interval from Q-wave to T-wave peak was also determined (QTpeak) and the QT/QTpeak ratio was calculated. Coronary perfusion pressure (cP) was also monitored to assess the change in coronary bed resistance.

A solution of pinacidil was infused, with a peristaltic pump, through a T-junction at very low flow rate (28 times lower than the total flow); the very small increase in the total flow rate induced when infusion was started did not elicit any detectable change of the parameters measured. The drug concentrations in the infusing solutions were calculated in order to obtain final pinacidil concentrations of 10, 30, 50 μM in the aortic cannula.

Study protocol

The effects of pinacidil on contractility ($+dP/dt_{max}$), coronary resistance (cP) and ECG intervals were evaluated at four

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driving frequencies: 2.5, 3, 3.5 and 4 Hz. The choice of such a narrow range of frequencies was justified for two technical reasons: (1) in some preparations a frequency lower than 2.5 Hz allows a spontaneous junctional rhythm to arise; (2) at frequencies higher than 4 Hz the stimulus artifact on ECG tracings can fall close to the end of preceding T wave, making it impossible to measure the QT interval.

Each preparation was first allowed to recover for 30–45 min with stimulation at 2.5 Hz and then frequency was increased in 0.5 Hz steps up to 4 Hz, leaving 5 min for stabilization at each frequency step. After 15 min exposure of the heart to a single drug concentration the same stimulation procedure was repeated. Four to six experiments were performed at each concentration. Repetition of the stimulation protocol *per se* was found to have no influence on the cardiac parameters measured.

Determination of ventricular fibrillation threshold

In a separate series of experiments an attempt was made to induce ventricular fibrillation (VF) by electrical stimulation in basal conditions and after 15 min exposure to 10, 30, 50 and 100 μM pinacidil ($n = 8$ at each concentration).

After removing the atrial free wall and septum, stimulating electrodes were fitted over the upper edge of the intraventricular septum and a stimulation protocol similar to that described by Almotrefi & Baker (1980) was applied. Driving frequency was suddenly increased from 2.5 to 20 Hz and current intensity was progressively increased either until VF occurred or an intensity 10 fold greater then the basal value was reached.

Statistical analysis

A paired sample t test was used to assess the significance of the changes induced by the different frequencies of stimulation and drug concentrations on the parameters measured. For all comparisons, $P \leqslant 0.05$ was the criterion for statistical significance.

Results

Effects on coronary tone, contractility and ECG intervals

At the stimulation rate of 2.5 Hz the mean control values (±s.e.) of Cp, dP/dt_{max}, PR interval, QRS interval, QT interval duration and QTpeak/QT ratio were 64.0 ± 2.2 mmHg, 1405 ± 86 mmHg s^{-1}, 48.6 ± 2.2 ms, 22.5 ± 0.4 ms, 180.1 ± 3.4 ms and 0.900 ± 0.004, respectively. As expected, when the driving frequency was increased in steps, $+dP/dt_{max}$ and PR increased while QT and cP decreased (Figure 1). QRS duration and QTpeak/QT ratio were rate-independent.

In the presence of pinacidil (10, 30 or 50 μM) coronary pressure and cardiac contractility decreased, QT shortened and PR lengthened in a dose-dependent way, while QRS duration was not modified. The effects of pinacidil were virtually identical at any frequency applied, hence only the effects observed as 2.5 Hz are shown in Figure 2. Although the narrow range of concentrations tested does not allow us to define E_{max} and EC_{50} for each parameter measured, the various concentration-effect curves appear clearly distinct; the mean percentage changes (±s.e.) achieved at the highest pinacidil concentration (50 μM) were −74.0% ± 2.2 for $+dP/dt_{max}$ −51.3% ± 3.6 for cP, −34.3% ± 4.2 for QT and +16.2 ± 2.6 for PR.

As well as shortening the QT interval duration, pinacidil changed the morphology of the T-wave (Figure 3) in that it shortened the QTpeak interval to a greater extent than the QT interval, thus decreasing the QTpeak/QT ratio. The effect on QTpeak/QT ratio was dose-dependent and, unlike that on QT interval, also rate-dependent at least at the highest concentration tested (50 μM). Figure 4 shows the effect of pinacidil on QTpeak/QT ratio at the driving frequencies of 2.5 Hz and 4 Hz.

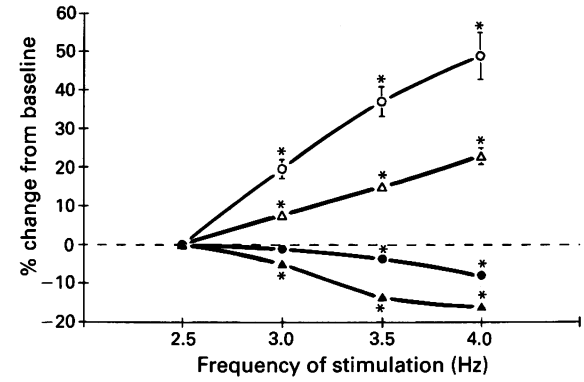

Figure 1 Frequency-dependence of the cardiac parameters of the guinea-pig isolated perfused heart measured under control conditions ($n = 16$). Changes are expressed as a percentage of the value observed at 2.5 Hz. Symbols: (○) $+dP/dt_{max}$; (△), PR interval; (●) coronary pressure; (▲), QT interval. Significant change from baseline: * $P \leqslant 0.05$.

Since the negative inotropic effect of pinacidil has been ascribed to the reduction of the plateau phase of the AP, due

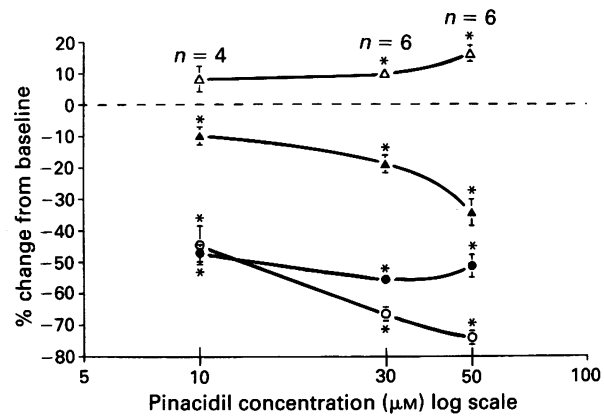

Figure 2 Effects of 10, 30 and 50 μM pinacidil on coronary pressure (●) $+dP/dt_{max}$ (○), QT interval (▲) and PR interval (△) in guinea-pig isolated perfused heart. Significant changes: * $P \leqslant 0.05$.

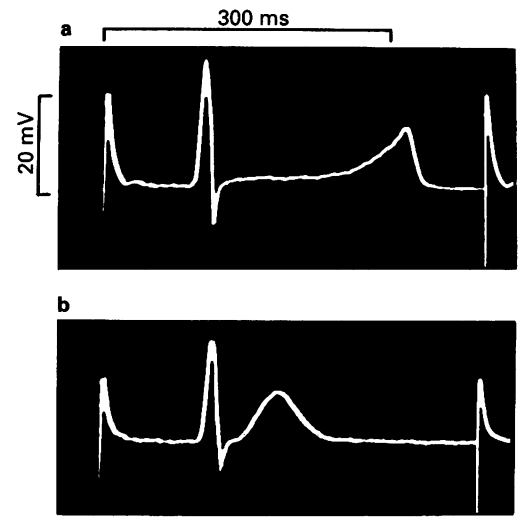

Figure 3 Pinacidil-induced ECG changes in guinea-pig isolated perfused heart. The drug lengthens PR interval, shortens QT interval and modifies QT-wave shape. (a) Control (2.5 Hz); (b) pinacidil 50 μM.

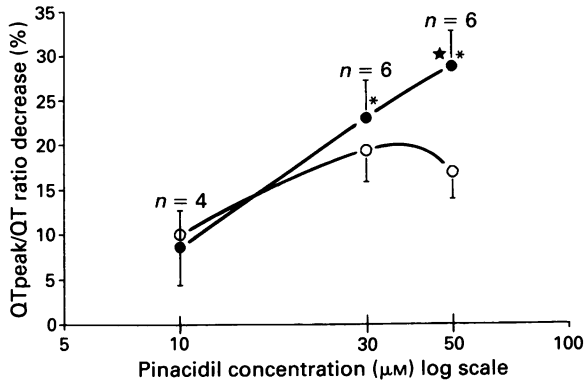

Figure 4 Effects of 10, 30 and 50 μM pinacidil on QTpeak/QT ratio at 2.5 Hz (○) and 4 Hz (●) in guinea-pig isolated perfused heart. Asterisks indicate the significance ($P \leq 0.05$) of the concentration-dependent changes and stars indicate the significance of the frequency-dependent changes.

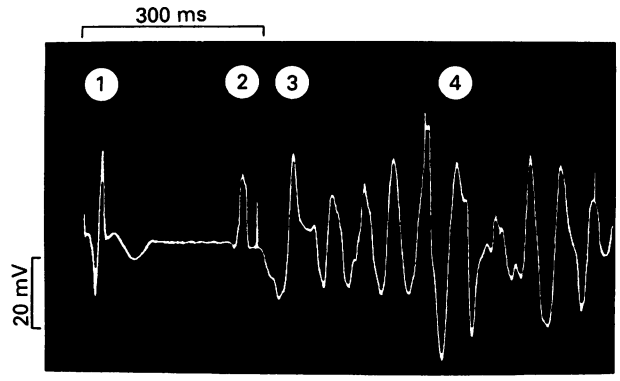

Figure 6 Ventricular fibrillation occurring after exposure to 50 μM pinacidil in guinea-pig isolated perfused heart. Numbers represent: (1) last normal beat; (2) first premature ventricular beat; (3) second premature ventricular beat rising from the T-wave; (4) ventricular fibrillation.

to the shortening of repolarization time (Smallwood & Steinberg, 1988), we analyzed the relationship between QT changes and $+dP/dt_{max}$ changes. Figure 5 shows that the decrease in $+dP/dt_{max}$ paralleled QT shortening only up to a 15–20% QT change, whereas greater QT variation was not associated with further appreciable $+dP/dt_{max}$ depression.

Taking into account the biological variability of QT duration in untreated hearts (160–215 ms at 2.5 Hz, 155–205 ms at 3 Hz, 145–190 ms at 3.5 Hz, 140–180 ms at 4 Hz) a further correlation was sought at each frequency step between pretreatment QT value and drug-induced QT shortening. The results indicate that QT shortening did not correlate with pretreatment QT duration at any dose level (data not shown).

Proarrhythmic effects

In 4 of 20 experiments, sustained ventricular fibrillation (VF) occurred after 16–28 min exposure to pinacidil (in one case at 30 μM and in three cases at 50 μM) and lasted until KCl (0.1 ml of a 0.54 M solution) was infused as a bolus into the aortic cannula. No clear relationship was noted between the onset of VF and the frequency applied (2.5–4 Hz). Further analysis of the ECG tracings revealed that VF was always preceded by a short run of premature ventricular beats, the last one rising from the peak of the T wave (R on T phenomenon) (Figure 6).

In order to study this proarrhythmic effect better, we tried to induce VF electrically by increasing, first the driving frequency (20 Hz), and then the stimulus current (see Methods). Under control conditions and after exposure to 10 μM pinacidil VF could never be induced. At a drug concentration of 30 μM, VF occurred in 5 out of 8 experiments at stimulation currents 2 to 5 times higher than the basal value. In 3 of 5 cases VF stopped spontaneously after 3–5 s (non-sustained VF), while in the other 2 cases the arrhythmia was still present after 5 min (sustained VF). At higher drug concentrations (50 and 100 μM) VF was always inducible and in every case the arrhythmia was of the sustained type. Apart from in one experiment at 50 μM concentration in which current intensity had to be increased 1.5 fold, in all the others it was sufficient to increase the driving frequency to 20 Hz in order to induce VF.

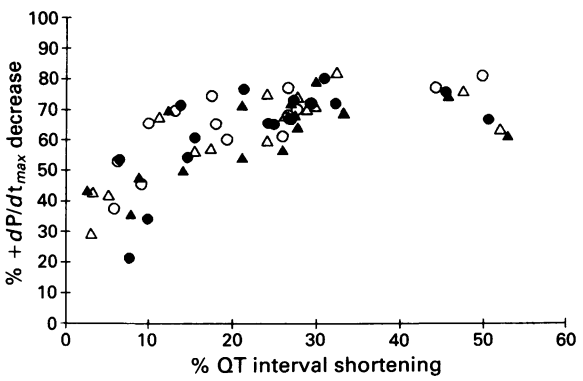

Figure 5 Relationship between QT interval and $+dP/dt_{max}$ changes induced by 10, 30 and 50 μM pinacidil in guinea-pig isolated perfused heart. Data obtained at different stimulation rates are shown: 2.5 Hz (○), 3 Hz (●), 3.5 Hz (△) and 4 Hz (▲).

Discussion

In agreement with the findings of other investigators (Kawashima & Liang, 1985; Longman *et al.*, 1988; Smallwood & Steinberg, 1988; Steinberg *et al.*, 1988; Nielsen *et al.*, 1989), our results show that in the range of concentrations between 10 and 50 μM, pinacidil decreases coronary tone and heart contractility, shortens ventricular repolarization time (QT) and prolongs AV conduction time (PR) without modifying intraventricular conduction time (QRS). While coronary vessels appear to be the tissue most sensitive to the actions of pinacidil, the parameter which undergoes the greatest percentage variation at the maximal dose tested is $+dP/dt_{max}$ (−74%). Such a marked negative inotropic effect is not generally seen *in vivo*, probably due to the lower doses employed and to the sympathetic activation induced by vasodilatation (Kawashima & Liang, 1985; Nichols *et al.*, 1986).

The mechanism by which pinacidil depresses heart contractility has not yet been clarified. It is generally accepted that drugs which shorten AP duration can reduce Ca-influx during the plateau phase (Sheu & Lederer, 1985). However, our data show that QT shortening greater than 15–20% is not accompanied by further substantial $+dP/dt_{max}$ depression, suggesting that the decrease of Ca-entry induced by AP shortening reaches a ceiling.

The magnitude of all the effects observed, except that on QTpeak/QT ratio (see below), was completely independent of driving frequency in the 2.5–4 Hz range. This behaviour is different from that of other drugs interacting with ionic channels, such as Na and Ca entry blockers (Hondeghem & Katzung, 1984) or some K channel blockers (Hondeghem & Snyders, 1990), which exhibit various patterns of use-dependence.

A possible explanation of the rate-independent effects of pinacidil may be that it binds to a voltage activated K channel in the heart with an affinity which is independent of the activation state of the channel. Alternatively, pinacidil could bind to a K channel which is not gated by the membrane potential of cardiac cells.

Recent experimental evidence (Fan *et al.*, 1990; Tzeng & Hoffman, 1990; Arena & Kass, 1989a,b) strongly suggests that the main site of action of pinacidil at the myocardial level is the ATP-sensitive K channel. The gating kinetics of such a

channel seem to be almost independent of membrane potential (Kakei et al., 1985) and this property is thus consistent with the use-independent effects of pinacidil. Furthermore, in normoxic conditions this K current is completely inhibited by high intracellular ATP levels (Noma, 1983) so that it does not normally contribute to cell repolarization. This feature could explain why the action of pinacidil on QT duration is independent of QT duration in the pretreatment period. In fact, if pinacidil were to act on a K current normally contributing to cell repolarization (e.g. the delayed rectifier) its effect should be less marked in preparations where K current is already high, namely where QT interval is shorter.

Another feature worth considering regarding the action of pinacidil is the change of T-wave morphology (Figure 3), as reflected by the decrease of QTpeak/QT ratio. It is difficult to understand why the effect of pinacidil on QTpeak interval is more marked than that on QT interval and why only the former is rate-dependent. A simple explanation may be that these two effects are the outcome of different ionic mechanisms.

It is generally acknowledged that T wave normally results from the asynchronous repolarization of the cells within the ventricular myocardium (Burgess, 1979). The first to repolarize are the epicardial cells, the last are the endocardial. Since the maximum voltage of T wave (T peak) occurs when most of the cells with short action potential duration (APD) are fully repolarized, a decrease in QTpeak interval should reflect a quicker repolarization of the epicardial cells. It has recently been shown that the epicardium (unlike the endocardium) has a large transient outward K current (I_{to}) (Litovsky & Antzelevitch, 1988; Wang et al., 1991) and that pinacidil can augment the Ca-dependent component of this current (I_{to2}) (Tzeng & Hoffmann, 1990). Thus, a reduction in the QTpeak/QT ratio could reflect a non-homogeneous drug-induced shortening of AP in the myocardial cell population.

Given that the dispersion of APD is a well-known arrhythmogenic condition (Kuo et al., 1983), it should be taken into account in explaining the occurrence of VF in some of our experiments. Finding this type of proarrhythmic effect in our model was worthy of note for at least two reasons: (1) To our knowledge it is the first time that pinacidil has been shown to promote VF under non ischaemic conditions. Wollenben et al. (1989) found that pinacidil reduces the time required for the Langendorff-perfused rat heart to develop VF following acute global ischaemia. Chi et al. (1990) were able to demonstrate that in infarcted dogs exposed to a second episode of acute ischaemia, pinacidil increases the likelihood of electrically inducing VF. In contrast, other authors (Nielsen et al., 1989) who tested the drug (0.15–100 μM) on perfused rabbit hearts in normoxic conditions did not observe any proarrhythmic effects. The reason for the discrepancy between these findings and our data is not clear but species-specific factors may be involved. (2) The guinea-pig heart is intrinsically resistant to electrically-induced VF because of its small size (short re-entry circuit length) and its relatively long QT interval (long refractory period) compared to that of other species with similar heart sizes (rats, for example). It is well recognized (Fozzard & Arnsdorf, 1986) that in the presence of a functional or anatomical circuit, re-entrant arrhythmias can occur only when: $RP < L/V$, where: RP = refractory period of the re-excited area; L = circuit length and V = slowest conduction velocity.

Pinacidil is able to create the functional substrate for re-entry simply by shortening refractoriness (QT interval), without delaying intraventricular conduction time (QRS). It is also conceivable that pinacidil may be responsible for the short runs of premature ventricular beats preceding the spontaneous VF episodes. In this respect it is noteworthy that Steinberg et al. (1988), studying the electrophysiological effects of pinacidil on canine Purkinje fibres, detected, as we did, the appearance of arrhythmias at concentrations $\geqslant 30\,\mu$M in about 20% of preparations.

The true clinical relevance of these experimental observations cannot be inferred with certainty. The highest concentration found in serum of hypertensive patients treated with pinacidil is about 30–40 times lower than that producing VF in our experiments (Carsen et al., 1983) and, so far, no proarrhythmic effects have been reported in man.

Nevertheless, in 30% of patients treated with pinacidil, T-wave changes were observed (Goldberg, 1988) and little is known about drug tolerability in the subgroup of hypertensive patients with ischaemic heart disease. Recent evidence indicates that a new KCO (SR 44866) increases long term mortality in rats with healed myocardial infarction (Mulder et al., 1990). It therefore seems reasonable to recommend careful surveillance, at least in patients suffering from myocardial ischaemia.

Finally, on mere experimental grounds, the combined exposure of guinea-pig heart to pinacidil, plus electrical stimulation, may represent a new, valuable model to induce sustained VF and study the effectiveness of antiarrhythmic drugs.

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8-Hydroxy-2-(di-n-propylamino)tetralin impairs spatial learning in a water maze: role of postsynaptic 5-HT_{1A} receptors

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1 The effect of 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT), a 5-HT_{1A} receptor agonist, on place navigation was studied by use of two spatial tasks in a water maze.

2 In the first experiment, rats treated subcutaneously with 100 and 300 (but not 30) $\mu g\,kg^{-1}$ 8-OH-DPAT were impaired in their ability to locate a hidden platform. The probe test confirmed the impairment of spatial navigation but the effect (time spent in the training quadrant) was quantitatively different, depending on whether 8-OH-DPAT was administered only before each training session, only before the probe test or in both conditions.

3 In the second experiment, rats received 150 μg 5,7-dihydroxytryptamine (5,7-DHT) intracerebroventricularly to destroy 5-hydroxytryptamine (5-HT)-containing neurones and 24 days later were examined for choice accuracy in a two-platform spatial discrimination task.

4 At 100 (but not 30) $\mu g\,kg^{-1}$ 8-OH-DPAT impaired rats' accuracy with no effect on latency and no errors of omission. In 5,7-DHT-treated rats, this dose had a greater effect, including errors of omission. Sham-operated rats injected with 300 $\mu g\,kg^{-1}$ 8-OH-DPAT were markedly impaired in accuracy but they had longer latencies and made more errors than controls. All the effects were increased in 5,7-DHT treated rats.

5 The results suggest that, at doses causing no apparent changes in motor behaviour or motivation, 8-OH-DPAT impairs spatial navigation by stimulating postsynaptic 5-HT_{1A} receptors in the rat brain.

Keywords: 8-OH-DPAT; 5-HT_{1A} receptors; spatial discrimination; rat

Introduction

Several studies suggest that stimulation of 5-HT_{1A} receptors in the brain causes various effects such as hypotension (Fozard *et al.*, 1987), hypothermia (Goodwin & Green, 1985), changes in motor behaviour and hyperphagia (Dourish *et al.*, 1985; Bendotti & Samanin, 1986), antidepressant-like (Cervo & Samanin, 1987) and anxiolytic-like activity (Engel *et al.*, 1984; Carli & Samanin, 1988). There is little information on the role of this 5-hydroxytryptamine (5-HT) receptor type in learning and memory processes. High densities of 5-HT_{1A} binding sites have been found in the hippocampus (Pazos *et al.*, 1988), an area traditionally linked to cognitive functions, particularly spatial memory (O'Keefe & Nadel, 1978; Morris *et al.*, 1982).

Post-training infusion of 5-HT in the hippocampus was reported to impair retention in a Y-maze brightness discrimination task (Wetzel *et al.*, 1980). In agreement with these findings, Altman *et al.* (1990) recently reported that 5-hydroxytryptaminergic deafferentiation of the hippocampus facilitated learning of a positively reinforced spatial discrimination task. These studies suggest that 5-HT in the hippocampus has a negative influence on memory processes but do not clarify which 5-HT receptor type is involved. 5-HT_{1A} receptors apparently exert a predominant inhibitory influence on the electrical activity of hippocampal pyramidal cells (Andrade & Nicoll, 1987; Sprouse & Aghajanian, 1988) but other 5-HT receptor types in the hippocampus have electrophysiological effects opposite to those of 5-HT_{1A} receptors (Colino & Halliwell, 1987; Chaput *et al.*, 1990).

The density of 5-HT_{1A} receptor binding is high in the nucleus raphe dorsalis (DR) (Pazos *et al.*, 1988). Stimulation of these receptors reduces the firing of 5-HT cells (Sprouse & Aghajanian, 1986) and diminishes the release of 5-HT in various terminal regions (Sharp *et al.*, 1989). There is no information on the role of presynaptic 5-HT_{1A} receptors on learning and memory. Electrical stimulation of the DR disrupts memory in a passive avoidance task by a 5-HT-dependent mechanism (Fibiger *et al.*, 1978). Therefore stimulation of pre- and postsynaptic 5-HT_{1A} receptors by appropriate agonists may affect rats' performance in learning and memory tasks.

8-Hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT), a specific agonist at 5-HT_{1A} receptors, was recently reported to reduce the efficiency of rats in a radial maze at 300 and 500 $\mu g\,kg^{-1}$ (Winter & Petti, 1987). These relatively high doses, however, cause profound changes in the animals' motor behaviour (Evenden & Angeby-Moller, 1990) making it difficult to assign the effects observed to specific disruption of memory processes. Recently 100 $\mu g\,kg^{-1}$ 8-OH-DPAT, administered before or immediately after the training trial, was found to impair rats' performance in a one-trial passive avoidance task; 30 $\mu g\,kg^{-1}$ 8-OH-DPAT administered before the training trial also impaired their performance (Carli *et al.*, 1992). These studies suggest that relatively low doses of 8-OH-DPAT interfere with mechanisms related to the acquisition and storage of memory of recent events. Spatial learning in the Morris water maze task was impaired by 200 $\mu g\,kg^{-1}$ 8-OH-DPAT while 60 $\mu g\,kg^{-1}$ had no effect (Hunter & Roberts, 1988). The dose and the test may therefore be critical to reveal the memory disruptive effect of 8-OH-DPAT.

In the present study we examined the effects of various doses of 8-OH-DPAT (starting from 30 $\mu g\,kg^{-1}$) in a Morris swim maze in which rats are required to learn the location of a spatially fixed platform. One purpose of this study was also to distinguish effects on acquisition from those on performance by administering 8-OH-DPAT at different times during the task.

To reduce the problems associated with drug effects on rats' performance, the effects of 8-OH-DPAT were also examined in a two-platform spatial discrimination task in the water maze, in which choice accuracy and latency can be measured

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separately (Hagan *et al.*, 1987). In order to clarify whether pre- or postsynaptic 5-HT$_{1A}$ receptors were involved in the effect of 8-OH-DPAT, this task was conducted in rats that had received 5,7-dihydroxytryptamine (5,7-DHT) intracerebroventricularly to destroy 5-HT-containing neurones.

Methods

Male albino rats (CD-COBS, Charles River, Italy), weighing 200–250 g, were housed in pairs and kept in a room at constant temperature (21 ± 1°C) and relative humidity (60%), with a regular light/dark schedule (07 h 00 min–19 h 00 min). Food (Altromin pellets for rats) and water were available *ad libitum*. All animals were handled for 2–3 days before starting the experiments.

Injection of 5,7-dihydroxtryptamine (5,7-DHT)

The rats, anaesthetized with Equithesin, were immobilized in a Kopf stereotaxic instrument. 5,7-Dihydroxytryptamine creatinine sulphate (5,7-DHT) 150 μg (calculated as a free base) was dissolved in 20 μl of ascorbic acid solution (1 mg ml^{-1}) and administered into the right lateral ventricle. Control animals received only the ascorbic acid solution (vehicle). To protect noradrenaline-containing neurones from the action of 5,7-DHT (Baumgarten *et al.*, 1973), 30 min before the injection of 5,7-DHT the rats were given desipramine 25 mg kg^{-1} intraperitoneally (i.p.), an inhibitor of noradrenaline uptake into nerve endings (Breese & Cooper, 1975). After surgery, animals were allowed to recover for 24 days during which time they were accustomed to being handled.

Forty-eight hours after the end of the experiments, rats treated with 5,7-DHT and their controls were killed by decapitation and their brains were rapidly removed for assay of 5-HT according to Achilli *et al.* (1985).

Morris water-maze

A circular 'swimming pool' measuring 1.5 m in diameter, 0.5 m high was used. The pool was filled to a depth of 0.29 m with water (26 ± 1°C) which was rendered opaque by the addition of a food dye (coffee color, Bayo, Italy). The water was changed daily. The pool was located centrally in a large room and was surrounded by various visual cues: a blackened window with a big white cross, a white wall with a big black cross, a long table, a door and a picture-covered wall with a rack for cages. The site of the objects could be occluded, when required, by black curtains around the maze. When open, the curtains were collected together at one corner of the room, so forming a prominent visual cue in themselves. The room was illuminated by a light bulb (100 W) located in the centre of the ceiling or by two overhead lamps (each 60 W) positioned and directed in such a way as to give diffuse illumination of the room.

The rats were placed in the pool and left to swim around until they found the single escape platform (11 × 11 cm) the top of which was made of transparent perspex, 'hidden' 1.5–2.0 cm below the water surface.

The pool surface was divided into four quadrants (training quadrant; adjacent left; adjacent right and opposite quadrant). There were four starting positions from which the rats were put into the water, arbitrarily designated by the cardinal points North, East, South and West (N, E, S, and W) around the circumference of the pool. Three concentric equal-area zones from the pool centre and separated by radii 0.58 PR (PR = pool radius) and 0.82 PR were defined on the pool surface.

The rat's behaviour was monitored by an overhead video camera (CCD, Sitel, Italy) linked to the VP112 scanning unit (HVS Image, U.K.) which in turn was connected to a Amstrad PC 1512 computer.

For all rats, training consisted of four trials each day for five days. The platform was fixed in one of the four quadrants throughout training. A training trial began with the experimenter lowering the animal into the pool, its head facing and close to the side wall, at position N, E, S or W. The timer was started (by a remote control connected to the computer) and the time the animal took to escape from the water onto the platform (escape latency) was recorded. If the animal did not find the platform in 120 s, it was picked up by the experimenter and put onto the platform. The rat remained on the platform for 15 s before being picked up and put in a holding cage for a 4–8 min inter-trial interval.

Twenty-four hours after the last training day, a probe test was done to assess the spatial bias of all trained animals. The hidden platform was removed from the pool and the rat was placed in it for a single 60 s period. The rats were placed in the pool from the starting point opposite their training quadrant. A record was made of the paths the animals followed searching for the platform. The software (HVS Image, U.K.) used by our monitoring set-up gave a precise measure of the path length swum, latency to reach the platform, speed and the % time swum by the rat in each quadrant and in each equal-area zone.

Escape latency (s), swimming distance (m), speed (m s^{-1}) were analysed by analysis of variance with factors: drug (between subjects) and days (within subjects), and *post hoc* comparisons between groups were analysed by Tukey's test. Probe test performance (swimming speed, % time swum in the training quadrant and % time swum in the outer equal-area zone) were analysed by analysis of variance and *post hoc* comparison with the controls (saline) by Dunnett's *t* test.

Two-platform spatial discrimination task

The apparatus was the same as in the place navigation task. Two visible platforms were used. The fixed one protruded 1.5–2.0 cm above the water surface. Its top was square (11 × 11 cm) and made of Perspex. The second platform was identical to the first and also protruded 1.5–2.0 cm above the water. It was made of the same material but was filled with expanded polystyrene. It was 'anchored' with thread to a solid movable base placed on the bottom of the pool. Thus one platform was rigid and provided support, the other sank when the rats attempted to climb on it. These platforms were both flat grey.

The rats were trained to swim to one grey escape platform while avoiding the second grey floating platform. For all rats, the rigid escape platform (correct) was always in the same location at the centre of one of the four quadrants. The floating platform (incorrect) was positioned, over successive trials, in a quasirandom sequence of eight locations around the pool, subject to the constraint that the spatial relationship between the platforms and starting positions did not consistently reward either right or left turning tendencies. The rigid platform was equally often the closest to the starting point as the one farthest away. They were sometimes close together, while in other trials they were far apart.

All rats were trained for 12 consecutive days, doing 10 trials each day. A trial began with the rat being placed in the pool while held at, and facing, the side wall. Eight possible starting locations were used in quasirandom sequence across trials. A trial ended when the rat escaped onto the rigid platform, where he was allowed to sit for 15 s before being returned to a holding cage before the next trial. The rats were trained in squads of four. Trials were run spaced with an inter-trial interval of approximately 2–4 min. Each rat's daily testing lasted approximately 30 min. The criterion was reached when performance had to average at least 80% correct over two days. A correct trial was one in which the rats climbed onto the rigid platform without touching the floating platform with

their forepaws or snout. The occasional incident of brushing past the floating platform in passing was not considered an error. If the rat did not choose to escape on either platform (correct or incorrect) in 60 s it was taken out of the pool and an omission error was scored. We measured (1) the first choice in each trial (correct/incorrect), (2) the latency to escape (s), and (3) the number of omissions.

Treatment schedules

In the Morris swim maze, in one experiment 8-OH-DPAT hydrobromide (R.B.I. Wayland, Massachusetts, U.S.A.) was dissolved in saline and injected subcutaneously (s.c.) 30 min before each acquisition training session and before the probe test. In order to distinguish effects on acquisition from those on performance, in another two experiments 8-OH-DPAT was administered before acquisition training sessions only or before the probe test only.

In the experiment in which choice procedure was used, sham-operated and 5,7-DHT-treated rats were injected s.c., starting 24 days after intracerebroventricular injection of 5,7-DHT, with saline or 8-OH-DPAT 30 min before each acquisition training session.

Statistics

Data, percentages of correct choices and latency to escape (s) from vehicle and 5,7-DHT groups were analysed separately by analysis of variance (two-way ANOVA with factors) days (within subject) and drug (between subject) and *post hoc* comparisons between groups were done with Tukey's test. Lesioned and unlesioned rats were also compared at each dose separately using a two-factor (days × lesion) ANOVA. The number of omissions on the first day of training was analysed by two-way analysis of variance and the *post hoc* comparisons by Tukey's test.

Results

Morris water maze acquisition

All animals improved their performance over the trials as shown by the progressive reduction in latency and distance swum to reach the platform (latency: $F_{4,112} = 90.2$, $P < 0.001$; distance: $F_{4,112} = 57.5$, $P < 0.001$). Administration of 8-OH-DPAT before each session dose-dependently affected rats' performance (latency: $F_{3,28} = 12.8$, $P < 0.001$; distance: $F_{3,28} = 13.8$, $P < 0.001$; speed: $F_{3,28} = 7.7$, $P < 0.001$). Animals treated with 8-OH-DPAT 300 $\mu g\,kg^{-1}$ swam significantly more than controls ($P < 0.01$; Tukey's test) for the whole period of training while in animals treated with 100 $\mu g\,mg^{-1}$ the effect was seen only on days 1, 2 and 3 ($P < 0.05$; Tukey's test). The impairment (days × group interaction, $F_{12,112} = 3.7$ $P < 0.01$) was less evident on measuring the latency to reach the platform; 300 $\mu g\,kg^{-1}$ 8-OH-DPAT caused a significant increase only on days 1, 2 and 3 ($P < 0.01$, Tukey's test) and 100 $\mu g\,kg^{-1}$ produced no effect. This can be explained by the fact that rats injected with 100 $\mu g\,kg^{-1}$ 8-OH-DPAT had a higher swimming speed from the first days and by the end of training all 8-OH-DPAT-treated animals swam faster than controls (significant days × group interaction $F_{12,112} = 2.9$, $P < 0.01$).

Morris water maze probe test

Figure 2a shows the results with animals which had received 8-OH-DPAT before each training session and before the probe test. The acquisition data for this group are shown in Figure 1. Only those treated with 300 $\mu g\,kg^{-1}$ were unable to locate the training quadrant ($F_{3,27} = 5.8$, $P < 0.01$). They spent significantly less time in the training quadrant than the other experimental groups ($P < 0.01$; Dunnett's t test). These animals tended to swim close to the side walls ($F_{3,27} = 4.7$, $P < 0.01$) as shown by the significantly higher time they spent in the outer zone of the pool ($P < 0.05$; Dunnett's t test). The swimming speed ($F_{3,27} = 9.5$, $P < 0.01$) of the animals treated with 100 and 300 $\mu g\,kg^{-1}$ 8-OH-DPAT was significantly higher than controls ($P < 0.01$; Dunnett's t test).

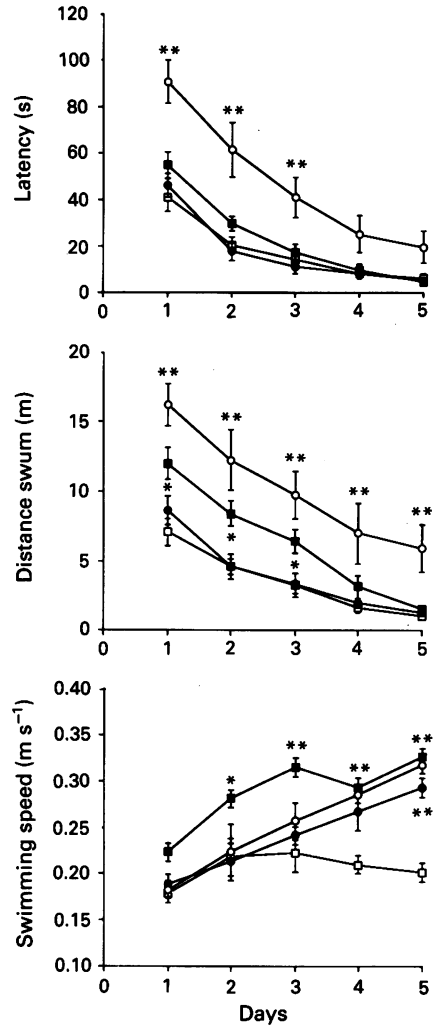

Figure 1 Acquisition of familiarity with the Morris swim maze by rats treated with 0 (□), 30 (●), 100 (■) or 300 (○) $\mu g\,kg^{-1}$ 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT). The animals received 8-OH-DPAT subcutaneously 30 min before each training session. Each value is the mean of 8 rats per group; vertical bars show s.e.means.
* $P < 0.05$; ** $P < 0.01$ vs. controls (Tukey's test).

When 8-OH-DPAT was administered only before the probe test (Figure 2b), all the doses significantly ($F_{3,28} = 6.9$, $P < 0.01$) reduced the time spent in the training quadrant ($P < 0.05$ for 30 $\mu g\,kg^{-1}$ and $P < 0.01$ for 100 and 300 $\mu g\,kg^{-1}$; Dunnett's t test). Only rats treated with 300 $\mu g\,kg^{-1}$ spent significantly more time than controls in the outer zone ($F_{3,28} = 6.2$, $P < 0.01$; $P < 0.01$ Dunnett's t test), whereas swimming speed ($F_{3,28} = 16.6$, $P < 0.001$) was higher in animals injected with 100 and 300 $\mu g\,kg^{-1}$ 8-OH-DPAT ($P < 0.05$ and $P < 0.01$ respectively, Dunnett's t test).

Figure 2c shows the results of the experiment in which the animals received 8-OH-DPAT only before each training session. The effects of 8-OH-DPAT on acquisition were not

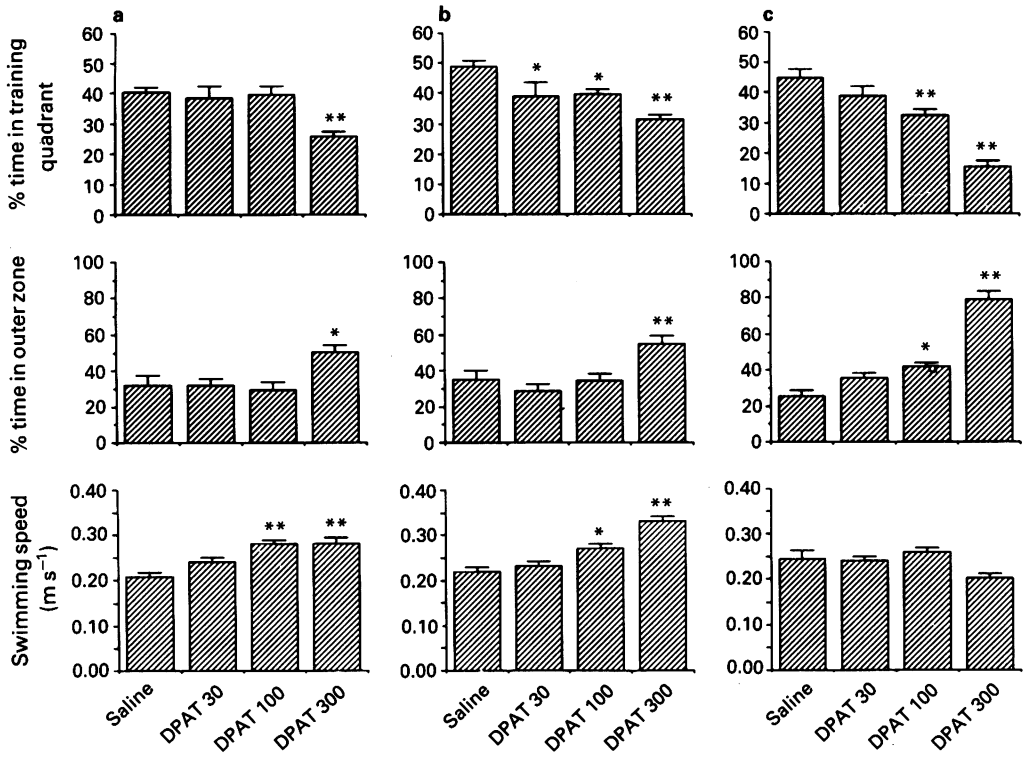

Figure 2 Behaviour in the probe test of rats given 0, 30, 100 and 300 $\mu g\,kg^{-1}$ 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) subcutaneously 30 min before each training session and before the probe test (*a*), only before the probe test (b) or only before each training session (c). Each value is the mean of 8 rats per group; s.e.mean shown by vertical bars.
* $P < 0.05$; ** $P < 0.01$ vs. controls (Tukey's test).

different from those shown in Figure 1. Animals' performance during the probe test was impaired after doses of 100 and 300 $\mu g\,kg^{-1}$ given only before each training session, (% time in training quadrant: $F_{3,27} = 28.4$, $P < 0.001$; % time in outer zone: $F_{3,27} = 55.0$, $P < 0.001$; $P < 0.01$, Dunnett's test). The time the animals treated with 300 $\mu g\,kg^{-1}$ 8-OH-DPAT spent in the training quadrant was markedly reduced ($P < 0.01$, Dunnett's *t* test) and they spent about 80% of their time in the outer zone of the pool ($P < 0.01$, Dunnett's *t* test). These effects were less marked but still significant with 100 $\mu g\,kg^{-1}$ ($P < 0.05$; Dunnett's *t* test) whereas the lowest dose caused no effect. No effect on swimming speed was seen with any dose in this condition ($F_{3,27} = 1.9$ NS).

Two-platform spatial discrimination task

The accuracy of controls improved over the days and approached asymptote on day 4 (Figure 3). 5,7-DHT lesion by itself did not significantly modify swim maze acquisition (days × lesion: $F_{11,165} = 1.4$, $P > 0.05$). 8-OH-DPAT significantly reduced the percentage of correct choices (accuracy) in vehicle- and 5,7-DHT-treated rats (days × drug: $F_{33,352} = 2.0$ and $F_{33,330} = 1.9$; $P < 0.01$ for vehicle and 5,7-DHT, respectively). Rats treated with 300 $\mu g\,kg^{-1}$ 8-OH-DPAT before each session made significantly more errors ($P < 0.01$; Tukey's test) than controls from day 3 to day 9 but by days 10–12 they did not differ from controls. In 5,7-DHT-treated rats, 300 $\mu g\,kg^{-1}$ 8-OH-DPAT caused more errors throughout the whole of training ($P < 0.01$; Tukey's test). A similar but less marked pattern of effects was seen with 100 $\mu g\,kg^{-1}$ 8-OH-DPAT ($P < 0.05$ and $P < 0.01$; Tukey's test). Animals treated with 100 $\mu g\,kg^{-1}$ showed more errors only on days 3 ($P < 0.01$) and 4 ($P < 0.05$ Tukey' test) in vehicle and days 1–7 in 5,7-DHT-treated animals (days 1, 6 and 7, $P < 0.05$; days 2–5, $P < 0.01$ Tukey's test).

A comparison between lesioned and unlesioned animals showed a significant days × lesion interaction at 300 ($F_{11,165} = 1.4$, $P < 0.05$) and 100 $\mu g\,kg^{-1}$ ($F_{11,176} = 2.0$, $P < 0.05$). No effect at any time or condition was seen with 30 $\mu g\,kg^{-1}$ 8-OH-DPAT.

Animals treated with 300 $\mu g\,kg^{-1}$ 8-OH-DPAT showed significantly higher latencies for most of the training time in vehicle (days × drug: $F_{33,352} = 3.1$, $P < 0.01$) and 5,7-DHT-treated animals (days × drug: $F_{33,330} = 2.5$, $P < 0.01$), whereas 100 $\mu g\,kg^{-1}$ significantly increased latencies only in 5,7-DHT-treated animals on days 1 and 2 ($P < 0.05$; Tukey's test) (Figure 3).

The animals treated with 300 $\mu g\,kg^{-1}$ tended to swim close to the side wall and made significantly more errors of omission on day 1 than controls (saline) in vehicle and 5,7-DHT-treated animals (treatment × drug: $F_{3,61} = 2.5$, $P < 0.05$); 100 $\mu g\,kg^{-1}$ caused more errors only on day 1 for 5,7-DHT treated animals ($P < 0.01$; Tukey's test).

The results on omissions by the various experimental groups on day 1 are shown in Table 1. At all doses, the incidence of errors of omission essentially disappeared from day 2

Table 1 Spatial discrimination task: errors of omission caused by 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) in vehicle and 5,7-dihydroxytryptamine (5,7-DHT)-treated rats

Drug	$\mu g\,kg^{-1}$	*Vehicle*	*5,7-DHT*
Saline	0	0.4 ± 0.1	0.6 ± 0.2
8-OH-DPAT	30	0.9 ± 0.2	0.9 ± 0.2
8-OH-DPAT	100	1.6 ± 0.5	3.7 ± 0.7**
8-OH-DPAT	300	3.7 ± 1.2**	6.3 ± 0.7**††

Values are mean ± s.e.mean of 7–9 rats per group.
The animals were injected intracerebroventricularly with 5,7-DHT (150 μg in 20 μl) or vehicle (ascorbic acid 0.1%) 24 days before the experiments.
** $P < 0.01$ vs. vehicle + saline or 5,7-DHT + saline (Tukey's test).
†† $P < 0.01$ vs. vehicle + 8-OH-DPAT 300 $\mu g\,kg^{-1}$ (Tukey's test).

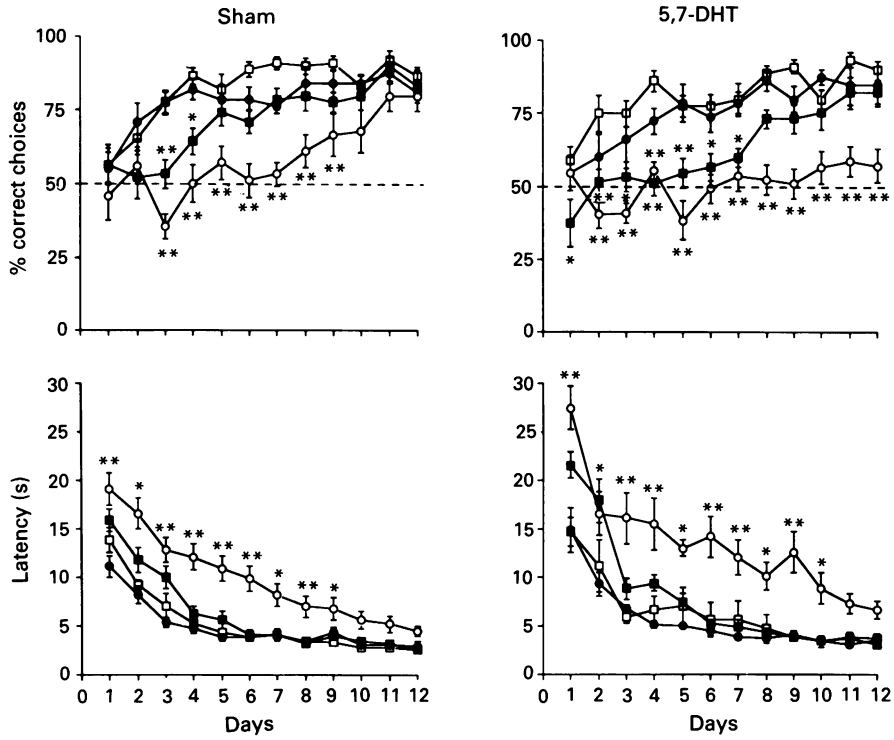

Figure 3 Spatial discrimination task: the effects of 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) on the percentage of correct choices and latency of rats injected intercerebroventricularly with vehicle (Sham) or 5,7-dihydroxytryptamine (5,7-DHT). 5,7-DHT (150 μg in 20 μl) or vehicle (ascorbic acid 0.1%) were administered 24 days before the beginning of the experiments. The animals received 0 (□), 30 (●), 100 (■) or 300 (○) $\mu g\,kg^{-1}$ 8-OH-DPAT subcutaneously 30 min before each acquisition session. Each value is the mean of 7–9 rats per group; vertical bars show s.e.means.
* $P < 0.05$; ** $P < 0.01$ vs. controls (Tukey's test).

in vehicle-treated animals while 5,7-DHT-treated animals given 300 $\mu g\,kg^{-1}$ 8-OH-DPAT still had about 20% omissions until day 4.

Brain levels of 5-HT were markedly lower (about 10% of controls) in 5,7-DHT-lesioned rats. Levels of 5-HT ($ng\,g^{-1}$; mean ± s.e.mean) were: vehicle + saline 471 ± 18; 5,7-DHT + saline 56 ± 6. No dose of 8-OH-DPAT significantly changed 5-HT levels in vehicle- or 5,7-DHT-treated rats (data not shown).

Discussion

8-OH-DPAT at doses of 100 and 300 $\mu g\,kg^{-1}$ administered 30 min before training sessions dose-dependently impaired place navigation in the Morris swim maze while 30 $\mu g\,kg^{-1}$ had no such effect. In rats given 300 $\mu g\,kg^{-1}$ 8-OH-DPAT, distances taken to reach the hidden platform were longer than those of controls for the whole period of training but latencies were significantly increased only in the initial sessions. Rats treated with 100 $\mu g\,kg^{-1}$ showed a significant increase in distance swum on days 1, 2 and 3.

The lack of effect of 100 $\mu g\,kg^{-1}$ 8-OH-DPAT on latencies may be explained by the higher swimming speed of these animals in the early sessions. By day 5, all the animals treated with 8-OH-DPAT showed a higher swimming speed with no significant effect on latency. At this time only rats treated with 300 $\mu g\,kg^{-1}$ 8-OH-DPAT swam longer distances. The results suggest that the distance swum is a more accurate measure of performance impairment during acquisition of a swim maze task when there are changes in animals' swimming speeds. All the animals treated with 8-OH-DPAT, like controls, clearly improved their performance over the trials. It is difficult, however, to establish whether the improvement was due to true spatial localization or alternative strategies which the animals used to reach the platform.

The probe test confirmed that 300 $\mu g\,kg^{-1}$ 8-OH-DPAT impaired spatial navigation since the animals spent significantly less time in the training quadrant. However, the drug was even more active when administered only before the probe test. Even 30 $\mu g\,kg^{-1}$ produced a significant effect in this condition. This raises the question whether acute effects of 8-OH-DPAT on motor performance were involved in the effect in the probe test. This is likely in view of the fact that visual inspection of their behaviour revealed that particularly rats treated with 300 $\mu g\,kg^{-1}$ tended to swim very close to the side walls. These animals also spent more time than the other experimental groups in the outer zone of the pool. An additional confounding variable was that animals receiving 100 and 300 $\mu g\,kg^{-1}$ before acquisition and before the probe test or only before the probe test swam faster than controls.

To clarify how the effects of 8-OH-DPAT during acquisition influenced animals' behaviour in the probe test, one group of rats received 8-OH-DPAT only before each training session and was tested in the probe test 24 h later. Animals treated with 100 and 300 $\mu g\,kg^{-1}$ remained in the training quadrant less than controls, spent more time in the outer zone of the pool and showed no change in their swimming speed during the probe test. Thus the effect on swimming speed seems related to the acute effects of 8-OH-DPAT, whereas the tendency to swim around in large circles, already observed in the early training sessions, is probably part of the strategy the animals adopt to improve their performance during acquisition.

The effects in the probe test in animals which had received 100 and 300 $\mu g\,kg^{-1}$ only during the acquisition stage, suggest that spatial localization was impaired in animals treated with 8-OH-DPAT.

Obviously, the effects observed in the Morris swim maze cannot be interpreted exclusively in terms of changes in learning and memory processes since sensory-motor abnormalities or changes in motivation could be involved in the effect of

8-OH-DPAT on latencies, distances swum or rats' behaviour during the probe test.

In a second experiment therefore we used a recently described procedure in which choice accuracy and latency could be measured separately (Hagan *et al.*, 1987). Moreover, motivational effects could be revealed by the number of omissions. In order to clarify whether pre- or postsynaptic 5-HT$_{1A}$ receptors were involved in the effect of 8-OH-DPAT on choice accuracy, this study was conducted in animals which had received 5,7-DHT intracerebroventricularly to destroy 5-HT-containing neurones.

Rats treated with 100 and 300 $\mu g\,kg^{-1}$ 8-OH-DPAT before each session made significantly more errors than saline-treated animals. That memory processes were disrupted is suggested by the fact that at 100 $\mu g\,kg^{-1}$ 8-OH-DPAT impaired the rats' accuracy with no effect on omissions or latencies in vehicle-treated animals. Moreover, 300 $\mu g\,kg^{-1}$ 8-OH-DPAT caused significantly more omissions in vehicle treated rats only on day 1 while accuracy was impaired until day 10. Animals treated with 300 $\mu g\,kg^{-1}$ 8-OH-DPAT also showed increased latencies. This effect could not be attributed to a reduction in motor activity since swimming speed was actually increased. As mentioned above, the animals treated with 300 $\mu g\,kg^{-1}$ tended to swim close to the side walls and this may have influenced the paths they used to reach the platform.

The effect of 8-OH-DPAT on choice accuracy was significantly potentiated in rats treated with 5,7-DHT. The greater effect of 100 $\mu g\,kg^{-1}$ 8-OH-DPAT in 5,7-DHT-treated rats could not be attributed only to sensory-motor disturbances or changes in motivation since the number of omissions was increased only on day 1, latencies were longer on days 1 and 2 but choice accuracy was impaired significantly until day 7. 5,7-DHT-treated rats, given 300 $\mu g\,kg^{-1}$ 8-OH-DPAT instead, showed not only longer latencies but also more omissions for most of the training period, suggesting that abnormalities in motor behaviour or motivation could have contributed to the results.

The results in the two-platform spatial discrimination task confirm that at 100 $\mu g\,kg^{-1}$, 8-OH-DPAT specifically interferes with the mechanisms involved in spatial learning. Although the lack of specific 5-HT$_{1A}$ receptor antagonists prevented us trying to prove it more directly, the fact that relatively low doses of 8-OH-DPAT were active and its relative specificity as a 5-HT$_{1A}$ receptor agonist (Hoyer, 1988) suggest that the compound impairs spatial navigation by an action on 5-HT$_{1A}$ receptors.

That 8-OH-DPAT may interfere with mechanisms related to the acquisition and storage of memory of recent events is also suggested by a recent study showing that 8-OH-DPAT affected rats' performance in a passive avoidance task by a mechanism which could be separated from effects on pain perception, general activity or emotional behaviour (Carli *et al.*, 1992).

High densities of 5-HT$_{1A}$ binding sites are found in the hippocampus, raphe nuclei and some layers of the cerebral cortex in the rat brain (Pazos *et al.*, 1988). The fact that 5,7-DHT treatment did not reduce but actually potentiated the effect of 8-OH-DPAT suggests that postsynaptic 5-HT$_{1A}$ receptors are involved in the ability of 8-OH-DPAT to impair spatial navigation. In view of the important role of hippocampus in the processes involved in spatial learning (O'Keefe & Nadel, 1978; Morris *et al.*, 1982), it is likely that 5-HT$_{1A}$ receptors in this region are involved in the effect of 8-OH-DPAT. Various studies suggest that supersensitivity of postsynaptic 5-HT receptors, including the 5-HT$_{1A}$ type, develops after 5,7-DHT (Nelson *et al.*, 1978; Zemlan *et al.*, 1983; Goodwin *et al.*, 1987). Although the density of hippocampal 5-HT$_{1A}$ binding sites is not increased in rats treated with 5,7-DHT (and may actually be reduced in the CA2/CA3 region) (Vergé *et al.*, 1986; Hensler *et al.*, 1991), a supersensitivity of some hippocampal 5-HT$_{1A}$ receptors following 5,7-DHT lesions of 5-hydroxytryptaminergic terminals cannot be excluded. Experiments on inhibition of forskolin-stimulated adenylate cyclase by 5-HT$_{1A}$ receptor agonists (De Vivo & Maayani, 1986) may help clarify this.

In conclusion, the present study indicates that 8-OH-DPAT, a specific 5-HT$_{1A}$ receptor agonist, impairs spatial learning by a mechanism which may involve postsynaptic 5-HT$_{1A}$ receptors, probably in the hippocampus. Measures aimed at changing the activity of postsynaptic 5-HT$_{1A}$ receptors in the hippocampus more directly will be necessary to clarify their role in spatial memory in rodents and, in general, in learning and memory processes in various tasks and species.

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Separation of putative a_{1A}- and a_{1B}- adrenoceptor mediated components in the tension response of the rat vas deferens to electrical field stimulation

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1 The effects of the putative α_{1B}-adrenoceptor antagonist, chloroethylclonidine (CEC), on tension responses of the rat isolated whole vas deferens to single and multiple pulses of electrical field stimulation have been evaluated by use of a microcomputer system which enables the averaging of like-responses throughout their time course.

2 CEC (10^{-7} to 3×10^{-6} M) selectively and in a concentration-dependent manner blocked the noradrenergic component of the response to a single field stimulus in the absence or presence of nifedipine (10^{-5} M, which blocked the purinergic but not the noradrenergic component of the response). The concentration-response curve of the vas to exogenously-applied noradrenaline (NA) was unaffected by CEC (10^{-6} M) but was flattened by nifedipine (10^{-5} M).

3 The tension response to 10 Hz trains of pulses was biphasic, with an early (<2 s) and a plateau (>4 s) phase. We deduce from our pharmacological analysis that the early phase contains a putative α_{1B}-adrenoceptor component (susceptible to CEC or prazosin but not to nifedipine) and a P_2-purinoceptor component (susceptible to suramin or nifedipine) whereas the plateau phase contains an α_{1A}-adrenoceptor component (susceptible to prazosin or nifedipine but not to CEC) and a P_2-purinoceptor component (susceptible to suramin or nifedipine).

4 We suggest that the putative α_{1B}-adrenoceptors may be functionally confined to the synaptic region whereas the putative α_{1A}-adrenoceptors are excluded from this region. Trains of pulses would allow NA to accumulate and spill out beyond the synaptic region to reach and activate the putative α_{1A}-adrenoceptors.

Keywords: Rat vas deferens; electrical field stimulation; α_1-adrenoceptor subtypes; chloroethylclonidine; suramin

Introduction

Three components have been described as contributing to the complex response of the rat vas deferens to electrical stimulation of its innervation (Brown *et al.*, 1983): a non-noradrenergic component, which is now thought to be purinergic (French & Scott, 1983; Sneddon & Westfall, 1984; Burnstock, 1990) and two noradrenergic components which differ in their susceptibility to nifedipine (Brown *et al.*, 1983). The noradrenergic phase of the response to a single stimulus is not impaired by nifedipine (French & Scott, 1981), whereas the noradrenergic component in the sustained response to trains of stimuli and the response to exogenous noradrenaline (NA) are both inhibited by nifedipine. These differences may reflect different subgroups of α_1-adrenoceptors, different topographical locations for the receptors and/or different transduction mechanisms (Brown *et al.*, 1983; McGrath, 1984).

More recently, Morrow & Creese (1986) have proposed a division of α_1-adrenoceptors into α_{1A} and α_{1B} subtypes on the basis of the complex displacement of [^{3}H]-prazosin binding by WB4101 and phentolamine. Minneman and co-workers (Han *et al.*, 1987a, b; Minneman *et al.*, 1988) have reported that chloroethylclonidine (CEC) binds specifically and irreversibly to the putative α_{1B} subtype. They have shown that the rat vas deferens possesses approximately equal proportions of these putative α_{1A}- and α_{1B}-adrenoceptor binding sites as distinguished by CEC (Han *et al.*, 1987b). In addition, they have shown that although CEC bound to the putative α_{1B}-adrenoceptor sub-type in rat vas deferens it did not block the mechanical response to exogenous NA thus implying that this response was due to activation of the α_{1A} subtype (Han *et al.*, 1987b).

The present study has investigated the effects of CEC and hence of putative α_{1B}-adrenoceptor blockade on the responses of the rat vas deferens to single and multiple pulse field stimulation. A microcomputer-controlled data collection and stimulus delivery system was used to average routinely the time course of individual responses from many tissues. This allowed the identification of drug-induced changes in the various components of the responses in whole vasa (Marshall & Spriggs, 1985). Separation of these components has been facilitated by the use of the P_2-purinoceptor antagonist, suramin (Dunn & Blakeley, 1988; Hoyle *et al.*, 1990; Leff *et al.*, 1990) which specifically inhibits purinergic responses in the rat vas deferens without affecting nifedipine-sensitive NA responses (Mallard *et al.*, 1989).

Methods

Whole vasa deferentia were dissected from mature male Wistar rats (250–400 g body weight; killed by stunning and exsanguination) and were stripped of adhering fat and mesenteric investment. Each vas deferens was suspended between parallel platinum wire electrodes in a 7 ml jacketed organ bath containing Krebs solution gassed with 5% CO_2 in O_2 at 37°C. Isometric tension was recorded with dynamometer 2 oz strain gauges and tissues were maintained at a resting tension of 0.5 g for the duration of experiments. Tissues were allowed to equilibrate for 40 min, with bath fluid changes every 10 min, before responses were elicited to challenges of field stimulation. Thereafter bath fluid was changed every 5 min.

A microcomputer simultaneously controlled data collection from, and stimulus delivery to 4 tissue baths. Analogue signals from the transducers were d.c.-amplified (Lectromed or Fylde) and low pass filtered (−3 db at 50 Hz) before being digitised to 12 bit resolution at a sampling frequency of 100 Hz for each transducer. The amplifier gain was adjusted to give a dynamic

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range of 9.8 g with a 2 mg resolution. Digitised data were displayed on a colour monitor immediately after collection and were stored on floppy disk to await numerical analysis. The microcomputer controlled the timing of the interval between successive challenges of field stimulation (set at 5 min); the frequency and number of pulses delivered in each challenge; the emptying and refilling of the baths via electro-mechanical valves and the dumping of digitised data to floppy disk. Baseline tension was recorded for 0.5 s before each response. Challenges of electrical field stimulation were applied as single pulses (SPFS), twin pulses (TPFS, with an inter-pulse interval of 3 to 120 s) or as trains of pulses (TP, at 10 Hz for 10 s duration). All stimulus pulses were of 0.8 ms pulse width and 40 V amplitude. Tissues exposed to single or twin pulse field stimulation were not exposed to trains of pulses.

Stored data were subsequently analysed by calculating the mean baseline tension preceding each challenge and subtracting it from the individual values comprising the complete time coure of the responses to field stimulation. The resultant values were scaled into grammes. The full time-courses of responses from individual tissues were then meaned at each sampled time-point, allowing comparison of the absolute tension responses between different treatment groups. All results presented are the mean of observations from 5 to 8 individual tissues. Estimates of variability were calculated in terms of s.e. mean and were usually within 5 to 10% of the mean value. Figure 1A shows a characteristic mean response ($n = 6$) of the rat vas deferens to a TPFS challenge together with its s.e.mean envelope. This error envelope is representative of those associated with the mean tension responses displayed in subsequent figures which do not include individual error envelopes due to the congestion they would create. The meaned data were smoothed, by a technique that preserved the shape of the responses but removed high frequency noise, and then plotted with either a Hewlett Packard HP7470A XY-plotter or a Brother HL-8e laser printer.

Unless stated otherwise, drugs were in contact with the tissues for 15 min at each concentration; concentration-response relationships were constructed cumulatively. One vas deferens from each pair was used as a control to monitor any time-dependent variation in the responses to field stimulation. When appropriate, the effects of drug vehicle were also monitored in control experiments. Unless stated otherwise, responses of time-based controls and vehicle controls showed no significant change over the duration of the experiments from control responses established at the start of each experiment.

Exogenous noradrenaline

Concentration-response curves to NA were constructed with a 7 min dose-cycle and allowing each response to achieve its maximum which resulted in agonist contact times of 30–60 s. Control curves were established for all tissues and a second curve established either in the absence (time-based controls) or presence of antagonist drug. Sigmoid curves were fitted to the individual response curves by a non-linear curve-fitting technique (GraphPad Inplot) and the calculated maximum responses and EC_{50} values compared by the paired Student's t test. No significant differences ($P < 0.05$) between control curves and time-based control curves were detected.

Drugs and solutions

The composition of the Krebs solution used was (mM): NaCl 119, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.0, $MgSO_4 \cdot 7H_2O$ 1.2. $CaCl_2 \cdot 2H_2O$ 2.5 and glucose 11.1.

Chloroethylclonidine (Research Biochemicals Inc.) and suramin (Bayer) were prepared as stock solutions at 10^{-2} M in distilled water; noradrenaline bitartrate (Sigma) stock solution contained 40 mM ascorbate.

Nifedipine (Bayer) was prepared as a 10^{-2} M stock solution in dimethyl sulphoxide (DMSO, BDH). Prazosin hydrochloride (Pfizer) was made up as a 10^{-2} M solution in methanol.

Dilutions of all drug stock solutions were made on a daily basis in distilled water and all drug additions were made manually with microlitre syringes.

Statistical analysis of drug effects on responses to electrical stimulation

Significant differences between mean responses at selected points along the time course of the responses were identified by either Student's t test or, where appropriate, 2-way analysis of variance (ANOVA) followed by Tukey's multiple range test.

Results

Responses to twin pulse field stimulation

The mechanical response of the rat vas deferens to the first stimulus of a TPFS challenge consisted of a large (maximum tension) first phase, peaking at 280–300 ms after the stimulus, followed by a second smaller phase peaking at 650 ms after the stimulus (Figure 1). The response to the second stimulus of the challenge differed in that the second phase progressively diminished as the inter-pulse interval was reduced (Figure 1B). With a 3 s inter-pulse interval the second phase of this response appeared to be absent. The 5 min interval between challenges was sufficient to ensure that the response to the first stimulus of a TPFS challenge was not modified by the preceding challenge, as shown by the superimposition of the responses in the left-hand panel of Figure 1B. We considered that this observation permits us the convenience of referring to the response to the first stimulus of TPFS as the response to a single stimulus (SPFS).

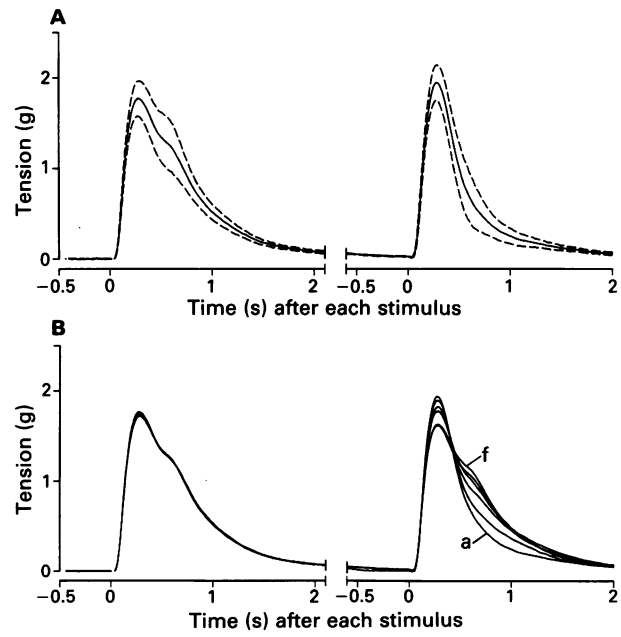

Figure 1 Full time-course tension responses of rat whole vas deferens to twin pulse field stimulation (TPFS) showing the characteristic biphasic response to the first stimulus; the first and second phases peak at 280 ms and 650 ms respectively after the stimulus. (A) With a 3 s inter-pulse interval; the mean response is shown by a continuous line and the s.e.mean envelope by broken lines ($n = 6$). (B) The effect of changing the inter-pulse interval from 3 to 120 s. Each line represents the mean from 6 different tissues. The second phase of the response to the second stimulus appears to be absent with an inter-pulse interval of 3 s (a) and progressively reappears as the inter-pulse interval is increased to 5, 10, 20, 60 and (f) 120 s. The six mean responses to the first stimulus of TPFS are all superimposed.

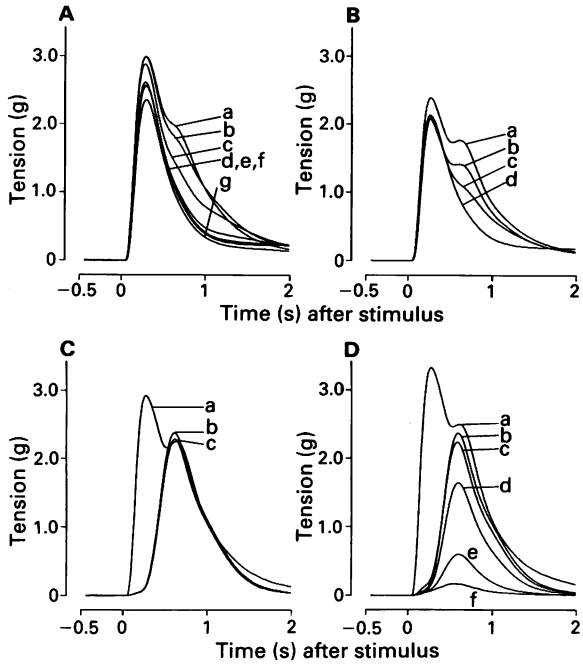

Figure 2 Full time-course responses of the rat vas deferens to single pulse field stimulation (SPFS). Each response is the mean of observations from 6 to 8 tissues. In each figure (a) represents the control response. (A) Responses after 5 (b), 10 (c), 15 (d), 20 (e) and 30 (f) min contact with 10^{-6} M chloroethylclonidine; (g) 30 min after washout. (B) responses after 15 min contact with chloroethylclonidine at a concentration of 10^{-7} M (b), 3×10^{-7} M (c) and 10^{-6} M (d). (C) Responses after 15 (b) and 90 (c) min contact with nifedipine at a concentration of 10^{-5} M. (D) Responses in the presence of nifedipine 10^{-5} M alone (b) and nifedipine (10^{-5} M) plus chloroethylclonidine at concentrations of 10^{-7} M (c), 3×10^{-7} M (d), 10^{-6} M (e) and 3×10^{-6} M (f).

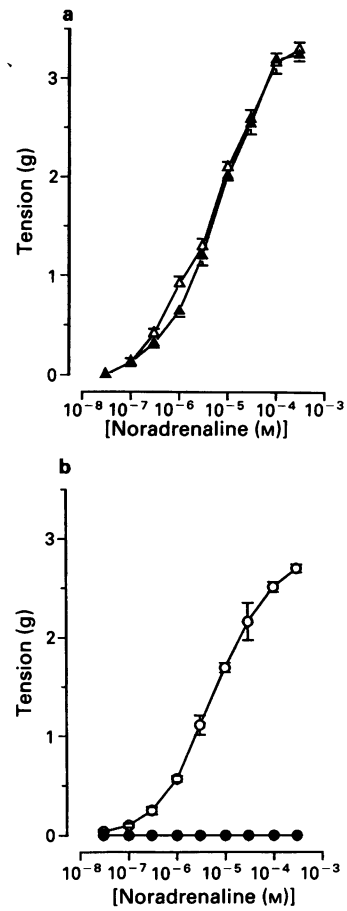

Figure 3 Effects of (a) chloroethylclonidine (10^{-6} M) and (b) nifedipine (10^{-5} M) on tension responses of the rat vas deferens to exogenous noradrenaline (NA). Concentration-responses curves to NA were constructed with 30–60 s contact times and a 7 min dose-cycle. Control curves (open symbols) were established for all tissues and a second curve (filled symbols) established either in the absence (time-based controls, not shown) or presence of drug. Student's t test showed no significant differences ($P > 0.05$) between control and time-based controls, nor between any of the controls and the curve in the presence of chloroethylclonidine. Values are mean (s.e.mean shown by vertical bars) from 6 observations.

Responses to SPFS

Chloroethylclonidine (10^{-6} M), which was without effect on the resting tension of the vas, inhibited the second phase of the response to SPFS, the effect being evident after 5 min contact and maximal after 15 min (Figure 2A). With 15 min contact times, chloroethylclonidine (10^{-7} to 10^{-6} M) induced a concentration-related inhibition of the second phase of the response (Figure 2B). Nifedipine (10^{-5} M) produced, within 15 min, a selective inhibition of the first phase of the response to SPFS but even after 90 min contact failed to reduce the magnitude of the second phase of the response (Figure 2C). In the presence of nifedipine (10^{-5} M), chloroethylclonidine exhibited a concentration-related inhibition of the second phase of the response to SPFS (Figure 2D).

Responses to exogenous noradrenaline

Chloroethylclonidine (10^{-6} M) failed to modify the concentration-response curve to exogenous NA in the rat vas deferens (Figure 3a), whereas nifedipine (10^{-5} M) abolished responses to exogenous NA (Figure 3b).

Responses to trains of pulses

The mechanical response of the rat vas deferens to trains of stimuli was biphasic (Figure 4). Both the early (<2 s) phase and the late plateau (>4 s) phase of the response to TP were reduced in a concentration-dependent manner by prazosin (10^{-8} to 3×10^{-7} M, Figure 4A). The two highest concentrations of prazosin caused approx. 50% block of both phases of the response to TP. CEC (3×10^{-6} M) also showed a concentration-related inhibition of the first phase of the response but in contrast to prazosin, CEC failed to impair the plateau phase of responses to TP (Figure 4B). Nifedipine induced concentration-related reductions in both phases of the response to TP but at a concentration of 3×10^{-6} M the plateau phase of the response was abolished whilst the first phase was inhibited by only about 50% (Figure 4C). Suramin reduced the early phase of the response to TP by 30% and the plateau phase by 50% (Figure 4D). When CEC was administered in the presence of suramin the remaining early phase of the response was extinguished whereas the plateau phase was not further impaired (Figure 4E). When CEC was administered in the presence of nifedipine both phases of the response were completely blocked (Figure 4F).

Discussion

There are two major findings from this work. Firstly, that CEC is an agent which selectively inhibits one of the three components (Brown *et al.*, 1983) of the nerve-mediated response of the rat vas deferens; and secondly that suramin selectively removes the purinergic component of the responses without impairing either of the noradrenergic components.

The noradrenergic component of the response to single pulse electrical field stimulation (SPFS)

It has been known for a decade that the noradrenergic component of the response to SPFS is resistant to inhibition by

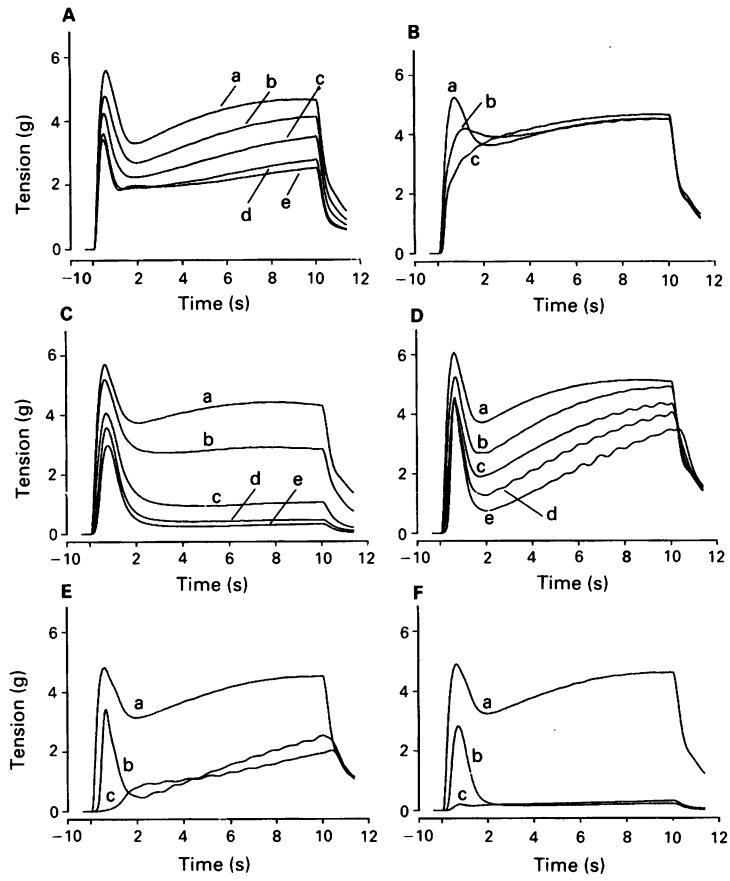

Figure 4 Effects of drugs on responses of rat whole vas deferens to 10 Hz trains of electrical field stimuli delivered for 10 s at 5 min intervals. Abscissae show time after the first stimulus of each train. Response (a) in each figure represents the mean control response. All responses shown are means obtained from 6 tissues. (A) Prazosin at 10^{-8} M (b), 3×10^{-8} M (c), 10^{-7} M (d) and 3×10^{-7} M (e); (B) chloroethylclonidine (CEC) at 10^{-6} M (b) and 3×10^{-7} M (c); (C) nifedipine at 10^{-7} M (b), 3×10^{-7} M (c), 10^{-6} M (d), and 3×10^{-6} M (e); (D) suramin at 3×10^{-5} M (b), 10^{-4} M (c), 3×10^{-4} M (d) and 10^{-3} M (e); (E) suramin at 10^{-3} M (b), suramin at 10^{-3} M + CEC at 3×10^{-6} M (c); (F) nifedipine at 10^{-5} M (b), nifedipine 10^{-5} M + CEC at 10^{-5} M (c).

nifedipine (French & Scott, 1981) whereas both the response to exogenous NA (Brown *et al.*, 1983) and the noradrenergic component (Swedin, 1971; Anton *et al.*, 1977; Brown *et al.*, 1979) in the plateau phase of responses to trains of stimuli are blocked (Brown *et al.*, 1983). McGrath (1982) had suggested the possibility of subgroups of α_1-adrenoceptors based on agonist/antagonist profiles and the discriminatory action of nifedipine led to speculation that, in the rat vas, neuronally-released NA may act on a different subset of receptors from that activated by exogenous NA and/or that different excitation-contraction coupling systems may be involved (Brown *et al.*, 1983). Because nifedipine acts by blocking calcium channels, its use cannot provide direct evidence for the identity of a receptor instrumental in opening the dihydropyridine-sensitive calcium channel. Indeed in the vas, nifedipine also blocks the phase of the response mediated through purinoceptors (French & Scott, 1981). CEC on the other hand provides direct evidence about receptors.

CEC is an alkylating agent which binds to α-adrenoceptors (Leclerc *et al.*, 1980). Minneman and colleagues (Han *et al.*, 1987a, b) consolidated the distinction between the α_{1A} and α_{1B} binding sites defined by Morrow & Creese (1986) by use of CEC which distinguished between these two α_1 sites, both with high affinity for prazosin, but with different affinities for WB4101. CEC appeared to bind selectively to Morrow & Creese's putative α_{1B}-adrenoceptor subtype which has a low affinity for WB4101. This sub-classification has been reinforced by work with various other antagonists or ligands including benoxathian, (+)-niguldipine and 5-methyl-urapidil (see Wilson *et al.*, 1991). Han *et al.* (1987b) reported that rat vas deferens contained approximately equal numbers of α_{1A}- and α_{1B}-adrenoceptor binding sites but that only the α_{1A} (not CEC-sensitive) binding site appeared to elicit contraction to exogenous NA. Exogenous NA produced an elevation in [^{3}H]-inositol phosphate accumulation in rat vas which was therefore attributed to activation of the α_{1B} site as it was blocked by CEC.

In the present experiments CEC selectively inhibited the noradrenergic phase of the response of the vas to a single pulse but, at the same concentration, did not affect the concentration-response curve to exogenous NA. The effect of CEC on the noradrenergic component of the response to single pulse field stimulation was indistinguishable from that of prazosin suggesting that only the CEC-sensitive α_1-adrenoceptor subtype is involved in this component. These observations provide direct evidence for two different α_1-adrenoceptors being involved in the responses of the vas. It would appear that the noradrenergic component of the response to single pulses may be due almost exclusively to activation of the putative α_{1B} subtype which in the rat vas appears to be coupled to an inositol phosphate transduction pathway whereas the response to exogenous NA is due to α_{1A}-mediated activation of dihydropyridine-sensitive calcium channels.

These results, in conjunction with our evidence that α_2-adrenoceptor-mediated feedback is of little importance in the loss of the noradrenergic component in the response to the second stimulus (Marshall & Spriggs, 1984; Mallard *et al.*, 1991), lead us to speculate that the loss of this noradrenergic component (Figure 1B) may be due to a transduction failure caused by depletion of an IP_3-sensitive calcium pool. This speculation is supported by the report of Berridge (1988) that the response (membrane potential spikes) of *Xenopus* oocytes to iontophoretically administered IP_3 underwent rapid desensitization when the IP_3 was given at 30 s intervals but that the spikes were reproducible in magnitude if the interval between IP_3 applications was extended to 2 min. This time-scale corresponds well with that determined in our vas experiments for

'desensitization' and recovery of the noradrenergic component of the response to the second pulse of twin pulse field stimulation.

The noradrenergic components of the response to trains of pulses

We have dissected pharmacologically the response of the whole vas deferens to 10 Hz trains of electrical field stimuli of 10 s duration. The character of the tension responses to trains of stimuli was essentially as described by Brown *et al.* (1983) consisting of a rapidly rising phase which peaks and then decays to reach a nadir at about 2 s after the start of stimulation; thereafter the tension was sustained or rose gradually to the end of the stimulation period. Both phases of the response to trains of pulses involved α_1-adrenoceptors as prazosin reduced both phases by approx. 50%, confirming Brown *et al.* (1979). CEC however abolished only the early peak and did not impair the plateau phase providing direct evidence that the putative α_{1B}-adrenoceptor is implicated only in the early phase of the response. This transient contribution of the α_{1B}-adrenoceptor activation is consistent with our hypothesis that the α_{1B}-adrenoceptor/transduction system in rat vas exhibits rapid 'desensitization' and that this 'desensitization' persists for the duration of the stimulation period.

Under identical experimental conditions nifedipine abolished the plateau phase of the response to trains leaving a residual early component which was completely blocked by CEC and which we therefore attribute to α_{1B}-adrenoceptor activation. The early phase of the response to trains was also inhibited by prazosin which is consistent with its lack of selectivity between the α_1-adrenoceptor subtypes.

Suramin selectively inhibits P_2-purinoceptors (Dunn & Blakeley, 1988; Hoyle *et al.*, 1990; Leff *et al.*, 1990) and its effect on responses of the rat vas to twin pulse field stimulation is similar to that of nifedipine (Mallard *et al.*, 1989). However, in contrast to nifedipine, suramin does not inhibit responses of the rat vas to exogenous NA (Mallard *et al.*, 1989) and so, for the first time, we have been able to demonstrate simultaneously (Figure 4E) the two noradrenergic components of Brown *et al.* (1983) in the response to trains of stimuli without the interference of a purinergic component. The addition of CEC in the continuing presence of suramin then selectively removed the early (putative α_{1B}) component leaving extant the previously unisolated dihydropyridine-sensitive (putative α_{1A}) component.

In conclusion, the early phase of the response to trains of pulses comprises the sum of a P_2-purinoceptor-mediated contraction (blocked by suramin or nifedipine) and an α_{1B}-adrenoceptor-mediated contraction (blocked by prazosin or CEC) whereas the plateau phase of the response comprises the sum of a P_2-purinoceptor-mediated contraction (blocked by suramin or nifedipine) and an α_{1A}-adrenoceptor-mediated contraction (blocked by prazosin or nifedipine). Thus neuronal NA released by a single impulse appears to act only on α_{1B}-adrenoceptors whereas that released by a train of impulses acts also on α_{1A}-adrenoceptors. One appealingly simple hypothesis to explain these observations is that α_{1B}-adrenoceptors are functionally confined to the synaptic region whereas α_{1A}-adrenoceptors are excluded from this region. Repetitive nerve stimulation with a train of impulses would allow NA to accumulate and spill out beyond the synaptic region to reach and activate the α_{1A}-adrenoceptors. The observation that the response to SPFS in the presence of the uptake$_1$- inhibitor, cocaine, possesses a nifedipine-sensitive noradrenergic component (Brown *et al.*, 1983) is consistent with this hypothesis.

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Anpirtoline, a novel, highly potent 5-HT_{1B} receptor agonist with antinociceptive/antidepressant-like actions in rodents

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1 The purpose of the present study was to relate the effects of the novel drug, anpirtoline, on 5-hydroxytryptamine (5-HT) receptor subtypes to its antinociceptive and antidepressant-like actions in rodents.

2 Binding assays with rat brain membranes have shown that anpirtoline bound with a much higher affinity to 5-HT_{1B} receptors (K_i = 28 nM) than to 5-HT_{1A} (K_i = 150 nM) and 5-HT_2 (K_i = 1.49 μM) receptors.

3 Like 5-HT, anpirtoline concentration-dependently inhibited forskolin-stimulated adenylate cyclase activity in homogenates from the rat substantia nigra. Both effects were not additive, and could be prevented by 5-HT_{1B} receptor antagonists such as propranolol and penbutolol.

4 In superfused rat and pig brain cortex slices preincubated with [^{3}H]-5-HT, the electrically evoked tritium overflow was inhibited by anpirtoline and 5-HT. Whereas 5-HT was equipotent in both tissues (EC_{50} = 69 nM), anpirtoline was markedly less potent in pig brain cortex slices (EC_{50} = 1190 nM) than in rat brain cortex slices (EC_{50} = 55 nM). The concentration-response curve for anpirtoline was shifted to the right by metitepine in both preparations.

5 In the social behaviour deficit test, anpirtoline and trifluoromethylphenyl-piperazine were effective in reversing the isolation-induced impairments in mice, an effect shown only by compounds with agonist properties at the 5-HT_{1B} receptor.

6 In the electrostimulated pain test using mice, anpirtoline dose-dependently increased the pain threshold with an ED_{50} of 0.52 mg kg^{-1}, i.p. The antinociceptive activity of anpirtoline was abolished by pretreatment with cyproheptadine or propranolol.

7 In the forced swimming test in rats, anpirtoline induced a dose-related increase in swimming activity. With an ED_{50} value of 4.6 mg kg^{-1}, i.p., anpirtoline was 4 times more potent than the two standard compounds imipramine and desipramine. The decrease of immobility time or the increase of active periods in this model of behavioural despair is suggested to be characteristic of antidepressant drugs.

8 Anpirtoline exhibits both antinociceptive and antidepressant-like activities in animals. It is probable that anpirtoline elicits these pharmacological effects via its agonist effect on 5-HT_{1B} and 5-HT_{1A} receptors.

Keywords: Anpirtoline; 5-hydroxytryptamine receptors; analgesia; antidepressant activity; 5-HT_{1B}-, 5-HT_{1D}-, 5-HT_{1A}-receptors

Introduction

Endogenous monoamines, particularly noradrenaline and 5-hydroxytryptamine (5-HT), have been studied in the context of both regulation of nociceptive threshold and the pathophysiology of depressive illness. In recent years, interest in 5-HT has undergone a renaissance. The discovery of new 5-HT receptors and our improved understanding of the functional role of these receptors have contributed to an intensification of research in this field (Whitaker-Azmitia & Peroutka, 1990).

The processing of pain impulses, their perception as painful information, and the organism's response to the perceived pain are highly complex and incompletely understood neurophysiological events. Alterations in brain 5-HT concentration caused by precursor loading or precursor restriction result in analgesia (Lin *et al.*, 1980) and hyperalgesia (Lytle *et al.*, 1975), respectively. It is also known that 5-HT *per se* and the stimulation of raphe nuclei where 5-hydroxytryptaminergic perikarya and dendrites are located increase the nociceptive threshold (Yaksh & Wilson, 1979). Moreover, the dorsal horn of the spinal cord is partly controlled by descending 5-hydroxytryptaminergic pathways (Le Bars, 1988).

Although there are some doubts concerning the altered monoamine metabolism in the pathophysiology of depression, it is likely that 5-HT may play a role in the development of this mental disorder (Meltzer & Lowy, 1987). Many antidepressants in current use such as imipramine, amitriptyline and fluoxetine block the neuronal uptake of 5-HT (Chouinard, 1985). It has also been demonstrated that administration of amitriptyline was associated with analgesic effects in patients with migraine or postherpetic neuralgia (Taub, 1973; Couch *et al.*, 1976; Watson *et al.*, 1982).

Because anpirtoline (6-chloro-2-[piperidyl-4-thiol]-pyridine hydrochloride (ASTA Medica AG), a new chemical entity (Figure 1), exhibited antinociceptive and antidepressant-like properties in preliminary (unpublished) studies in rats and mice, further investigations have been carried out to examine the possible involvement of 5-hydroxytryptaminergic neurotransmission in the central mechanisms of action of this drug. *In vitro* studies on the possible interaction of anpirtoline with various 5-HT receptor subtypes and extensive behavioural studies strongly suggested that the clearcut antinociceptive and antidepressant-like effects of anpirtoline in mice and rats probably involved its agonist properties toward 5-HT_{1B} and/or 5-HT_{1A} receptors.

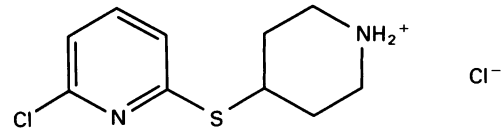

Figure 1 Chemical structure of anpirtoline.

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Methods

In vitro *studies*

Receptor binding assays Adult male Sprague-Dawley rats weighing ~250 g were killed by decapitation and membranes from pooled hippocampi, striata or anterior cortices were prepared as described elsewhere (Hall *et al.*, 1985). Three subtypes of 5-HT receptors were investigated by use of binding assays: 5-HT$_{1A}$ with [^{3}H]-8-hydroxy-2-(di-n-propylamino) tetralin ([^{3}H]-8-OH-DPAT) (100 Ci mmol^{-1}, Service des Molécules Marquées, CEA, Saclay, France) as radioligand; 5-HT$_{1B}$ with [^{3}H]-5-HT (12 Ci mmol^{-1}, Amersham, Buckinghamshire, U.K.) plus 0.1 μM 8-OH-DPAT and 0.1 μM mesulergine to prevent the binding of the ^{3}H-amine to 5-HT$_{1A}$ and 5-HT$_{1C}$ sites, respectively; 5-HT$_2$ with [^{3}H]-ketanserin (66.4 Ci mmol^{-1}, Amersham). Hippocampal, striatal and cortical membranes were used for studies on 5-HT$_{1A}$, 5-HT$_{1B}$ and 5-HT$_2$ binding sites, respectively, following the protocols described in detail elsewhere (Adrien *et al.*, 1989).

The inhibition of the specific binding of each radioligand by anpirtoline was examined with at least 10 different concentrations of the drug, between 0.1 nM and 0.1 mM. Calculations of IC_{50} and n_H values were described by McPherson (1983). The equation of Cheng & Prusoff (1973) was used for converting the IC_{50}s into K_is.

Measurements of adenylate cyclase activity Adenylate cyclase assays were performed on aliquots (10 μl, corresponding to about 5 μg of membrane protein) of substantia nigra homogenates (from male Sprague-Dawley rats) incubated for 20 min at 30°C with 0.5 mM [^{32}P]-alpha-ATP (10–20 Ci mmol^{-1}, Amersham) in the presence of 10 μM forskolin, 10 μM GTP and 0.1 M NaCl as described in detail elsewhere (Hamon *et al.*, 1988). The enzymatic activity is expressed as nmol [^{32}P]-adenosine 3':5'-cyclic monophosphate ([^{32}P]-cyclic AMP) formed per mg protein and per 20 min. All assays were performed as triplicates, and each experiment was repeated at least twice.

Investigations on brain slices Cerebral cortex slices (0.3 mm thick, 3 mm in diameter) were prepared from male Wistar rats and from pigs of either sex. The slices were incubated for 60 min in physiological salt solution at 37°C (for composition, see Schlicker *et al.*, 1989a) containing 0.1 μM [^{3}H]-5-HT (12.5–30.0 Ci mmol^{-1}, New England Nuclear, Dreieich, Germany). Subsequently, the slices were superfused with physiological salt solution at 1 ml min^{-1} (37°C; 110 min). Two periods of electrical field stimulation were applied to each slice after 40 and 90 min of superfusion; rectangular pulses of 50 mA and 2 ms were administered at a frequency of 3 Hz. At the end of the superfusion, the radioactivity of the slices (solubilized with Soluene, Packard, Downers Grove, IL, U.S.A.) and superfusate samples was determined by liquid scintillation counting (for details of the method, including calculation of stimulation-evoked tritium overflow, see Schlicker *et al.*, 1989a,b).

In vivo *studies*

Electrostimulated pain-test The electrostimulation test was performed using the procedure described by Blake *et al.* (1963). The test drug(s) or vehicle were administered to adult male NMRI mice and male Sprague-Dawley rats. After 30 min, each animal was placed in an arena in which square wave electrical stimulation could be applied via the mesh floor. The current was raised until the animal responded vocally. The antinociceptive activity of the compounds was expressed as the ratio (× 100) of the current (in mA) provoking a response in drug- and vehicle-treated animals.

Forced swimming test A situation of behavioural despair was created in the forced swimming test with female Sprague-Dawley rats weighing 100–150 g. The rats were placed in a container containing about 15 l of water at 25°C. Unlike the method originally described by Porsolt *et al.* (1977) the swimming movements instead of the time of immobility were registered. The swimming activity of the rats was measured by an electronic motor activity monitor (Rhema Labortechnik, Hochheim, F.R.G.). This device records the changes of electrical inductance in an electrical field evoked by swimming movements of the animal. The mean activity over a 5 min test period caused by swimming or diving of the animals is expressed in number of impulses min^{-1}.

Social behaviour deficit test The behavioural deficit test was performed according to Frances (1988) with the male NMRI mice (25–35 g) housed either in groups of 10 per cage or isolated (1 per cage) for 1 week before being tested. One group-housed mouse and one isolated mouse identically treated either with saline or with the test compound were placed together under an inverted glass beaker (diameter = 10 cm). For both mice the number of attempts to escape from this situation were counted for 3 min. The activity of the isolated mice is expressed as a percentage related to their group-housed counterparts. In this experiment a non-significant difference between grouped and isolated mice stands for the reversal of isolation-induced deficit by a compound.

Statistical analysis

In *in vivo* investigations, effective doses (ED_{50}) were calculated by regression analysis. To compare two mean values, Student's *t* test for unpaired samples was used (Cavalli-Sforca, 1969).

In *in vitro* studies, results are given as means ± s.e.mean. For comparison of mean values, Student's *t* test was used; Bonferroni's procedure was employed if two or more experimental groups were compared with the same control series. The apparent pA_2 value for metitepine (against anpirtoline) was calculated according to Furchgott (1972).

Drugs

Anpirtoline HCl was synthesized by the Department of Chemical Research, ASTA Medica AG, Frankfurt, Germany. In addition, the following drugs were used: cyproheptadine HCl, imipramine HCl, desipramine HCl, (±)-propranolol (Sigma, Munich, Germany); tramadol (Tramal, Chemie Grünenthal, Aachen, Germany); buprenorphine (Temgesic, Boehringer Mannheim, Mannheim, Germany); citalopram HCl (Lundbeck, Copenhagen, Denmark); indalpine (Rhône-Poulenc Santé, Gennevilliers, France); metitepine maleate (Hoffmann-La Roche, Basel, Switzerland); phentolamine methane sulphonate (CIBA-Geigy, Wehr, Germany); 5-hydroxytryptamine creatinine sulphate (Merck, Darmstadt, Germany); mesulergine (Sandoz, Basel, Switzerland); trifluoromethylphenyl-piperazine (TFMPP, Interchim, Montluçon, France); (±)-penbutolol (Hoechst, Puteaux, France); MK-212 (6-chloro-2-(1-piperazinyl) pyrazine HCl, Merck, Sharp and Dohme, Munich, Germany); 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT, Research Biochem. Inc., Natick, Ma, U.S.A.); cinanserin (Squibb, Princeton, New Jersey, U.S.A.).

Results

Receptor binding studies

Anpirtoline bound differentially to the three 5-HT receptor subtypes 5-HT$_{1A}$, 5-HT$_{1B}$ and 5-HT$_2$. The drug exhibited a higher affinity for 5-HT$_{1B}$ sites (K_i = 28 ± 4 nM, n = 3) in striatal membranes than for 5-HT$_{1A}$ sites (K_i = 150 ± 17 nM, n = 3) in hippocampal membranes. As shown in Figure 2, its potency in inhibiting [^{3}H]-ketanserin specific binding to cortical membranes was markedly less than against [^{3}H]-8-OH-

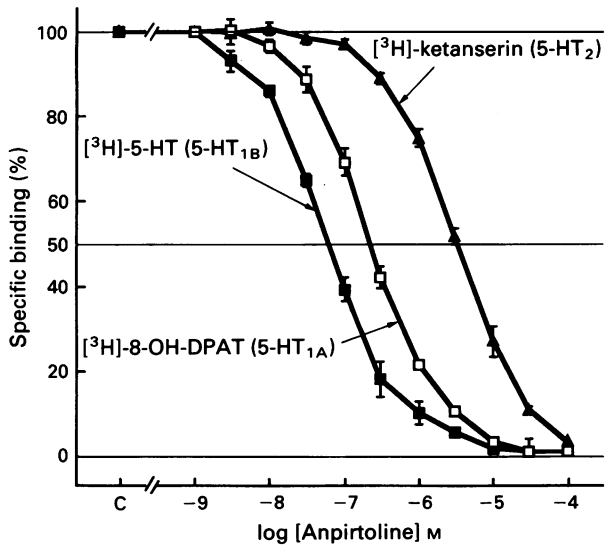

Figure 2 Concentration-dependent inhibition by anpirtoline of the specific binding of [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) to 5-HT_{1B} sites, [^{3}H]-8-hydroxy-2-(di-n-propylamino)tetralin ([^{3}H]-8-OH-DPAT) to 5-HT_{1A} sites, and [^{3}H]-ketanserin to 5-HT_2 sites, in rat brain membranes: Binding assays were performed with: 5-HT_{1B} sites—striatal membranes, 2.3 nM [^{3}H]-5-HT plus 0.1 μM 8-OH-DPAT and 0.1 μM mesulergine; non specific binding was determined in the presence of 10 μM 5-HT; 5-HT_{1A} sites—hippocampal membranes, 0.8 nM [^{3}H]-8-OH-DPAT; non specific binding was determined in the presence of 10 μM 5-HT; 5-HT_2 sites—cortical membranes, 0.8 nM [^{3}H]-ketanserin; non specific binding was determined in the presence of 1 μM cinanserin. Each point is the mean (vertical bars show s.e.mean) of triplicate determinations in a typical experiment (100%: 5-HT_{1B}: 102.6 fmol [^{3}H]-5-HT specifically bound mg^{-1} membrane protein; 5-HT_{1A}: 112.6 fmol [^{3}H]-8-OH-DPAT mg^{-1} membrane protein; 5-HT_2: 147.6 fmol [^{3}H]-ketanserin mg^{-1} membrane protein). This experiment was repeated 3 times for the calculation of K_i values indicated in the text.

DPAT and [^{3}H]-5-HT specific binding to 5-HT_{1A} and 5-HT_{1B} sites, respectively, indicating a lower affinity (K_i = 1.49 ± 0.22 μM, n = 3) for 5-HT_2 sites. In all cases, concentration-dependent inhibitions of the specific binding of ^{3}H-ligands occurred with apparent Hill coefficients close to 1.0.

Adenylate cyclase

As shown in Figure 3, the inhibition of forskolin-stimulated adenylate cyclase activity by 5-HT was concentration-dependent, with a maximal effect (−22%) for 1–10 μM of the indoleamine, and an EC_{50} of about 20 nM. Anpirtoline also exerted a concentration-dependent inhibition of [^{32}P]-cyclic AMP formation, but to a lesser extent than 5-HT. Thus only a 13% reduction of the enzymatic activity was observed in the presence of 10 μM anpirtoline (Figure 3).

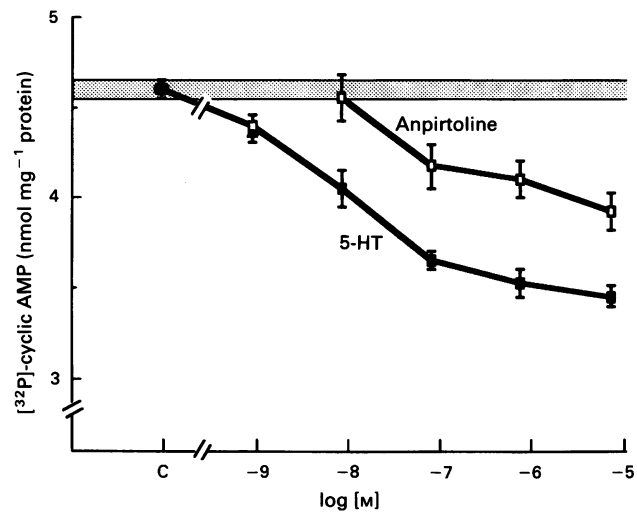

Figure 3 Concentration-dependent inhibition of forskolin-stimulated adenylate cyclase activity by 5-hydroxytryptamine (5-HT, ■) and anpirtoline (□) in rat substantia nigra homogenates. Assays were performed with 0.5 mM [^{32}P]-alpha-ATP, 10 μM forskolin, 10 μM GTP and 0.1 M NaCl (see Hamon *et al.*, 1988), plus various concentrations of 5-HT, or anpirtoline (abscissa scale). The formation of [^{32}P]-cyclic AMP is expressed as nmol mg^{-1} protein 20 min^{-1} (at 30°C). Each point represents the mean (vertical bars show s.e.mean) of triplicate determinations in three independent experiments. The grey area corresponds to the range of control values (assays without 5-HT or anpirtoline, C on the abscissa scale).

As expected from an effect through the stimulation of 5-HT receptors, the inhibition of forskolin-stimulated adenylate cyclase by anpirtoline was not additive with that due to a saturating concentration (1 μM) of 5-HT (Table 1). Furthermore, the mixed 5-HT_{1A}, 5-HT_{1B} receptor antagonists (±)-penbutolol (1 μM) and (±)-propranolol (1 μM) prevented the inhibitory effect of both 5-HT (1 μM) and anpirtoline (10 μM) (Table 1). Finally, the selective 5-HT_{1A} receptor agonist, 8-OH-DPAT (10 nM-1 μM) did not affect the formation of [^{32}P]-cyclic AMP by substantia nigra homogenates.

In vitro [^{3}H]-5-hydroxytryptamine release from cortex slices

The effect of anpirtoline on the electrically evoked tritium overflow from rat and pig brain cortex slices was compared to that of 5-HT which had previously been examined under identical experimental conditions (Schlicker *et al.*, 1989a,b). The superfusion medium contained an inhibitor of 5-HT uptake (10 μM citalopram or 10 μM indalpine) and, in experiments on pig brain cortex slices, phentolamine (3.2 μM).

Table 1 Blockade by penbutolol and propranolol of the inhibitory effect of anpirtoline and/or 5-hydroxytryptamine (5-HT) on forskolin-stimulated adenylate cyclase in substantia nigra homogenates and lack of effect of 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) on the enzymatic activity

		[^{32}P]-cyclic AMP (nmol mg^{-1} protein)		
Addition		*None*	*Penbutolol* (1 μM)	*Propranolol* (1 μM)
None		4.36 ± 0.11	4.52 ± 0.12	4.55 ± 0.13
Anpirtoline	10 μM	3.81* ± 0.11	4.31 ± 0.10	4.36 ± 0.09
5-HT	1 μM	3.54* ± 0.06	4.26 ± 0.12	4.30 ± 0.14
Anpirtoline + 5-HT	10 μM + 1 μM	3.62* ± 0.10	4.38 ± 0.11	4.32 ± 0.12
8-OH-DPAT	1 μM	4.30 ± 0.08	4.56 ± 0.14	4.58 ± 0.11

Assays were performed as described in the legend to Figure 3. Each value (in nmol [^{32}P]-cyclic AMP synthesized mg^{-1} protein 20 min^{-1} at 30°C) is the mean ± s.e.mean of triplicate determinations in at least three separate experiments.
* $P < 0.05$ when compared to [^{32}P]-cyclic AMP formation in the absence of agonists (no addition).

Table 2 Antinociceptive activity of anpirtoline in comparison with buprenorphine and tramadol in electrostimulated pain test in mice and rats

Compound	n *Species*	*Doses* (mg kg^{-1})	*Route*	*Nociceptive threshold* (% of controls[1])	ED_{50}[2] (mg kg^{-1})
Anpirtoline	10 mice	1.5	p.o.	121 ± 4*	2.8
		3.0	p.o.	152 ± 7**	(2.3–3.4)
		6.0	p.o.	184 ± 6***	
Anpirtoline	10 rats	1.0	p.o.	127 ± 7*	1.86
		2.0	p.o.	148 ± 6**	(1.4–2.4)
		4.0	p.o.	184 ± 6***	
Tramadol	10 mice	10.0	p.o.	114 ± 3 NS	25.7
		20.0	p.o.	139 ± 5**	(22.3–28.6)
		40.0	p.o.	168 ± 8**	
Anpirtoline	10 mice	0.25	i.p.	125 ± 4**	0.52
		0.5	i.p.	150 ± 3**	(0.41–0.63)
		1.0	i.p.	171 ± 5***	
Buprenorphine	10 mice	0.05	i.p.	122 ± 4**	0.1
		0.1	i.p.	150 ± 7**	(0.08–0.12)
		0.2	i.p.	170 ± 6***	
Anpirtoline + cyproheptadine	10 mice	6.0	p.o.	117 ± 3#	—
		12.0	i.p.		
Anpirtoline + propranolol	10 mice	6.0	p.o.	121 ± 5#	—
		5.0	i.p.		

[1] Control animals received 1% methocel p.o. or 0.9% NaCl i.p. Values represent means ± s.e.mean of n animals. * $P < 0.005$; ** $P < 0.01$; *** $P < 0.001$, NS, not significantly different from nociceptive threshold of methocel- (p.o.) or saline- (i.p.) treated animals (= 100%).
# $P < 0.01$ significantly different from anpirtoline 6 mg kg^{-1}, p.o., alone.
Significance determined by Student's t test.
[2] ED_{50} is given with 95% confidence limits.

Anpirtoline did not affect basal tritium efflux (results not shown), but concentration-dependently inhibited the electrically evoked tritium overflow from rat (Figure 4a) and pig (Figure 4b) brain cortex slices. The maximum inhibitory effects were approximately 55 and 50%, respectively (Figure 4). The maximum degree of inhibition obtainable with 5-HT tended to be higher (by 65–70%) but the difference between the maximal effects due to both compounds was not significant ($P > 0.05$). The respective pEC_{50} values for anpirtoline and 5-HT were 7.25 and 7.16 with rat brain cortex slices and 5.93 and 7.16 with pig brain cortex slices. The concentration-response curve for anpirtoline was shifted to the right by metitepine in either tissue (Figure 4). The apparent pA_2 values of metitepine were 7.03 and 6.71 for rat and pig brain cortex slices, respectively.

Effect of anpirtoline on nociception

As shown in Table 2, both oral and intraperitoneal administration of anpirtoline resulted in a dose-dependent increase in the nociceptive threshold of electrical stimulation in mice and/or rats. After intraperitoneal administration, the antinociceptive-like activity of 0.25–1.0 mg kg^{-1} anpirtoline was similar to that induced by 0.05–0.2 mg kg^{-1} buprenorphine, a strong centrally acting antinociceptive opiate compound (Nickel & Aledter, 1987). Via the oral route, anpirtoline was more potent than tramodol (also a strong analgesic of the opiate type) as doses about 10 times higher of the latter drug were required to produce the same increase in the nociceptive threshold as anpirtoline (Table 2; see also Nickel & Aledter, 1987). The antinociceptive activity of anpirtoline was not influenced by naloxone or idazoxan (not shown). However, it was abolished after pretreatment of the animals with cyproheptadine or propranolol (Table 2).

Antidepressant-like activity of anpirtoline

Anpirtoline significantly increased the swimming activity in female rats with an ED_{50} of 4.6 mg kg^{-1}, i.p. The two refer-

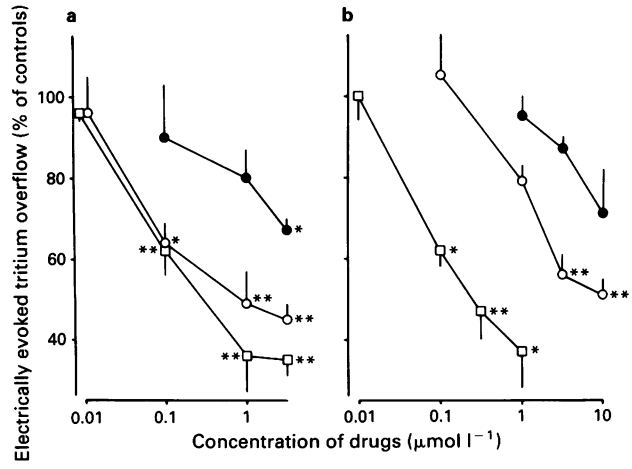

Figure 4 Effects of anpirtoline (○) and 5-hydroxytryptamine (□)(5-HT) on the electrically evoked tritium overflow from superfused rat (a) and pig (b) brain cortex slices, and interaction of anpirtoline with metitepine (●). Anpirtoline or 5-HT was added to the medium from the 62nd min of superfusion onward whereas the following drugs were present throughout the superfusion: rat brain cortex slices (a)—10 μM citalopram (5-HT uptake inhibitor) and, if relevant, 3.2 μM metitepine; pig brain cortex slices (b)—10 μM indalpine (5-HT uptake inhibitor), 3.2 μM phentolamine, and, if relevant, 1 μM metitepine. Tritium overflow was evoked twice, after 40 and 90 min of superfusion (S_1, S_2), and the ratio of the overflow evoked by S_2 over that evoked by S_1 was determined (S_2/S_1). The evoked tritium overflow is given as a percentage of the ratio S_2/S_1 in the corresponding controls. Tritium overflow evoked by S_2 in the control series related to anpirtoline was (in % of tissue tritium): 6.78 ± 0.69 and 11.17 ± 1.83 (means ± s.e.mean, $n = 7$ each, $P < 0.05$) in rat brain cortex slices superfused in the absence and presence of metitepine, respectively. The respective values for pig brain cortex slices were 3.54 ± 0.49 and 5.67 ± 0.89 (means ± s.e.mean, $n = 7$ each, $P < 0.05$). Means of 4–9 experiments (s.e.mean shown by vertical lines); * $P < 0.05$; ** $P < 0.005$.

Table 3 Antidepressant-like activity of anpirtoline in the forced swimming test in rats

Compound	n	Doses ($mg\,kg^{-1}$)	Route	Animal activity (% of controls)	ED_{50}[1] ($mg\,kg^{-1}$)
Anpirtoline	10	2	i.p.	111.5 NS	4.6
	10	4	i.p.	144.2**	(4.1–5.2)
	10	8	i.p.	177.5**	
Imipramine	10	20	i.p.	150**	—
Desipramine	10	20	i.p.	156***	—

** $P < 0.01$; *** $P < 0.001$, NS, not significantly different from saline-treated rats (100%); *n*, number of rats. Significance determined by Student's *t* test

[1] ED_{50} is given with 95% confidence limits.

ence compounds imipramine ($20\,mg\,kg^{-1}$, i.p.) and desipramine ($20\,mg\,kg^{-1}$, i.p.), as expected, also increased the swimming activity by 50% and 56% over the controls, respectively (Table 3).

Social behaviour

In the social behaviour deficit test, active compounds show a bell-shaped dose-response curve (Frances, 1988). Saline-treated isolated mice make about half as many attempts to find a way out of a confined situation as do saline-treated grouped mice. Active compounds are able to reverse this behavioural deficit when the treated isolated mice act similarly to their group-housed counterparts within the same trial (Frances, 1988). In this study, imipramine ($8–32\,mg\,kg^{-1}$, i.p.) and desipramine ($5–20\,mg\,kg^{-1}$, i.p.) were not able to reverse the isolation-induced behavioural deficit or even increased it (Table 4). In contrast, anpirtoline (2.5 mg and $5\,mg\,kg^{-1}$, i.p.) and the 5-HT_{1B} receptor agonist, TFMPP (Titeler *et al.*, 1987; Schechter, 1988), at $1–4\,mg\,kg^{-1}$, i.p., increased the number of escape attempts in isolated mice to a level not significantly different from that of grouped mice (Table 4). As shown in Table 4, pretreatment with the mixed 5-HT_{1A}, 5-HT_{1B} receptor antagonist (±)-propranolol ($5\,mg\,kg^{-1}$, i.p.) prevented the effects of anpirtoline and TFMPP in this test.

Discussion

Anpirtoline, a new chemical entity which is not related to known analgesic and/or antidepressant drugs, has been demonstrated to have a relatively high affinity for 5-HT_{1B} binding sites and to influence forskolin-stimulated adenylate cyclase activity in the rat substantia nigra. In agreement with previous data (Bouhelal *et al.*, 1988; Hamon *et al.*, 1990b), the 5-HT_{1A} receptor agonist, 8-OH-DPAT, did not mimic 5-HT, but mixed 5-HT_{1A}, 5-HT_{1B} receptor antagonists such as (±)-penbutolol and (±)-propranolol prevented the inhibitory effect of both the indoleamine and anpirtoline on this enzymatic activity. These results further supported the view that the 5-HT receptor negatively coupled with adenylate cyclase in the rat substantia nigra is the 5-HT_{1B} receptor subtype. Indeed, Bouhelal *et al.* (1988) and Hamon *et al.* (1990b) have already shown that in addition to 5-HT, 5-HT_{1B} receptor agonists such as RU-24969, TFMPP and CGS-12066B inhibited forskolin-stimulated adenylate cyclase activity in the rat substantia nigra. Accordingly, anpirtoline could be considered as a 5-HT_{1B} receptor agonist in the rat brain. Although the inhibition of forskolin-activated adenylate cyclase was less with anpirtoline than with 5-HT at the highest concentration tested ($10\,\mu M$), it cannot be concluded that anpirtoline is a partial agonist because, in contrast to what is expected for such a drug, anpirtoline ($10\,\mu M$) did not reduce the inhibitory effect of 5-HT on adenylate cyclase activity (Table 1).

Further characterization of anpirtoline was carried out in two functional models, i.e. in superfused rat and pig brain cortex slices preincubated with [^{3}H]-5-HT. The electrically evoked tritium overflow in either tissue is tetrodotoxin-sensitive and Ca^{2+}-dependent and, thus, represents quasi-physiological 5-HT release (Fink *et al.*, 1988; Göthert, 1980). 5-HT release in either tissue is subject to modulation via inhibitory presynaptic autoreceptors (Moret, 1985; Timmer-

Table 4 Effects of anpirtoline and other drugs on isolation-induced social behaviour deficit in mice

Compound	n	Dose ($mg\,kg^{-1}$, i.p.)	Number of escape attempts (mean ± s.e.mean) Grouped mice	Isolated mice	%
NaCl 0.9%	99	—	32.6 ± 1.5	17.5 ± 0.9***	54
Imipramine	8	8	45.6 ± 7.2	19.0 ± 2.9**	42
	8	16	38.4 ± 4.1	22.9 ± 5.0*	60
	8	32	51.4 ± 4.0	7.9 ± 3.0***	15
Desipramine	8	5	34.1 ± 6.6	11.6 ± 2.7**	34
	8	10	27.9 ± 3.0	16.0 ± 2.7*	57
	8	20	29.9 ± 4.3	8.4 ± 1.1***	28
Anpirtoline	21	1.25	31.2 ± 1.9	21.9 ± 2.4**	70
	21	2.5	31.3 ± 2.4	27.7 ± 3.5 NS	89
	21	5	31.7 ± 3.6	24.8 ± 3.2 NS	78
TFMPP	8	1	45.9 ± 4.5	46.8 ± 10.5 NS	102
	8	2	32.6 ± 3.7	44.5 ± 5.4 NS	136
	8	4	34.8 ± 4.7	29.0 ± 3.6 NS	83
Propranolol + anpirtoline	8	5 + 2.5	24.9 ± 4.2	10.6 ± 2.7*	43
Propranolol + TFMPP	8	5 + 2	33.0 ± 4.4	22.7 ± 3.4*	69

Duration of isolation: 7 days; significance determined by Student's *t* test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not significantly different from grouped controls; for each condition, the percentage of escape attempts in isolated vs. grouped mice is indicated in the right hand column. *n*, number of mice.

TFMPP = trifluoromethylphenyl-piperazine.

mans & Thoolen, 1987; Middlemiss, 1988; Starke *et al.*, 1989). The autoreceptor could be classified as a 5-HT$_{1B}$ receptor in the rat brain and as a 5-HT$_{1D}$ receptor in the pig brain (Engel *et al.*, 1986; Schlicker *et al.*, 1989b; Hoyer & Middlemiss, 1989; Starke *et al.*, 1989). Like 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor agonists, anpirtoline inhibited the electrically evoked tritium overflow from rat and pig brain cortex slices. Furthermore, the rightward shift of its concentration-response curve by metitepine in either tissue supported the view that this effect was mediated via the stimulation of 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors, respectively. The potency of anpirtoline was similar to that of 5-HT in rat brain cortex slices, but markedly lower (by more than one order of magnitude) than that of the indoleamine in pig brain cortex slices.

Of the three behavioural tests which have been investigated in the present study, two are especially responsive to 5-HT$_{1B}$ receptor agonists, i.e. the isolation-induced social behaviour deficit in mice (Frances, 1988; Frances *et al.*, 1990) and the vocalization elicted by nociceptive electrical stimulation (see Hamon *et al.*, 1990a). In agreement with the inferences from the *in vitro* studies, anpirtoline also behaved as a 5-HT$_{1B}$ receptor agonist in these two tests since it reversed the isolation-induced social behaviour deficit in mice, as did the 5-HT$_{1B}$ receptor agonist TFMPP (Titeler *et al.*, 1987; Schechter, 1988; see Table 4), and increased the nociceptive threshold in the electrostimulated pain-test, as previously found with the potent 5-HT$_{1B}$ receptor agonists, TFMPP and *m*-chlorophenyl-piperazine (Zemlan *et al.*, 1988). In addition to 5-HT$_{1B}$ receptor agonists, opiates and α_2-adrenoceptor agonists also exhibit antinociceptive properties in relevant animal tests (Sullivan *et al.*, 1987; Yaksh & Stevens, 1988). However, opiate receptors and α_2-adrenoceptors were very probably not involved in the antinociceptive effect of anpirtoline since neither the opiate receptor antagonist, naloxone, nor the α_2-adrenoceptor antagonist, idazoxan, reversed this effect. Conversely, 5-HT receptor antagonists such as cyproheptadine and (±)-propranolol abolished the anpirtoline effect. Similarly (±)-propranolol prevented the reversal by anpirtoline of isolation-induced social behaviour deficit in mice. Although further investigations have to be performed with selective 5-HT$_{1B}$ receptor antagonists (which, unfortunately, are not yet available) for a definitive demonstration, the data obtained so far strongly support the view that anpirtoline acts *in vitro* and *in vivo* as 5-HT$_{1B}$ receptor agonist in both rats and mice.

Another interesting property of anpirtoline is its antidepressant-like action in the forced swimming test (Porsolt *et al.*, 1977) in rats. Indeed, anpirtoline was at least as potent as classical antidepressants such as imipramine and desipramine in reducing the immobility time. However the efficient doses of anpirtoline in this test (2–8 mg kg^{-1}, i.p.) were generally higher than those required to elicit antinociceptive activity (0.25–1.0 mg kg^{-1}, i.p.; Table 2) and reversing the isolation-induced social behaviour deficit (maximal reversion was obtained with 2.5 mg kg^{-1}, i.p. of anpirtoline, see Table 4). These data are compatible with the possibility that different receptors might be responsible for the antidepressant-like action of anpirtoline on the one hand and its antinociceptive effect and the ability to reverse isolation-induced social behaviour deficit on the other hand. Interestingly, the binding studies have shown that anpirtoline bound not only to 5-HT$_{1B}$ receptors but also to 5-HT$_{1A}$ receptors, and the ratio of its respective K_i values for these two receptors: K_i(5-HT$_{1A}$)/K_i(5-HT$_{1B}$) = 5.4, was approximately in the same range as that of the efficient doses in the forced swimming test over those in the other two behavioural tests. As 5-HT$_{1A}$ receptor agonists exhibit potent antidepressant-like activity in relevant tests in rodents (Wieland & Lucki, 1990; Martin *et al.*, 1990; Martin & Puech, 1991), it might be tentatively proposed that the stimulation of central 5-HT$_{1A}$ receptors accounted for the antidepressant-like properties of anpirtoline. However, further studies are necessary to prove or reject this hypothesis.

In conclusion, anpirtoline is a new 5-HT receptor agonist acting preferentially on central 5-HT$_{1B}$ receptors in rodents. Its potent antinociceptive and antidepressant-like properties and ability to reverse isolation-induced social behaviour deficit further support the theory that central 5-HT neurotransmission plays a major role in the control of pain and mood (see Meltzer & Lowy, 1987; Le Bars, 1988).

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Effect of K^+ channel-modulating drugs on the vasoconstrictor responses of leukotrienes C_4, D_4 and angiotensin II in the guinea-pig isolated perfused heart

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1 The vascular actions of leukotrienes C_4 (LTC_4) and LTD_4 in the guinea-pig isolated perfused heart were studied in the presence of potassium (K^+) channel modulatory compounds.

2 Cromakalim (0.35–10 μM), a K^+ channel activator, inhibited the vasoconstrictor responses of LTC_4 (30 pmol), LTD_4 (30 pmol) and angiotensin II (AII) (100 pmol) in a concentration-dependent manner.

3 Glyceryl trinitrate (10 mg l^{-1}) and vasoactive intestinal peptide (10 nM) induced a similar vasodilator action to cromakalim in the isolated heart but had no effect on responses to LTC_4 and LTD_4.

4 The inhibitory action by cromakalim (10 μM) on the LTC_4 (30 pmol) response could be reversed in the presence of an equimolar concentration of glibenclamide. However, glibenclamide (10 μM) only partially restored the LTD_4 (30 pmol) actions.

5 Galanin (10 nM) and charybdotoxin (60 nM) had no effect on the vascular responses to LTC_4 and LTD_4 (30 pmol).

6 Inhibition by cromakalim of coronary vasospasm induced by vascular LTC_4, LTD_4 and AII appears to be separate from its vasodilator action and it is postulated that a cromakalim-sensitive mechanism in the coronary vasculature is important in the vasoconstrictor responses to LTC_4, LTD_4 and AII.

Keywords: Coronary vasculature; cromakalim; glibenclamide; peptido-leukotrienes; angiotensin II

Introduction

The peptido-leukotriene C_4 (LTC_4) and its metabolite LTD_4, are among the most potent vasoconstrictors in the coronary vasculature in a number of species (Burke *et al.*, 1982), including man (Marone *et al.*, 1988). It has been proposed that the leukotrienes are involved in coronary disease (Ganz & Alexander, 1985). Their action in the coronary vessels appears to be primarily a direct one and involves the entry of extracellular calcium, since calcium channel blockers inhibit the vasoconstrictor effect of the leukotrienes in these vessels (Lefer & Yanagisawa, 1986; Stanton & Piper, 1987).

Nelson *et al.* (1990) have proposed that the voltage-sensitive calcium channels, which are affected by changes in cellular membrane potential may play a major role in vascular tone. Membrane potential can be regulated by K^+ channels, of which there are at least eight different types in the heart (Carmeliet, 1989). Particular interest has centred on the ATP-sensitive K^+ (K_{ATP}) channels due to their ability, on activation, to cause smooth muscle relaxation (Quast & Cook, 1989). K_{ATP} channels have been identified in vascular smooth muscle (Standen *et al.*, 1989), cardiac muscle (Noma, 1983), skeletal muscle (Spruce *et al.*, 1985) and pancreatic β-cells (Cook & Hales, 1984). In the vasculature it is thought that many synthetic and endogenous vasodilators act by opening these K_{ATP} channels (Nelson *et al.*, 1990) and in the coronary arteries the vasodilatation observed when the intravascular oxygen tension decreases appears to be due to activation of these K_{ATP} channels (Daut *et al.*, 1990).

The compounds which modulate K^+ channels include cromakalim, an activator of K^+ channels, and glibenclamide, which blocks the K_{ATP} channels. Evidence has accumulated showing that the actions of these drugs occur at the K_{ATP} channels (Quast & Cook, 1989), although it has been suggested that cromakalim interacts in the vasculature with the delayed-rectifier K^+ channel (Beech & Bolton, 1989). The calcium-sensitive K^+ channels, which can be blocked by charybdotoxin (CTX) (Vazquez *et al.*, 1989), have also been proposed as the site of action of cromakalim (Gelband *et al.*, 1989). Recent evidence has also indicated that cromakalim may exert a direct inhibitory action on intracellular calcium movement (Bray *et al.*, 1991).

Cromakalim has been shown to protect ischaemic/reperfused myocardial tissue (Grover *et al.*, 1990). Therefore, in the present study, the effect of K^+ channel modulatory drugs on the leukotriene-induced constrictor actions in the coronary vasculature of the isolated heart were investigated.

Methods

Langendorff-perfused heart preparations

Male guinea-pigs (Dunkin-Hartley; 350–450 g) were killed by cervical dislocation; the heart was removed and placed in cold Krebs-Henseleit buffer. The heart was immediately perfused, by the modified Langendorff technique, via retrograde cannulation of the aorta with Krebs solution gassed with a mixture of 95% O_2:5% CO_2. The heart was perfused with a constant flow of 8 ml min^{-1} and the variable perfusion pressure (PP) was measured with a pressure transducer (Druck type PDCR-75). Each heart was allowed to equilibrate for 15 min before treatment and for at least 15 min between each bolus dose of agonist.

K^+ channel regulatory compounds

All of the compounds used were infused into the perfusing buffer at least 10 min before agonist administration.

Cromakalim (M&B 44809) was dissolved in a small amount of ethanol and then diluted in distilled water. The final concentration of ethanol (0.1%) had no effect on the heart. Glibenclamide was dissolved in a minimum amount of dimethyl sulphoxide (DMSO) and then in Krebs buffer. The vasoconstrictor responses of the leukotrienes in the presence of the final concentration of DMSO alone (0.015%) were not significantly different from those responses observed in control hearts (data not shown).

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All the other compounds used, galanin and vasoactive intestinal peptide (VIP), were dissolved in distilled water.

Radioimmunoassay

In order to determine the basal and leukotriene-stimulated release of thromboxane (Tx) A_2 from the isolated heart preparation, a radioimmunoassay (RIA) for TxB_2, the stable metabolite of TxA_2, was performed. Effluent was collected at 1 min intervals before and after administration of LTC_4 and LTD_4 (30 pmol). Following partial purification with C_{18} SEP-PAK cartridges the RIA for TxB_2 was performed according to the method of Jose *et al.* (1976).

Materials

The following compounds were used; LTC_4 and LTD_4 (Merck Frosst, Canada), U46619 (11α,9α-epoxymethano-prostaglandin H_2, Upjohn), endothelin-1 (Peninsula), angiotensin II (Sigma), glyceryl trinitrate (Schwarz Pharmaceuticals), cromakalim (May & Baker), glibenclamide (Sigma), galanin (Peninsula), vasoactive intestinal peptide (Sigma), charybdotoxin (Bachem), dimethyl sulphoxide (Sigma), TxB_2 antisera (a gift from Dr J. Salmon, Wellcome Laboratories), [^{3}H]-TxB_2 (NEN).

Statistical analysis

The arithmetic means and their standard errors are quoted. Differences were analysed with Student's *t* test for unpaired data. To analyse data expressed as % a probit transformation was first performed, then Student's *t* test was used. $P < 0.05$ was considered significant. $n = 4$ unless stated otherwise.

Results

Effect of K^+ channel regulating compounds

In the present study a single bolus dose of LTC_4 and LTD_4 was used to provide a standard vasoconstrictor response in the coronary circulation of the isolated heart. This dose (30 pmol) has been previously shown (McLeod & Piper, 1991) to induce a submaximal constrictor action in the guinea-pig isolated heart. Cromakalim (0.35–10 μM) dose-dependently inhibited the vasoconstrictor responses to LTC_4 and LTD_4 (30 pmol) (Figure 1). IC_{50} values were 1.8 μM and 0.19 μM for LTC_4 and LTD_4 respectively. The leukotriene responses in the presence of cromakalim (0.35–10 μM) were slower in attaining their peak (0.5 min in controls compared with 1 min in the presence of cromakalim) (Figure 1). The lowest concentration of cromakalim significantly inhibited the response to LTD_4 (30 pmol), although it had little action on effects of LTC_4 (30 pmol). Cromakalim produced a vasodilator effect, with cromakalim (10 μM) reducing the basal PP from 43.4 ± 4.2 mmHg to 24.5 ± 1.4 mmHg. The inhibition of responses to leukotrienes observed with cromakalim was not due to physiological antagonism caused by its vasodilator action since glyceryl trinitrate (GTN) (10 mg l^{-1}) and VIP (10 nM), both vasodilators, produced similar reductions in resting PP (23.2 ± 3.7 mmHg and 26.3 ± 3.5 mmHg respectively) but had no effect on the peak leukotriene responses (Table 1).

Cromakalim (3.5 μM) also significantly reduced the vascular actions of U46619 (300 pmol), a TxA_2-mimetic, endothelin-1 (ET-1) (30 pmol) and AII (100 pmol) (Figure 2). These doses produced equieffective vasoconstrictor actions (24.6 ± 1.9 mmHg, 27.2 ± 2.3 mmHg and 37 ± 3.4 mmHg respectively) compared with LTC_4 and LTD_4 (30 pmol) (23.4 ± 3.9 mmHg and 29.5 ± 3.1 mmHg respectively). However, a differential inhibitory action by cromakalim was apparent. Cromakalim (3.5 μM) induced a 78 ± 1.6%, 88 ± 4.6% and 90 ± 1.8% inhibition of responses to LTC_4

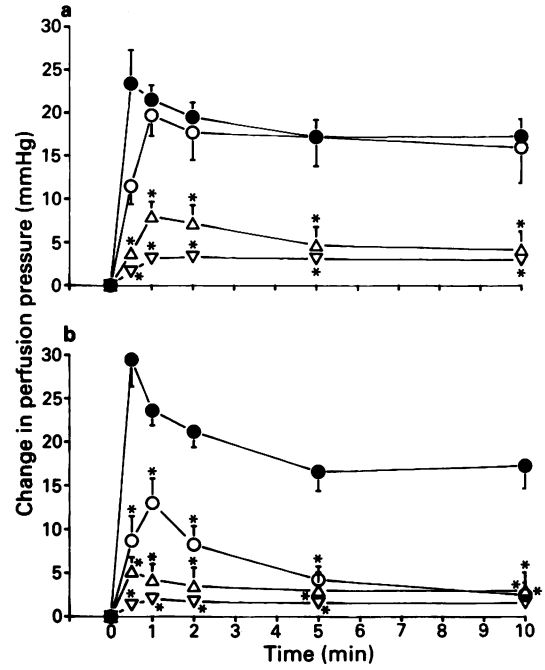

Figure 1 Dose-dependent actions of cromakalim (● control; ○ 0.35 μM; △ 3.5 μM; ▽ 10 μM) on the time-related changes in perfusion pressure induced by (a) leukotriene C_4 (LTC_4, 30 pmol) and (b) LTD_4 (30 pmol).
* $P < 0.05$ compared with controls; $n = 4$–8.

Table 1 Action of glyceryl trinitrate (GTN), vasoactive intestinal peptide (VIP), galanin and charybdotoxin (CTX) on the peak vasoconstrictor actions of leukotriene C_4 (LTC_4) and LTD_4

	Peak changes in perfusion pressure (mmHg)	
Treatment	LTC_4 (30 pmol)	LTD_4 (30 pmol)
Control	23.4 ± 3.9	29.5 ± 3.1
GTN (10 mg l^{-1})	23.2 ± 3.7	32.2 ± 5.7
VIP (10 nM)	31.3 ± 0.8	29.0 ± 5.1
Galanin (10 nM)	23.2 ± 5.0	31.6 ± 5.0
CTX (60 nM)	28.2 ± 3.1	34.5 ± 1.5

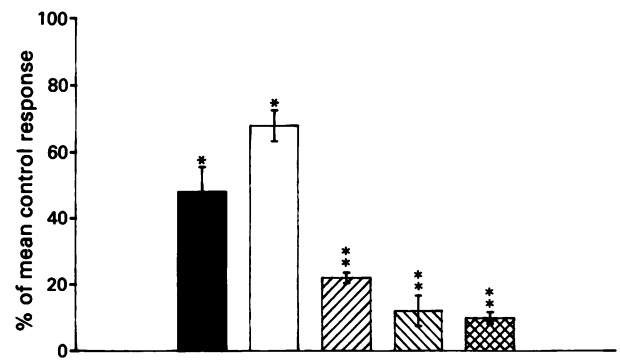

Figure 2 Histogram showing the effect of cromakalim (3.5 μM), as a % of mean control response, on the responses to U46619 (300 pmol) (■), endothelin-1 (30 pmol) (□), leukotriene C_4 (LTC_4, 30 pmol) (▨), LTD_4 (30 pmol) (▧) and angiotensin II (100 pmol) (▩). Mean control values are 100%.
* $P < 0.05$ compared with mean control response; ** $P < 0.05$ compared with U46619 response in the presence of cromakalim; $n = 4$.

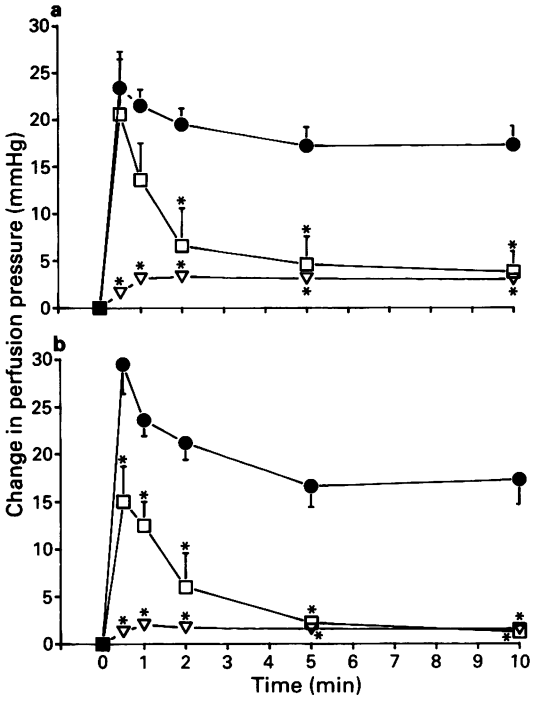

Figure 3 Time course of the vasoconstriction induced by (a) leukotriene C_4 (LTC_4, 30 pmol) and (b) LTD_4 (30 pmol) (●) in the presence of cromakalim alone (10 μM) (▽), and with glibenclamide (10 μM) (□). * $P < 0.05$ compared with controls; $n = 5$–9.

(30 pmol), LTD_4 (30 pmol) and AII (100 pmol) respectively, compared with 52 ± 7.7% and 32 ± 4.6% attenuation of U46619 (300 pmol) and ET-1 (30 pmol) effects (Figure 2).

The maximal inhibitory action by cromakalim (10 μM) of the LTC_4 (30 pmol)-induced vasoconstrictor effects could be reversed after incubation in the presence of an equimolar concentration of glibenclamide. However, glibenclamide (10 μM) could only partially restore the LTD_4 response (Figure 3). Although the peak leukotriene vasoconstrictor effects could be restored, the responses were not as prolonged as observed with controls (Figure 3). Glibenclamide (10 μM) alone caused a vasoconstrictor effect with an increase in the basal PP from 43.4 ± 4.2 mmHg to 54.4 ± 1.9 mmHg. This concentration of glibenclamide had no effect on the vasoconstrictor action of U46619 (300 pmol). A peak increase in PP of 36.7 ± 3.1 mmHg was induced by U46619 alone compared with 36.2 ± 3.6 mmHg in the presence of glibenclamide (10 μM).

By use of RIA a basal release of TxB_2 was detected from the guinea-pig isolated heart (0.5 ± 0.06 ng min^{-1}; $n = 6$). However, no change in TxB_2 generation occurred up to 1 min post-administration of LTC_4 or LTD_4 (30 pmol) (0.5 ± 0.05 and 0.46 ± 0.15 ng ml^{-1} respectively).

Galanin (10 nM) had no effect on either the basal PP (38.3 ± 2.6 mmHg compared with 43.4 ± 4.2 mmHg) or the vasoconstrictor actions of LTC_4 and LTD_4 (30 pmol) (Table 1).

CTX (60 nM) had no action on the resting PP (39.8 ± 4.6 mmHg compared with 43.4 ± 4.2 mmHg) and also did not significantly affect the peak vasoconstrictor responses to LTC_4 and LTD_4 (30 pmol) (Table 1).

Discussion

Cromakalim has previously been found to induce vasodilatation of dog mesenteric artery (Masuzawa *et al.*, 1990) and coronary arteries both *in vivo* (Giudicelli *et al.*, 1990) and *in vitro* (McPherson & Angus, 1990). This vasodilator action by cromakalim is associated with hyperpolarization of smooth muscle possibly caused by direct activation of K_{ATP} channels (Standen *et al.*, 1989). In the present study, cromakalim (0.35–10 μM) caused a reduction in the resting tone of the coronary vessels in the guinea-pig isolated perfused heart. In the presence of cromakalim the vasoconstrictor responses to LTC_4, LTD_4 and AII were significantly reduced by between 80–90% of control responses. Cromakalim would appear to have a differential inhibitory action on the leukotriene responses since with the lowest concentration of cromakalim a much greater attenuating effect was observed on LTD_4 responses than on those to LTC_4. The reversal, by cromakalim, of LTD_4-induced contractions of guinea-pig trachea has been observed previously (O'Donnell & Zeng, 1990). The AII response in rat aorta has also been found to be sensitive to cromakalim (Cook *et al.*, 1988). Attenuation of the vasoconstrictor responses to LTC_4, LTD_4 and AII may have, at least partially, been due to the vasodilatation induced by cromakalim since the contractile responses to U46619 and ET-1 were also significantly reduced, although not to the same extent (30–50% of controls) as the leukotrienes and AII. Attenuation of ET-1-induced actions by cromakalim has been previously reported in rat aorta (Kim *et al.*, 1989). However, in the presence of equiactive concentrations of GTN and VIP, vasodilators which are independent and dependent on K^+ channel activation respectively (Bray *et al.*, 1987; Standen *et al.*, 1989), no effect on the vascular responses induced by LTC_4 and LTD_4 was observed. These results suggest that the inhibitory capacity of cromakalim is not due to a physiological antagonism associated with its vasodilator action. It therefore appears that there is a specific cromakalim-sensitive element in the leukotriene vasoconstrictor response.

The sulphonylurea drug glibenclamide, appears to be a selective blocker of K_{ATP} channels (Sturgess *et al.*, 1985), although Cocks *et al.* (1990) found that high concentrations of glibenclamide antagonized the action of U46619. In the present study, however, glibenclamide (10 μM) had no action against the vascular response to U46619 (300 pmol). The presence of glibenclamide (10 μM) caused an increase in the resting PP of the coronary vessels, in contrast to the rabbit isolated aorta where glibenclamide had no effect on resting tension (Quast & Cook, 1988). It has been postulated that under normal physiological conditions the probability of K_{ATP} channels being in an open state is very low, since the intracellular concentration of ATP will be buffered by creatine phosphate at a level in excess of that required to close the channels (Davies, 1990). The fact that glibenclamide in our system was able to cause a constrictor action indicates that some of the K_{ATP} channels were in an open state. This suggests that, of the K^+ channels present in the vasculature, the K_{ATP} channels in particular may be involved in the maintenance of resting tone of the vasculature within the isolated perfused heart. However, both low intracellular pH (Davies, 1990) and hypoxia within the coronary arteries (Daut *et al.*, 1990) have been reported to lead to an increase in the probability of open K_{ATP} channels. Both these situations could arise in the isolated perfused heart causing the probability of open K_{ATP} channels to be higher than in the normal physiological state.

In the presence of glibenclamide (10 μM) the attenuation of LTC_4- and LTD_4-induced responses by cromakalim (10 μM) was reversed. These results suggest that glibenclamide-sensitive K^+ channels, possibly K_{ATP} channels, were involved in the cromakalim-induced effect. It is possible that glibenclamide affects the LT vascular responses by antagonising the actions of TxA_2 which may be released upon LT stimulation. However, neither LTC_4 nor LTD_4 generate the release of TxA_2 from guinea-pig isolated heart, a finding which is in agreement with previous reports (Terashita *et al.*, 1981). The peak LTC_4 response was restored back to control values in the presence of cromakalim and glibenclamide and a much quicker recovery phase was apparent. This indicates that the

cromakalim-inhibitory effect on the peak LTC_4 response, but not the latter phase, involved a glibenclamide-sensitive mechanism. Both the initial and later phases of the LTD_4 response were only partially reversed implying that the action of cromakalim on the LTD_4 response was not solely due to interaction with glibenclamide-sensitive K^+ channels. Although it has been proposed that cromakalim acts directly in the vasculature as an activator of K_{ATP} channels (Standen *et al.*, 1989) other K^+ channels have been reported to be affected by cromakalim. Beech & Bolton (1989) found that the characteristics of the K^+ channel which cromakalim interacted with bore more relation to the delayed-rectifier K^+ channel than K_{ATP} channel. However, cromakalim has also been reported to modulate calcium-sensitive K^+ channels (Gelband *et al.*, 1989; Okabe *et al.*, 1990) as well as inactivating voltage-sensitive calcium channels (Okabe *et al.*, 1990). Both LTC_4 and LTD_4 vasoconstrictor responses in the coronary vessels are sensitive to blockers of the voltage-sensitive calcium channels (Lefer & Yanagisawa, 1986; Stanton & Piper, 1987). It is therefore possible that cromakalim could be inhibiting the LTD_4 vascular response, and the later phase of the LTC_4 effect, by activating calcium-sensitive K^+ channels and inhibiting voltage-sensitive calcium channels. CTX is a potent blocker of calcium-sensitive K^+ channels in vascular smooth muscle (Vazquez *et al.*, 1989). If the vascular responses to leukotrienes did involve calcium-sensitive K^+ channels then it would be expected that an augmented leukotriene response would be observed in the presence of CTX. However, in the present study, CTX (60 nM), a concentration found to be effective in guinea-pig trachea (Jones *et al.*, 1990), had no significant action on the vasoconstrictor effects of LTC_4 and LTD_4, although the LTD_4 response was slightly potentiated. A higher concentration may be required in order to observe a significant augmentation of the LT response. Bray *et al.* (1991) reported that the ability of cromakalim to relax noradrenaline-induced contractions in rabbit aorta may be due to cromakalim having a direct inhibitory action on intracellular calcium movement. This could be another possible mode of action by which cromakalim has its selective inhibitory action on the responses of LTC_4 and LTD_4.

Galanin is thought to act by opening K^+ channels (Ahren *et al.*, 1988). At a concentration previously observed to be effective (Dunne *et al.*, 1989) galanin had no effect on the resting PP or peak leukotriene responses. In pancreatic β-cells, galanin causes the opening of K_{ATP} channels (De Weille *et al.*, 1988). Although, the vasodilator effect of galanin in rat mesenteric artery is not mediated via the activation of K_{ATP} channels (Nakayama *et al.*, 1991). Galanin had no effect in the guinea-pig isolated heart indicating that the K_{ATP} channels present in the coronary vasculature differ from those in the pancreatic β-cells, and that the action of galanin is tissue-specific.

In conclusion, the present study shows that cromakalim, a hyperpolarizing agent, inhibits the vasoconstrictor responses to LTC_4, LTD_4 and AII in the coronary vessels of the guinea-pig isolated perfused heart. This effect on actions of leukotrienes appears to be mediated through a glibenclamide-sensitive K^+ channel, possibly the K_{ATP} channel, although other ion channels may also be involved in the inhibitory action on LTD_4 responses. This suggests that a cromakalim-sensitive mechanism, present in the coronary vasculature of guinea-pig isolated heart, is important in the vasoconstrictor responses of LTC_4 and LTD_4.

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The role of nitric oxide in the regional vasodilator effects of endothelin-1 in the rat

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1 The role of nitic oxide (NO) derived from L-arginine in the regional vasodilator effects of endothelin-1 has been investigated in anaesthetized, spontaneously hypertensive (SH) rats in which autonomic reflexes were abolished by ganglion blockade. The experimental design incorporated animals infused with phenylephrine to mimic the peripheral vasoconstrictor effects of the NO biosynthesis inhibitors and a single dose per animal paradigm to obviate problems of tachyphylaxis to the vasodilator effects of endothelin-1.

2 Infusion of the inhibitor of NO synthase, N-monomethyl-L-arginine (L-NMMA) at a dose ($5\,mg\,kg^{-1}\,min^{-1}$) which maximally raised blood pressure did not influence either the fall in blood pressure or the vasodilator responses induced in the hindquarters and carotid vascular beds by endothelin-1 ($1\,nmol\,kg^{-1}$, i.v.). The duration (but not the initial magnitude) of the vasodepressor response to endothelin-1 was however significantly attenuated (by 49%) during infusion of the more potent inhibitor of NO synthase, N^G-nitro-L-arginine methyl ester (L-NAME), $2\,mg\,kg^{-1}\,min^{-1}$.

3 Increasing the dose of L-NAME to 10 and $25\,mg\,kg^{-1}\,min^{-1}$ significantly attenuated, but did not abolish, the falls in blood pressure and hindquarters vasodilator responses to acetylcholine, $1\,\mu g\,kg^{-1}$, and endothelin-1, $1\,nmol\,kg^{-1}\,min^{-1}$. The effects were selective in that vasodepressor responses to the endothelium-independent vasodilator, sodium nitroprusside, $1\text{-}10\,\mu g\,kg^{-1}\,min^{-1}$, were unaltered.

4 The data indicate that NO generated *de novo* from L-arginine mediates a significant component of the vasodilator effect of endothelin-1 in the anaesthetized, ganglion-blocked SH rat. However, a major component of the vasodepressor effects of both endothelin-1 and acetylcholine may occur independently of this mechanism.

Keywords: N^G-monomethyl-L-arginine; nitric oxide; spontaneously hypertensive rats; endothelin-1; regional vasodilatation

Introduction

Prominent regional vasodilator responses to endothelin-1 were described soon after its discovery (Yanagisawa *et al.*, 1988) in anaesthetized rats (Wright & Fozard, 1988) and cats (Lippton *et al.*, 1988). Such responses were subsequently confirmed in rats (see, e.g., Madeddu *et al.*, 1989; Winquist *et al.*, 1989; Hoffmann *et al.*, 1989; 1990; Gardiner *et al.*, 1990a; Le Monnier de Gouville *et al.*, 1990b; Ohlstein *et al.*, 1990) and cats (Minkes & Kadowitz, 1989; Le Monnier de Gouville *et al.*, 1990a; Minkes *et al.*, 1990) and also demonstrated to occur in dogs (Lippton *et al.*, 1989; Otsuka *et al.*, 1990; Tsuchiya *et al.*, 1990), rabbits (Cocks *et al.*, 1989; Hof *et al.*, 1989; Lippton *et al.*, 1991a), primates (Clozel & Clozel, 1989; Lopez *et al.*, 1990) and man (Clarke *et al.*, 1989; Kiowski *et al.*, 1991). Vasodilator responses to endothelin-1 have equally been demonstrated in a variety of isolated blood vessel preparations (see, e.g., Cocks *et al.*, 1989; Folta *et al.*, 1989; Randall *et al.*, 1989; Warner *et al.*, 1989; Baydoun *et al.*, 1990; Hasunuma *et al.*, 1990; Kitazumi *et al.*, 1990; Perreault & De Marte, 1991).

A number of different mechanisms has been invoked to explain the vasodilator effects of endothelin-1. *In vitro* data suggest the presence of the endothelium is obligatory (Cocks *et al.*, 1989; Folta *et al.*, 1989; Randall *et al.*, 1989; Sakata *et al.*, 1989; Warner *et al.*, 1989; Douglas & Hiley, 1990). The release of vasodilator products of cyclo-oxygenase activity has been implicated (Folta *et al.*, 1989) as has the involvement of ATP-dependent K^+ channels (Hasunuma *et al.*, 1990). However, data obtained *in vivo* suggest that vasodilator products of cyclo-oxygenase do not mediate the regional vasodilator responses to endothelin-1 in rats (Gardiner *et al.*, 1990b; Ohlstein *et al.*, 1990; Wright & Fozard, 1990), rabbits (Lippton *et al.*, 1991a), cats (Minkes *et al.*, 1990), dogs (Hoffmann *et al.*, 1989) or monkeys (Lopez *et al.*, 1990). Moreover, activation of ATP-dependent K^+ channels appears to be a mechanism selective for pulmonary vessels (Hasunuma *et al.*, 1990; Lippton *et al.*, 1991b) and is not the mechanism of the systemic vasodilator effects in either rats (Le Monnier de Gouville *et al.*, 1990b) or cats (Lippton *et al.*, 1991b).

The data implicating endothelium derived nitric oxide (NO) in the vasodilator response to endothelin-1 are equivocal. Thus, evidence both for (Warner *et al.*, 1989; Douglas & Hiley, 1990; Kitazumi *et al.*, 1990; Perreault & De Marte, 1991) and against (Baydoun *et al.*, 1990; Folta *et al.*, 1990) the concept has been obtained from *in vitro* experiments. Similarly, whereas Whittle *et al.* (1989) reported significant inhibition of the vasodepressor effects of endothelin-1 in anaesthetized rats by N-monomethyl-L-arginine (L-NMMA), a selective inhibitor of NO formation, Gardiner *et al.* (1989) found no attenuation of either the vasodepressor or the regional vasodilator effects of endothelin-1 following L-NMMA in conscious rats. However, in a subsequent study, also in conscious rats, Gardiner *et al.* (1990c) showed that N^G-nitro-L-arginine methyl ester (L-NAME), a more potent inhibitor of NO biosynthesis than L-NMMA (Moore *et al.*, 1990), attenuated the vasodilator effects of endothelin-1.

The objective of the present study was to explore in detail the effect of inhibition of NO biosynthesis from L-arginine with L-NMMA and L-NAME on the regional vasodilator effects of endothelin-1 *in vivo*. The anaesthetized, spontaneously hypertensive (SH) rat treated with a ganglion blocking agent (Wright & Fozard, 1988; 1990) provides a robust and reproducible model for this purpose. The experimental design incorporated animals infused with phenylephrine to mimic the peripheral vasoconstrictor effects of the NO biosynthesis inhibitors and a single dose per animal paradigm to obviate problems of tachyphylaxis to the vasodilator effects of endothelin-1 (Le Monnier de Gouville *et al.*, 1990a, b).

Methods

In vivo *preparations*

Male SH rats weighing 339–393 g supplied by Biological Research Laboratories, Füllinsdorf, Switzerland, were used in

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this study. Rats were anaesthetized with Inaktin, 120 mg kg^{-1}, i.p. A tracheotomy was performed and catheters inserted into the right carotid artery for recording blood pressure and heart rate and into the jugular veins for drug administration. In some rats, pulsed Doppler flow probes (Haywood *et al.*, 1981) were placed around the left carotid artery and, through a midline abdominal incision, around the left renal artery, the superior mesenteric artery and the lower abdominal aorta just above the iliac bifurcation (equated to hindquarters blood flow). The incisions were closed and parameters allowed to stabilize for 1 h. Following the stabilization period, the antonomic ganglia were blocked with mecamylamine (0.25 mg kg^{-1} infused intravenously over 15 min).

Experimental design

One hour after the end of the infusion of mecamylamine, rats were infused with either phenylephrine, L-NMMA or L-NAME at doses specified below. The maximum duration of any infusion did not exceed 22 min and in each case the cardiovascular effects were maximal at 4 min and maintained for the duration of the infusion. For this reason the cardiovascular effects during the infusion (see Table 1) are given as the change at 4 min. Three series of experiments were carried out. In the first, involving infusions of phenylephrine, 5–10 μg kg^{-1} min^{-1}, L-NMMA, 5 mg kg^{-1} min^{-1} or L-NAME, 2 mg kg^{-1} min^{-1}, a bolus injection of endothelin-1 1 nmol kg^{-1}, which is just submaximal, was administered 4 min after the start of the infusion. In a second series of experiments involving infusions of phenylephrine, 10 μg kg^{-1} min^{-1} or L-NAME, 10 and 25 mg kg^{-1} min^{-1}, a submaximal dose of acetylcholine (1 μg kg^{-1}) was given 4 min after the start of the infusion followed by a bolus injection of endothelin, 1 nmol kg^{-1}, 2 min later. A representative tracing illustrating this latter experimental design is presented in Figure 1. In a third series of experiments carried out in animals without flow probes, the effects of acetylcholine 0.1, 0.3 and 1 μg kg^{-1}, sodium nitroprusside, 1, 3 and 10 μg kg^{-1} and endothelin-1 1 nmol kg^{-1} were monitored during infusions of phenylephrine, 5 μg kg^{-1} min^{-1} or L-NAME, 10 mg kg^{-1} min^{-1}. In each experimental series, only one infusion/injection sequence was carried out in each animal.

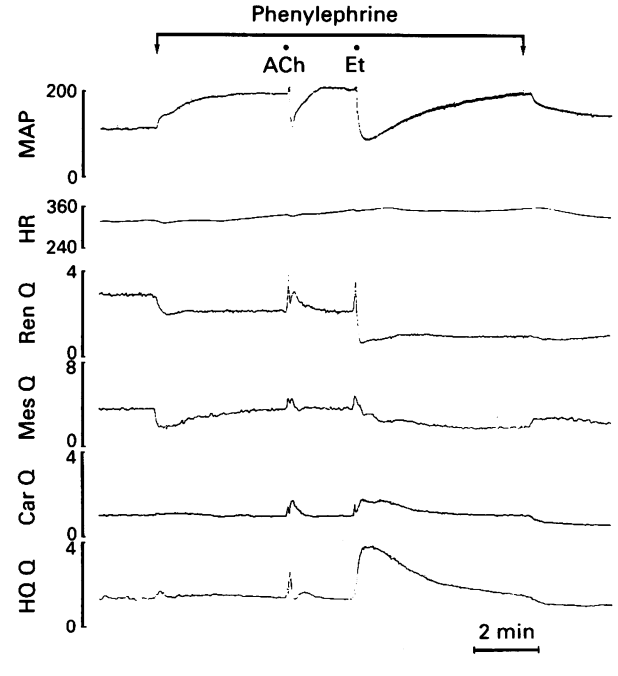

Figure 1 Representative chart record to illustrate the experimental design used to evaluate the effects of N^G-nitro-L-arginine methyl ester (L-NAME) on the responses to acetylcholine (ACh) and endothelin (Et). Since inhibitors of NO synthase induce peripheral vasoconstriction, control animals were infused with phenylephrine, 10 μg kg^{-1} min^{-1} (illustrated), to increase vascular tone in the preparations to the extent seen with L-NAME (see Table 1). Comparisons could then be made between responses to bolus injections of ACh (1 μg kg^{-1}) and Et (1 nmol kg^{-1}) administered during infusions of either phenylephrine or L-NAME. Variables recorded: mean arterial pressure (MAP; mmHg), heart rate (HR; b min^{-1}) and renal (Ren), mesenteric (Mes), carotid (Car) and hindquarters (HQ) blood flows (Q; kHz).

Drugs

Porcine endothelin-1 (Peptide Institute Inc., Osaka, Japan) was dissolved in acetic acid (10 mM) to 10^{-5} M and stored in aliquots at −20°C. Other drugs were freshly prepared each day and diluted in 0.9% w/v NaCl solution before use. N^G-monomethyl-L-arginine citrate (L-NMMA) was supplied by Ultrafine Chemicals, Salford, UK, N^G-nitro-L-arginine methylester (L-NAME), sodium nitroprusside and acetylcholine chloride were purchased from Sigma. Mecamylamine hydrochloride was the kind gift of Merck, Sharpe and Dohme. Phenylephrine hydrochloride was obtained from Serva.

Statistical analysis

All data are expressed as mean values ± s.e.mean of n individual determinations. Student's t test was used to evaluate the significance of a difference between group means.

Results

Haemodynamic effects of phenylephrine, L-NMMA and L-NAME: effects on endothelin-1

The data from the first series of experiments in which the effects of endothelin-1 were compared during infusions of phenylephrine, 5–10 μg kg^{-1} min^{-1}, L-NMMA, 5 mg kg^{-1} min^{-1} or L-NAME, 2 mg kg^{-1} min^{-1} are presented in Figure 2 and Table 1. All the infused drugs gave rapid, marked and sustained rises in mean arterial pressure (MAP) and decreases in conductance in the renal, mesenteric, carotid and hindquarters vascular beds; the effects were maximal 4 min after the start of the infusion, illustrated for phenylephrine in Figure 1. In general, the conductance changes were somewhat less with phenylephrine than with either L-NMMA or L-NAME despite the increases in MAP being similar (Table 1). Heart rate (HR) changed minimally during infusion of L-NMMA and L-NAME (data not illustrated) but increased by approx. 50 b min^{-1} following phenylephrine (evident from the 0 time values in Figure 2).

Endothelin-1, 1 nmol kg^{-1}, injected 4 min after the start of the infusion of phenylephrine induced a pronounced fall in MAP accompanied by increases in conductance in the carotid and hindquarters vascular beds and slight vasoconstrictor responses in the renal and mesenteric vascular beds (Figure 2a). Following L-NMMA, the fall in MAP induced by endothelin-1 was similar to that seen during phenylephrine infusion. The vasodilator responses in the carotid and hindquarters vascular beds were somewhat larger than those seen during phenylephrine infusion and there was a trend towards vasodilatation in the mesenteric artery bed. The renal vasoconstrictor response to endothelin-1 was more marked during infusion of L-NMMA than phenylephrine (Figure 2b). The hypotensive response to endothelin-1 during infusion with L-NAME was of similar magnitude to that seen in the presence of phenylephrine (Figure 2a and c) but of significantly shorter duration (mean time to return 50% towards baseline [$t_{1/2}$] during phenylephrine infusion was 86 ± 4 s (n = 5) and during L-NAME, 44 ± 5 s (n = 4) (P < 0.01)). The conductance changes in the carotid mesenteric and hindquarters vascular

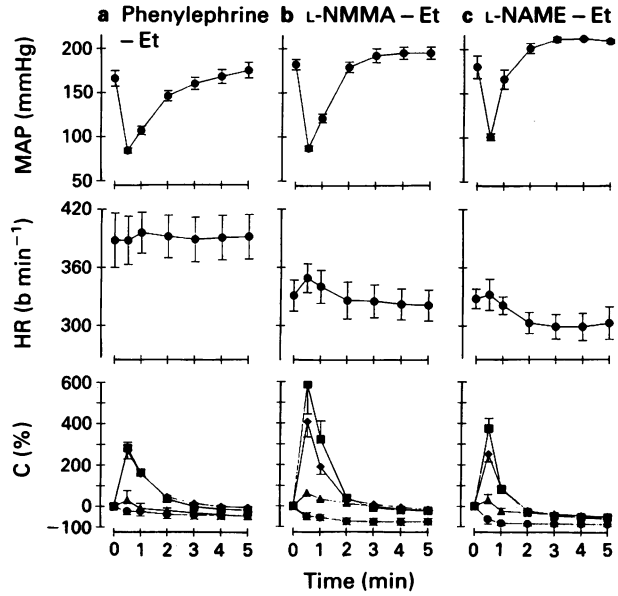

Figure 2 Circulatory changes following administration of endothelin (Et), 1 nmol kg^{-1} (i.v. bolus), to anaesthetized, ganglion-blocked SH rats infused with phenylephrine, 5–10 μg kg^{-1} min^{-1} (a) N-monomethyl-L-arginine (L-NMMA) 5 mg kg^{-1} min^{-1} (b) or N^{G}-nitro-L-arginine methyl ester (L-NAME) 2 mg kg^{-1} min^{-1} (c). Variables are mean arterial pressure (MAP, mmHg), heart rate (HR, b min^{-1}) and % change in vascular conductance (C) in renal (●), mesenteric (▲), carotid (◆) and hindquarters (■) beds. The abscissa scale represents time in min after injection of endothelin. Points represent mean values of 5 (MAP, HR) or 3–5 (C) individual values, s.e.mean shown by vertical bars.

beds were similar in the phenylephrine- and L-NAME-infused animals but the renal vasoconstrictor response to endothelin-1 was substantially greater in the latter than the former (Figure 2c).

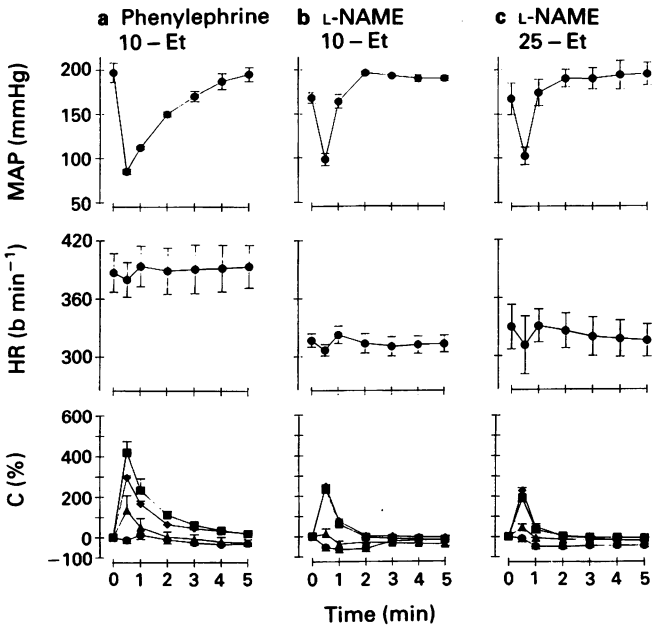

Figure 3 Circulatory changes following administration of endothelin Et (Et), 1 nmol kg^{-1} (i.v. bolus), to anaesthetized, ganglion-blocked SH rats infused with phenylephrine, 10 μg kg^{-1} min^{-1} (a) N^{G}-nitro-L-arginine methyl ester (L-NAME), 10 mg kg^{-1} min^{-1} (b) or N-monomethyl-L-arginine (L-NAME) 25 mg kg^{-1} (c). For further details see legend to Figure 2. Symbols as in Figure 2.

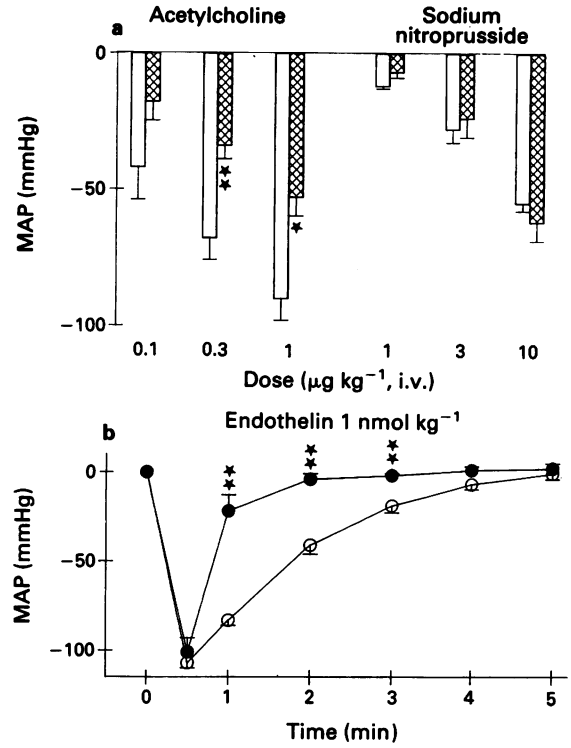

Figure 4 Changes in mean arterial pressure (MAP, mmHg) induced by bolus i.v. injections of acetylcholine, 0.1–1 μg kg^{-1}, sodium nitroprusside, 1–10 μg kg^{-1} (a) and endothelin, 1 nmol kg^{-1} (b) during infusion with either phenylephrine, 5 μg kg^{-1} min^{-1} (open columns (a); ○, (b); $n = 3$–5 in (a) and (b) or N^{G}-nitro-L-arginine methyl ester (L-NAME, 10 mg kg^{-1} min^{-1} (cross-hatched column (a); ●, (b), $n = 4$ in (a) and (b)). *, ** indicate a significant difference ($P < 0.05$; 0.01, respectively) from the value in the phenylephrine series.

Effects of higher doses of L-NAME

The data from the second series of experiments in which the effects of acetylcholine and endothelin-1 were compared during infusions of phenylephrine, 10 μg kg^{-1} min^{-1} or L-NAME, 10 and 25 mg kg^{-1} min^{-1} are presented in Table 1 and Figures 1 and 3. In this series of experiments, the MAP changes measured 4 min after phenylephrine exceeded those of the first series and were significantly higher than those produced by either dose of L-NAME. Changes in conductance in the renal, mesenteric, carotid and hindquarters vascular beds were similar in the phenylephrine- and L-NAME-infused animals (Table 1). The haemodynamic changes induced by L-NAME were broadly similar at each infusion dose (Table 1).

The hypotensive response to acetylcholine, 1 μg kg^{-1} min^{-1}, elicited 4 min following the start of the infusion of phenylephrine, was 81 ± 2 mmHg, $n = 4$. It was reduced slightly but significantly during infusion with L-NAME 10 mg kg^{-1} min^{-1} (to 66 ± 4 mmHg, $n = 4$) and to a similar extent (65 ± 8 mmHg, $n = 3$) during the higher dose. The vasodilator effects of acetylcholine in the mesenteric and carotid vascular beds were unaffected by infusion of L-NAME, 10 or 25 mg kg^{-1} min^{-1}; in contrast vasodilator responses in the renal and hindquarter vascular beds were significantly attenuated (by 30–40%) and to a similar extent following each dose of L-NAME (data not illustrated).

The changes in MAP and regional conductances induced by endothelin-1 during infusion with phenylephrine were similar to those recorded in the first series except that frank vasodilatation was seen initially in the mesenteric artery bed and little further vasoconstriction was induced in the renal vasculature (compare Figures 2a and 3a). Both the hypotension and the peripheral vasodilator responses to endothelin-1 were attenuated during infusion of L-NAME compared to those obtained

Table 1: Haemodynamic effects of phenylephrine, N-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine methyl ester (L-NAME) in anaesthetized SH rats treated with a ganglion blocking agent

	First series									*Second series*								
	Phenylephrine ($5–10\,\mu g\,kg^{-1}\,min^{-1}$)			*L-NMMA* ($5\,mg\,kg^{-1}\,min^{-1}$)			*L-NMMA* ($2\,mg\,kg^{-1}\,min^{-1}$)			*Phenylephrine* ($10\,\mu g\,kg^{-1}\,min^{-1}$)			*L-NMMA* ($10\,mg\,kg^{-1}\,min^{-1}$)			*L-NMMA* ($25\,mg\,kg^{-1}\,min^{-1}$)		
	Baseline	4′	*n*	Baseline	4′	*n*	Baseline	4′	*n*	Baseline	4′	*n*	Baseline	4′	*n*	Baseline	4′	*n*
MAP	110 ± 11	167 ± 9	5	121 ± 3	182 ± 6	4	126 ± 8	180 ± 13	4	110 ± 8	208 ± 6	4	114 ± 7	170 ± 8	4	115 ± 8	169 ± 15	3
HR	352 ± 13	388 ± 28	5	337 ± 18	332 ± 16	4	343 ± 15	328 ± 10	3	334 ± 15	369 ± 14	4	361 ± 10	328 ± 8	4	346 ± 18	337 ± 21	3
RenC	2.0 ± 0.9	−40 ± 10	4	3.5 ± 1.1	−57 ± 7	4	3.8 ± 1.6	−60 ± 2	3	1.9 ± 0.3	−58 ± 3	4	1.5 ± 0.4	−53 ± 4	3	6.1 ± 1.0	−67 ± 3	3
MesC	3.3 ± 0.9	−17 ± 14	5	5.1 ± 0.5	−45 ± 3	4	2.4 ± 0.3	−53 ± 3	4	4.5 ± 0.5	−57 ± 6	4	4.7 ± 2.0	−62 ± 4	3	3.7 ± 0.9	−59 ± 2	3
CarC	1.4 ± 0.2	−34 ± 8	5	1.1 ± 0.2	−48 ± 3	4	0.9 ± 0.2	−53 ± 3	4	1.3 ± 0.2	−58 ± 5	4	1.3 ± 0.3	−70 ± 3	4	1.1 ± 0.2	−64 ± 1	3
HQC	1.8 ± 0.1	−23 ± 7	5	1.3 ± 0.1	−42 ± 6	4	1.1 ± 0.1	−35 ± 2	4	1.0 ± 0.1	−40 ± 6	4	1.1 ± 0.2	−38 ± 3	4	1.4 ± 0.2	−40 ± 6	3

MAP = mean arterial pressure in mmHg, HR = heart rate in $b\,min^{-1}$; Renal (Ren), mesenteric (Mes), carotid (Car) and hindquarters (HQ) conductances (C) – baseline values in conductance units ($100\,[kHz\,mmHg^{-1}]$), 4 min values are % changes. Values are means ± s.e.mean of *n* individual observations.

during infusion of phenylephrine (Figure 3). When the dose-response relationship for L-NAME is considered, it is clear that with respect to inhibition of the fall in MAP induced by endothelin-1, the 10 and 25 mg kg^{-1} min^{-1} doses of L-NAME were not more effective than the 2 mg kg^{-1} min^{-1} dose (compare Figures 3b and c with Figure 2c). The vasodilator effects of endothelin-1 in the carotid and particularly the hindquarters vascular beds were inhibited dose-dependently by L-NAME (Figures 3b and c); however, the change between the 10 and 25 mg kg^{-1} min^{-1} was small and clear residual vasodilator responses remained in both beds (Figure 3).

Selectivity of action of L-NAME

To assess the selectivity of action of L-NAME, a third series of experiments were carried out in anaesthetized, ganglion-blocked SHR set up for recording blood pressure and heart rate but without placement of the flow probes. Bolus injections of acetylcholine, 0.1, 0.3 and 1 μg kg^{-1}, sodium nitroprusside, 1, 3 and 10 μg kg^{-1} and endothelin-1, 1 nmol kg^{-1}, were given sequentially during infusions of phenylephrine, 5 μg kg^{-1} min^{-1} or L-NAME, 10 mg kg^{-1} min^{-1}; which raised MAP from 150 $\pm$ 3 to 208 $\pm$ 6 (n = 5) and from 159 $\pm$ 10 to 212 $\pm$ 6 (n = 4) mmHg, respectively. The results are presented in Figure 4. Responses to acetylcholine were significantly smaller during infusion of L-NAME than during infusion of phenylephrine; in contrast responses to sodium nitroprusside were closely similar in the two experimental situations (Figure 4a). In confirmation of the earlier result (see Figure 3a and b) the magnitude of the depressor response to endothelin-1 was unchanged during perfusion with L-NAME although the response was of significantly shorter duration (Figure 4b).

Discussion

The present experiments were designed specifically to explore the role of NO derived from L-arginine in the vasodilator response to endothelin-1 in the rat by use of two selective inhibitors of NO synthase, L-NMMA and L-NAME (Rees *et al.*, 1989b; Moore *et al.*, 1990). Since both L-NMMA and L-NAME cause marked rises in blood pressure in rats (Gardiner *et al.*, 1989; 1990c; d; Fozard & Part, 1990), presumably a consequence of removing vasodilator NO 'tone', responses to endothelin-1 were compared to those obtained in animals in which vasoconstriction was induced by infusion of phenylephrine. Although there were minor discrepancies, the haemodynamic changes induced by the NO biosynthesis inhibitors and phenylephrine were broadly similar (Table 1).

The first point to be made is that L-NMMA at a dose (5 mg kg^{-1} min^{-1}) that raises blood pressure to maximum levels in this preparation (see Table 1 and Fozard & Part 1990) caused no inhibition of the vasodepressor responses to endothelin-1 (Figure 2). Since comparisons were made with responses obtained during phenylephrine infusion, the lack of effect cannot be explained by inhibition being offset by the augmenting effects of an elevated vasoconstrictor tone (see for example, Rees *et al.*, 1989b; Gardiner *et al.*, 1990c; van Gelderen *et al.*, 1991). Rather the data would point to the possibility of the vasodilator response to endothelin-1 being independent of NO newly synthesized from L-arginine. However, a discrepancy between the potency of NO biosynthesis inhibitors to increase blood pressure and that to inhibit responses to endothelium-dependent vasodilators such as substance P, bradykinin and acetylcholine has been noted previously (see for example Aisaka *et al.*, 1989; Whittle *et al.*, 1989; Gardiner *et al.*, 1990c; 1991). Thus, an alternative explanation might be that the dose of inhibitor required to block evoked NO release is higher than that needed to block basal release. This would be supported by the fact that the more potent NO synthase inhibitor, L-NAME, attenuated significantly the duration of the vasodepressor response to endothelin-1 (Figure 2).

To evaluate this hypothesis further, a second series of experiments was carried out in which doses of 10 and 25 mg kg^{-1} min^{-1} of L-NAME were infused and in which a comparison with acetylcholine was included. The results were clear-cut in that both the vasodepressor and the hindquarters vasodilator responses to acetylcholine and endothelin-1 were attenuated following L-NAME, 10 mg kg^{-1} min^{-1} and that minimal further inhibition occurred with the higher dose (Figure 4). Significantly, the higher doses of L-NAME were no more effective against the vasodepressor effect of endothelin-1 than was the smaller dose of 2 mg kg^{-1} min^{-1} (Figure 2c). The data presented in Figure 4 confirm the inhibitory effect of L-NAME 10 mg kg^{-1} min^{-1} on endothelin-1 and acetylcholine and show the effect to be selective since responses to the endothelium-independent vasodilator, sodium nitroprusside, remain unchanged. These observations indicate that NO generated by *de novo* synthesis contributes in part to the vasodepressor effects of endothelin-1 and acetylcholine but that a major component of the response in each case may occur independently of this mechanism. In the case of endothelin-1, it is clearly the initial phase of the fall in blood pressure which appears not to involve NO.

Since we have no independent measure of NO generation from L-arginine, it cannot be concluded with certainty that the resistance of the endothelin and acetylcholine vasodilator response to blockade by L-NAME indicates an NO-independent mechanism. However, it might be expected that with the high, supramaximal doses of L-NAME used, NO synthase would be completely blocked. Moreover, with respect to endothelin-1, there are several potential candidates for the mechanism of the component of the vasodilator response resistant to NO biosynthesis inhibition. One possibility is the generation of vasodilator products of cyclo-oxygenase which has been demonstrated to occur *in vitro* following endothelin-1 (De Nucci *et al.*, 1988; Rae *et al.*, 1989; Rakugi *et al.*, 1989). However, this seems not to be important *in vivo* since the vasodepressor and/or regional vasodilator effects of endothelin-1 were resistant to blockade by indomethacin in rats (Gardiner *et al.*, 1990b; Ohlstein *et al.*, 1990; Wright & Fozard, 1990) and other species (Hoffmann *et al.*, 1989; Lopez *et al.*, 1990; Minkes *et al.*, 1990; Lippton *et al.*, 1991a). A second possible mechanism may be advanced based on the observation that pulmonary vasodilatation induced by endothelin-1 in the rat (Hasunuma *et al.*, 1990) and cat (Lippton *et al.*, 1991b) can be inhibited by glibenclamide, an antagonist of ATP-dependent K^+ channels. The observation would be consistent with the release of endothelium-derived hyperpolarizing factor (EDHF; see Taylor & Weston, 1988), the actions of which are known to be attenuated by ATP-dependent K^+ channel blockade (Standen *et al.*, 1989; Brayden, 1990), However, whilst the vasodilator effects of endothelin-1 in discrete vascular beds, notably the lung could conceivably be the consequence of such a mechanism, this is unlikely to be a major factor. Thus, the vasodepressor response to endothelin-1 in the anaesthetized rat was totally unaffected by glibenclamide, at a dose (20 mg kg^{-1}) adequate to block the hypotensive response to cromakalim (Le Monnier de Gouville *et al.*, 1990b). A final possibility, a direct hyperpolarizing effect of endothelin-1, has been recorded from smooth muscle cells in culture (Van Renterghem *et al.*, 1988; Hu *et al.*, 1991). Whilst this could conceivably result in direct smooth muscle, relaxation, it should be noted that such direct (i.e., endothelium-independent) vasodilator responses have up to the present not been reported.

With respect to acetylcholine, the present data are consistent with a growing number of studies showing a resistance of its vasodilator response *in vivo* to blockade by inhibitors of NO synthase (see for example Aisaka *et al.*, 1989; Gardiner *et al.*, 1990c; Rees *et al.*, 1989a; Ross *et al.*, 1991; Van Gelderen *et al.*, 1991). Our data further indicate the effect to be regional

in that vasodilatation in the hindquarters vascular bed had a clear L-NAME-sensitive component whereas that in the carotid and mesenteric beds had not. A possible explanation for the residual response to acetylcholine is the release of EDHF (Brayden, 1990).

In conclusion, the present data indicate that NO generated *de novo* from L-arginine mediates a significant component of the vasodilator effect of endothelin-1 in the anaesthetized, ganglion-blocked rat. Blockade by the NO biosynthesis inhibitor, L-NAME, is manifested selectively against the later phase of the vasodepressor response to endothelin-1 and the vasodilator response in the hindquarters vascular bed. The limited blockade of endothelin-1 plus the need for doses higher than those required to raise blood pressure maximally may explain the inconsistencies in the literature with respect to the effects of NO biosynthesis inhibitors on vasodilator responses to endothelin-1 *in vivo*.

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The effect of adrenoceptor antagonists on the ileal brake mechanism in the rat

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1 Studies were carried out in the rat to investigate the effect of adrenoceptor antagonists on stomach to caecum transit time under control conditions and during ileal infusion of Intralipid. Stomach to caecum transit time (SCTT) of the head of the meal was measured by use of environmental hydrogen analysis and the distribution of the meal was assessed by a scintigraphic technique.

2 Four adrenoceptor antagonists were used in these studies, the α_1 antagonist prazosin, the α_2 antagonist, idazoxan, the β_1 antagonist atenolol and the β_2 antagonist ICI 118551.

3 None of the antagonists affected SCTT of the head of the meal during ileal infusion of saline. However, the α_1 and β_1 antagonists significantly reversed ($P < 0.05$) the delay in SCTT induced by ileal infusion of Intralipid whereas the α_2 antagonist, idazoxan, potentiated this delay ($P < 0.05$).

4 Study of the distribution of the radiolabelled meal showed that the Intralipid delayed SCTT by slowing both gastric emptying ($P < 0.05$) and small bowel transit ($P < 0.05$).

5 Prazosin delayed gastric emptying under control conditions ($P < 0.001$) but did not alter significantly the effect of ileal lipid on the distribution of the meal, 100 min or 200 min after gavage. Idazoxan delayed gastric emptying during ileal infusion of saline ($P < 0.05$) and produced a proximal shift in the geometric centre of the meal in the small intestine ($P < 0.001$) and despite the apparent potentiation of the ileal brake effect indicated by the hydrogen analysis, idazoxan increased the amount of radioactivity in the colon 200 min after gavage ($P < 0.05$).

6 The meal distribution was more compatible with the hydrogen analysis after administration of the β-adrenoceptor antagonists. The reversal of the lipid-induced delay in SCTT caused by atenolol was associated with more radioactivity in the large intestine 200 min after the gavage. ICI 118551 had no significant effects on either the distribution of the meal or the SCTT of the head of the meal.

7 In conclusion, the data confirm that the sympathetic nervous system normally modulates or mediates the mechanisms that influence gastrointestinal transit in the rat and suggest that these mechanisms may be involved in the ileal brake effect. Nevertheless the data also suggest that simple measurement of the transit of the head of the meal by use of environmental hydrogen analysis may sometimes give a misleading impression of the action of drugs on gastrointestinal transit of the bulk of a test meal.

Keywords: Stomach to caecum transit; gastric emptying; ileal brake; adrenoceptor antagonists

Introduction

Ileal infusion of triglyceride emulsions delay gastric emptying and small bowel transit in human volunteers (Spiller *et al.*, 1984; Read *et al.*, 1984) and stomach to caecum transit time in rats (Brown *et al.*, 1987). The mechanism by which this effect occurs remains largely unknown. Spiller and colleagues showed that in normal volunteers the inhibition of small bowel motor activity induced by infusion of fatty acids into the small intestine, 170 cm from the teeth was associated with elevation of the gastrointestinal peptides neurotensin and enteroglucagon (Spiller *et al.*, 1984). However, data from our laboratory showed that the ileal infusion (205 cm) of Intralipid did not result in increased plasma levels of neurotensin and enteroglucagon, but jejunal infusion of Intralipid caused an increase in plasma levels of both hormones without delaying small bowel transit (Read *et al.*, 1984). A series of studies carried out in human volunteers indicated that intravenous administration of the opiate antagonist, naloxone, partially reversed the triglyceride-induced delay in small bowel transit time of a meal (Kinsman & Read, 1984) but not the delay in gastric emptying of the meal (Welch *et al.*, 1987), suggesting that the delay in small bowel transit was mediated at least in part through endogenous opiates. These results were essentially confirmed in an animal model (Brown & French, 1989).

Adrenoceptor antagonists can reduce the effects of stress on gastric emptying and small bowel transit (Stanghellini *et al.*, 1984; O'Brien *et al.*, 1987) and orocaecal transit time can be delayed by the β-agonist isoprenaline (McIntyre *et al.*, 1987). The aims of these experiments were to investigate if the sympathetic nervous system mediates the delay in stomach to caecum transit time produced by ileal infusion of Intralipid by studying the effect of α_1-, α_2-, β_1- and β_2-adrenoceptor antagonists on stomach to caecum transit time and the gastrointestinal distribution of a radiolabelled meal.

Methods

Animals

Experiments were carried out on a total of 112 adult male albino rats weighing between 250–300 g and obtained from Sheffield Field Laboratories. The rats were equipped with chronic indwelling ileal cannulae and housed singly in cages. The animals were deprived of food (Diet 86, Oxoid, London) 18 h before the experiment but water was available *ad libitum*. Animals were allowed at least one week postoperative recovery before any experiments were carried out.

Preparation of the test meal

'Californian' white beans (H.J. Heinz Co. Ltd., Hayes, Middlesex), were washed to remove the tomato sauce and

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homogenised with a little water. Lactose (May & Baker Ltd., Dagenham) was added to produce a concentration of 10% w/v. The radioactive test meal was produced in the same way except 1 mBq ^{99m}Technitium-sulphur colloid (Amersham International PLC, Amersham, Bucks.) was added to the water used to homogenize the beans.

Surgical procedure

A plastic cannula (Silastic ID 0.02 in, OD 0.037 in, Dow Corning Corp, Medical Products, Midland, Michigan, U.S.A.), 25 cm in length was implanted in the ileum of animals, under barbiturate anaesthesia (Sagatal 60 mg kg^{-1}; May & Baker Ltd., Dagenham). The abdomen was opened via a midline incision and the cannula placed in the ileal lumen approximately 20 cm proximal to the ileo-caecal junction. The intestinal wound was closed with a purse-string suture around the cannula, making sure the lumen of the cannula was not occluded. Sufficient cannula was left free in the abdominal cavity to allow the gastrointestinal tract full mobility. The intestinal incision was closed in two separate layers, the muscle and the skin with a sterile braided silk suture 5–0 (Mersilk, Ethicon, Edinburgh). A small square of nylon mesh was secured 2–3 cm for the end of the cannula with silicon glue (Medical adhesive type A, Dow Corning Corp., Medical Products, Midland, Michigan, U.S.A.). The cannula was tunnelled subcutaneously from an abdominal stab wound to the midscapular region where it was exteriorised via a cutaneous puncture wound, the piece of nylon mesh lying under the skin acting as an anchorage point, as the regenerating tissues under the skin formed a platform over the mesh. Each rat was allowed a post-operative recovery period of one week before any experimetal procedures were performed. Every day a small volume of saline (0.3 ml) was infused into the ileum of each rat to ensure the cannula remained patent.

Preparation of test substances

The drugs used in the study were the selective α_1-adrenoceptor antagonist prazosin (0.5 mg kg^{-1}; Sigma Chemical Co., Dorset), the selective α_2-adrenoceptor antagonist, idazoxan (5 mg kg^{-1}; Reckitt & Coleman, Hull) the selective β_1-adrenoceptor antagonist, atenolol (2 mg kg^{-1}; Sigma Chemical Co., Dorset) and the selective β_2 adrenoceptor antagonist ICI 118551 (4 mg kg^{-1}; ICI Pharmaceuticals, Cheshire). All except ICI 118551 were dissolved in saline and injected subcutaneously in a volume of 0.25 ml of saline 30 min before the start of the experiment. ICI 118551 was suspended in 0.5% solution of carboxymethyl cellulose and given to the rats orally by gavage in a 2 ml volume; this compound is more active on the gastrointestinal tract when given orally than when injected subcutaneously (Bilski, ICI, personal communication). The doses of drugs chosen for this study are within the range used for these sub-classes of receptors and have previously been shown to antagonize either gastrointestinal motility patterns or an appropriate dose of an agonist (Bilski *et al.*, 1983; Sagrada *et al.*, 1987; Fioramonti *et al.*, 1989). The triglyceride emulsion, Intralipid (Kabi Vitrum, Bucks.) was used as the stimulus for the ileal brake mechanism.

Experimental protocol

(i) *Environmental hydrogen technique* A total of 18 animals were used for this part of the study. Experiments were performed on batches of 6 animals simultaneously. For each of the drugs used in the study apart from the β_2-antagonist, the following four experiments were conducted on each of the animals: (i) saline injection and ileal saline infusion; (ii) saline injection and ileal Intralipid (20%) infusion; (iii) drug injection and ileal saline infusion and, (iv) drug injection and ileal Intralipid infusion. Four experiments were also carried out to test the β_2-antagonist, ICI 118551, except the vehicle was administered orally (2 ml) with and without the drug.

The effect of each of these combinations (i–iv; Latin square design) on the passage of the head of a bean meal through the stomach and small intestine was investigated with an adaptation of the breath hydrogen technique for use in small mammals (Brown *et al.*, 1987).

After fasting for 18 h, rats were placed in Bollman restraining cages and given either placebo or active drug; 30 min later rats were connected to the infusion pump (Braun, Germany) by a metal connector and plastic tubing. Solutions of lipid or saline were infused into the ileum at a rate of 0.3 ml h^{-1} for 30 min. Then the rats were given 5 ml of the bean/lactose test meal by gavage and placed in the perspex chambers. The perspex chambers provided a controlled environment from which the hydrogen concentrations could be monitored easily and solutions infused into the animals without causing them any disturbance. An explanation of the environmental hydrogen technique is described in detail by Brown *et al.* (1987). The infusion tube was attached to a pulley system allowing the animal free movement within the chamber. The infusion continued for a further 165 min after gavage.

Stomach to caecum transit time (SCTT) of the head of the bean/lactose test meal was defined as the time taken from gavage, to an increase in the hydrogen concentrations in the rats environment of 2 p.p.m. sustained for at least three consecutive readings taken at 10 min intervals (Brown *et al.*, 1987). It was assumed that this occurred when the unabsorbable carbohydrate component of the meal reached the colon and was fermented by the colonic bacteria (Bond & Levitt, 1975; Read *et al.*, 1980).

(ii) *Radiolabelled meal technique* The remaining 94 animals were divided into five groups, control animals (20), those to be given prazosin (19), idazoxan (19) atenolol (20) and ICI 118551 (16). These groups were then further subdivided into 4 subgroups: those to be given drug injection and ileal saline killed at (i) 100 min and (ii) 200 min, and those to be given drug injection and ileal Intralipid killed at (iii) 100 min and (iv) 200 min. Animals were killed by exposure to cyclopropane gas (BOC Gases Ltd.) in a closed chamber. Surgical anaesthesia was rapidly obtained and death occurred within 30 s without causing any obvious signs of trauma to the internal organs (Paton & Payne, 1968; Atkinson & Lee, 1973).

After death, the gastrointestinal tract was ligated at the lower oesophageal sphincter, the pylorus and the ileo-caecal valve, completely removed from the animal and transferred to a longitudinal perspex trough filled with warm saline. Care was taken in handling the intestine to avoid stretching and displacement of luminal contents. The trough was pulled by an electric motor at a rate of 10 cm min^{-1} at a constant depth of 46 mm under a crystal scintillation detector (type DMI-2 nuclear Enterprises Ltd, Edinburgh) fitted with a 6 mm slit collimator and connected to a counter ratemeter (type MS 310E, J & P Engineering Ltd., Reading). The resultant radioactivity profile was displayed on a chart recorder also running at 10 cm min^{-1} and recorded on a computer for further analysis. The remainder of the carcass was monitored with a scintillation meter to ensure all radiation used was contained within the excised gastrointestinal tract.

The radioactivity profiles from the chart recorder were divided into 12 sections. These consisted of the stomach, caecum and 10 equal sections of the small intestine from the pylorus to the ileo-caecal valve. The area under each section was obtained gravimetrically from the computer and expressed as a % of the total radioactivity present. The 'geometric centre' (GC) of the small intestinal distribution of the ^{99m}Tc-sulphur colloid at each time interval for each meal was calculated from the following equation (Miller *et al.*, 1981):

$$GC = \text{sum of (fraction of } ^{99m}\text{Tc/segment} \times \text{segment number)}$$

Table 1 Effect of ileal saline or Intralipid infusions on stomach to caecum transit time (SCTT) of the head of a meal during administration of adrenoceptor antagonists

	Saline infusion		*Intralipid infusion*	
	Saline injection	*Drug injection*	*Saline injection*	*Drug injection*
Drug				
Prazosin (6)	107.0 ± 4.0	88.3 ± 4.8	170.0 ± 5.8	130.0 ± 16.7*
Idazoxan (7)	107.0 ± 4.9	112.9 ± 10.4	168.6 ± 8.3	202.9 ± 10.9*
Atenolol (6)	108.3 ± 7.5	109.2 ± 6.6	181.7 ± 3.1	101.7 ± 9.8*
ICI 118551 (6)	103.3 ± 8.1	117.5 ± 5.3	168.3 ± 7.0	173.3 ± 10.9

Values represent mean ± s.e. for SCTT in min.
Numbers of animals used are in parentheses.
* Represents statistically significant effects of the drug compound with saline administration.

Statistical analysis

The difference in transit times during injection of the different drugs was assessed with the Mann Whitney U test for non-parametric independent data. The results from the radioactive studies were expressed as means and standard error of the means. The degree of statistical significance was assessed by the Mann-Whitney U test for non-parametric data.

Results

Effect of ileal infusion of Intralipid

Ileal infusion of Intralipid significantly delayed the passage of the head of a bean/lactose meal through the stomach and small intestine compared with ileal infusion of saline. Not only was there a delay in the arrival of the head of the meal in the caecum (Intralipid vs saline; mean ± s.e.mean, 168 ± 8 vs 107 ± 4 min, $n = 26$, $P < 0.001$), but there was more of the meal in the stomach and duodenum ($P < 0.01$) and less in the ileum and colon ($P < 0.001$) 200 min after gavage and there was a proximal shift in the distribution of the meal in the small intestine ($P < 0.05$; Table 2). Similar trends in the distribution of the meal were seen at 100 min after gavage.

Effect of α_1-adrenoceptor antagonist

Prazosin had no effect on the SCTT of the head of the meal during ileal infusion of saline (Table 1), although analysis of the distribution of the meal showed that more of the meal was in the stomach at 100 min after gavage (Table 3). The amount of the meal in the colon at this time and the geometric centre in the small intestine were unchanged. There were no changes in the distribution of the meal at 200 min.

Prazosin significantly reversed the delay in SCTT of the head of the meal induced by Intralipid (Table 1), but did not significantly influence the distribution of the meal in the gastrointestinal tract at 100 min or 200 min (Table 3).

Effect of α_2-adrenoceptor antagonist

Idazoxan had no significant effect on SCTT of the head of the meal when saline was infused into the ileum. It did however alter the distribution of the meal with more radioactivity in the stomach at 100 and 200 min after gavage (Table 4), less in the colon at 200 min and a proximal shift in the geometric centre in the small intestine at 200 min (Table 4).

In contrast to prazosin, idazoxan significantly increased the Intralipid induced delay in SCTT of the head of the meal (Table 1). There were however, no significant changes in the distribution of the bulk of the meal in the stomach and small intestine at 100 min and 200 min but there was an increase in the amount of radioactivity in the colon at 200 min (Table 4).

Effect of β_1-adrenoceptor antagonist

During ileal infusion of saline, atenolol did not alter the SCTT of the head of the meal, but it did cause a small but significant

Table 2 Effect of ileal infusion of lipid on the gastrointestinal distribution of a meal, 100 and 200 min after gavage

	Saline infusion	*Lipid infusion*	P *value*
100 min			
Stomach	12.2 ± 1.7	18.6 ± 2.6	<0.05
Duodenum	13.3 ± 1.9	16.7 ± 1.8	
Proximal SI	12.2 ± 0.9	6.8 ± 0.7	<0.001
Mid SI	7.2 ± 0.4	7.0 ± 0.4	
Distal SI	5.2 ± 1.0	7.7 ± 1.2	<0.05
Colon	1.1 ± 0.2	0.0 ± 0.0	<0.001
GC of SI	3.7 ± 0.2	4.0 ± 0.4	
200 min			
Stomach	6.0 ± 0.6	15.0 ± 2.8	
Duodenum	7.3 ± 1.8	15.9 ± 0.4	<0.001
Proximal SI	7.5 ± 0.8	8.2 ± 0.5	<0.05
Mid SI	7.1 ± 0.4	7.9 ± 0.4	
Distal SI	9.9 ± 0.6	6.7 ± 0.4	<0.001
Colon	6.8 ± 0.8	1.4 ± 0.5	<0.001
GC of SI	4.8 ± 0.2	4.1 ± 0.2	<0.05

% distribution of radiation in each region is expressed as the mean ± s.e.mean ($n = 5$).
GC of SI: geometric centre of the small intestine: see text.

Table 3 The effect of the α_1-adrenoceptor antagonist, prazosin, on gastrointestinal distribution of a meal during ileal infusion of saline and lipid

	Saline infusion		*Lipid infusion*	
	Saline injection	*Prazosin injection*	*Saline injection*	*Prazosin injection*
100 min				
Stomach	12.2 ± 1.7	28.1 ± 7.0**	18.6 ± 2.6	19.0 ± 4.0
Colon	1.1 ± 0.2	1.0 ± 0.6	0.0 ± 0.0	0.0 ± 0.0
GC of SI	3.7 ± 0.2	3.6 ± 0.5	4.0 ± 0.2	3.8 ± 0.4
200 min				
Stomach	6.0 ± 0.6	7.0 ± 0.9	15.0 ± 2.8	19.1 ± 3.5
Colon	6.8 ± 0.8	6.5 ± 0.3	1.4 ± 0.5	1.2 ± 0.2
GC of SI	4.8 ± 0.2	4.7 ± 0.2	4.1 ± 0.2	3.7 ± 0.3

Mean ± s.e.mean % radiation in stomach and colon.
% distribution of radiation in each region is expressed as the mean ± s.e.mean ($n = 5$).
GC of SI: geometric centre of small intestine, see text.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; statistical significances using Mann Whitney U test for non-parametric data.

Table 4 The effect of the α_2-adrenoceptor antagonist, idazoxan, on gastrointestinal distribution of a meal during ileal infusion of saline and lipid

	Saline infusion		*Lipid infusion*	
	Saline injection	*Idazoxan injection*	*Saline injection*	*Idazoxan injection*
100 min				
Stomach	12.2 ± 1.7	21.9 ± 2.9*	18.6 ± 2.6	17.5 ± 2.0
Colon	1.1 ± 0.2	1.8 ± 0.6	0.0 ± 0.0	0.0 ± 0.0
GC of SI	3.7 ± 0.2	3.6 ± 0.2	4.0 ± 0.4	3.7 ± 0.09
200 min				
Stomach	6.0 ± 0.6	15.5 ± 2.5**	15.0 ± 2.8	12.3 ± 1.6
Colon	6.8 ± 0.8	12.8 ± 3.1*	1.4 ± 0.5	5.1 ± 1.5*
GC of SI	4.8 ± 0.2	3.8 ± 0.2**	4.1 ± 0.2	4.4 ± 0.2

% distribution of radiation in the stomach and small intestine is expressed as the mean ± s.e.mean ($n = 5$).
GC of SI: geometric centre of small intestine.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; statistical significances using Mann Whitney U test for non-parametric data.

Table 5 The effect of a β_1-adrenoceptor antagonist, atenolol, on gastrointestinal distribution of a meal during ileal infusion of saline and lipid

	Saline infusion		*Lipid infusion*	
	Saline injection	*Atenolol injection*	*Saline injection*	*Atenolol injection*
100 min				
Stomach	12.2 ± 1.7	15.7 ± 3.5	18.6 ± 2.6	17.7 ± 1.4
Colon	1.1 ± 0.2	2.6 ± 0.8***	0.0 ± 0.0	1.5 ± 0.2**
GC of SI	3.7 ± 0.2	4.0 ± 0.2	4.0 ± 0.4	3.7 ± 0.1
200 min				
Stomach	6.0 ± 0.6	5.3 ± 0.7	15.0 ± 2.8	13.6 ± 2.3
Colon	6.8 ± 0.8	8.0 ± 1.1	1.4 ± 0.5	2.4 ± 0.4
GC of SI	4.8 ± 0.2	5.0 ± 0.3	4.1 ± 0.2	3.8 ± 0.2

% distribution of radiation in the stomach and small intestine is expressed as the mean ± s.e.mean ($n = 5$).
GC of SI: geometric centre of small intestine.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; statistical significances using Mann Whitney U test for non-parametric data.

Table 6 The effect of the α_2-adrenoceptor antagonist ICI 118551, on gastrointestinal distribution of a meal during ileal infusion of saline and lipid

	Saline infusion		*Lipid infusion*	
	Saline injection	*ICI 118551 injection*	*Saline injection*	*ICI 118551 injection*
100 min				
Stomach	12.2 ± 1.7	11.1 ± 1.2	18.6 ± 2.6	15.7 ± 3.2
Colon	1.1 ± 0.2	0.9 ± 0.1	0.0 ± 0.0	1.1 ± 0.3
GC of SI	3.7 ± 0.2	3.9 ± 0.2	4.0 ± 0.4	4.1 ± 0.2
200 min				
Stomach	6.0 ± 0.6	7.1 ± 0.8	15.0 ± 2.8	15.6 ± 3.5
Colon	6.8 ± 0.8	7.3 ± 0.3	1.4 ± 0.5	1.6 ± 0.4
GC of SI	4.8 ± 0.2	4.9 ± 0.2	4.1 ± 0.2	3.7 ± 0.1

% distribution of radiation in the stomach and small intestine is expressed as the mean ± s.e.mean ($n = 5$).
GC of SI: geometric centre of small intestine.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; statistical significances using Mann Whitney U test for non-parametric data.

increase in the proportion of the meal in the colon at 100 min after gavage (Table 5).

Atenolol reversed the delay in SCTT of the head of the meal induced by Intralipid, and this was associated with the presence of some of the meal in the colon at 100 min after gavage ($P < 0.01$). There were no other significant changes in the distribution of the meal.

Effect of β_2-adrenoceptor antagonist

ICI 118551, did not alter the SCTT of the head of the meal or the meal distribution either during ileal infusion of saline or during ileal infusion of Intralipid (Tables 1 and 6).

Discussion and conclusions

The results of environmental hydrogen analysis suggest that the sympathetic nervous system either mediates or modulates the ileal brake mechanism in the rat. Selective α_1- and β_1-adrenoceptor blockers both reverse the lipid-induced delay in the arrival of the head of the meal in the caecum, while the α_2-adrenoceptor inhibitor potentiates the delay, an effect compatible with an action of presynaptic α_2-adrenoceptors. None of the antagonists altered the stomach to caecum transit time of the head of the test meal during ileal infusion of saline. These data are compatible with the possibility that ileal infusion of lipid delays gastrointestinal transit via the peripheral sympathetic ganglia.

The time at which the head of a meal, introduced into the stomach, arrives at the caecum does not necessarily indicate the transit of the bulk of the meal through the stomach and small intestine. This measurement could conceal important effects on gastric or intestinal motility or, even worse, give a misleading impression of major effects when none exist. To investigate these possibilities, we carried out a parallel series of experiments to measure the gastrointestinal distribution of a similar radiolabelled test meal at 100 and 200 min after gavage. Although the measurements were quite different, it is unlikely that methodological differences caused the meal to be handled differently in each experiment. The composition of the meals used in each experiment were identical except for the radiolabel, solutions were introduced into the ileum and drugs administered in exactly the same dose and route. Furthermore we have previously shown that the method of killing the animals to estimate distribution does not induce movement of gastrointestinal contents (Brown, 1988). Thus the results from the two methods should be directly comparable and the combined data should therefore provide a more complete insight into the effect of nutrients and drugs on the movement of a meal through the stomach and smalll intestine.

Although none of the drugs affected SCTT of the head of the meal during control infusion of saline into the ileum, both α_1- and α_2-receptor antagonists delayed gastric emptying, suggesting a basal or meal-induced sympathetic tone acting on α-adrenoceptors in the stomach to enhance gastric emptying. Idazoxan also caused a proximal shift of the meal in the small intestine suggesting a tonic sympathetic influence on α_2-adrenoceptors to accelerate small bowel transit. In addition the β_1-antagonist, atenolol, significantly increased the proportion of the meal in the caecum at 100 min after gavage.

These results suggest that under some circumstances, the head of a meal may be processed by the gut in a quite different manner from the bulk of the meal. A small portion of any meal usually empties from the stomach almost as soon as it is ingested (Jian *et al.*, 1984) and may pass fairly quickly down the small intestine, stimluating the nutrient receptors that then control the gastric emptying, digestion and small bowel transit of the rest of the meal. Thus, it is quite possible for a drug to delay gastric emptying and slow the passage of the bulk of the meal through the small intestine but to have no effect on the passage of the first portion of the meal.

The data from this study confirm the action of Intralipid on the stomach to caecum transit time of the head of the meal and show that this is associated with delays in gastric emptying and small bowel transit. The effects of the β-adrenoceptor antagonists on SCTT of the head of the meal and distribution of the meal complement each other. The β_2-antagonist has no effect on either meal transit or meal distribution while the acceleration in the arrival of the head of the meal at the caecum induced by the β_1-antagonist, atenolol, is associated with the presence of radioactivity in the caecum at 100 min after gavage. It is in the effects of the α-antagonists that the data from the two sets of experiments seem at variance. The reversal in the lipid-induced delay in SCTT of the head of the meal is not associated with any change in meal distribution apart from a small but non significant proximal shift in the geometric centre of the meal in the small intestine. Similarly the potentiation of the ileal brake effect on the head of the meal is not associated with any corresponding change in the distribution of the meal. In fact, there is more radioactivity in the colon at 200 min after gavage and not less.

In conclusion, our data suggest that the sympathetic nervous system normally modulates or mediates the mechanisms that influence gastrointestinal transit in the rat and may be involved in the ileal brake effect. The results from both the environmental hydrogen analysis and the meal distribution indicate that the sympathetic nervous system maintains a basal tone during control SCTT and that the delay in both gastric emptying and SCTT may be mediated at least in part through this branch of the autonomic nervous system. The catecholamines adrenaline and noradrenaline have different affinities for the subtypes of adrenoceptors: α_1- and β_1-receptors preferentially responding to noradrenaline (Lands *et al.*, 1967) whereas α_2- and β_2-receptors preferentially respond to adrenaline (Ariens & Simons, 1983). In our studies, blockade of α_1- and β_1-receptors results in inhibition of the ileal brake, whereas the α_2 blockade enhances the effects of ileal lipid infusion, when analysing the head of the meal data. This may suggest that the ileal brake is mediated through the release of endogenous noradrenaline, since blockade with the selective antagonists abolishes the effects of ileal lipid. They nevertheless indicate that simple measurement of the transit of the head of the meal by use of environmental hydrogen analysis may under some circumstances, give a misleading impression of the action of drugs on gastrointestinal transit of the bulk of a test meal.

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Platelet-activating factor (PAF) plays an important role in the immediate asthmatic response in guinea-pig by augmenting the response to histamine

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1 To investigate the role of platelet activating factor (PAF) in the immediate asthmatic response, we examined the bronchial reactivity to histamine after administration of PAF to guinea-pigs or antigen challenge to passively sensitized guinea-pigs.

2 A bolus injection of PAF (20–40 ng kg^{-1}), which did not cause a significant increase in intrathoracic pressure (ITP), augmented the bronchial response to histamine almost 8 fold. This airway hyperreactivity was observed even 1 min after PAF treatment.

3 A subthreshold dose of antigen (0.01 mg kg^{-1}, i.v.) also provoked hyperreactivity to histamine, which became significant 6 and 11 min after the antigen treatment.

4 The specific PAF-antagonists, SM-10661 and CV-6209 (i.v.) dose-dependently inhibited both PAF- and antigen-induced airway hyperreactivities to histamine.

5 These results suggest that PAF plays an important role in antigen-induced acute airway responses by augmenting the activities of spasmogens.

Keywords: PAF; SM-10661; immediate asthmatic response in guinea-pig

Introduction

Platelet activating factor (PAF) is a phospholipid mediator released from various inflammatory cells and has been proposed as a mediator of asthma because of its capacity to mimic a range of asthmatic symptoms (Morley *et al.*, 1984). Sensitized guinea-pig lung releases PAF and its inactive metabolite lyso-PAF (Fitzgerald *et al.*, 1986). PAF can therefore be generated in sensitized guinea-pig lung in response to antigen challenge. In guinea-pigs, PAF induces acute bronchospasm (Vargaftig *et al.*, 1980) and causes intrathoracic accumulation of neutrophils and platelets (Dewar *et al.*, 1984).

Airway hyperreactivity is one of the characteristics of asthma which becomes more pronounced following inhalation of allergen by atopic subjects (Cockcroft *et al.*, 1977). Mazzoni *et al.* (1985) reported that airway responsiveness to bombesin increased after a 1 h infusion of PAF (600 ng kg^{-1} h^{-1}) in guinea-pigs. It was revealed that inhalation of PAF caused sustained airway hyperreactivity to methacholine or histamine in man and guinea-pigs (Cuss *et al.*, 1986; Coyle *et al.*, 1988). Allergen-induced hyperresponsiveness 24 h after allergen inhalation in an allergic rabbit was modified by the PAF-antagonist, BN 52021 (Coyle *et al.*, 1989). These results indicate that PAF is involved in airway hyperreactivity. It has been presumed that airway hyperreactivity is a consequence of inflammatory events. It is thought that obstruction of the airway lumen by activation of various leucocytes, mucosal swelling and impaired mucus clearance play important roles in the occurrence of airway hyperreactivity (Cockcroft, 1983). However, physical obstruction due to inflammatory events in the airways needs several hours or days to become evident.

Recently, we observed that the selective PAF receptor antagonist, SM-10661 ((±)-*cis*-3,5-dimethyl-2-(3-pyridyl)-thiazolidin-4-one hydrochloride) inhibited not only PAF-induced increases in intrathoracic pressure (ITP) in anaesthetized guinea-pigs, but also rapid ITP increases by antigen challenge to passively sensitized guinea-pigs, although it had no effect on histamine-induced increase in ITP (Uchida *et al.*, 1989). These results led us to speculate the existence of interaction between PAF and histamine.

In this study, we investigate whether PAF-induced hyperreactivity to histamine can occur within several minutes and examine the role of this response in the immediate anaphylactic bronchospasm.

Methods

Animals

Male Dunkin-Hartley guinea-pigs (Japan SLC, Inc.) weighing 430–500 g were used throughout this study. The animals were sensitized by intravenous injection of 0.85 mg kg^{-1} rabbit anti-egg albumin anti-serum (Seikagaku kogyo Co., Ltd., Japan) 18–24 h before antigen challenge.

Measurement of airway responses

Prior to the operation, the animals were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.p.) and sodium phenobarbitone (120 mg kg^{-1}, i.p.) and the trachea, carotid artery and jugular vein were cannulated for measurement of airway obstruction, systemic blood pressure and for the introduction of drugs respectively. Gallamine trietide (10 mg kg^{-1}, i.m.) was injected to prevent spontaneous breathing. The guinea-pigs were ventilated with a rodent ventilator (Harvard Inc.) with room air supplemented with oxygen at 60 strokes min^{-1} with a stroke volume of 3–7 ml. The body temperature was maintained at 37°C with a heating pad throughout the experiment. Airway obstruction was measured according to the method of Konzett & Rössler (1940) using a differential pressure transducer (Ugo Basile) connected by the side-arm of the tracheal cannula to a bronchospasm transducer and expressed as ITP in cmH_2O. The intratracheal cannula was clipped before and after the experiment to obtain the maximal response.

Airway hyperreactivity induced by PAF

Histamine (3 μg kg^{-1}), PAF (20 ng kg^{-1}) or leukotriene D_4 (0.07 nmol kg^{-1}) was injected via the jugular vein and the ITP was measured as a prechallenge value. Then PAF (10–40 ng kg^{-1}, i.v.) was injected 15 min later. The histamine (3 μg kg^{-1}, i.v.), PAF (20 ng kg^{-1}) or leukotriene D_4

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($0.07\,nmol\,kg^{-1}$) were injected 1, 6 and 11 min after the PAF administration. For the measurement of the duration of the PAF effect, histamine was administered at 31 and 61 min after PAF injection. For the control, saline containing 0.25% bovine serum albumin (BSA) was used instead of PAF. The phasic blood pressure and heart rate were continuously monitored via a heparinized arterial cannula connected to a pressure transducer and a recorder, respectively.

Airway hyperreactivity induced by antigen

Fifteen minutes after measurement of the prechallenge value by histamine as stated above in passively sensitized guinea-pigs, the animals were challenged intravenously with antigen (ovalbumin, $0.01\,mg\,kg^{-1}$). Histamine reactivity was measured 1, 6, 11, 31 and 61 min after antigen administration. For the control, saline was used instead of ovalbumin.

Effect of PAF-antagonists on the hyperreactivity induced by PAF or antigen

SM-10661 or CV-6209 was injected into the jugular vein 2 min before PAF or antigen administration. Histamine reactivity was estimated 6 min after PAF or antigen administration and % inhibition was calculated according to the equation shown below.

Increase in ITP induced by histamine, PAF, leukotriene D_4 or antigen

Normal or passively sensitized, anaesthetized guinea-pigs were injected intravenously with histamine, PAF, leukotriene D_4 or ovalbumin. The ITP peak value was measured as the % increase in ITP.

Effect of PAF or antigen on circulating cell count

Blood samples ($20\,\mu l$) of normal or passively sensitized, anaesthetized guinea-pigs were drawn from cannula in the jugular vein before and after intravenous administration of PAF ($40\,ng\,kg^{-1}$) or ovalbumin ($0.01\,mg\,kg^{-1}$). After appropriate dilution, platelet and white blood cell counts were measured by Sysmex Microcell Counter F-800 (Toha Iyoh Denshi, Japan).

Materials

Histamine dihydrochloride (Nakalai Tesque Japan Inc.), leukotriene D_4 (Peptide Institute, Inc., Japan), sodium pentobarbitone (Pittmanmoor, U.S.A.), sodium phenobarbitone (Wako Junyaku, Japan), gallamine trietide and fatty acid-free bovine serum albumin (Sigma) were dissolved in physiological saline. Alkyl-PAF (mixture of C_{16} and C_{18}, Funakoshi, Japan) was dissolved in 0.25% BSA saline buffer. SM-10661 ((±)-cis-3,5-dimethyl-2-(3-pyridyl)thiazolidin-4-one hydrochloride) was synthesized in the synthetic division of Sumitomo Pharmaceuticals Co., Ltd. CV-6209 (2-[N-acetyl-N-(2-methoxy-3-octadecylcarbamoyloxypropoxycarbonyl) ammomethyl]-1-ethylpyridinium chloride) was a gift from Takeda Chemical Industries. SM-10661 was dissolved in saline and the pH was adjusted to between 6 and 7 with 1 N sodium hydroxide. CV-6209 was dissolved in 0.25% BSA-saline.

Statistical analysis

Data are presented as the mean ± s.e. of n observations. The % increase in ITP, % augmentation and % inhibition by the drugs were calculated according to the following formulae.

$$\%\ \text{increase in ITP} = \left[\frac{\text{airway response by histamine (cm)} - \text{baseline value (cm)}}{\text{occlusion value (cm)} - \text{baseline value (cm)}}\right] \times 100$$

$$\%\ \text{augmentation} = \left[\frac{\%\ \text{increase in ITP to histamine after PAF or antigen administration}}{\%\ \text{increase in ITP to histamine before PAF or antigen administration}} - 1\right] \times 100$$

$$\%\ \text{inhibition} = \left[1 - \frac{\text{airway response by histamine under treatment with drug (cm)} - \text{baseline value (cm)}}{\text{airway response by histamine under treatment with vehicle (cm)} - \text{baseline value (cm)}}\right] \times 100$$

Significance was assessed by Dunnett's test and a P value of less than 0.05 was considered significant.

Results

Airway hyperreactivity to histamine induced by PAF

Administration of histamine caused a dose-dependent increase in ITP as shown in Figure 1. The ITP was elevated about 1.3 ± 0.3, 4.3 ± 1.1 or 80.4 ± 3.2% above the baseline at doses of 1, 3 or $10\,\mu g\,kg^{-1}$ respectively. Histamine, $3\,\mu g\,kg^{-1}$ caused a small but significant change in ITP and returned to the initial level within 1 min. This response was reproducible by repeated administration from −15 to 31 min, and the response was small but significantly increased at 61 min (Figure 2a). Intravenous administration of PAF also caused a dose-dependent increase in ITP and the ED_{50} value was $86.5\,ng\,kg^{-1}$ (Figure 3a). At a dose of less than $50\,ng\,kg^{-1}$, the ITP returned to the baseline level within 1 min. However, at more than $50\,ng\,kg^{-1}$, the increase in ITP was observed for over 1 min. After the administration of $40\,ng\,kg^{-1}$ of PAF, which by itself caused a detectable but insignificant increase in

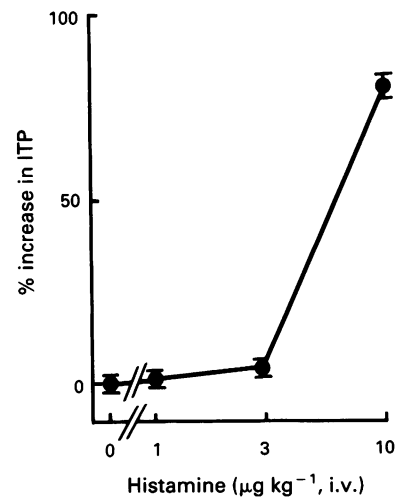

Figure 1 Dose-dependent increase in intrathoracic pressure (ITP) in response to histamine administration in anaesthetized guinea-pigs. ITP was measured by the Konzett-Rössler method. Each value represents the mean of $n = 4$; s.e.mean shown by vertical bars.

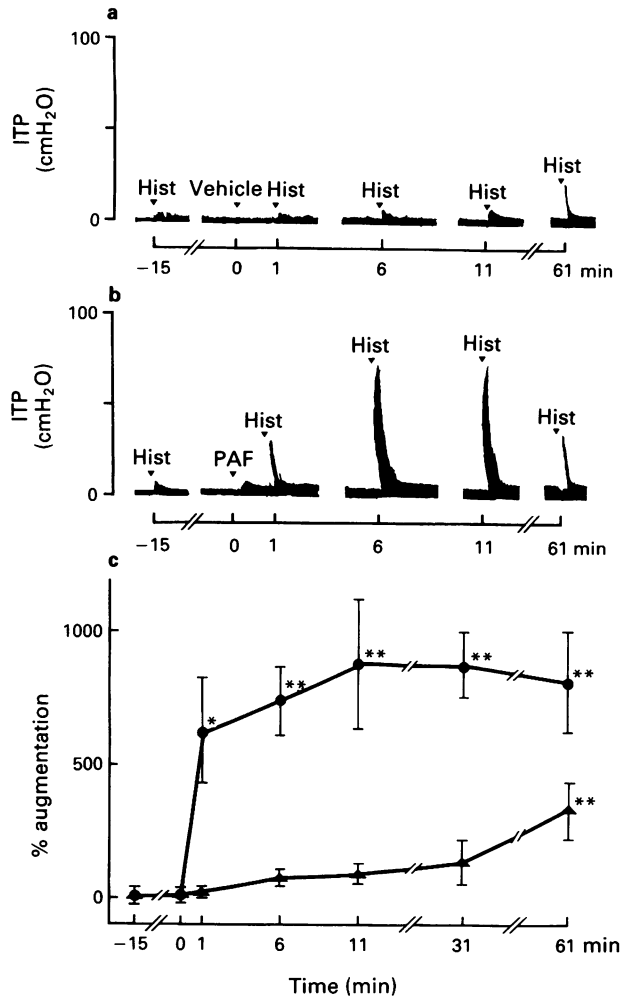

Figure 2 Time course of increase in intrathoracic pressure (ITP) in response to histamine before and after PAF treatment: (a) and (b) are representative traces of histamine (Hist) ($3\,\mu g\,kg^{-1}$, i.v.) responsiveness before and after a bolus injection of 0.25% BSA-saline or $40\,ng\,kg^{-1}$ of PAF to anaesthetized guinea-pigs, respectively. Control value of % increase in ITP in response to histamine was 3.8 ± 0.5 and % increase in ITP to PAF was 7.8 ± 1.5 ($n = 9$). (c) Represents the time course of the % increase in ITP in response to histamine ($3\,\mu g\,kg^{-1}$, i.v.) after intravenous (●) PAF ($40\,ng\,kg^{-1}$, $n = 9$) or (▲) 0.25% BSA-saline ($n = 5$) administration. Each value is expressed as % augmentation according to Methods and represents the mean with s.e. shown by vertical bars. Statistical significance; $^*P < 0.05$; $^{**}P < 0.01$. Dunnett's test compared with the control value.

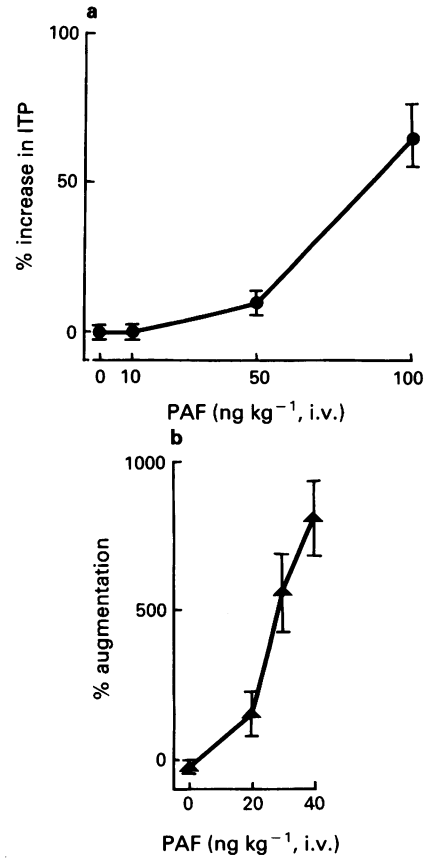

Figure 3 Dose-response of (a) PAF-induced increase in intrathoracic pressure (ITP), ($n = 7$–8) and (b) increase in ITP in response to $3\,\mu g\,kg^{-1}$ of histamine 6 min after PAF administration ($n = 4$–6) in guinea-pigs. Each value represents the mean with s.e. shown by vertical bars.

ITP, the response to histamine ($3\,\mu g\,kg^{-1}$) was augmented significantly. The % augmentation at 1, 6, 11, 31 and 61 min was 620 ± 200, 745 ± 125, 880 ± 247, 875 ± 125 and 810 ± 191 respectively (Figure 2b and 2c). Smaller doses of PAF (20 and $30\,ng\,kg^{-1}$) resulted in similar but smaller responses than that of $40\,ng\,kg^{-1}$ of PAF. At a dose of $30\,ng\,kg^{-1}$, the responses at 6 and 11 min were significant and % augmentation was 565 ± 122 and 523 ± 103 respectively. At $20\,ng\,kg^{-1}$ of PAF, the responses increased by 153 ± 101 and 246 ± 171% at 6 and 11 min, both of which were not significant. Thus these responses were induced dose-dependently at doses between 20 and $40\,ng\,kg^{-1}$ of PAF. The dose-response curve at 6 min is shown in Figure 3b.

Smaller doses of histamine induced smaller increases in ITP after PAF treatment and when larger doses of histamine were used, it was impossible to obtain the % augmentation because the increase in ITP induced by histamine alone exceeds 100%. We therefore used a combination of $40\,ng\,kg^{-1}$ of PAF and $3\,\mu g\,kg^{-1}$ of histamine throughout the experiment.

Effects of PAF-antagonists on PAF-induced airway hyperreactivity

The effects of two structurally unrelated PAF-antagonists were examined on PAF-induced hyperreactivity to histamine. Both PAF-antagonists did not affect the baseline level or influence the response of histamine (data not shown). When SM-10661 was administered intravenously 2 min before PAF, the hyperreactivity to histamine was inhibited dose-dependently. The ED_{50} value of SM-10661 at 6 min was $2.7\,mg\,kg^{-1}$ (Figure 4a). Another PAF-antagonist, CV-6209, also dose-dependently inhibited the PAF-induced hyperreactivity to histamine with an ED_{50} value of $0.002\,mg\,kg^{-1}$ (Figure 4b).

Airway hyperreactivity to leukotriene D_4 or PAF induced by PAF

Intravenous administration of leukotriene D_4 to anaesthetized normal guinea-pigs caused a dose-dependent increase in ITP (Figure 5a). Administration of leukotriene D_4 at a dose of $0.07\,nmol\,kg^{-1}$ caused a small but significant increase in ITP which returned to initial levels within 1 min. After treatment with $40\,ng\,kg^{-1}$ of PAF, the % augmentation of ITP in response to the same dose of leukotriene D_4 was 193 ± 56, 3987 ± 1246 and 4698 ± 701 at 1, 6 and 11 min respectively, and the values at 6 and 11 min were significant (Figure 5b). Similarly, the responses to $20\,ng\,kg^{-1}$ of PAF itself, which did not cause significant increase in ITP, were also markedly increased and the % augmentation was 1244 ± 697, 3316 ± 860 and 3711 ± 960 at 1, 6 and 11 min respectively

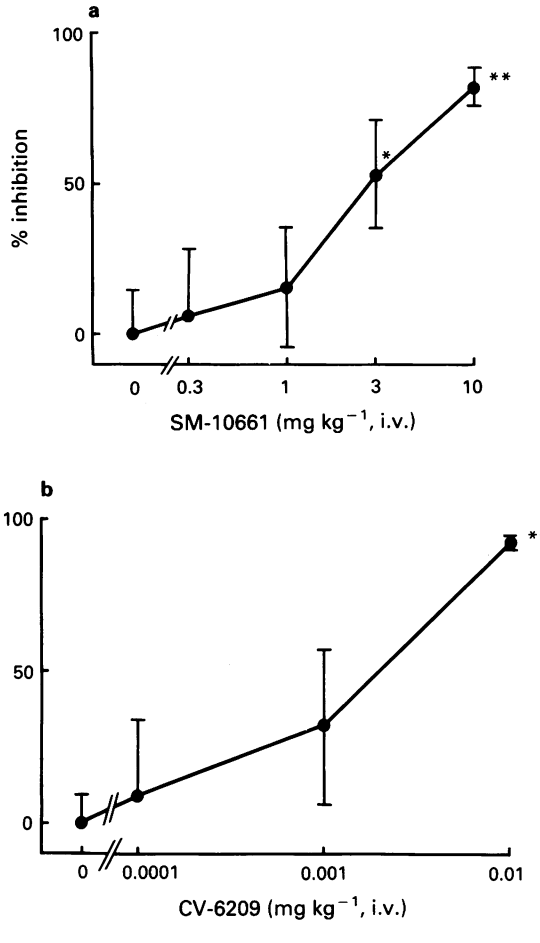

Figure 4 Inhibitory effects of PAF-antagonists on PAF (40 ng kg^{-1}, i.v.)-induced hyperreactivity to histamine in anaesthetized guinea-pigs at 6 min. PAF-antagonists were administered intravenously 2 min before PAF injection. The responses to histamine were dose-dependently inhibited by pretreatment with (a) SM-10661 (n = 8–12; control, n = 14) or (b) CV-6209 (n = 5–7; control, n = 10). Each value represents the mean with s.e. shown by vertical bars. Statistical significance; $^*P < 0.05$; $^{**}P < 0.01$. Dunnett's test compared with the vehicle-treated group.

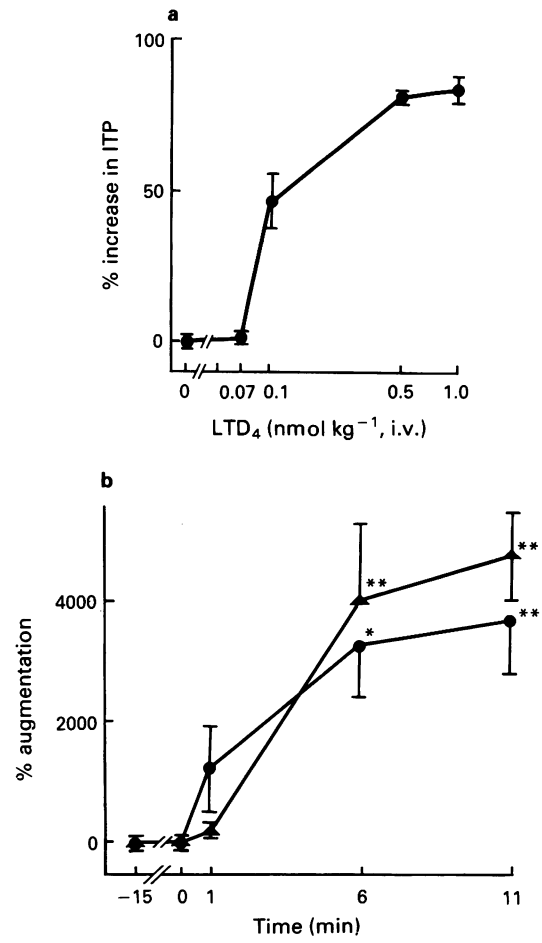

Figure 5 (a) Dose-response to leukotriene D_4 (LTD_4)-induced increase in intrathoracic pressure (ITP) in anaesthetized guinea-pigs. (b) Time course of airway hyperreactivity to leukotriene D_4 (▲) or PAF (●), induced by PAF-treatment. Leukotriene D_4 (0.07 nmol kg^{-1}) or PAF (20 ng kg^{-1}) were injected 15 min before and 1, 6 and 11 min after PAF (40 ng kg^{-1}) was administered to anaesthetized guinea-pigs. The prevalue of the % increase in ITP induced by leukotriene D_4 or PAF was 1.4 ± 0.3 or 1.0 ± 0.02, respectively. Each value represents the mean (with s.e. shown by vertical bars) of n = 5. Statistical significance; $^*P < 0.05$; $^{**}P < 0.01$. Dunnett's test compared with the prevalue.

after PAF treatment as shown in Figure 5b. Larger doses of PAF provoked more than 100% increase in ITP as was seen with a large dose of histamine.

Airway hyperreactivity to histamine induced by ovalbumin in passively sensitized guinea-pigs

Figure 6a shows the dose-response curve of the increase in ITP by antigen administration. Ovalbumin-induced increases in ITP were apparent at doses over 0.02 mg kg^{-1}, i.v., in passively sensitized guinea-pigs. The degree of response to histamine (3 μg kg^{-1}) was the same as in nonsensitized guinea-pigs (Figure 7a). Administration of 0.01 mg kg^{-1} of antigen to passively sensitized guinea-pigs caused no marked increase in ITP (1.6 ± 0.3%, n = 4) and the baseline values did not change (Figure 7b). When histamine was administered 1 min after 0.01 mg kg^{-1} of antigen, the response was almost the same as the pre-challenge value. The increases in ITP caused by histamine returned to the baseline level within 1 min. However, the responses to histamine at 6, 11, 31 and 61 min after antigen treatment were far greater than the response before antigen administration. The % augmentation of ITP at 6, 11, 31 and 61 min was 1811 ± 418, 1045 ± 226, 1333 ± 478 and 1913 ± 554, respectively (Figure 7b and c). The dose-response curve of % augmentation to antigen at 6 min is shown in Figure 6b.

Effects of PAF-antagonists on airway hyperreactivity to histamine induced by ovalbumin administration in passively sensitized guinea-pigs

When SM-10661 (i.v.) was administered 2 min before antigen treatment, the hyperreactivity to histamine was inhibited dose-dependently (Figure 8a). The ED_{50} value at 6 min was 23.0 mg kg^{-1}. Another PAF-antagonist, CV-6209, also inhibited the response dose-dependently with an ED_{50} of 1.4 mg kg^{-1} (Figure 8b).

Effect of PAF or antigen on circulating cell count

The circulating platelet and white cell counts in normal guinea-pigs 15 min before PAF administration were (42.6 ± 1.7) × 10^4 μl^{-1} and (33.6 ± 5.9) × 10^2 μl^{-1}, respectively. One minute after PAF injection, platelet and white blood cell counts decreased to (25.8 ± 3.1) × 10^4 μl^{-1} and (23.2 ± 3.5) × 10^2 μl^{-1}, respectively. The decrease of platelet count is statistically significant ($P < 0.01$, n = 5). Both platelet and white blood cell counts gradually recovered with time. In passively sensitized guinea-pigs, circulating platelet and white

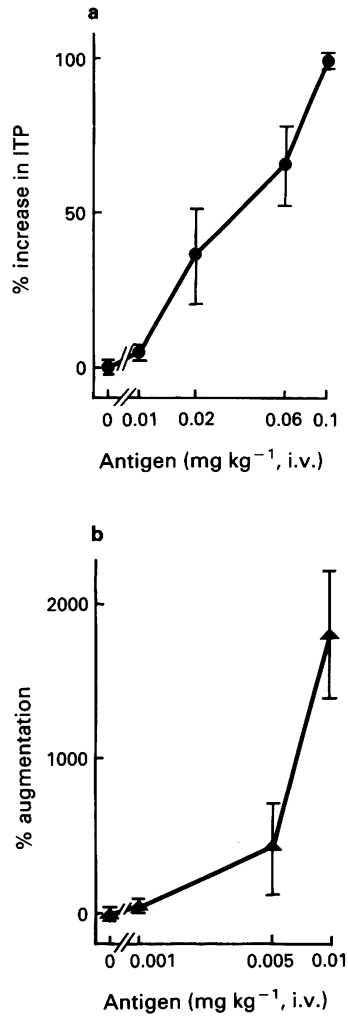

Figure 6 Dose-response to (a) ovalbumin-induced increase in intrathoracic pressure (ITP) 4 min after ovalbumin challenge ($n = 4$–6) and (b) increase in ITP in response to histamine (3 μg kg^{-1}, i.v.) 6 min after ovalbumin administration ($n = 4$–6) in passively sensitized anaesthetized guinea-pigs. Each value represents the mean with s.e. shown by vertical bars.

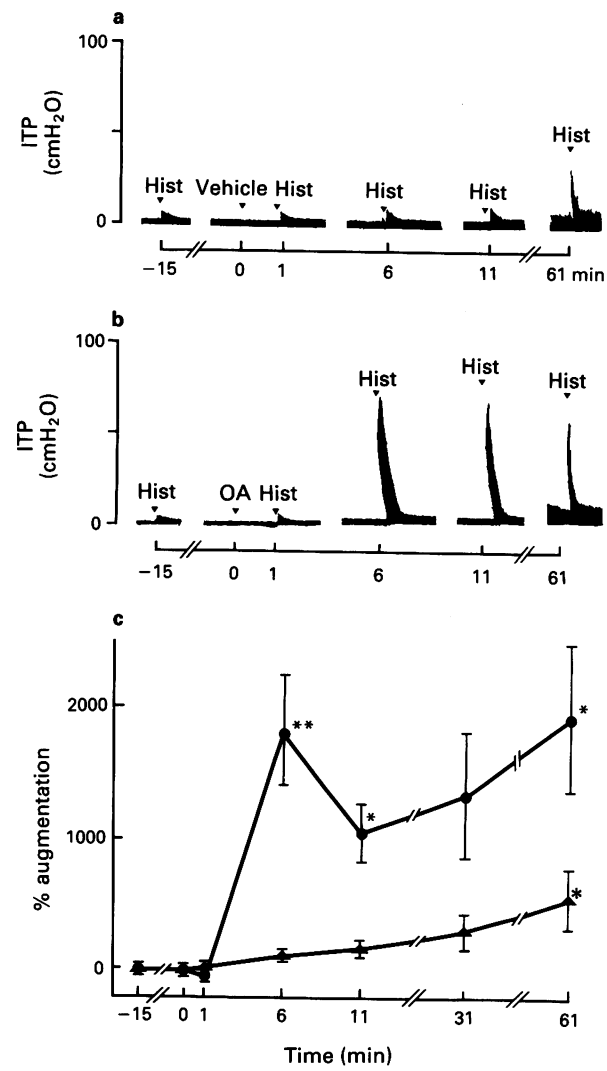

Figure 7 Time course of the increase in intrathoracic pressure (ITP) in response to histamine before and after ovalbumin treatment: (a) and (b) are representative traces of histamine (3 μg kg^{-1}, i.v.) responsiveness before and after a bolus injection of saline or 0.01 mg kg^{-1} of ovalbumin to passively sensitized, anaesthetized guinea-pigs, respectively. The prevalue of the % increase in ITP in response to histamine was 3.3 ± 0.6 and the % increase in ITP to antigen was 0.4 ± 0.2 ($n = 9$). (c) Represents the time course of the % augmentation of ITP in response to histamine (3 μg kg^{-1}, i.v.) after intravenous (●) ovalbumin (0.01 mg kg^{-1}, $n = 9$) or (▲) saline ($n = 8$) administration. Each value is expressed as % augmentation according to Methods and represents the mean with s.e. shown by vertical bars. Statistical significance; $^*P < 0.05$, $^{**}P < 0.01$. Dunnett's test compared with the prevalue.

blood cell counts 15 min before antigen administration were (45.2 ± 2.8) × 10^4 μl^{-1} and (39.3 ± 4.2) × 10^2 μl^{-1}, respectively. One minute after antigen administration, these cell counts did not change. However, at 6 min after antigen treatment, platelet and white blood cell counts decreased to (12.0 ± 1.2) × 10^4 μl^{-1} ($P < 0.01$, $n = 4$) and (23.0 ± 2.6) × 10^2 μl^{-1} ($P < 0.05$, $n = 4$), respectively. Both cells gradually recovered with time.

Discussion

We examined airway reactivity to histamine after PAF or antigen treatment to investigate the role of PAF in antigen-induced bronchial responses in passively sensitized guinea-pigs.

We showed that PAF, at doses of above 50 ng kg^{-1}, induced an increase in ITP in guinea-pigs. This result is almost comparable with the data reported by Vargaftig *et al.* (1980). Because of this PAF-induced increase in ITP, PAF has been considered a mediator of immediate asthmatic response.

We observed that a small dose of PAF, which provokes marginal increases in ITP, induced airway hyperreactivity to histamine, leukotriene D_4 and PAF within 1 min. These results suggest that PAF induces a nonspecific airway hyperreactivity in nonsensitized guinea-pigs. Anderson *et al.* (1988) reported that similar airway hyperreactivity to histamine was induced by 20 ng kg^{-1} of PAF in guinea-pigs. Our data were correlated with the potentiation rate at this dose, but we showed that larger doses of PAF provoked more marked increases in ITP in response to spasmogens. In addition, we observed that PAF-induced hyperreactivity to histamine lasted for as long as 61 min. In man, such a lasting airway hyperreactivity is likely to augment the responses to subsequent antigen stimulation in asthmatic patients.

Mazzoni *et al.* (1985) found that continuous administration of PAF (600 ng kg^{-1} h^{-1}) induced airway hyperreactivity to bombesin. We have not yet examined the potentiation of the response to bombesin, but we noted that a bolus injection of PAF was sufficient to induce hyperreactivity to histamine. Furthermore, we showed that a lower dose of PAF was sufficient to induce hyperreactivity.

The specific PAF antagonist, SM-10661 (Komuro *et al.*, 1990), administered 2 min before PAF injection dose-

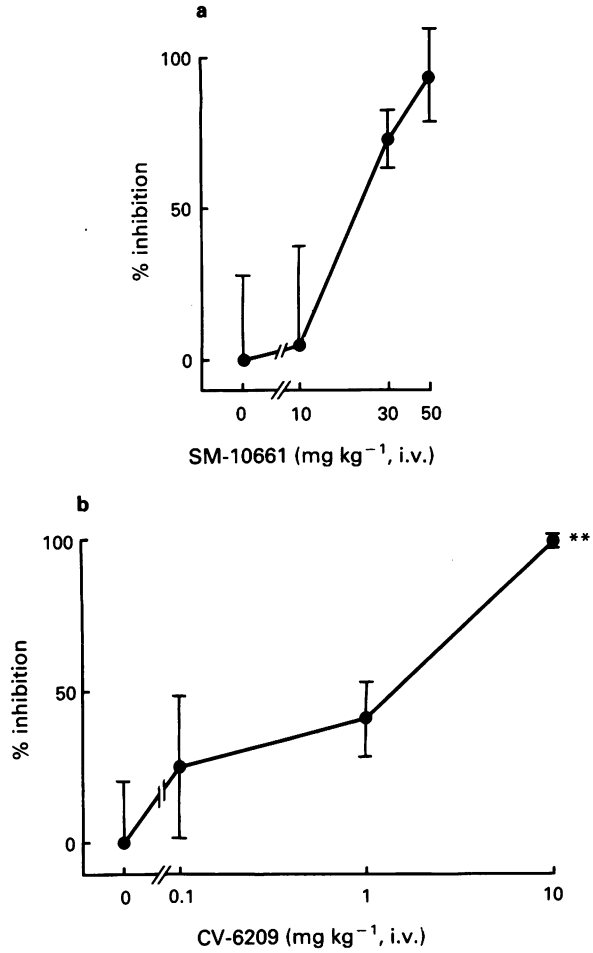

Figure 8 Inhibitory effects of PAF-antagonists on ovalbumin (0.01 mg kg^{-1}, i.v.)-induced hyperreactivity to histamine in passively sensitized, anaesthetized guinea-pigs at 6 min. PAF antagonists were administered intravenously 2 min before ovalbumin injection. The responses to histamine were dose-dependently inhibited by pretreatment with (a) SM-10661 ($n = 5$–9; control, $n = 8$) or (b) CV-6209 ($n = 5$–8; control, $n = 7$). Each value represents the mean with s.e. shown by vertical bars. Statistical significance; $^*P < 0.05$, $^{**}P < 0.01$. Dunnett's test compared with the vehicle treated group.

dependently inhibited the hyperreactivity to histamine. In previous studies, we showed that SM-10661 inhibited PAF-induced platelet aggregation but did not influence ADP-, collagen- or calcium ionophore A-23187-induced platelet aggregation at concentrations up to 400 μM. SM-10661 at doses of up to 100 mg kg^{-1} also did not inhibit airway responses induced by histamine (8 μg kg^{-1}, i.v.) or leukotriene D_4 (2.0 nmol kg^{-1}, i.v.). Furthermore it influenced neither the antigen-induced release of histamine nor leukotriene D_4 from passively sensitized guinea-pig chopped lung at concentrations up to 10^{-4} M (Uchida *et al.*, 1989). Another specific PAF-antagonist, CV-6209 (Terashita *et al.*, 1987), also inhibited PAF-induced hyperreactivity to histamine. Therefore, this PAF-induced airway hyperreactivity to spasmogens seems to be mediated via PAF receptors.

We found that the administration of antigen, at levels which did not cause an increase in ITP, to passively sensitized guinea-pigs, induced hyperreactivity to histamine 6 min after antigen treatment. The dose of antigen required to induce hyperreactivity to histamine was about one tenth the dose of antigen which alone induced the increase in ITP. The hyperreactivity was also observed for 61 min after the antigen administration. Popa *et al.* (1973) also reported that 30 min to 3 h after antigen-induced apparent bronchospasm, hyperreactivity to histamine occurred in sensitized guinea-pigs. Non-specific airway hyperreactivity to i.v. histamine or acetylcholine which was elicited by aerosol allergen challenge in sensitized guinea-pigs 24 h after challenge, persisted for at least 48 h and was inhibited by PAF-antagonist, ketotifen or methylprednisolone (Havill *et al.*, 1990). In the present experiments, we found that the hyperreactivity was induced within 6 min after the administration of ovalbumin, at a dose which was insufficient to induce an increase in ITP. Our results suggest that the hyperreactivity is induced earlier after antigen administration than has been known so far.

The PAF-antagonists, SM-10661 and CV-6209, inhibited the antigen-induced hyperreactivity to histamine. These results may suggest that PAF is involved in this response. The antigen may induce the synthesis of PAF, which induces hyperreactivity, but the amount of histamine and leukotrienes released by antigen administration are not enough to induce increases in ITP. In contrast to the PAF-induced hyperreactivity to histamine, augmentation of histamine response at 1 min after antigen administration was not observed. It was presumed that mast cell or basophil activation accounted for the symptoms of allergic asthma. It is known that the time-courses of extracellular appearance of lipid derived mediators such as PAF, leukotriene C_4, leukotriene B_4 and thromboxane A_2 are superimposable and there is a short lag period until these mediators are released after antigen challenge (Mencia-Huerta *et al.*, 1983). This may explain why the hyperreactivity to histamine could not be observed 1 min after the antigen treatment. It should also be noted that there is a difference between the doses of PAF-antagonists required to inhibit PAF- and antigen-induced hyperreactivity. In addition, the ratio of inhibitory potency of the two PAF-antagonists on PAF and antigen were not the same. Bussolino *et al.* (1986) reported that intracellularly synthesized PAF does not tend to be released from the cell. Stewart *et al.* (1988) and Stewart & Dusting (1990) found that PAF receptor antagonists inhibited bradykinin-induced prostacyclin (PGI_2) generation in bovine arteriole endothelial cells whereas exogenous PAF failed to stimulate PGI_2 synthesis. They suggested the existence of external and internal PAF receptors or receptor subtypes. Hwang (1988) also described the existence of PAF receptor subtypes. Therefore, the differences in amounts of SM-10661 and CV-6209 required for PAF- and antigen-induced hyperreactivity may be due to these mechanisms. These points require further investigation.

There have been several reports stating that PAF can induce airway hyperreactivity to spasmogens in man (Cuss *et al.*, 1986; Rubin *et al.*, 1987), monkeys (Patterson *et al.*, 1987), dogs (Chung *et al.*, 1986) or sheep (Christman *et al.*, 1987). Although the exact mechanism of hyperreactivity has not yet been elucidated, PAF-induced inflammatory events, such as leucocyte invasion of the thorax, oedema formation and impaired mucus clearance are thought to have important roles (Lellouch-Tubiana *et al.*, 1988). Despite a lack of correlation between airway hyperreactivity and the presence of eosinophils in bronchial alveolar fluids (Havill *et al.*, 1990), the role of eosinophils has generally been a topic of much attention (Durham & Kay, 1985; Sanjar *et al.*, 1989). Cockcroft *et al.* (1983) and Sanjar *et al.* (1989) found that the accumulation of sufficient eosinophils in the airway lumen requires more than 1 h or days. However, we observed that PAF or antigen administration induced the decrement of circulating white blood cells at 1 min or 6 min respectively, which implies the accumulation of white blood cells in the airway. So, the role of white blood cells such as eosinophils cannot be neglected in the rapid PAF- or antigen-induced hyperreactivity which we observed. PAF can activate guinea-pig platelets and Coyle *et al.* (1988) reported that the platelet-depletion of the ragweed-sensitized rabbit, inhibited the development of bronchial hyperresponsiveness 24 h after antigen exposure which was thought to be the consequence of the eosinophil accumulation. Platelet aggregates and migrated polymorphonuclear neutrophils were seen in the constricted arterioles 1 min after the intravenous administration of 100 ng kg^{-1} of PAF (Lellouch-Tubiana *et al.*, 1985). In the present study, we observed that the circulating platelet number decreases either

1 min after PAF injection or 6 min after antigen administration which correlates well with the development of airway hyperreactivity. These results may indicate the importance of platelets in the development of airway hyperresponsiveness. The precise role of platelets or white blood cells in PAF- or antigen-induced immediate airway response should be investigated. It is also necessary to examine whether antigen-induced thrombocytopaenia and leucopaenia are PAF-dependent.

PAF-antagonists reportedly inhibit late anaphylactic bronchospasm in guinea-pigs (Hutson *et al.*, 1988; Uchida *et al.*, 1989) and sheep (Tomioka *et al.*, 1989). These results indicated that PAF is involved in late anaphylactic response. In the present experiments, we revealed that PAF also may play an important role in the immediate anaphylactic response by elevating reactivity to spasmogens.

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British Journal of Pharmacology

VOLUME 105 (3) MARCH 1992

R.K. Waite & S. Holly. Training and employment in pharmacology. Fourth updating survey on the organisation of pharmacology in Great Britain: summary of findings 507

SPECIAL REPORTS

P.J. Birch, S.M. Harrison, A.G. Hayes, H. Rogers & M.B. Tyers. The non-peptide NK_1 receptor antagonist, (±)-CP-96,345, produces anti-nociceptive and anti-oedema effects in the rat 508

V.J. Harrison, A. Randriantsoa & P. Schoeffter. Heterogeneity of endothelin-sarafotoxin receptors mediating contraction of pig coronary artery 511

M. Shoaib & I.P. Stolerman. MK801 attenuates behavioural adaptation to chronic nicotine administration in rats 514

PAPERS

A. Rubino, S. Amerini, F. Ledda & L. Mantelli. ATP modulates the efferent function of capsaicin-sensitive neurones in guinea-pig isolated atria 516

M.K. Pugsley, W.P. Penz, M.J.A. Walker & T.M. Wong. Cardiovascular actions of the κ-agonist, U-50,488H, in the absence and presence of opioid receptor blockade 521

F. Lembeck, J. Donnerer, M. Tsuchiya & A. Nagahisa. The non-peptide tachykinin antagonist, CP-96,345, is a potent inhibitor of neurogenic inflammation 527

M. Schwanstecher, S. Löser, Ch. Brandt, K. Scheffer, F. Rosenberger & U. Panten. Adenine nucleotide-induced inhibition of binding of sulphonyl-ureas to their receptor in pancreatic islets 531

F. Sannajust, C. Barrès, E. Koenig-Bérard & J. Sassard. Sympathoinhibitory effects of rilmenidine may be mediated by sites located below the brainstem 535

F. Sannajust, C. Cerutti, E. Koenig-Bérard & J. Sassard. Influence of anaesthesia on the cardiovascular effects of rilmenidine and clonidine in spontaneously hypertensive rats 542

S.N. Russell, S.V. Smirnov & P.I. Aaronson. Effects of BRL 38227 on potassium currents in smooth muscle cells isolated from rabbit portal vein and human mesenteric artery 549

C.G. Sobey, R.A. Dalipram, G.J. Dusting & O.L. Woodman. Impaired endothelium-dependent relaxation of dog coronary arteries after myocardial ischaemia and reperfusion: prevention by amlodipine, propranolol and allopurinol 557

M.J. Neal & J.R. Cunningham. Effect of sulphur containing amino acids on [^{3}H]-acetylcholine release from amacrine cells of the rabbit retina 563

P.T. Männistö, P. Tuomainen & R.K. Tuominen. Different *in vivo* properties of three new inhibitors of catechol *O*-methyltransferase in the rat 569

R. Schulz, E. Nava & S. Moncada. Induction and potential biological relevance of a Ca^{2+}-independent nitric oxide synthase in the myocardium 575

S.J. De Kimpe, D. Van Heuven-Nolsen & F.P. Nijkamp. Bovine polymorphonuclear leukocytes increase sensitivity to noradrenaline in isolated mesenteric arteries 581

M. Takei, K. Endo & K. Takahashi. Mechanism of action of MY-1250, an active metabolite of Repirinast, in inhibiting histamine release from rat mast cells 587

X.-J. Xu, Z. Wiesenfeld-Hallin, J. Hughes, D.C. Horwell & T. Hökfelt. CI988, a selective antagonist of cholecystokinin$_B$ receptors, prevents morphine tolerance in the rat 591

Y. Nakazato, H. Hayashi & H. Teraoka. Comparisons of the effects of ryanodine on catecholamine secretion evoked by caffeine and acetylcholine in perfused adrenal glands of the guinea-pig 597

M.J. Sumner, W. Feniuk, J.D. McCormick & P.P.A. Humphrey. Studies on the mechanism of 5-HT_1 receptor-induced smooth muscle contraction in dog saphenous vein 603

T. Aikawa, K. Sekizawa, M. Morikawa, S. Itabashi, H. Sasaki & T. Takishima. The role of cyclic AMP in non-adrenergic non-cholinergic contraction in guinea-pig bronchi 609

Y. Endo & M. Nakamura. The effect of lipopolysaccharide, interleukin-1 and tumour necrosis factor on the hepatic accumulation of 5-hydroxytryptamine and platelets in the mouse 613

W.-S.F. Wong, S.L. Bloomquist, A.M. Bendele & J.H. Fleisch. Pharmacological and histological examinations of regional differences of guinea-pig lung: a role of pleural surface smooth muscle in lung strip contraction 620

T.R. Walker & S.P. Watson. Okadaic acid inhibits activation of phospholipase C in human platelets by mimicking the actions of protein kinases A and C 627

H.Y. Jin, M. Katori, M. Majima & N. Sunahara. Increased secretion of glandular-kallikrein in the bronchial washings induced by intravenous injection of leukotriene C_4 in guinea-pigs 632

J. Nicholls, S.M.O. Hourani & I. Kitchen. Characterization of P_1-purinoceptors on rat duodenum and urinary bladder 639

G.R. Martin, M.L. Bolofo & H. Giles. Inhibition of endothelium-dependent vasorelaxation by arginine analogues: a pharmacological analysis of agonist and tissue dependence 643

R.E. Widdop, S.M. Gardiner, P.A. Kemp & T. Bennett. The influence of atropine and atenolol on the cardiac haemodynamic effects of N^G-nitro-L-arginine methyl ester in conscious, Long Evans rats 653

J. Connolly, J. Boulter & S.F. Heinemann. α4-2β2 and other nicotinic acetylcholine receptor subtypes as targets of psychoactive and addictive drugs 657

S.J. Taylor & G.J. Kilpatrick. Characterization of histamine-H_3 receptors controlling non-adrenergic non-cholinergic contractions of the guinea-pig isolated ileum 667

M.R. Zarrindast & A. Jamshidzadeh. Inhibitory effect of morphine on yawning induced by cholinoceptor and dopamine D_2 receptor activation in rats 675

D.N. Brindley, P. Hales, A.I.I. Al-Sieni & J.C. Russell. Sustained decreases in weight and serum insulin, glucose, triacylglycerol and cholesterol in JCR: LA-corpulent rats treated with D-fenfluramine 679

A.B. Hawcock, J.C. Barnes & A.D. Michel. Pharmacological characterisation of angiotensin-induced depolarizations of rat superior cervical ganglion *in vitro* 686

M. D'Amato, D. Currò, P. Montuschi, G. Ciabattoni, E. Ragazzoni & R.A. Lefebvre. Release of vasoactive intestinal polypeptide from the rat gastric fundus 691

Y. Nasa, K. Ichihara, R. Yoshida & Y. Abiko. Positive inotropic and negative chronotropic effects of (−)-*cis*-diltiazem in rat isolated atria **696**

F. Karoum & M.F. Egan. Dopamine release and metabolism in the rat frontal cortex, nucleus accumbens, and striatum: a comparison of acute clozapine and haloperidol **703**

I. Th. Lippe & P. Holzer. Participation of endothelium-derived nitric oxide but not prostacyclin in the gastric mucosal hyperaemia due to acid back-diffusion **708**

R. Padrini, S. Bova, G. Cargnelli, D. Piovan & M. Ferrari. Effects of pinacidil on guinea-pig isolated perfused heart with particular reference to the proarrhythmic effect **715**

M. Carli & R. Samanin. 8-Hydroxy-2-(di-n-propylamino)tetralin impairs spatial learning in a water maze: role of postsynaptic 5-HT_{1A} receptors **720**

N.J. Mallard, R.W. Marshall, A.J. Sithers & T.L.B. Spriggs. Separation of putative α_{1A}- and α_{1B}-adrenoceptor mediated components in the tension response of the rat vas deferens to electrical field stimulation **727**

E. Schlicker, U. Werner, M. Hamon, H. Gozlan, B. Nickel, I. Szelenyi & M. Göthert. Anpirtoline, a novel, highly potent 5-HT_{1B} receptor agonist with antinociceptive/antidepressant-like actions in rodents **732**

J.D. McLeod & P.J. Piper. Effect of K^+ channel-modulating drugs on the vasoconstrictor responses of leukotrienes C_4, D_4 and angiotensin II in the guinea-pig isolated perfused heart **739**

J.R. Fozard & M.-L. Part. The role of nitric oxide in the regional vasodilator effects of endothelin-1 in the rat **744**

N.J. Brown, R.D.E. Rumsey, C. Bogentoft & N.W. Read. The effect of adrenoceptor antagonists on the ileal brake mechanism in the rat **751**

S. Morooka, M. Uchida & N. Imanishi. Platelet-activating factor (PAF) plays an important role in the immediate asthmatic response in guinea-pig by augmenting the response to histamine **756**

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Y. Nasa, K. Ichihara, R. Yoshida & Y. Abiko. Positive inotropic and negative chronotropic effects of (−)-*cis*-diltiazem in rat isolated atria **696**

F. Karoum & M.F. Egan. Dopamine release and metabolism in the rat frontal cortex, nucleus accumbens, and striatum: a comparison of acute clozapine and haloperidol **703**

I. Th. Lippe & P. Holzer. Participation of endothelium-derived nitric oxide but not prostacyclin in the gastric mucosal hyperaemia due to acid back-diffusion **708**

R. Padrini, S. Bova, G. Cargnelli, D. Piovan & M. Ferrari. Effects of pinacidil on guinea-pig isolated perfused heart with particular reference to the proarrhythmic effect **715**

M. Carli & R. Samanin. 8-Hydroxy-2-(di-n-propylamino)tetralin impairs spatial learning in a water maze: role of postsynaptic 5-HT_{1A} receptors **720**

N.J. Mallard, R.W. Marshall, A.J. Sithers & T.L.B. Spriggs. Separation of putative α_{1A}- and α_{1B}-adrenoceptor mediated components in the tension response of the rat vas deferens to electrical field stimulation **727**

E. Schlicker, U. Werner, M. Hamon, H. Gozlan, B. Nickel, I. Szelenyi & M. Göthert. Anpirtoline, a novel, highly potent 5-HT_{1B} receptor agonist with antinociceptive/antidepressant-like actions in rodents **732**

J.D. McLeod & P.J. Piper. Effect of K^+ channel-modulating drugs on the vasoconstrictor responses of leukotrienes C_4, D_4 and angiotensin II in the guinea-pig isolated perfused heart **739**

J.R. Fozard & M.-L. Part. The role of nitric oxide in the regional vasodilator effects of endothelin-1 in the rat **744**

N.J. Brown, R.D.E. Rumsey, C. Bogentoft & N.W. Read. The effect of adrenoceptor antagonists on the ileal brake mechanism in the rat **751**

S. Morooka, M. Uchida & N. Imanishi. Platelet-activating factor (PAF) plays an important role in the immediate asthmatic response in guinea-pig by augmenting the response to histamine **756**

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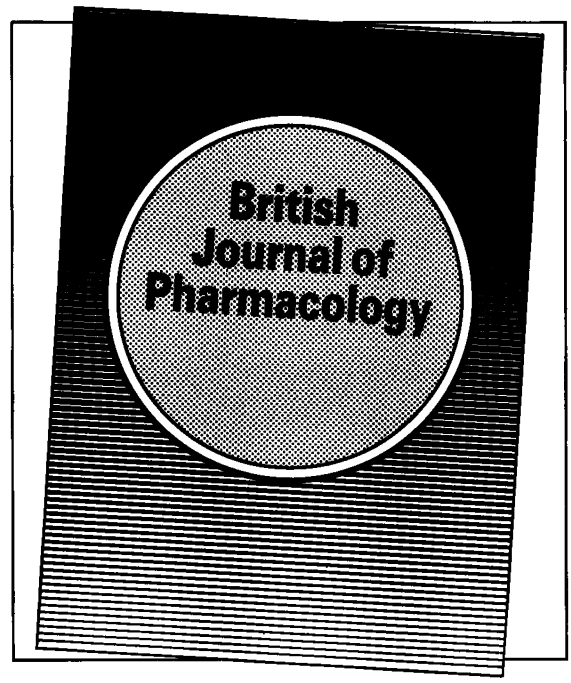

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R.K. Waite & S. Holly. Training and employment in pharmacology. Fourth updating survey on the organisation of pharmacology in Great Britain: summary of findings 507

SPECIAL REPORTS

P.J. Birch, S.M. Harrison, A.G. Hayes, H. Rogers & M.B. Tyers. The non-peptide NK_1 receptor antagonist, (±)-CP-96,345, produces anti-nociceptive and anti-oedema effects in the rat 508

V.J. Harrison, A. Randriantsoa & P. Schoeffter. Heterogeneity of endothelin-sarafotoxin receptors mediating contraction of pig coronary artery 511

M. Shoaib & I.P. Stolerman. MK801 attenuates behavioural adaptation to chronic nicotine administration in rats 514

PAPERS

A. Rubino, S. Amerini, F. Ledda & L. Mantelli. ATP modulates the efferent function of capsaicin-sensitive neurones in guinea-pig isolated atria 516

M.K. Pugsley, W.P. Penz, M.J.A. Walker & T.M. Wong. Cardiovascular actions of the κ-agonist, U-50,488H, in the absence and presence of opioid receptor blockade 521

F. Lembeck, J. Donnerer, M. Tsuchiya & A. Nagahisa. The non-peptide tachykinin antagonist, CP-96,345, is a potent inhibitor of neurogenic inflammation 527

M. Schwanstecher, S. Löser, Ch. Brandt, K. Scheffer, F. Rosenberger & U. Panten. Adenine nucleotide-induced inhibition of binding of sulphonyl-ureas to their receptor in pancreatic islets 531

F. Sannajust, C. Barrès, E. Koenig-Bérard & J. Sassard. Sympathoinhibitory effects of rilmenidine may be mediated by sites located below the brainstem 535

F. Sannajust, C. Cerutti, E. Koenig-Bérard & J. Sassard. Influence of anaesthesia on the cardiovascular effects of rilmenidine and clonidine in spontaneously hypertensive rats 542

S.N. Russell, S.V. Smirnov & P.I. Aaronson. Effects of BRL 38227 on potassium currents in smooth muscle cells isolated from rabbit portal vein and human mesenteric artery 549

C.G. Sobey, R.A. Dalipram, G.J. Dusting & O.L. Woodman. Impaired endothelium-dependent relaxation of dog coronary arteries after myocardial ischaemia and reperfusion: prevention by amlodipine, propranolol and allopurinol 557

M.J. Neal & J.R. Cunningham. Effect of sulphur containing amino acids on [^{3}H]-acetylcholine release from amacrine cells of the rabbit retina 563

P.T. Männistö, P. Tuomainen & R.K. Tuominen. Different *in vivo* properties of three new inhibitors of catechol *O*-methyltransferase in the rat 569

R. Schulz, E. Nava & S. Moncada. Induction and potential biological relevance of a Ca^{2+}-independent nitric oxide synthase in the myocardium 575

S.J. De Kimpe, D. Van Heuven-Nolsen & F.P. Nijkamp. Bovine polymorphonuclear leukocytes increase sensitivity to noradrenaline in isolated mesenteric arteries 581

M. Takei, K. Endo & K. Takahashi. Mechanism of action of MY-1250, an active metabolite of Repirinast, in inhibiting histamine release from rat mast cells 587

X.-J. Xu, Z. Wiesenfeld-Hallin, J. Hughes, D.C. Horwell & T. Hökfelt. CI988, a selective antagonist of cholecystokinin$_B$ receptors, prevents morphine tolerance in the rat 591

Y. Nakazato, H. Hayashi & H. Teraoka. Comparisons of the effects of ryanodine on catecholamine secretion evoked by caffeine and acetylcholine in perfused adrenal glands of the guinea-pig 597

M.J. Sumner, W. Feniuk, J.D. McCormick & P.P.A. Humphrey. Studies on the mechanism of 5-HT_1 receptor-induced smooth muscle contraction in dog saphenous vein 603

T. Aikawa, K. Sekizawa, M. Morikawa, S. Itabashi, H. Sasaki & T. Takishima. The role of cyclic AMP in non-adrenergic non-cholinergic contraction in guinea-pig bronchi 609

Y. Endo & M. Nakamura. The effect of lipopolysaccharide, interleukin-1 and tumour necrosis factor on the hepatic accumulation of 5-hydroxytryptamine and platelets in the mouse 613

W.-S.F. Wong, S.L. Bloomquist, A.M. Bendele & J.H. Fleisch. Pharmacological and histological examinations of regional differences of guinea-pig lung: a role of pleural surface smooth muscle in lung strip contraction 620

T.R. Walker & S.P. Watson. Okadaic acid inhibits activation of phospholipase C in human platelets by mimicking the actions of protein kinases A and C 627

H.Y. Jin, M. Katori, M. Majima & N. Sunahara. Increased secretion of glandular-kallikrein in the bronchial washings induced by intravenous injection of leukotriene C_4 in guinea-pigs 632

J. Nicholls, S.M.O. Hourani & I. Kitchen. Characterization of P_1-purinoceptors on rat duodenum and urinary bladder 639

G.R. Martin, M.L. Bolofo & H. Giles. Inhibition of endothelium-dependent vasorelaxation by arginine analogues: a pharmacological analysis of agonist and tissue dependence 643

R.E. Widdop, S.M. Gardiner, P.A. Kemp & T. Bennett. The influence of atropine and atenolol on the cardiac haemodynamic effects of N^G-nitro-L-arginine methyl ester in conscious, Long Evans rats 653

J. Connolly, J. Boulter & S.F. Heinemann. α4-2β2 and other nicotinic acetylcholine receptor subtypes as targets of psychoactive and addictive drugs 657

S.J. Taylor & G.J. Kilpatrick. Characterization of histamine-H_3 receptors controlling non-adrenergic non-cholinergic contractions of the guinea-pig isolated ileum 667

M.R. Zarrindast & A. Jamshidzadeh. Inhibitory effect of morphine on yawning induced by cholinoceptor and dopamine D_2 receptor activation in rats 675

D.N. Brindley, P. Hales, A.I.I. Al-Sieni & J.C. Russell. Sustained decreases in weight and serum insulin, glucose, triacylglycerol and cholesterol in JCR: LA-corpulent rats treated with D-fenfluramine 679

A.B. Hawcock, J.C. Barnes & A.D. Michel. Pharmacological characterisation of angiotensin-induced depolarizations of rat superior cervical ganglion *in vitro* 686

M. D'Amato, D. Currò, P. Montuschi, G. Ciabattoni, E. Ragazzoni & R.A. Lefebvre. Release of vasoactive intestinal polypeptide from the rat gastric fundus 691

Y. Nasa, K. Ichihara, R. Yoshida & Y. Abiko. Positive inotropic and negative chronotropic effects of (−)-*cis*-diltiazem in rat isolated atria 696

F. Karoum & M.F. Egan. Dopamine release and metabolism in the rat frontal cortex, nucleus accumbens, and striatum: a comparison of acute clozapine and haloperidol 703

I. Th. Lippe & P. Holzer. Participation of endothelium-derived nitric oxide but not prostacyclin in the gastric mucosal hyperaemia due to acid back-diffusion 708

R. Padrini, S. Bova, G. Cargnelli, D. Piovan & M. Ferrari. Effects of pinacidil on guinea-pig isolated perfused heart with particular reference to the proarrhythmic effect 715

M. Carli & R. Samanin. 8-Hydroxy-2-(di-n-propylamino)tetralin impairs spatial learning in a water maze: role of postsynaptic 5-HT_{1A} receptors 720

N.J. Mallard, R.W. Marshall, A.J. Sithers & T.L.B. Spriggs. Separation of putative α_{1A}- and α_{1B}-adrenoceptor mediated components in the tension response of the rat vas deferens to electrical field stimulation 727

E. Schlicker, U. Werner, M. Hamon, H. Gozlan, B. Nickel, I. Szelenyi & M. Göthert. Anpirtoline, a novel, highly potent 5-HT_{1B} receptor agonist with antinociceptive/antidepressant-like actions in rodents 732

J.D. McLeod & P.J. Piper. Effect of K^+ channel-modulating drugs on the vasoconstrictor responses of leukotrienes C_4, D_4 and angiotensin II in the guinea-pig isolated perfused heart 739

J.R. Fozard & M.-L. Part. The role of nitric oxide in the regional vasodilator effects of endothelin-1 in the rat 744

N.J. Brown, R.D.E. Rumsey, C. Bogentoft & N.W. Read. The effect of adrenoceptor antagonists on the ileal brake mechanism in the rat 751

S. Morooka, M. Uchida & N. Imanishi. Platelet-activating factor (PAF) plays an important role in the immediate asthmatic response in guinea-pig by augmenting the response to histamine 756

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